30. 3′-(β-D-Glycopyranosyloxy)flavylium Ions: Synthesis and Investigation of Their Properties in Aqueous Solution. Hydrogen Bonding as a Mean of Colour Variation

by **Hakima El Hajji**[a]), **Olivier Dangles**[b])*, **Paulo Figueiredo**[a]), and **Raymond Brouillard**[a])

[a]) Laboratoire de Chimie des Polyphénols, Université Louis Pasteur, 1, rue Blaise Pascal, F-67008 Strasbourg
[b]) Laboratoire de Chimie Organique III, Université Claude Bernard, 43, boulevard du 11 Novembre 1918, F-69622 Villeurbanne

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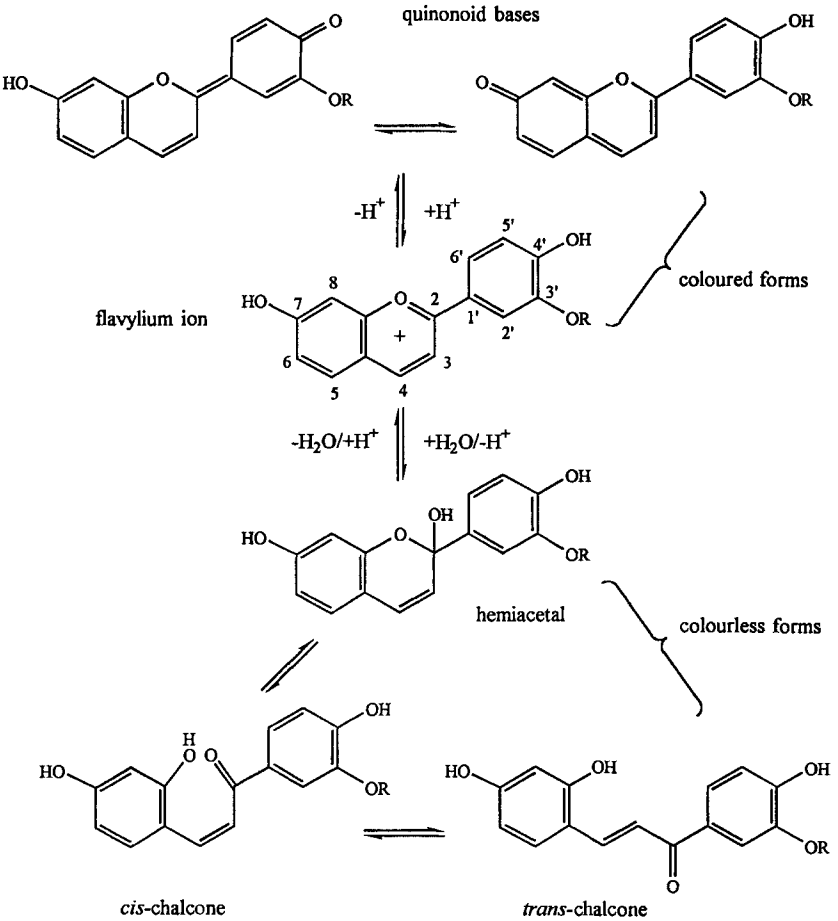
This work describes a straightforward synthesis of two 3′-(β-D-glycopyranosyloxy)flavylium ions thought to be good models of natural anthocyanins (pigments). For both pigments and for the non-glycosylated flavylium ion taken as a reference, H_2O addition and proton-transfer reactions as well as formation of molecular complexes with chlorogenic acid and caffeine (copigmentation) are quantitatively investigated in mildly acidic aqueous solution. A remarkable affinity of caffeine for the *trans*-chalcone form of the pigments is demonstrated. Moreover, the differences in the flavylium pK_a values are interpreted in terms of possible intramolecular H-bonding between the glycosyl residue and the chromophore. The discussion is then extended to a series of malonylated anthocyanins recently reported for their unusual pigmentation properties. A possible role for the malonyl group (frequently encountered in the structure of naturally occurring anthocyanins) in plant colour expression is outlined for the first time.

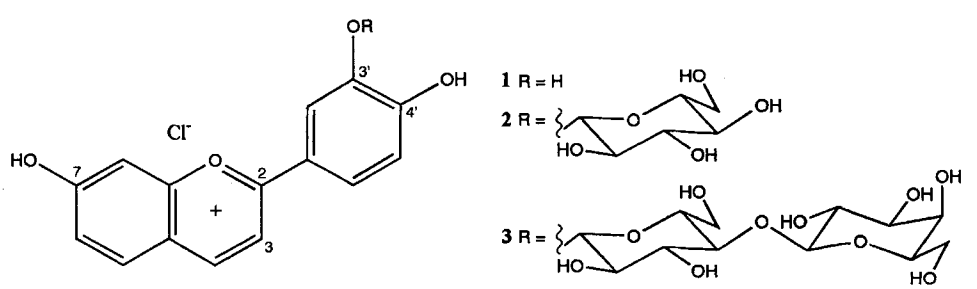
Introduction. – Anthocyanins are coloured flavonoids (polyphenols) widespread in the plant kingdom, especially in the vacuoles of the epidermal cells of flower petals [1] [2]. Their bright orange, red, purple or blue colours make them key elements in the plant-insect interaction leading to pollination [3]. The anthocyanin chromophore is a flavylium (= 2-phenyl-1-benzopyrylium) ion diversely substituted at the 3, 3′, 4′, 5, 5′ and 7 positions by OH, MeO, and glycosyloxy groups. Beyond the variety of flavylium chromophores, the multiplicity of natural colours largely rests on the ability of anthocyanins to form molecular complexes with other natural, usually colourless, polyphenols (copigments) as well as metal complexes with Al^{3+}, Fe^{3+}, Mg^{2+}, *etc.* [1] [2]. Moreover, the variety of vacuolar pHs (typically ranging from 3 to 7) allows conversion of flavylium ions into neutral and eventually anionic quinonoid bases according to proton-transfer reactions which take part in colour diversity (*Scheme 1*).

In a previous work [4], we showed that the synthesis of 3-methoxy- and 3-(β-D-glucopyranosyloxy)flavylium ions (including the naturally occurring pigment callistephin) allowed a systematic investigation of the influence of the flavylium substitution pattern, especially the glycosyl moiety, on the proton transfer, water addition and copigmentation reactions. This work is now extended to two 3′-(β-D-glycopyranosyloxy)flavylium ions and the corresponding non-glycosylated ion (pigments **1**–**3**). The relatively simple, easily synthesized pigments **2** and **3** are thought to be good models for more complex 3′-(β-D-glycopyranosyloxy)flavylium ions frequently encountered in plants [2] [5]. The influence of the glycosyl residues on the thermodynamics of proton transfer through

Scheme 1. *Structural Transformations of Flavylium Ions in Mildly Acidic Aqueous Solution*

