# 145. Synthesis of 3-Methoxy- and 3-(β-D-Glucopyranosyloxy)flavylium Ions. Influence of the Flavylium Substitution Pattern on the Reactivity of Anthocyanins in Aqueous Solution

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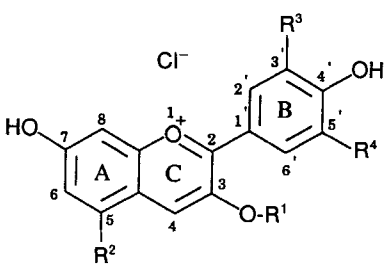
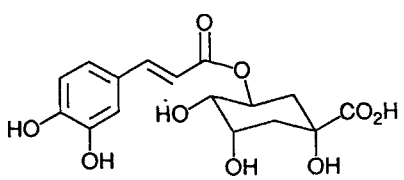
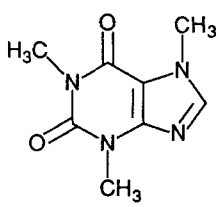
The synthesis of 3-glycosyloxylated flavylium ions (anthocyanins), in particular of callistephin (**4**), a natural anthocyanin, is described. The structural transformations in aqueous solution and molecular complexation with chlorogenic acid (**7**) and caffeine (**8**) of the synthesized pigments **3** and **4** are investigated and compared to those of the corresponding 3-methoxyflavylium ions **1** and **2** and to those of oenin (**5**) and malvin (**6**), two very common natural anthocyanins. The results are discussed in terms of the role played by the glycosyloxy residues in the chemical properties of anthocyanins. Anthocyanin molecular complexation (copigmentation) is quantitatively investigated by UV/VIS spectroscopy and $^1$H-NMR. In particular, the UV/VIS spectroscopic data are interpreted using a general theoretical treatment, which, *e.g.*, allows to demonstrate the formation of molecular complexes between the colourless forms of an anthocyanin and **8**.

**Introduction.** – Glycosyloxylated flavylium ions are widespread in plants and constitute the main coloured forms of anthocyanins which are natural pigments from the flavonoid family (polyphenols). In naturally occurring anthocyanins, the flavylium ( = 2-phenyl-1-benzopyrylium) chromophore is substituted in various ways by OH, MeO, and β-glycosyloxy groups (see, *e.g.*, **1**–**6**). These pigments are largely responsible for the red, purple, and blue colours displayed in flowers, fruits, and leaves and have developed sophisticated *in vivo* mechanisms for colour stabilization and variation based on the structural transformations of flavylium ions in aqueous solution as a function of pH,




**7** chlorogenic acid




**8** caffeine

| | **1** | **2** | **3** | **4** | **5** | **6** |
|---|---|---|---|---|---|---|
| $R^1$ | Me | Me | β-D-Glc | β-D-Glc | β-D-Glc | β-D-Glc |
| $R^2$ | H | OH | H | OH | OH | β-D-GlcO |
| $R^3$ | H | H | H | H | MeO | MeO |
| $R^4$ | H | H | H | H | MeO | MeO |

molecular complexation (copigmentation), and metal complexation [1] [2]. Recent publications from our group have thrown the basis of a quantitative description of these mechanisms [3–5].

Although extraction and purification of natural anthocyanins are now well-established procedures, they are still time-consuming and necessarily lead to limited amounts of pigment. Therefore, we typically use not only naturally occurring anthocyanins from plant extracts (some being commercially available in small quantities) but also synthetic non-glycosyloxylated flavylium ions that can be prepared on the g-scale and have proved good models of plant pigments. However, there is a need for a simple chemical synthesis giving reasonable amounts of pure glycosyloxylated flavylium ions in order to get closer to the natural case and to study systematically the influence of the flavylium substitution pattern on the chemical reactivity of natural anthocyanins in $H_2O$. In particular, the role played by the glycosyloxy residues, a point largely neglected so far, is expected to be highlighted from a comparison between a glycosyloxylated flavylium ion and the corresponding pigment in which the glycosyloxy group is replaced by a MeO group. Moreover, to our knowledge, no report on the chemical synthesis of glycosyloxylated flavylium ions has appeared since the works of *Robinson* and coworkers in the early thirties [6].

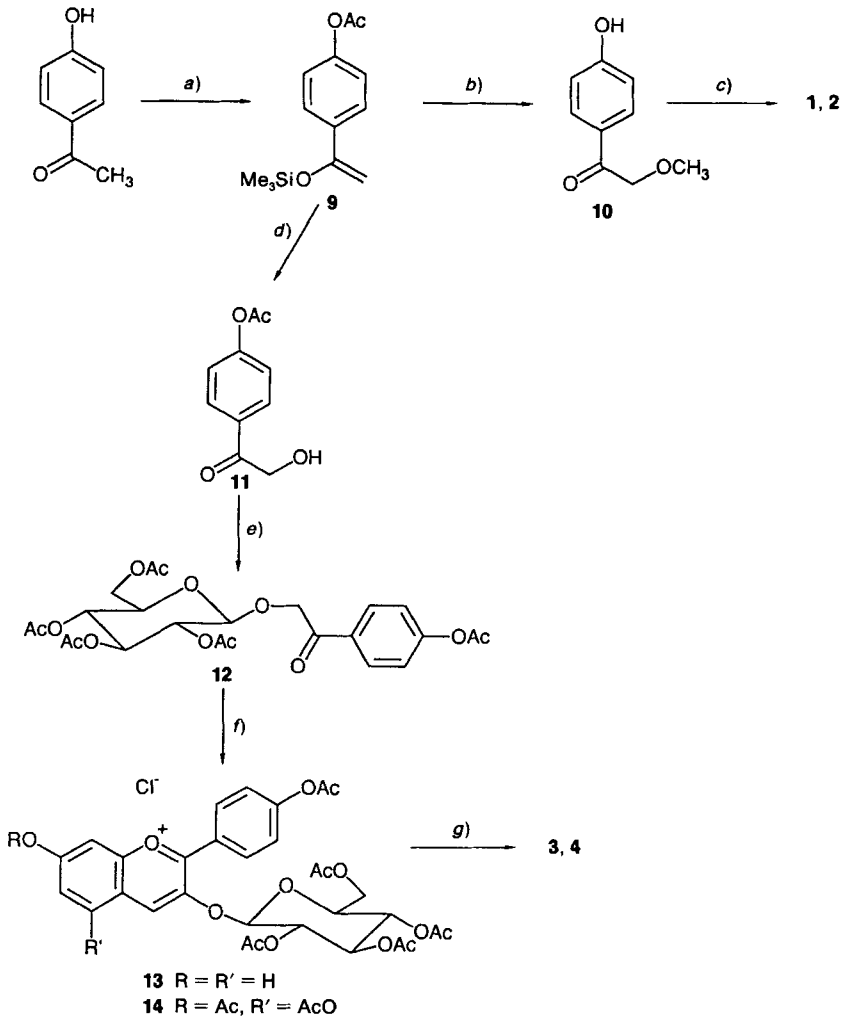
The presence of a $\beta$-glycosyloxy group at the 3-position of the flavylium nucleus is a characteristic common to all naturally occurring anthocyanins and is known to provide stability to the chromophore which, in its 3-OH form, suffers irreversible degradation in the weakly acidic to neutral conditions found in the natural medium [7]. Therefore, we here essentially report on the synthesis of two 3-($\beta$-glycosyloxy)flavylium ions, *i.e.*, 5-deoxycallistephin ( = 3-($\beta$-D-glucopyranosyloxy)-4′,7-dihydroxyflavylium chloride; **3**) and callistephin ( = 3-($\beta$-D-glucopyranosyloxy)-4′,5,7-trihydroxyflavylium chloride; **4**), this latter compound being a natural anthocyanin.