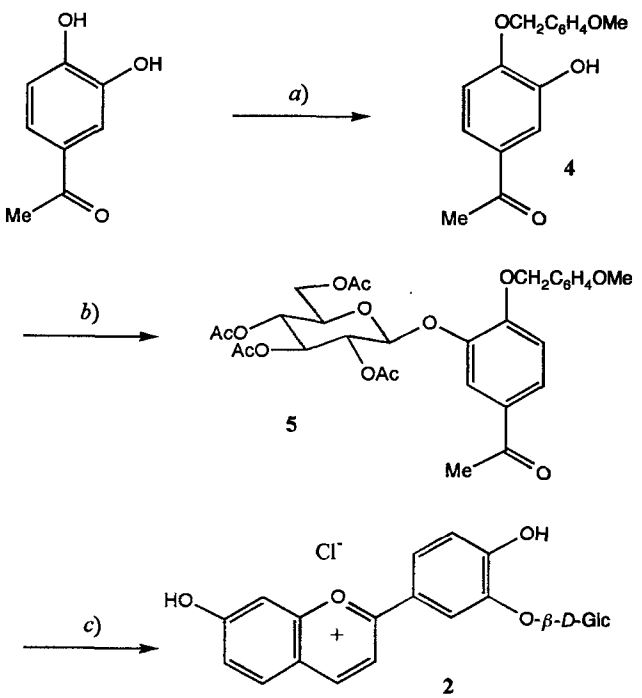


intramolecular H-bonding with the chromophore will be discussed and the discussion extended to the case of malonylated anthocyanins which were recently shown to display very unusual acid-base properties [6].



Results and Discussion. – 1. *Synthesis of the Flavylium Ions* (see *Scheme 2*). For the glycosidation of 3,4-dihydroxyacetophenone on its 3-OH group, the preliminary selective protection of the more acidic 4-OH group was required. In the first step, 3,4-dihydroxyacetophenone was thus converted into the corresponding 4-(4-methoxybenzyl) ether. The position of the 4-methoxybenzyloxy group was confirmed by ^{1}H-NMR (NOE connectivities between H–C(5) and the methylene and aromatic protons of the 4-methoxyphenyl group). Glycosidation of the 3-OH group was then attempted under acidic conditions using the following glycosyl donors: 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide activated by $Hg(CN)_2$ [7], penta-*O*-acetyl-D-glucose or 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate activated by BF_3 [8], phenyl (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl) sulfoxide activated by trimethylsilyl triflate [9]. All gave unsatisfactory yields. The best results (60–70% of pure β-D-anomer) were obtained when **4** was reacted with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide under phase-transfer conditions in a mixture of CH_2Cl_2 and saturated aqueous K_2CO_3 solution using tris[2-(2-methoxyethoxy)ethyl]amine as the phase-transfer agent [10]. The moderate alkaline conditions allowed to minimize the side reactions of the bromide, *i.e.*, hydrolysis and β-elimination. In the following step, **5** was condensed with 2,4-dihydroxybenzaldehyde under anhydrous acidic conditions which simultaneously cleaved the 4-methoxyphenyl group. After removal of the acetyl groups (MeONa in $MeOH/CHCl_3$), pigment **2** was

Scheme 2. *Chemical Synthesis of Pigment* **2**



a) 1. NaH, THF; 2. 4-methoxybenzyl bromide. *b*) 2,3,4,6-Tetraacetyl-β-D-glucopyranosyl bromide, CH_2Cl_2, sat. aq. K_2CO_3 soln., tris[2-(2-methoxyethoxy)ethyl]amine, 40°. *c*) 1. 2,4-Dihydroxybenzaldehyde, HCl, AcOEt; 2. MeONa, $MeOH/CHCl_3$ 3:1; 3. 1M HCl.

obtained and purified by column chromatography (reversed phase (*C-18*) silica). The same procedure was applied to the synthesis of pigment **3**, whereas simple condensation of 3,4-dihydroxyacetophenone with 2,4-dihydroxybenzaldehyde yielded pure **1**.

2. *Structural Transformations of the Flavylium Ions.* In addition to proton-transfer reactions, flavylium ions are susceptible to H_2O addition in their electrophilic 2-position leading to an epimeric mixture of colourless hemiacetals whose central ring quickly opens up to give a *cis*-chalcone in slow equilibrium with its *trans*-isomer (*Scheme 1*) [11]. This process, usually referred to as 'hydration', causes weakly acidic solutions of common anthocyanins to fade rapidly. It can, however, be efficiently countered by complexation phenomena selectively stabilizing the coloured forms (flavylium ion and quinonoid bases): conversion of 3′,4′-dihydroxyflavylium ions into highly coloured metal chelates [12], formation of hydrophobic stacks between the coloured forms and colourless aromatic compounds [13] (copigmentation) which tends to remove H_2O from the surface of the chromophores. Both processes are accompanied by large bathochromic shifts in the VIS absorption band of the pigment and thus eminently participate in colour variation.

The structural transformations of flavylium ions in weakly acidic aqueous solution can be conveniently reduced to the two following reactions: the conversion of the flavylium ion into a tautomeric mixture of quinonoid bases resulting from deprotonation of the OH group at position 4′ or 7 (overall thermodynamic constant K_a) and the hydration of the flavylium ion into a mixture of colourless (or pale-yellow) hemiacetals and chalcones (overall thermodynamic constant K_h).

Flavylium ions unsubstituted at position 3 are more resistant to H_2O addition at the 2-position [14] than naturally occurring anthocyanins which all have a 3-glycosyloxy group [11a–c]. In particular, the kinetics of H_2O addition for pigments **1**–**3** is much slower than that of proton transfer so that the pK_a and pK_h values can be determined according to the simple procedure described in the *Exper. Part.*

Re-acidification to pH *ca.* 1 of weakly acidic equilibrated solutions quickly converts the hemiacetal and *cis*-chalcone forms back to the flavylium ion, thus resulting in a quasi-instantaneous hyperchromic effect. In a second step, more colour can be gained from the slow *trans*-chalcone → flavylium ion conversion. From the amplitudes of the two consecutive colour gains, the percentage of *trans*-chalcone can be estimated [14]. Thus, the *trans*-chalcone could be demonstrated to be by far the most abundant colourless form (70 to 75% of the total concentration of colourless forms) of pigments (**1**–**3**). This result confirms previous observations with other flavylium ions unsubstituted at position 3 [15], such as the 4′,7-dihydroxyflavylium ion, and contrasts sharply with the case of natural anthocyanins (3-(glycosyloxy)flavylium ions) for which the hemiacetal is the dominant colourless form.

The pK_h values (see *Table*) and the relative amounts of *trans*-chalcone are almost identical for pigments **1**–**3**. Thus, the glycosyl groups of pigments **2** and **3** have little influence on the thermodynamics of H_2O addition and the relative distribution of the colourless forms.