In a second part, the structural transformation of pigments **3** and **4** ($H_2O$ addition, proton transfer) as well as their ability to form vertical stacking molecular complexes (copigmentation) with chlorogenic acid (**7**) and caffeine (**8**) is studied and compared to the case of the corresponding 3-methoxyflavylium ions **1** and **2**. The copigment-promoted changes in the VIS-absorption spectrum of the pigments are investigated under strongly acidic and weakly acidic conditions and quantitatively interpreted by means of a general theoretical treatment; thus the taking part of the anthocyanin colourless forms in the anthocyanin-caffeine complexation are highlighted. These investigations are extended to two very abundant natural anthocyanins, *i.e.*, oenin ( = 3-($\beta$-D-glucopyranosyloxy)-4′,5,7-trihydroxy-3′,5′-dimethoxyflavylium chloride; **5**), ubiquitous in red wines and malvin ( = 3,5-bis($\beta$-D-glucopyranosyloxy)-4′,7-dihydroxy-3′,5′-dimethoxyflavylium chloride; **6**).

**Results and Discussion.** – 1. *Synthesis of Flavylium Ions.* The synthetic pathway is summarized in *Scheme 1.* Acetylation [8] of 4-hydroxyacetophenone followed by trimethylsilylation [9] of the enol form gave **9** in 80% overall yield. Then, **9** was treated by (diacetoxyiodo)benzene (instead of iodosobenzene, *cf.* [10]) in MeOH in the presence of $BF_3$ and deacetylated to afford **10** in 40% overall yield. Flavylium chlorides **1** and **2** were then obtained simply upon condensation at 0° of **10** with 2,4-dihydroxybenzaldehyde (yield 85%) and 2,4,6-trihydroxybenzaldehyde (yield 75%), respectively.

Alternatively, **9** was treated by 3-chloroperbenzoic acid and then citric acid to give **11** (overall yield 50%) [11]. Several procedures were tried for the glycosylation of **11**. The use

*Scheme 1*



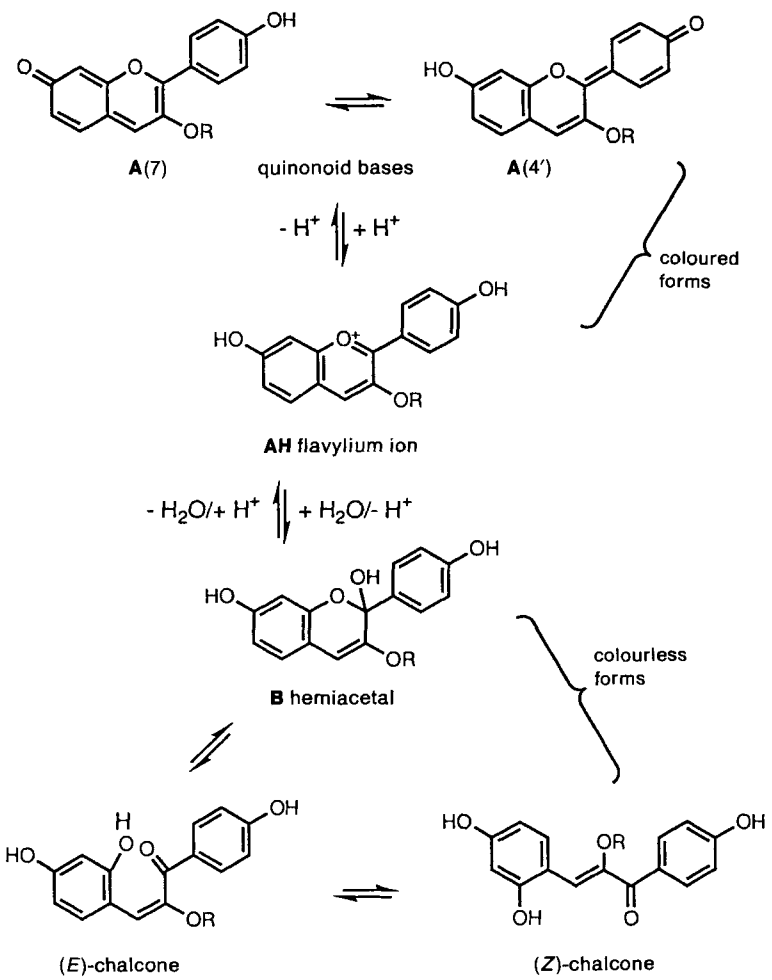

*a*) 1. $Ac_2O$, 4-(dimethylamino)pyridine, $CHCl_3$; 2. $Me_3SiCl$, $Et_3N$, DMF, 85°. *b*) 1. $PhI(OAc)_2$, MeOH; $BF_3$, $Et_2O$; 2. $NaHCO_3$, $H_2O$, MeOH. *c*) 2,4-Dihydroxybenzaldehyde, HCl, AcOEt, 0° (→ **1**); 2,4,6-trihydroxybenzaldehyde, HCl, HCOOH, 0° (→ **2**). *d*) 1. 3-Chloroperbenzoic acid, $CH_2Cl_2$; 2. citric acid, MeOH. *e*) 2,3,4,6-Tetraacetyl-β-D-glucopyranosyl bromide, AgTfO, $CH_2Cl_2$, 0° or $Hg(CN)_2$, toluene, reflux. *f*) 2,4-Dihydroxybenzaldehyde, HCl, AcOEt, −10° (→ **13**); 2,4-diacetoxy-6-hydroxybenzaldehyde, HCl, AcOEt, −10° (→ **14**). *g*) 1. KOH, $H_2O$/MeOH 1:1; 2. 1M HCl.