The pK_a values appear much more sensitive to glycosidation of the 3′-OH group. E.g., the β-D-glucopyranosyl group of **2** lowers the pK_a by more than 0.4 unit with respect to the corresponding non-glycosylated pigment **1**. Possible intramolecular H-bonding processes can be put forward to interpret this difference. H-Bonding between the highly acidic 4′-OH group (donor) and the much more electron-rich 3′-OH group (acceptor)

Table. *Thermodynamic Constants of the Structural Transformations and Copigmentation Reactions of Pigments* **1**–**3** (25°, 0.5M ionic strength)

	1	**2**	**3**
pK_a	4.35 (0.07)	3.92 (0.07)	4.65 (0.09)
pK_h	2.91 (0.02)	2.95 (0.02)	2.99 (0.02)
K_1 (chlorogenic acid)[a])	91 (9)	96 (4)	114 (6)
β_{12} (chlorogenic acid)[b])	1438 (540)	[d])	[d])
K_1' (chlorogenic acid)[c])	100 (27)	[d])	[d])
K_1 (caffeine)[a])	33 (4)	42 (3)	28 (1)
K_1' (caffeine)[c])	97 (5)	57 (4)	169 (25)

[a]) K_1 1:1 flavylium/copigment binding constant.
[b]) β_{12}, overall 1:2 flavylium/copigment binding constant.
[c]) K_1', 1:1 colourless forms/copigment binding constant.
[d]) No detectable binding, standard deviations in brackets.

may occur in flavylium ion **1**, although probably counterbalanced by H-bonding between the poorly acidic 3′-OH group (donor) and the 4′-keto group (acceptor) of the quinonoid base formed upon deprotonation of the OH group at position 4′. The overall effect on the thermodynamics of proton transfer is expected to be small. Indeed, the 4′,7-dihydroxyflavylium ion (pK_a 4.30 at 25° and ionic strength lower than 10^{-2} M [15]) and pigment **1** have almost the same acidity. In pigment **2**, only H-bonding in the flavylium ion is to be considered although the H-bond acceptor capability of the 3′-(β-D-glucopyranosyloxy) group is expected to be somewhat weaker than that of a OH group (a glycosyl group can be considered electron-withdrawing because of the cumulative electron-withdrawing effect of the sugar OH groups in addition to electronic effects peculiar to acetal groups (*exo*-anomeric effect [16])). As a result of its selective stabilization through H-bonding, flavylium ion **2** would be predicted to be less acidic than flavylium ion **1**, whereas the opposite is observed experimentally. In addition, comparison of the ^{1}H-NMR spectra of pigments **1** and **2** recorded under the same conditions (D_2O + 2% DCl) shows that the signals of the flavylium protons exhibit almost the same chemical shifts, thus indicating close electron distributions in both flavylium ions. Consequently, the higher acidity of pigment **2** more probably points to a specific stabilization of one of the quinonoid bases (probably, the 4′-keto tautomer) that could be achieved through H-bonding between the keto group and one of the sugar OH groups. This was somewhat supported by molecular-modeling calculations which gave an optimized structure for the 4′-keto quinonoid base of pigment **2** in which *O*–C(4′) and the proton of the glucopyranosyl 2-OH group are distant by 0.223 nm (*Fig. 1*).

More surprisingly, passing from **2** to **3**, *i.e.*, replacing the β-D-glucopyranosyl group by a lactosyl (β-D-galactopyranosyl-1 → 4-β-D-glucopyranosyl) group, raises the pK_a by more than 0.7 unit. Therefore, although apparently remote from the flavylium moiety, the D-galactopyranosyl group strongly lowers the acidity of the phenolic protons. In this case, formation of a H-bond between one O-atom of the galactopyranosyl moiety (acceptor) and one of the two phenolic OH groups of the flavylium nucleus (donor) may by suggested. Consistent with this view, the two-dimensional ROESY spectrum of **3** shows several non-trivial long-range connectivities between some sugar protons (H–C(5)(Glc),

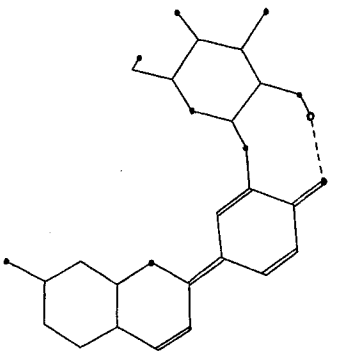


Fig. 1. *Optimized structure of the 4'-keto quinonoid form of pigment* **2** (semi-empirical quantum-mechanics calculations (AM1) *in vacuum*, HyperChem program). o = H-Atom involved in H-bonding, ● = O-atom.

some protons among H–C(3)(Glc), H–C(4)(Glc), H–C(5)(Gal), H–C(6)(Gal) and H'–C(6)(Gal)), and the flavylium protons H–C(2'), H–C(6'), and possibly H–C(5) (signal overlappings in the flavylium and sugar regions precludes the complete attribution of the proton pairs in NOE relationship). In addition, molecular-modeling experiments gave a low-energy conformation for pigment **3** in which the disaccharide moiety was folded toward the chromophore with the galactopyranosyl O–C(6) (acceptor) at H-bonding distance (0.211 nm) from the flavylium phenolic 7-OH proton (donor) (*Fig. 2*). Molecular modeling also demonstrated H-bonding between the galactopyranosyl 6-OH group (donor) and the glucopyranosyl O–C(3) (acceptor) that is susceptible to increase the H-bond acceptor capability of the galactopyranosyl O–C(6) and thus strengthen H-bonding with the flavylium 7-OH proton (cooperative H-bonds).

It must also be noted that, because of its axial 4-OH group, D-galactose displays a relatively hydrophobic α face (C–H groups at positions 3, 4, and 5) which can develop strong *van der Waals* contacts with aromatic surfaces. This is, *e.g.*, very well demonstrated in the highly refined X-ray crystal structure of the L-arabinose-arabinose binding protein complex which shows a tryptophan residue of the protein closely stacked on the α face of the sugar (L-arabinose formally derives from D-galactose on replacement of the

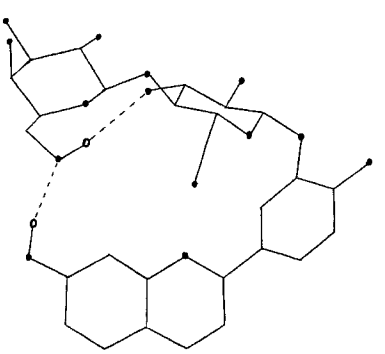
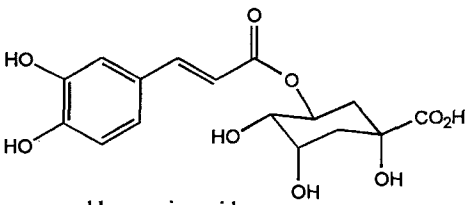


Fig. 2. *Optimized structure of the flavylium form of pigment* **3** (semi-empirical quantum-mechanics calculations (AM1) *in vacuum*, HyperChem program). o = H-Atom involved in H-bonding, ● = O-atom.

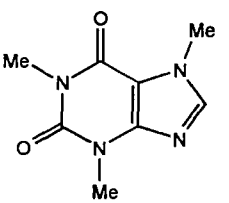
CH_2OH group by a H-atom) [17]. Similarly, intramolecular H-bonding in pigment **3** may be assisted by hydrophobic galactose-flavylium interactions.

3. *Copigmentation.* In copigmentation experiments, large copigment-to-pigment ratios (higher than 10) are typically used. Consequently, the concentration of copigmentation complexes is much higher than that of noncovalent homo- and/or heterodimers (flavylium-flavylium, flavylium-chalcone, chalcone-chalcone) which would eventually form upon weak self-association of the pigment [18]. Indeed, quantitative interpretation of the spectroscopic data could be achieved without taking into account weak self-association of the pigment.

Copigmentation of pigments **1**–**3** was investigated with chlorogenic acid (= 5-caffeoylquinic acid) and caffeine. Both compounds are very abundant in plants and highly H_2O-soluble, and they display planar structures with delocalized π electrons which are appropriate for vertical stacking onto the flavylium moiety. In addition, chlorogenic acid is a natural copigment of anthocyanins.



chlorogenic acid



caffeine

The *Table* shows that copigmentation of the glycosylated pigments **2** and **3** by chlorogenic acid essentially consists in the formation of 1:1 flavylium-copigment complexes responsible for both hyperchromic and bathochromic shifts observed in the VIS spectrum of the pigments upon copigment addition. This is the behaviour typically observed with natural anthocyanins [13]. Moreover, molecular-modeling calculations point to a glycosyl moiety lying well out of the flavylium plane and probably bringing steric hindrance on one face of the flavylium moiety, thus precluding 1:2 binding. By contrast, pigment **1** with its two equivalent faces, is able to accommodate two copigment molecules, and both 1:1 and 1:2 bindings are observed. These results are in line with those obtained with 3-methoxy- and 3-(β-D-glucopyranosyloxy)flavylium ions [4]. Unexpectedly, correct fitting of the VIS absorbance *vs.* copigment-concentration plot recorded in mildly acidic aqueous solution requires the hypothesis of 1:1 binding between chlorogenic acid and the colourless forms of **1**. This probably indicates that poorly substituted (non-glycosylated) planar chalcone forms are able to stack on the 3,4-dihydroxycinnamic (caffeic) moiety of chlorogenic acid. Addition of caffeine to mildly acidic solutions of pigments **1**–**3** caused fading throughout the investigated range of copigment concentration. This means that caffeine binds to the colourless chalcone form more strongly than to the highly coloured flavylium ion. The corresponding changes in the UV/VIS spectrum of pigment **3** are shown in *Fig. 3*. The UV band characteristic of the chalcone form (λ_{max} 375 nm) is strongly intensified at the expense of the flavylium VIS band (λ_{max} 465 nm). Both bands, however, are bathochromically shifted because of simultaneous π-stacking of caffeine onto the flavylium and chalcone forms. Fitting of the VIS ab-

sorbance *vs.* copigment-concentration plots demonstrated 1:1 binding and allowed to estimate the corresponding binding constants (*Table*). The binding selectivity, measured by the K_1'/K_1 ratio, is maximum for pigment **3** whose colourless forms (mainly the *trans*-chalcone) bind to caffeine six times as strongly as the flavylium ion. Two similar colour-damaging intermolecular complexation processes were previously reported: the inclusion of poorly substituted synthetic and naturally occurring flavylium ions into the macrocyclic cavity of β-cyclodextrin [19] and the association between caffeine and the 4′,7-dihydroxy-3-methoxyflavylium ion which, because of unique chalcone-caffeine 1:2 binding, caused colour loss at high caffeine/pigment molar ratios [4]. Copigmentation of pigments **1**–**3** by caffeine is, however, the first example of a 1:1 vertical stacking interaction that is selective of the colourless forms and causes colour loss in the whole range of copigment concentration. This unusual behaviour reflects the particular properties of flavylium ions unsubstituted at position 3 whose planar polarizable *trans*-chalcone forms are present in concentration large enough for them to efficiently compete with the corresponding flavylium forms for stacking on the caffeine molecule. Moreover, we have demonstrated that copigmentation of the natural anthocyanin malvin by caffeine is stronger with the neutral quinonoid bases than with the flavylium ion [13 b, c]. Therefore, it seems that the neutral planar forms of anthocyanins display a particular affinity towards caffeine, whereas the positively charged nucleus of the flavylium ion only weakly interacts with the electron-poor purine nucleus of caffeine.

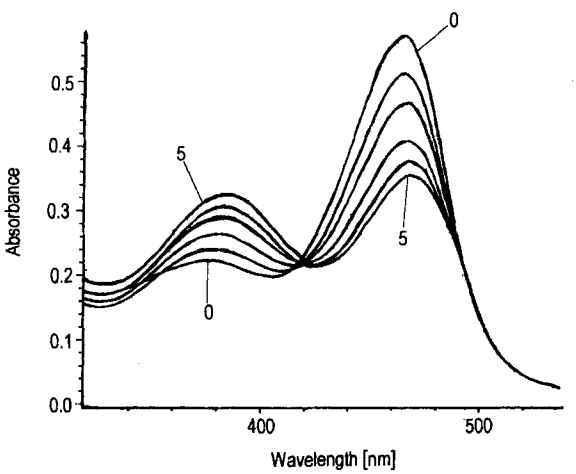
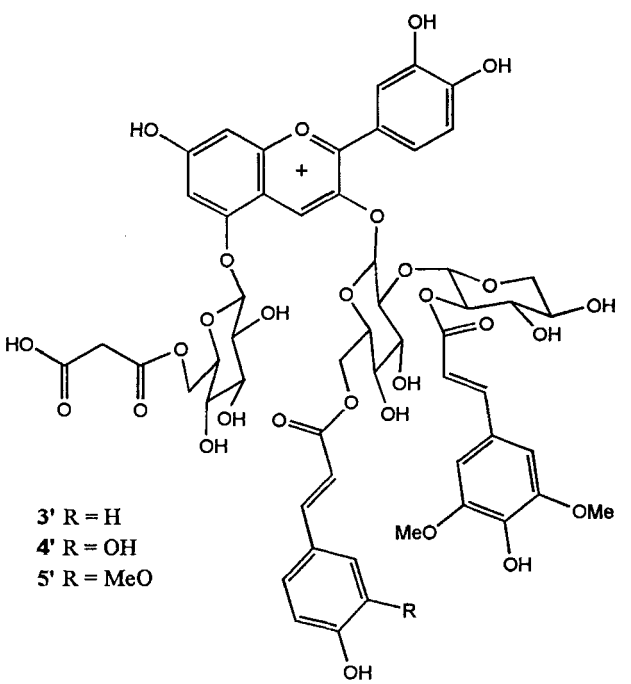


Fig. 3. *Changes in the UV/VIS spectrum of pigment* **3** *as a function of caffeine concentration* (25°, 0.5M ionic strength, pH 2.9, pigment concentration $6.5 \cdot 10^{-5}$ M caffeine/pigment molar ratios: 0 (spectrum 0), 25, 50, 100, 150, 200 (spectrum 5)).