of penta-*O*-acetyl-D-glucose as the glycosyl donor in the presence of hard *Lewis* acids such as $BF_3$ or $SnCl_4$ for activation proved unsuccessful, probably because of chelation (deactivation) of the *Lewis* acid by the α-hydroxyketo group of **11**. More surprisingly, phenyl-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)sulfoxide, a powerful glycosyl donor when activated by trimethylsilyl triflate [12], did not react either. Finally, the best results were obtained with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (prepared in

90% yield according to [13]), activated by soft *Lewis* acids such as mercury or silver salts under rigorously anhydrous conditions (see *Exper. Part*): silver triflate in $CH_2Cl_2$ at 0° [14] and mercury cyanide in toluene at reflux [15] gave similar results, *i.e.*, a 40% yield of **12** after chromatography. HCl-Promoted aldol-type condensation of **12** in aprotic medium at −10° with 2,4-dihydroxybenzaldehyde and 2,4-diacetoxy-6-hydroxybenzaldehyde gave **13** and **14**, respectively, which were deacetylated to the target compounds **3** (65% from **12**) and **4** (40% from **12**), respectively. The 2,4,6-trihydroxybenzaldehyde was not suitable for the condensation (poor electrophilicity and solubility), and condensation temperatures above −10° favoured acidolysis of the glycosidic bond, whereas temperatures below −10° reduced the yields.

2. *Transformations of the Flavylium Ions.* The tautomeric quinonoid bases **A** and the hemiacetal **B** are the kinetic and thermodynamic products, respectively, in the structural transformations of a flavylium ion **AH** (see *Scheme 2*) [16]. Hemiacetal **B** is in a fast

*Scheme 2*



cycle-chain tautomeric equilibrium with the (*E*)-chalcone, itself in very slow *cis-trans* equilibrium with the corresponding (*Z*)-form [17]. In strongly acidic aqueous solution, an anthocyanin is in the pure flavylium form **AH**, whereas at equilibrium under weakly acidic conditions (pH *ca.* 3), a mixture of **AH** and hemiacetal **B** is present, the chalcones being usually minor components. The quinonoid bases **A** which appear above pH 4 remain in low concentration at equilibrium (at least, in the case of common natural anthocyanins) because of their thermodynamic instability with respect to hemiacetal **B**. *For simplification, we generally consider the overall conversion of* **AH** *into the colourless forms* (hemiacetal and chalcones) taken as a whole *in the notation* **B**. This reversible fading process is usually called 'hydration of the flavylium ion'. The thermodynamic constant of the **AH**/**A** proton transfer ($K_a$) and the thermodynamic constant of the hydration ($K_h$) are defined as $a_H \cdot$ **[A]**/**[AH]** and $a_H \cdot$ **[B]**/**[AH]**, respectively, $a_H$ being $10^{-pH}$ (proton activity). The p$K_h$ and p$K_a$ values are reported in *Table 1* for **1** and **3**–**6** (no values for **2** because of its poor solubility). The p$K_h$ and p$K_a$ values of 3-(β-D-glucopyranosyloxy)flavylium ion **3** are markedly lower than those of the corresponding 3-methoxy derivative **1**, thus pointing out that a glycosyloxy group is less effective than a MeO group at stabilizing the positively charged flavylium chromophore. In fact, part of the electron-density of the '*exo*'-anomeric O-atom is delocalized towards the pyranose ring (the so-called '*exo*'-anomeric effect [18]) so that its electron-donating ability towards the pyrylium moiety is probably reduced. Thus, the replacement of a MeO group by a β-D-glucopyranosyloxy

Table 1. *–log Values for the Thermodynamic Constants of the Hydration* (p$K_h$) *and Proton Transfer* (p$K_a$) *Equilibria* (25°, 0.5M ionic strength, unless otherwise specified)

| | **1** | **2** | **3** | **4** | **5** | **6** |
|---|---|---|---|---|---|---|
| p$K_h$ | 2.50 (±0.03) | [a]) | 1.90 (±0.03) | 2.86 (±0.03) | 2.70 (±0.01) | 1.75 (±0.01) |
| p$K_a$ | 5.04 (±0.03) | [a]) | 4.50 (±0.04) | 4.14 (±0.03) | 4.25[b]) | 4.0[c]) |

[a]) Not determined because of the poor solubility of **2** in weakly acidic aqueous solution (neutral forms predominant).
[b]) From [16b] (0.2M ionic strength).
[c]) From [16c] (0.2M ionic strength).

group at the 3-position of a flavylium chromophore enhances the electrophilic character at C(2), resulting in the formation of larger amounts of hemiacetal at equilibrium. Moreover, the $^1$H-NMR signal of H–C(4) of callistephin (**4**) appears 0.27 ppm higher than H–C(4) of **2**, thus confirming that the electron-donating effect of the MeO group *cis* to H–C(4) in **2** is stronger than that of the β-D-glucopyranosyloxy group in **4**. Interestingly, the replacement of H–C(5) of **3** by a OH–C(5) (see **4**) increases the p$K_h$ value by *ca.* 1 unit and, therefore, largely improves the resistance of the chromophore against hydration. Clearly, the lone pairs of the OH O-atom at C(5) of **4** are strongly conjugated with the benzopyrylium chromophore. This stabilizing effect is lost upon glycosylation of OH–C(5) (see **6**), and almost the same difference in the p$K_h$ values (1 unit) is found when passing from **5** to **6**. This once more demonstrates the powerful electron-withdrawing effect of the pyranose ring on the '*exo*'-anomeric O-atom.

3. *Self-association of Flavylium Ions.* Noncovalent dimerization of common natural flavylium ions were detected by $^1$H-NMR measurements [19]. In the concentration range

used for UV/VIS spectroscopic investigations (total concentration of pigment lower than $10^{-4}$ M), this phenomenon can be safely neglected, particularly in weakly acidic solution where the flavylium ion is usually a minor anthocyanin form. Stronger self-association could occur in the case of non-glycosyloxylated flavylium ions, as recently pointed out for the 3′,4′,7-trihydroxy-3-methoxyflavylium ion [5]. As for **1**, the VIS-absorbance *vs.* pigment-concentration plot (*Beer*'s plot) recorded on strongly acidic $10^{-6}$ to $5 \cdot 10^{-5}$ M solutions of pigment was found almost linear. The value of the corresponding dimerization constant ($K_d$) could, however, be roughly estimated upon curve-fitting of the *Beer*'s plot [5]: $K_d = 2.1\ (\pm 1.3) \cdot 10^3\ M^{-1}$ at 25° and 0.5M ionic strength. The poor solubility of **2** in aqueous solution did not permit to estimate the corresponding $K_d$ value.