4. *A Model for the Role of the Malonyl (= Carboxyacetyl) Group of Malonylated Anthocyanins.* The differences in acidity between flavylium ions **1**–**3** suggest that H-bonding between the chromophore and the glycosyl moieties of anthocyanins, favouring either the usually red flavylium form or the purple quinonoid bases, may be another mechanism of colour variation in plants. In a recent work [6], three structurally related malonylated anthocyanins, the pigments **3′**–**5′**, were reported to possess extraordinary

acid-base properties allowing the formation of quinonoid bases in fairly acidic aqueous solution (pH *ca.* 2). These pigments are cyanidin (= 3,3′,4′,5,7-pentahydroxyflavylium) derivatives which all bear a β-D-glucopyranosyl group at the 5-OH and a sambubiosyl (= 2-*O*-(β-D-xylopyranosyl)-β-D-glucopyranosyl) group at the 3-OH. In addition, they are acylated by a malonyl group at the 6-position of the 5-β-D-glucopyranosyl group and by two aromatic acyl groups derived from cinnamic acid at the 6-gluco and 2-xylo positions of the sambubiosyl residue. Non-acylated pigment **1′** and pigment **2′**, which corresponds to demalonylated pigment **5′** (*Formulae* not shown; *cf.* [6]), were also investigated for comparison purposes. The authors describe striking changes in the VIS spectrum of pigments **3′**–**5′** as a function of pH which cannot be satisfactorily interpreted by the simple assumption of flavylium-quinonoid base proton transfer and H_2O addition. These unusual spectral changes are: a colour loss when the pH is raised from 0.7 to 1.5 and the appearance of a gradually increasing shoulder on the right side of the flavylium VIS absorption band when the pH is raised from 2.1 to 3.5. The first phenomenon was attributed to an intramolecular copigmentation process (hydrophobic stacking of the cinnamyl moieties on both sides of the flavylium moiety) that would be unfavourable in highly acidic aqueous solution (pH < 0.7) because of the disorder generated in the molecular network of liquid H_2O by high proton concentrations but would take place at less acidic pH (with a simultaneous reduction in the flavylium molar absorption coefficient) following a postulated solvent reorganisation. The second phenomenon was ascribed to the formation of the quinonoid bases (usually occurring at pH higher than 4) resulting from deprotonation of the copigmentation complex. Since non-malonylated pigment **2′** did not significantly depart from normal behaviour, it was concluded that the



6-malonyl group of the β-D-glucopyranosyl group at the 5-position of the cyanidin moiety played an important (although not completely elucidated) role in the structural and conformational transformations of pigments **3′**–**5′** [6].

We now wish to propose an alternative mechanism which fully accounts for the experimental observations without requiring the hypothesis of changes in the structure of liquid H_2O. The new approach, which rests on the acid-base and H-bonding properties of the malonyl group, has an additional advantage: it accounts for the large differences (up to 16 nm) in the wavelengths of flavylium absorption maximum at pH 0.9 between pigments **3′**–**5′** and non-acylated pigment **1′** which point to intramolecular copigmentation still occurring in highly acidic aqueous solution.

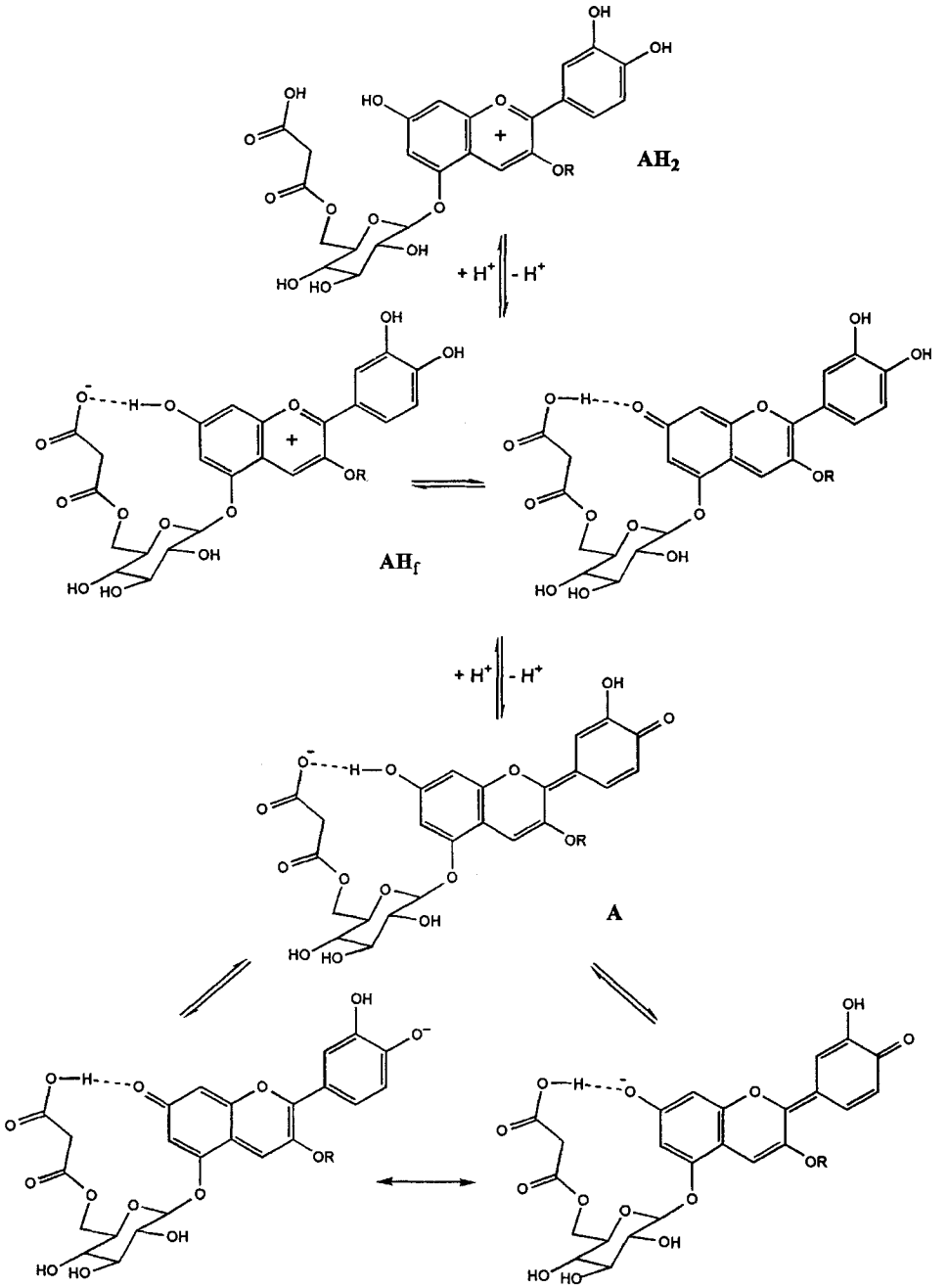
The whole scheme of the structural transformations of pigments **3′**–**5′** is as follows (see *Scheme 3* for a simplified representation of the structures involved): *a*) A proton transfer reaction occurs between the cationic form $\mathbf{AH_2}$ (malonyl group in its neutral protonated form) of flavylium ions **3′**–**5′** and the corresponding zwitterionic form **AH** (malonyl group in its anionic deprotonated form). *b*) An equilibrium is established between **AH** in an 'open' conformation (no contact between the malonate and flavylium moieties) and **AH** in a 'folded' conformation (noted as $\mathbf{AH_f}$) in which the malonate and flavylium moieties are H-bonded. $\mathbf{AH_f}$ is a mixture of two tautomeric forms, one having a zwitterionic malonate-flavylium structure and the other having a neutral malonic-quinonoid structure. *c*) A proton transfer reaction takes place between $\mathbf{AH_f}$ and the anionic form **A** which again can be depicted by two tautomeric forms, one having a neutral quinonoid chromophore and an anionic malonate group and the other having an anionic quinonoid chromophore (itself represented as a mixture of two 4′-keto and 7-keto mesomeric forms) and a neutral malonic group. *d*) Addition of H_2O to $\mathbf{AH_f}$ gives hemiacetal **B** (*cf. Scheme 1*; anionic malonate group).