4. *Copigmentation.* 4.1. *General.* The bright colours produced by anthocyanins in the plant kingdom are proofs that the colour loss due to flavylium hydration is not definitive. There are two main reasons for this: first, hydration involves chemical reactions that are all reversible, and second, the coloured and colourless forms are differentiated enough for complexation processes selectively involving the coloured forms to take place and restore colour. Copigmentation is the most important of these phenomena and consists in the formation of vertical stacking complexes between a colourless molecule having a planar $\pi$-electron-rich moiety (the copigment) and the coloured anthocyanin forms [1–3] [20]. Unlike the colourless forms, the coloured forms have almost planar chromophores with a strongly delocalized system of $\pi$-electrons which allows a good molecular contact with the copigment molecules. Therefore, the copigment competes with $H_2O$ for the flavylium chromophore and shifts the overall equilibrium between coloured and colourless forms towards the selectively complexed coloured forms, thus providing colour (*Fig. 1a*). In addition, the wavelength of maximum absorption of the copigmentation complex in the VIS range is longer than that of the free flavylium (probably a consequence of reduced local polarity around the flavylium ion upon complexation) so that copigmentation is effective at both colour stabilization and variation. Under strongly acidic conditions, hydration does not take place, and copigmentation is essentially manifested by its bathochromic effect, generally accompanied by a slight extinction in the flavylium absorption band (*Fig. 1b*).

Recent works in our group pointed out the influence of temperature, solvent composition, and copigment structure on the copigmentation magnitude [3] [20a, c]. By contrast, little work was published so far on the influence of the pigment structure. The simple comparison of the colour gains obtained when a given copigment is added to solutions of different pigments (pH, temperature, pigment and copigment concentrations being held constant) is of little value, because differences in those colour gains can be attributed not only to differences in pigment-copigment binding energy but also to differences in the ability of the flavylium ions to convert into colourless compounds. Much more reliable information can be drawn when VIS-absorbance *vs.* copigment-concentration plots including a large number of points (more than ten) are interpreted within a theoretical frame including the copigmentation equilibria and the hydration equilibrium whose thermodynamic constant has to be previously estimated. This approach was used throughout this work. Upon investigation of the copigmentation of **1** by chlorogenic acid (**7**; see below, *Fig. 2*) and caffeine (**8**), nonclassical behaviours were observed which prompted us to refine the theoretical treatment and take into account possible molecular complexation between the colourless forms and the copigment as well

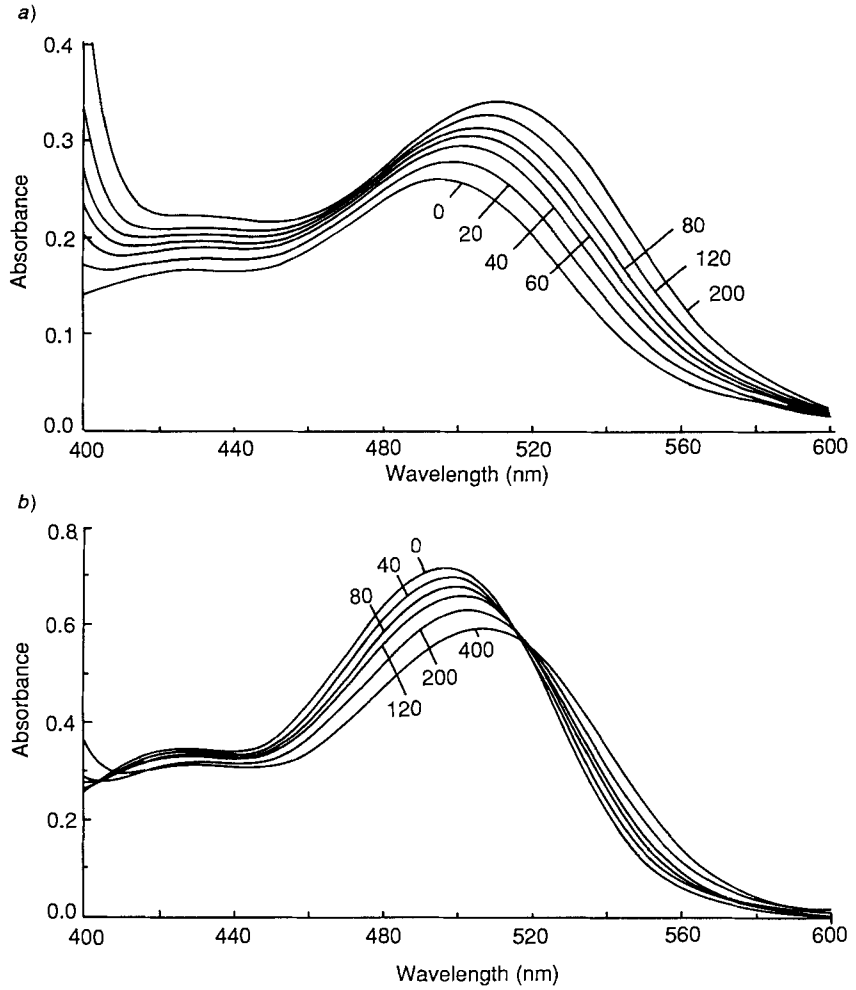



Fig. 1. *Changes in the VIS spectrum of callistephin* (**4**) *as a function of the chlorogenic-acid* (**7**) *concentration* (25°, 0.5M ionic strength). *a*) pH 3.04, concentration of **4** $10^{-4}$ M. *b*) 0.2M HCl, concentration of **4** $2.5 \cdot 10^{-5}$ M. The figures are the molar ratios **7**/**4**.

as ternary complexes involving two copigment molecules per pigment molecule. Moreover, the spectroscopic data were collected in a large range of copigment concentration, firstly, under strongly acidic conditions to study the copigmentation of the flavylium ion and secondly, under weakly acidic conditions to detect the possible contribution of the colourless forms in the pigment-copigment interaction. In that latter case, the pH of investigation was kept at a value which was at least 1 pH unit lower than the $pK_a$ value of the flavylium ion under scrutiny so that the free and complexed quinonoid bases could be safely neglected.

From now on, the following notations will be used: **AH** (free flavylium), **B** (colourless forms), **L** (copigment), **AHL** and $\mathbf{AHL_2}$ (1:1 and 1:2 copigmentation complexes of the flavylium ion), **BL** and $\mathbf{BL_2}$ (1:1 and 1:2 copigmentation complexes of the colourless

forms). In the more general case, the VIS absorbance can be written as: $A = \varepsilon_{AH} \cdot [\mathbf{AH}] + \varepsilon_{AHL} \cdot [\mathbf{AHL}] + \varepsilon_{AHL_2} \cdot [\mathbf{AHL_2}]$, the parameters noted $\varepsilon$ representing the molar absorption coefficients of the quoted species (the optical pathlength is omitted). The total concentration of anthocyanin expresses as: $c = [\mathbf{AH}] + [\mathbf{AHL}] + [\mathbf{AHL_2}] + [\mathbf{B}] + [\mathbf{BL}] + [\mathbf{BL_2}]$. The mass law applied to the complexation reactions gives: $K_1 = [\mathbf{AHL}]/([\mathbf{AH}] \cdot [\mathbf{L}])$, $K_2 = [\mathbf{AHL_2}]/([\mathbf{AHL}] \cdot [\mathbf{L}])$, $\beta_{12} = [\mathbf{AHL_2}]/([\mathbf{AH}] \cdot [\mathbf{L}]^2) = K_1 K_2$, $K_1' = [\mathbf{BL}]/([\mathbf{B}] \cdot [\mathbf{L}])$, $K_2' = [\mathbf{BL_2}]/([\mathbf{BL}] \cdot [\mathbf{L}])$, and $\beta_{12}' = [\mathbf{BL_2}]/([\mathbf{B}] \cdot [\mathbf{L}]^2) = K_1' K_2'$, the parameters noted $K$ and $\beta$ being the stepwise and the overall binding constants, respectively. Finally, the total concentration $L_t$ of copigment being at least 10 times as large as that of anthocyanin in all experiments, we may write: $[\mathbf{L}] = L_t$. Those relations and the mass-law expression for the hydration equilibrium ($K_h$ expression) can be combined to *Eqn. 1*, $A_0$, $A_1$, and $A_2$ being $\varepsilon_{AH} \cdot c$, $\varepsilon_{AHL} \cdot c$ and $\varepsilon_{AHL_2} \cdot c$, respectively. $A_0$ can be estimated by multiplying the VIS absorbance in the absence of copigment by $1 + K_h \cdot 10^{pH}$. In strongly acidic solution, the pigment is under a pure flavylium form, and *Eqn. 1* can be simplified to *Eqn. 2*. In this case, $A_0$ is directly the VIS absorbance in the absence of copigment. The values for $A_1$, $A_2$, $K_1$, $\beta_{12}$, $K_1'$, and $\beta_{12}'$ are the results of curve-fitting procedures.

$$A = \frac{A_0 + A_1 K_1 L_t + A_2 \beta_{12} L_t^2}{1 + K_1 L_t + \beta_{12} L_t^2 + K_h 10^{pH}(1 + K_1' L_t + \beta_{12}' L_t^2)} \quad (1)$$

$$A = \frac{A_0 + A_1 K_1 L_t + A_2 \beta_{12} L_t^2}{1 + K_1 L_t + \beta_{12} L_t^2} \quad (2)$$

4.2. *Copigmentation by Chlorogenic Acid* (**7**). Cinnamic-acid moieties are known to interact with flavylium ions either in an intramolecular way in sophisticated anthocyanins bearing aromatic-acid residues on their glycosyl moieties [2] [4] or in an intermolecular way. Examples of the latter case are the quinic esters of hydroxylated cinnamic acids which are efficient natural copigments of anthocyanins. Among them, chlorogenic acid (5-*O*-caffeoylquinic acid; **7**) is the most abundant in plants and forms 1:1 molecular complexes with the coloured forms of malvin (**6**) [3] [20a,c,e]. In the case of **1**, however, the VIS-absorbance *vs.* copigment concentration ($L_t$) plots could not be satisfactorily fitted against the theoretical law with the simple hypothesis of flavylium-chlorogenic acid 1:1 binding (*Eqns. 1* and *2* with $K_1' = 0, \beta_{12} = \beta_{12}' = 0$), and a very significant improvement occurred, when additional 1:2 binding was taken into account (*Eqns. 1* and *2* with $K_1' = 0, \beta_{12}' = 0$, *Fig. 2*). An iterative procedure was used to determine the binding constants: a trial value for $K_1$ was first estimated from measurements at the lowest **7**/**1** molar ratios allowing a first set of values for $A_1/A_0$, $A_2/A_0$, and $\beta_{12}$ to be determined from a curve-fitting of the data gained in strongly acidic solution. The values for $A_1/A_0$ and $A_2/A_0$ were then used to get refined values for $K_1$ and $\beta_{12}$ from a curve-fitting of the data gained in weakly acidic solution. The refined values for $K_1$ and $\beta_{12}$ were then used in a curve-fitting of the data gained in strongly acidic solution to get refined values for $A_1/A_0$ and $A_2/A_0$, and the procedure was repeated until the changes in the values for the parameters became smaller than the corresponding standard deviations. Thus, it could be demonstrated that the 1:1 copigmentation complex is able to accommodate a second copigment molecule with a binding constant $K_2$ of *ca.* 80 $M^{-1}$, leading to a 1:2 copigmentation complex in which the flavylium ion is probably intercalated between the caffeoyl

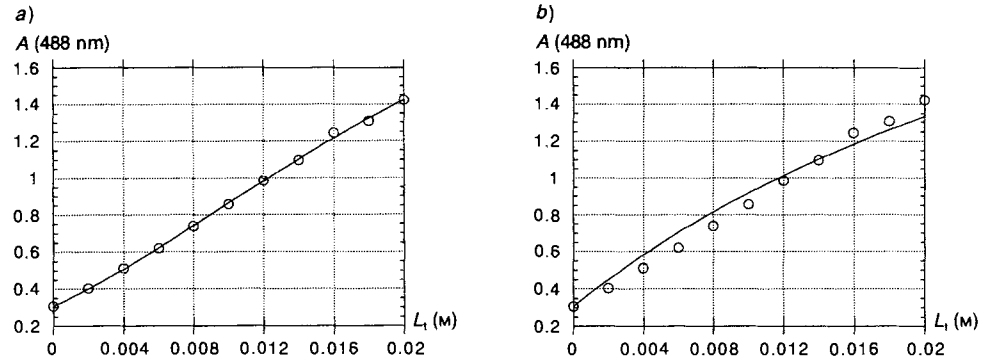



Fig. 2. *VIS-Absorbance* vs. *chlorogenic-acid* (**7**) *concentration* ($L_t$) *plot for* **1** (pH 3.7, 25°, 0.5M ionic strength, concentration of **1** $10^{-4}$ M). *a*) Curve-fitting assuming flavylium-chlorogenic acid 1:1 and 1:2 bindings. *b*) Curve-fitting assuming 1:1 binding.