For each coloured form ($\mathbf{AH_2}$, **AH**, $\mathbf{AH_f}$, **A**), conformations in which the aromatic acyl residues are stacked on the chromophore (intramolecular copigmentation) are assumed to be dominant [20]. This is, *e.g.*, well evidenced for $\mathbf{AH_2}$ by the very significant spectral differences between pigments **3′**–**5′** and pigment **1′** at pH 0.9 [6].

The VIS absorbance of a highly acidic pigment solution (pH < 1, pure $\mathbf{AH_2}$) is $D_0 = \varepsilon_{\mathbf{AH_2}}\, c$ (c, total pigment concentration; ε_i molar absorption coefficient of the species i; optical pathlength omitted). In less acidic pigment solution (pH > 1.5), $\mathbf{AH_2}$ is a minor negligible component, and the VIS absorbance (at the same wavelength) and total pigment concentration become $D = \varepsilon_{\mathbf{AH}}[\mathbf{AH}] + \varepsilon_{\mathbf{AH_f}}[\mathbf{AH_f}] + \varepsilon_{\mathbf{A}}[\mathbf{A}]$ and $c = [\mathbf{AH}] + [\mathbf{AH_f}] + [\mathbf{A}] + [\mathbf{B}]$, respectively. Combining the above equations with the expression of the thermodynamic constants of the four equilibria (K_{a1}, K, K_{a2} and K_{h2}, resp.) and identifying $\varepsilon_{\mathbf{AH_2}}$ and $\varepsilon_{\mathbf{AH}}$ leads to *Eqn. 1* ($r = \varepsilon_{\mathbf{AH_f}}/\varepsilon_{\mathbf{AH}}$; $r_{\mathbf{A}} = \varepsilon_{\mathbf{A}}/\varepsilon_{\mathbf{AH}}$; a_{H^+}, proton activity) which is mathematically equivalent to the equation used to fit the D_0/D *vs.* $1/[H^+]$ plot in the former model [6]. However, the acidity-dependent conformational equilibrium between 'open' and 'folded' flavylium ions (thermodynamic constant K_1) postulated in the former model is replaced here by the conformational equilibrium between **AH** and $\mathbf{AH_f}$ (thermodynamic constant K) and the former constants K_a^{CP} and K_h^{CP} must be replaced by K_{a2} and K_{h2}, respectively. This mathematical equivalence still holds for the expression of the apparent rate constant (first order) of H_2O addition as a function of $[H^+]$.

$$D_0/D = \frac{1 + K + K(K_{a2} + K_{h2})/a_{H^+}}{1 + rK + r_a K K_{a2}/a_{H^+}} \qquad (1)$$

Scheme 3. *Postulated Acid-Base Forms of Pigments* **3′–5′** *in Strongly Acidic to Mildly Acidic Aqueous Solution*



In the frame of this new approach, the unusual spectral changes observed with pigments **3′–5′** can be readily interpreted: in strongly acidic solution, pigments **3′–5′** possess a flavylium chromophore and a neutral protonated malonyl group ($\mathbf{AH_2}$). When the pH is raised from 0.7 to 1.5, deprotonation of the malonyl carboxy group occurs giving zwitterion **AH**. Deprotonation of $\mathbf{AH_2}$ is driven by the thermodynamically favoured ($K = 8-9$) conformational folding of **AH** to give $\mathbf{AH_f}$ which is a mixture of two H-bonded tautomeric forms stabilized by H-bonding. The zwitterionic form displays a H-bond between its carboxylate group (acceptor) and one of the highly acidic phenolic OH groups of the flavylium moiety (donor), presumably the 7-OH group [6]. In the neutral form, H-bonding occurs between a protonated malonyl group and the 7-keto group of the quinonoid chromophore. Semi-empirical quantum-mechanics calculations were carried out to determine the most probably structure for $\mathbf{AH_f}$. The 4′,7-dihydroxyflavylium ion and its 7-keto quinonoid base were taken as models for the chromophore of pigments **3′–5′**, the monomethyl ester of malonic acid and its carboxylate anion mimicking the malonyl moiety. The calculations starting from both the flavylium-malonic and quinonoid-malonate structures led to the same optimized structure (as if both structures were in fact mesomeric forms with a negligible energy barrier between them) having a quinonoid chromophore with a charge distribution almost identical to that of the 7-keto quinonoid tautomer. The only significant deviation concerns *O*–C(7) and is attributable to H-bonding (calculated atomic charges on *O*–C(7): –0.350, –0.202 and 0.311 for $\mathbf{AH_f}$, the 4′,7-dihydroxyflavylium ion and its 7-keto quinonoid base, resp.). Similarly, the charge distribution of the malonyl moiety was much closer to that of the protonated species. In addition, the distance between the bridging H-atom and *O*–C(7) was calculated to be 0.207 nm, whereas the distance between the same H-atom and the closest O-atom of the malonyl moiety amounted to 0.098 nm (*ca.* the O–H bond length in the monomethyl ester of malonic acid). Consequently, the structure of $\mathbf{AH_f}$ is much better depicted by the neutral tautomeric form than by the zwitterionic one. This is experimentally confirmed by the shoulder (typical of quinonoid-base absorption) already appearing on the right side of the VIS absorption band around pH 1.5 for pigments **3′–5′** [6].

It may be surprising at first sight that deprotonation of a carboxy group may occur under such acidic conditions (the pK_a of the carboxy group would be *ca.* 1, *i.e.*, roughly 2 units lower than that of the monoethyl ester of malonic acid whose value is 3.15 at 25° and 1M ionic strength [21]). However, it must be remembered that the relatively unpolar environment produced by two aromatic acyl residues stacked on the chromophore may considerably strengthen electrostatic interactions such as ion pairing and H-bonding. In our case, electrostatic interactions and hydrophobic stacking are assumed to act cooperatively in order to considerably lower the pK_a of the malonyl carboxy group. Similarly, in enzymatic cavities of low local dielectric constant, the pK_a values of acidic or basic amino-acid residues involved in H-bonding and ionic interactions may be very different from those of the corresponding amino acids or peptides in aqueous solution[1]). It can also be pointed out that the carboxy group of free histidine has a pK_a value as low as 1.8 because of favourable intramolecular electrostatic interactions of the carboxylate with the

[1]) For a recent example of an enzymatically perturbed pK_a value (*ca.* 6) of the ε-amino group of an active-site lysine residue, see [22].

α-ammonium and imidazolium groups. In pigments **3′**–**5′**, the groups that stabilize the carboxylate are the malonic ester group and a highly acidic phenolic group of the positively charged flavylium moiety.

Finally, the drop in VIS absorption intensity ($r < 1$) may be ascribed to the partial quinonoid structure of the chromophore as well as to possible tighter packing of the aromatic acyl residues around the chromophore since copigmentation complexes usually have smaller molar absorption coefficients than the corresponding flavylium ions [13].