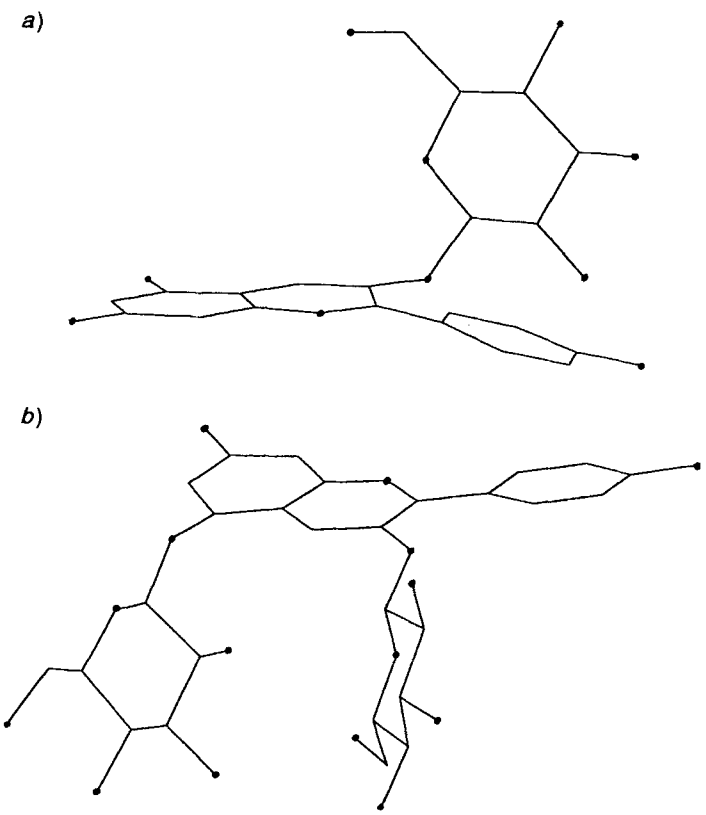



Fig. 3. *Minimal-energy conformations* a) *of the 3-(β-*D*-glucopyranosyloxy)-4',5,7-trihydroxyflavylium ion* **4** *and* b) *of the 3,5-bis(β-*D*-glucopyranosyloxy)-4',7-dihydroxyflavylium ion* **6** (with $R^3 = R^4 = H$ instead of MeO; HyperChem program, MM+ force field, solvent simulation: periodic boxes of 265 and 517 $H_2O$ molecules, respectively). ● = O-Atom.

moieties. So far, such sandwich complexes could be evidenced only in the case of intramolecular copigmentation in polyacylated anthocyanins [4].

In the case of the glycosyloxylated pigments **3**–**6**, the same analysis did not permit to detect the presence of 1:2 copigmentation complexes. The most simple explanation for this would be that the glycosyloxy residues bring some steric hindrance to one of the two faces of the chromophore and thus oppose the approach of a second copigment molecule. This was supported by molecular-modeling calculations on 3-(β-D-glucopyranosyloxy)-4′,5,7-trihydroxyflavylium ion (**4**) and 3,5-bis(β-D-glucopyranosyloxy)-4′,7-dihydroxyflavylium ion (**6** with $R^3 = R^4 = H$ instead of MeO) that yielded preferential conformations in which the glycosyloxy groups lay markedly out of the benzopyrylium plane (*Fig. 3*). In addition, the most stable conformation of the latter pigment was obtained, when the two glycosyloxy residues adopted a *cis*-arrangement with respect to the benzopyrylium plane. Thus, in 3-(glycosyloxy)- and 3,5-di(glycosyloxy)flavylium ions, the glycosyloxy groups seem to achieve a significant differentiation between the two faces of the chromophore, one being much more accessible to the copigment molecule. The $K_1$ values are remarkably close in the series of pigments investigated (except for **3** which interacts somehow more weakly with the copigment), *i.e.*, flavylium-chlorogenic acid 1:1 binding is little influenced by the substitution pattern of the flavylium ion (*Table 2*). In particular, the $K_1$ values for **5** and **6** are almost the same, thus indicating that the glycosyloxy group at C(5) in **6** does not oppose the binding of chlorogenic acid (**7**). This is consistent with both glycosyloxy groups being located on the same side of the chromophore in **6**. Moreover, the fact that **5** and **6** have the same intrinsic affinity for **7** shows that the larger colour gains usually observed in the copigmentation of anthocyanin 3,5-diglycosides with respect to the corresponding 3-monoglycosides (at the same pH) essentially reflect differences in the thermodynamics of the hydration process. Finally, none of the pigments investigated in this work could be demonstrated to interact with **7** through its colourless forms.

Table 2. *Values for the Binding Constants of the Anthocyanin-Chlorogenic Acid* (**7**) *Complexation* (25°, 0.5M ionic strength; for definition of $K_1$ and $\beta_{12}$, see text)

| | **1** | **2** | **3** | **4** | **5** | **6** |
|---|---|---|---|---|---|---|
| $K_1$ ($M^{-1}$) | 186 (±9) | 163 (±37)[a] | 78 (±11)[a]<br>58 (±2)[b] | 126 (±7)[a]<br>137 (±8)[b] | 182 (±4)[a]<br>187 (±9)[b] | 172 (±5)[a]<br>200 (±15)[b] |
| $\beta_{12}$ ($M^{-2}$) | $16.2 (\pm 1.2) \cdot 10^3$ | [c] | [c] | [c] | [c] | [c] |

[a] Calculated from spectral measurements in 0.2M HCl.
[b] Calculated from spectral measurements in weakly acidic formate buffers (for **1**, see text).
[c] No detectable 1:2 binding.

4.3. *Copigmentation by Caffeine* (**8**). Caffeine is a common purine able to form copigmentation complexes with malvin (**6**) [3] [20c,e]. However, its seemingly weak interaction with the 7-hydroxy-3,4-dimethoxyflavylium ion [20f] led us to question whether the glycosyloxy groups of natural anthocyanins could play a determining role in the copigmentation driving force. The theoretical treatment presented in this work which is more general and based on a larger set of experimental data allowed us to clarify

this point. Copigmentation of **1** by caffeine (**8**) was investigated first as a function of the copigment concentration. In strongly acidic solution, the usual behaviour was observed *i.e.*, a bathochromic effect in the pigment VIS band accompanied by a small decrease in the absorbance value at the absorption maximum [20c, e]. The VIS-absorbance (at a given wavelength) *vs.* caffeine (**8**)-concentration plot could be satisfactorily reproduced with the assumption of simple 1:1 flavylium-caffeine binding (*Eqn. 2* with $\beta_{12} = 0$). By contrast, a similar investigation under weakly acidic conditions gave a curve passing through a maximum (*Fig. 4a*), *i.e.*, the ability of **8** to regenerate colour from an initially poorly coloured solution of **1** was optimal at a certain concentration of **8** (roughly, $2.5 \cdot 10^{-2}$ M for a $10^{-4}$ M total concentration of pigment). Beyond this critical concentration, further addition of **8** resulted in a colour loss. At very large concentrations of **8** (beyond $7 \cdot 10^{-2}$ M), the colour was even paler than that of the initial solution without copigment. Such a phenomenon is unique so far and is a clear evidence for the taking part of the colourless forms in copigmentation by caffeine (**8**) upon formation not only of 1:1 but also of 1:2 copigmentation complexes. Indeed, using the values for $K_1$ and $A_1/A_0$ determined in strongly acidic solution, a curve-fitting of the VIS-absorbance *vs.* caffeine-concentration plot against a simplified *Eqn. 1* ($\beta_{12} = 0$) gave fairly accurate values for $K_1'$ and $\beta_{12}'$ (*Table 3*). However, these thermodynamic parameters concern the colourless forms taken as a whole, and additional work was needed to see, *e.g.*, whether both hemiacetal and chalcones were involved in the interaction with **8**.

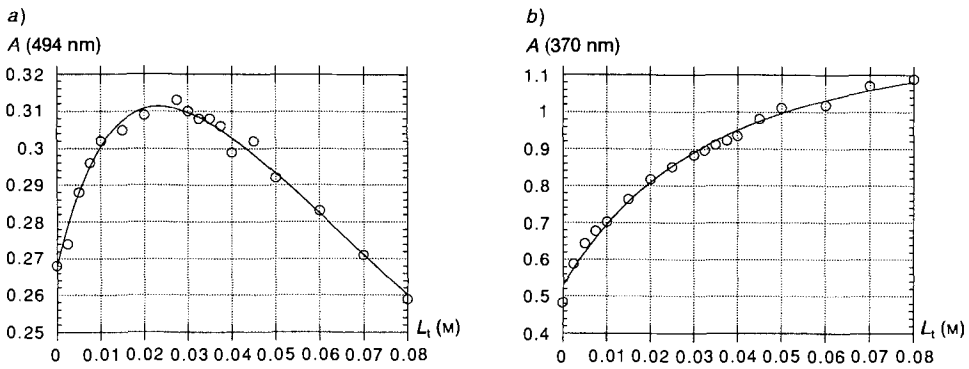



Fig. 4. *Absorbance* vs. *caffeine* (**8**) *concentration plots for* **1** (pH 3.7, 25°, 0.5M ionic strength, concentration of **1** $10^{-4}$ M). *a*) Detection in the VIS range (488 nm). *b*) Detection in the UV range (370 nm). The solid lines are the results of curve-fittings assuming flavylium-caffeine 1:1 binding and 1:1 and 1:2 bindings between **8** and the colourless forms of **1**.

Table 3. *Values for the Binding Constants of the Anthocyanin-Caffeine* (**8**) *Complexation* (25°, 0.5M ionic strength; for definition of $K_1$, $K_1'$, and $\beta_{12}'$, see text)

| | **1** | **2** | **3** | **4** | **5** | **6** |
|---|---|---|---|---|---|---|
| $K_1$ ($M^{-1}$) | 47 (±2) | 18 (±8) | 38 (±1) | 49 (±6) | 74 (±3) | 59 (±1) |
| $K_1'$ ($M^{-1}$) | 28 (±1) | [a]) | 48 (±1) | 19 (±1) | 0–5 | 10 (±1) |
| $\beta_{12}'$ ($M^{-2}$) | 270 (±8) | [a]) | [b]) | 226 (±27) | 301 (±15) | [b]) |

[a]) Not determined because of the poor solubility of pigment **2** in weakly acidic aqueous solution.
[b]) No detectable 1:2 binding.

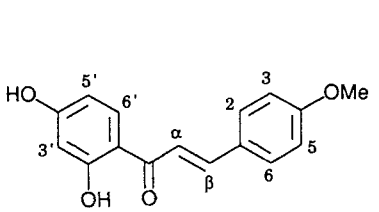
Evidence for the participation of the (*Z*)-chalcone was obtained by UV/VIS spectroscopy upon shifting the analytical wavelength from the VIS to the UV range at a value for which the flavylium ion and the (*Z*)-chalcone are the sole light-absorbing species (370 nm; steric repulsions enforce a non-planar conformation for the (*E*)-chalcone which, therefore, absorbs at a lower wavelength than the corresponding (*Z*)-isomer [21]). In that case, the absorbance *vs.* caffeine-concentration plot no longer displays a maximum and consists in a monotonously increasing curve (*Fig. 4b*). This shows that the (*Z*)-chalcone is formed in the presence of large concentrations of **8** and that its absorption outweighs the decreasing absorption of the flavylium ion. Moreover, the UV-absorbance *vs.* caffeine-concentration plot could be conveniently fitted against a modified *Eqn. 1* using the values for $K_1$, $K_1'$, and $\beta_{12}'$ determined from the spectroscopic investigation in the VIS range and taking into account the absorption of the free and complexed (*Z*)-chalcone forms (*Fig. 4b*).

Additional evidence for the participation of the (*Z*)-chalcone in copigmentation by caffeine (**8**) was gained by a $^1$H-NMR investigation in $D_2O$/($D_6$)DMSO 1:1 of 2′,4′-dihydroxy-4-methoxychalcone (**15**) which is not liable to cyclize back to hemiacetal and flavylium forms and is thus more amenable to $^1$H-NMR analysis. Despite the fact that organic cosolvents in large concentration tremendously weaken hydrophobic-type complexation, significant diamagnetic shifts occurred for the resonances of the vinylic and

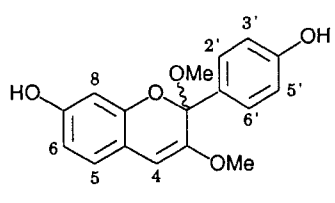
Table 4. *Influence of Caffeine* (**8**) *on the $^1$H-NMR Spectrum of 2′,4′-Dihydroxy-4-methoxychalcone* (**15**, 200 MHz, ($D_6$)DMSO/$D_2O$ 1:1, 27°, chalcone concentration $8 \cdot 10^{-3}$ M)[a])

| | H–C(6′) | H–C(α), H–C(β) | H–C(2), H–C(6) | H–C(3), H–C(5) | H–C(5′) | H–C(3′) |
|---|---|---|---|---|---|---|
| $\delta_0$ [ppm] | 7.92 | 7.67[b]) | 7.63[b]) | 6.94 | 6.37 | 6.21 |
| $\delta$ [ppm] | 7.81 | 7.58 | 7.48 | 6.89 | 6.31 | 6.13 |
| $\Delta\delta$ [Hz] | 22 | 18[b]) | 30[b]) | 10 | 12 | 16 |

[a]) $\delta_0$ = Chemical shift in the absence of **8**, $\delta$ = chemical shift in the presence of 17 equiv. of **8**, $\Delta\delta = \delta_0 - \delta$.
[b]) Rough estimates, because the corresponding signals overlap.




**15** 2′,4′-dihydroxy-4-methoxychalcone




**16** (MeOH/**1** adduct)

aromatic protons of chalcone **15** when **8** was added (*Table 4*). This is evidence of vertical stacking interactions taking place between the planar $\pi$-electron-rich moieties of **8** and **15**.