H-Bonding between the protonated malonyl group and the 7-keto group of the quinonoid chromophore in the major tautomeric form of $\mathbf{AH_f}$ is expected to lower the electronic density on the chromophore and may favour deprotonation of the 4′-OH group (*Scheme 3*). This would result in the formation of structure **A** which like $\mathbf{AH_f}$ displays two tautomeric forms: the first one has an anionic deprotonated malonyl group (malonate) and a neutral quinonoid chromophore which is written under the 4′-keto tautomeric form to allow H-bonding between the malonate group and the acidic 7-OH group. The second has a neutral protonated malonic group and an anionic quinonoid chromophore which itself may be written as a mixture of two mesomeric 7-keto- and 4′-keto forms. Semi-empirical quantum-mechanics calculations predict that the latter form could be more stable. Indeed, the electronic distribution in the optimized structure points to an anionic quinonoid chromophore with a dominant 4′-keto structure. *E.g.*, the atomic charges on *O*–C(7) and *O*–C(4′) in **A** are −0.455 and −0.405, respectively, whereas their common value in the free anionic quinonoid base is −0.413. In addition, the bridging proton lies 0.099 nm from the closest malonyl O-atom and 0.197 nm from *O*–C(7). The latter distance is significantly smaller than that calculated for $\mathbf{AH_f}$ (0.207 nm). This strengthening of H-bonding between the malonyl residue and the chromophore upon deprotonation may be the main driving force for the formation of **A** under remarkably acidic conditions.

Once more, acyl groups apparently remote from the chromophore turn out to have a determinant influence on the structural transformations of anthocyanins. It is particularly striking that proton transfer on a malonyl group acylating a glycosyl residue could be detected by UV/VIS spectroscopy owing to noncovalent interactions between the chromophore and the malonyl group. Whereas aromatic acyl groups interact with the chromophore through hydrophobic vertical stacking [1] [2] (intramolecular copigmentation) and, thereby, deeply modify the thermodynamics and the kinetics of H_2O addition [20] [23], malonyl groups seem to be able (at least in the presence of aromatic acyl groups) to form strong H-bonds with quinonoid chromophores. The concomitant reduction of the flavylium-quinonoid base pK_a value allows the quinonoid forms of anthocyanins to take part in the expression of natural colours, even in fairly acidic aqueous solution, and confers an explicit function to malonyl residues frequently encountered in the structure of natural anthocyanins [2] [5].

Experimental Part

General. Caffeine and chlorogenic acid were purchased from *Roth*, Karlsruhe, Germany.

Reversed-phase HPLC of pigments **1**–**3**: *Merck C-8* column (5 μm, 125 × 4 mm), flow rate 0.5 ml/min, gradient elution from 10% solvent *A* (5% HCO_2H in MeCN/H_2O 1:1)/90% solvent *B* (5% HCO_2H in H_2O) at time zero to 100% solvent *A* after 60 min; *Spectra-Physics* apparatus equipped with a *Hewlett-Packard* diode array detector typically monitoring at 260 and 500 nm.

UV/VIS Spectra: *Hewlett-Packard* diode-array spectrometer fitted with a quartz cell (optical pathlength, 1 cm) equipped with a stirring magnet; the temp. in the cell was measured with a *Comark* thermocouple and kept at 25° ± 0.1° by means of a *Lauda* water-thermostated bath; ionic strength fixed at 0.5M by NaCl; λ_{max} in nm.

^{1}H-NMR Spectra: at 27° on 200-, 400-, or 500-MHz-*Bruker* apparatus; chemical shifts δ in ppm rel. to external $SiMe_4$; internal references: $CHCl_3$ (δ 7.26), MeOH (δ 3.30) or MeCN (δ 1.94); coupling constants (J) in Hz, signal assignment for **1**–**3** from two-dimensional COSY and ROESY spectra (NOE connectivities: H–C(4) and H–C(5), H–C(3) and H–C(2′) (H–C(6′)), H–C(2′) and H–C(1) (Glc).

3′,4′,7-Trihydroxyflavylium Chloride (= *2-(3,4-Dihydroxyphenyl)-7-hydroxy-1-benzopyrylium Chloride*; (**1**). A soln. of 3,4-dihydroxyacetophenone (12 g, 79 mmol) and 2,4-dihydroxybenzaldehyde (10.9 g, 79 mmol) in distilled AcOEt (100 ml) was cooled to 0°. HCl (generated by action of 98% H_2SO_4 on NaCl) was gently bubbled through the soln. for 90 min. The mixture was kept at – 18° for 3 days and then filtered. More precipitate was collected upon concentration of the filtrate and subsequent addition of Et_2O. After washing with AcOEt and drying under vacuum, the combined solid fractions were found to be HPLC-pure **1** (yield 75%). UV/VIS (0.2M HCl): 473. ^{1}H-NMR (200 MHz, CD_3OD/DCl 98:2): 8.98 (*d*, *J* = 8.7, H–C(4)); 8.21 (*d*, *J* = 8.7, H–C(3)); 8.10 (*d*, *J* = 8.8, H–C(5)); 7.97 (*dd*, *J* = 8.8, 2.3, H–C(6′)); 7.81 (*d*, *J* = 2.3, H–C(2′)); 7.48 (*d*, *J* = 2.1, H–C(8)); 7.39 (*dd*, *J* = 8.7, 2.1, H–C(6)); 7.09 (*d*, *J* = 8.7, H–C(5′)). FAB-MS (positive mode): 255.

3-Hydroxy-4-(4-methoxybenzyloxy)acetophenone (= *1-[3-Hydroxy-4-(4-methoxybenzyloxy)phenyl]ethanone*; **4**). A soln. of 3,4-dihydroxyacetophenone (1 g, 6.57 mmol) in DMF (20 ml) was added dropwise to a suspension of NaH (0.32 g, 13.12 mmol) in DMF (30 ml). When H_2 evolution ceased, 4-methoxybenzyl bromide (0.9 ml, 5.74 mmol) was added. The mixture was stirred for 24 h at r.t. Then H_2O (20 ml) was slowly added and the mixture extracted with Et_2O. The aq. phase was acidified to pH 3.8 and extracted with AcOEt. The AcOEt phase was dried ($MgSO_4$) and evaporated. Pure **4** was obtained upon crystallization in AcOEt/hexane 1:1. Yield 75%. ^{1}H-NMR (200 MHz, $CDCl_3$): 7.54 (*d*, *J* = 1.7, H–C(2)); 7.52 (*dd*, *J* = 8.9, 1.7, H–C(6)); 7.35 (*d*, *J* = 8.6, H–C(2′), H–C(6′)); 6.96 (*d*, *J* = 8.9, H–C(5)); 6.94 (*d*, *J* = 8.6, H–C(3′), H–C(5′)); 5.72 (*s*, OH); 5.10 (*s*, CH_2); 3.83 (*s*, MeO); 2.54 (*s*, Me).

*4-(4-Methoxybenzyloxy)-3-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyloxy)acetophenone* (= *1-[4-(4-Methoxybenzyloxy)-3-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyloxy)phenyl]ethanone*; **5**). Tris[2-(2-methoxyethoxy)ethyl]-amine (0.35 ml, 1.1 mmol) and **4** (0.45 g, 1.65 mmol) were dissolved in sat. aq. K_2CO_3 soln. (9.5 ml) and vigorously stirred under Ar. A soln. of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl bromide (0.45 g, 1.1 mmol) in CH_2Cl_2 (9.5 ml) was added and the mixture stirred at 40° for 3 days (final pH 12.6). After addition of H_2O (10 ml), the aq. phase was washed twice with CH_2Cl_2 and the combined org. phase successively washed with 0.5% NaOH soln., H_2O, 2% HCl soln., and H_2O and evaporated. Pure **5** was isolated upon crystallization in AcOEt/hexane 1:1. Yield 60%. ^{1}H-NMR (200 MHz, $CDCl_3$): 7.75 (*d*, *J* = 2.0, H–C(2)); 7.66 (*dd*, *J* = 8.5, 2,0, H–C(6)); 7.33 (*d*, *J* = 8.6, H–C(2′), H–C(6′)); 6.97 (*d*, *J* = 8.5, H–C(5)); 7.1 (*d*, *J* = 8.6, H–C(3′), H–C(5′)); 5.27 (*d*, *J* = 6.4, H–C(1)(Glc)); 5.21 – 5.03 (*m*, H–C(2)(Glc), H–C(3)(Glc), H–C(4)(Glc)); 5.08 (*s*, CH_2); 4.60 (*dd*, *J* = 11.0, 7.2, 1H–C(6)(Glc)); 4.22 (*dd*, *J* = 11.0, 5.4, 1H–C(6)(Glc)); 3.82 (*m*, H–C(5)(Glc)); 3.81 (*s*, MeO); 2.53 (*s*, Me); 2.40–2.17 (4*s*, 4 Ac).

3′-(β-D-Glucopyranosyloxy)-4′,7-dihydroxyflavylium Chloride (= *2-[3-(β-D-Glucopyranosyloxy)-4-hydroxyphenyl]-7-hydroxy-1-benzopyrylium Chloride*; **2**). Equimolar amounts (0.2 mmol) of **5** and 2,4-dihydroxybenzaldehyde were condensed in AcOEt under HCl bubbling at – 5° for 1 h. The mixture was kept at – 18° for one week and then evaporated. Et_2O was added, and HPLC of the precipitate revealed complete deprotection of the 4′-OH group as well as partial deacetylation (mixture of two flavylium ions). The red powder was then dissolved in MeOH/$CHCl_3$ 3:1 and brought to pH 9 (wet pH paper) with a MeOH soln. of MeONa. The mixture was stirred at r.t. for 30 min, then carefully acidified to pH 1 with 1M HCl, kept at 4° for 12 h and evaporated. Chromatography on small column (*C-18* reversed-phase silica) gave HPLC-pure **2**. Yield 50%. UV/VIS (0.2M HCl): 465. ^{1}H-NMR (500 MHz, D_2O/CD_3CN/DCl 96:2:2): 8.81 (*d*, *J* = 8.7, H–C(4)); 7.93 (*d*, *J* = 8.7, H–C(3)); 7.90 (*d*, *J* = 9.2, H–C(5)); 7.79 (*dd*, *J* = 8.6, 2.1, H–C(6′)); 7.75 (*d*, *J* = 2.1, H–C(2′)); 7.23 (br. *d*, *J* = 9.2, H–C(6)); 7.22 (br. *s*, H–C(8)); 6.89 (*d*, *J* = 8.6, H–C(5′)); 4.93 (*d*, *J* = 7.2, H–C(1)(Glc)); 3.90 (*dd*, *J* = 12.1, 0.8, 1H–C(6)(Glc)); 3.74–3.67 (*m*, H–C(5)(Glc), 1H–C(6)(Glc)); 3.60 – 3.53 (*m*, H–C(2)(Glc), H–C(3)(Glc)); 3.42 (*t*, *J* = 9,2, H–C(4)(Glc)). Electrospray-MS (positive mode): 417.

3′-β-(D-Galactopyranosyl-β-1,4-D-glucopyranosyloxy)-4′,7-dihydroxyflavylium Chloride (= *2-[3-β-(D-Galactopyranosyl-β-1,4-D-glucopyranosyloxy)-4-hydroxyphenyl]-7-hydroxy-1-benzopyrylium Chloride*; **3**). As described for **2**, with hepta-D-acetyl-β-D-lactopyranosyl bromide instead of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl bromide. UV/VIS (0.2M HCl): 465. ^{1}H-NMR (400 MHz, D_2O/CD_3CN 7:3 + 2% DCl): 8.90 (*d*, *J* = 8.7, H–C(4)); 8.05 (*d*, *J* = 8.7, H–C(3)); 7.99 (*d*, *J* = 9.0, H–C(5)); 7.95 (*dd*, *J* = 8.6, 2.2, H–C(6′)); 7.93 (*d*, *J* = 2.2, H–C(2′)); 7.35 (*d*, *J* = 2.1, H–C(8)); 7.31 (*dd*, *J* = 9.0, 2.1, H–C(6)); 7.04 (*d*, *J* = 8.6, H–C(5′)); 5.09 (*d*, *J* = 7.7,

H–C(1)(Glc)); 4.37 (*d*, $J = 7.7$, H–C(1)(Gal)); 3.97 (br. *d*, $J = 12.0$, 1H–C(6)(Glc)); 3.82 (br. *d*, $J = 3.3$, H–C(4)(Gal)); 3.80–3.74 (*m*, H–C(5)(Glc), 1H–C(6)(Glc)); 3.73–3.57 (*m*, H–C(2)(Glc), H–C(3)(Glc), H–C(4)(Glc), H–C(5)(Gal), 2H–C(6)(Gal)); 3.56 (*dd*, $J = 7.7$, 3.3, H–C(3)(Gal)); 3.47 (*t*, $J = 7.7$, H–C(2)(Gal)). Electrospray-MS (positive mode): 579.

Structural Transformations of Flavylium Ions. The pK_a and pK_h values (see *General Part* for definitions) can be determined according to the following procedure: dil. NaOH solns. (1 ml) are quickly added to pigment solns. (1 ml, pH 2 to 2.5, flavylium form). Final pH values range from 3 to 6. The UV/VIS spectra of the solns. at different pH values are recorded a few seconds after mixing, and the VIS absorbance (at a fixed wavelength) is plotted against pH to give the pK_a value. After a few hours, the UV/VIS spectra of fully equilibrated solns. of coloured and colourless forms are recorded and a similar plot yields the pK_h value.

Copigmentation Experiments. The UV/VIS spectra of equilibrated pigment solns. were recorded for various copigment concentrations at two pH values: pH *ca.* 1 (pure flavylium ion) and pH *ca.* 3.5 (mixture of flavylium ion and colourless forms). The VIS absorbance *vs.* copigment concentration curves were then analysed according to a previously described procedure [4] taking into account 1:1 and 1:2 binding for the flavylium ion and the colourless forms.

Data Analysis. The curve fittings were carried out on a *Macintosh-II-SI* computer using the Kaleida Graph program. Standard deviations are reported.

Molecular Modeling. Molecular-modeling calculations on pigments **2** and **3** were run *in vacuo* on a *Pentium-90* PC using the HyperChem program (*Autodesk*, Sausalito, California) with the MM+ force field, the geometry-optimization procedure being repeated with different sets of input data files for the torsion angles about the glycosidic bonds. The low-energy conformations showing possible H-bonding between the glycosyl residue and the chromophore were further refined from semi-empirical quantum-mechanics calculations with the AM1 parametrization. The charge distributions and bond lengths of H-bonded flavylium-malonate structures were deduced from semi-empirical quantum-mechanics calculations *in vacuo* (AM1).

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