As for the hemiacetal of **1**, it was quantitatively generated in its methylated form **16** (not liable to further isomerization into chalcones) upon dissolving **1** in MeOH in the presence of 1 equiv. of NaOH. Here again, addition of **8** was found to displace the $^1$H-NMR signals of **16**, especially those of the benzopyrane moiety, towards lower $\delta$ values (*Table 5*).

Table 5. *Influence of Caffeine* (**8**) *on the* $^1$*H-NMR Spectrum of the Methanol/***1** *Adduct* **16** (200 MHz, $D_2O/CD_3OD$ 2:1, 27°, substrate concentration: $2 \cdot 10^{-2}$ M[a])

| | H–C(2′), H–C(6′) | H–C(5) | H–C(3′), H–C(5′) | H–C(6) | H–C(8) | H–C(4) |
|---|---|---|---|---|---|---|
| $\delta_0$ [ppm] | 7.36 | 7.05 | 6.85 | 6.52 | 6.44 | 6.09 |
| $\delta$ [ppm] | 7.33 | 6.93 | 6.81 | 6.40 | 6.37 | 5.99 |
| $\Delta\delta$ [Hz] | 6 | 24 | 8 | 24 | 14 | 20 |

[a]) $\delta_0$ = Chemical shift in the absence of **8**, $\delta$ = chemical shift in the presence of 12.6 equiv. of **8**, $\Delta\delta = \delta_0 - \delta$.

Therefore, all colourless forms seem to be involved in copigmentation by caffeine (**8**) and thus compete with the flavylium ion for the copigment. At relatively low copigment/pigment molar ratios, competition is in favour of the flavylium ion which is capable of stronger 1:1 binding with **8** than the colourless forms, and **8** behaves like any copigment, *i.e.*, it induces a colour gain in the pigment solution. At higher copigment/pigment molar ratios, competition turns in favour of the colourless forms which, unlike the flavylium ion, can be involved in 1:2 binding with **8**. Among the colourless forms, the (*Z*)-chalcone, which bears two aromatic rings remote from each other, seems more fitted for 1:2 binding than the corresponding hemiacetal. This is somewhat supported by $^1$H-NMR analysis. *E.g.*, both aromatic rings in 2′,4′-dihydroxy-4-methoxychalcone (**15**) experience the caffeine ring-current effect to almost the same extent, whereas the resonances of the B-ring protons in the methyl acetal **16** are much less displaced than those of the benzopyrane moiety upon addition of **8**. Moreover, 1:2 binding in anthocyanin chalcones was already demonstrated in the case of inclusion into $\beta$-cyclodextrin [20g]. The investigation of the glycosyloxylated pigments showed that the copigmentation of the colourless forms by **8** was a general phenomenon. Although this interaction was not strong enough in the glycosyloxylated pigments to cause colour loss at large concentrations of **8**, it was to be taken into account to correctly interpret the VIS-absorbance *vs.* caffeine-concentration plots obtained in weakly acidic solution. Whereas the flavylium-caffeine interaction appeared as a 1:1 association for all pigments **1**–**6**, the stoichiometry of the binding between the colourless forms and caffeine (**8**) was found much more dependent on the anthocyanin substitution pattern. The greater contrast was observed between **5** and **6** whose structures only differ by the nature of the substituent at C(5) (OH in **5**, $\beta$-D-glucopyranosyloxy in **6**): whereas the only detectable interaction between the colourless forms of **6** and **8** is a weak 1:1 binding ($K_1' = 10\ M^{-1}$), the colourless forms of **5** essentially interact with **8** upon strong complexation of two copigment molecules ($\beta_{12}' = 420\ M^{-2}$), 1:1 binding being hardly significant ($K_1' = 0$–$5\ M^{-1}$). Everything happens as if **8**, which is known to form weak non-covalent self-aggregates in aqueous solution [22], was able to interact with the colourless forms of **5** through its dimeric form. Such complexation phenomena markedly limit the ability of caffeine (**8**) to restore colour in weakly acidic solutions of non-glycosyloxylated and 3-monoglycosyloxylated anthocyanins.

This work was carried out in 'Le laboratoire de chimie des polyphénols' directed by Prof. *R. Brouillard* who is gratefully acknowledged for his encouragements and his comments.

## Experimental Part

*UV/VIS-Absorption Spectra. Hewlett-Packard* diode-array spectrometer; quartz cell ($d$ = 1 cm) equipped with a stirring magnet, thermostated at 25 (±0.1)° by means of a *Lauda* water-thermostated bath; temp. measured with a *Comark* thermocouple; ionic strength fixed at 0.5M by NaCl, $\lambda_{max}(\varepsilon)$ in nm.

*HPLC Analysis. Spectra-Physics* apparatus, equipped with a *Hewlett-Packard* diode-array detector typically monitoring at 260 and 500 nm; reversed-phase HPLC analysis of the glycosyloxylated flavylium ions on a *Merck C-8* column (5 μm, 125 mm × 4 mm); typical flow rate, 0.5 ml/min; typical gradient elution: from *A* (5% $HCO_2H$ in MeCN/$H_2O$ 1:1)/*B* (5% $HCO_2H$ in $H_2O$) 1:9 at time zero to 100% *A* after 60 min.

*NMR Analysis. Bruker SY 200* (200 MHz) or *Bruker AM 400* (400 MHz); at 27°, chemical shifts $\delta$ in ppm with respect to $SiMe_4$ as external standard (internal ref. MeOH, $\delta$ 3.30 ppm); coupling constants $J$ in Hz.

*pH Measurements. Metrohm* model *654* pH meter fitted with a small combined glass electrode; buffers for calibration, pH 7 and pH 4 *Aldrich* standards.

*Data Analysis.* Curve fittings with a *Macintosh IIsi* computer using the *Kaleida Graph* program; standard deviations are reported.

*Molecular-Modeling Calculations. Compaq ProLinea 4/50* PC using the HyperChem program (*Autodesk*, Sausalito, California) in the MM+ parametrization, the geometry optimization procedure being repeated with different sets of input data files. In the case of a diglycosylated flavylium ion, *e.g.*, calculations were run from different initial values for the torsion angles about the C(3)–O and C(5)–O bonds which define the position of the glycosyloxy groups with respect to the almost planar chromophore and for the torsion angle about the C(2)–C(1′) bond which defines the position of the B ring with respect to the benzopyrylium moiety. In a first step, the energy-minimization procedures were carried out in vacuum. The most stable conformation obtained that way was then put in a periodic box of a few hundred $H_2O$ molecules and further minimized.

*Transformations of Flavylium Ions.* The value of the thermodynamic constant ($K_h$) of the overall hydration equilibrium connecting the flavylium ion and the mixture of colourless forms (hemiacetal and chalcone forms) was gained from recording the VIS absorbance at the wavelength of the flavylium absorption maximum on fully equilibrated solns. of pigment at different pH values. The value of the thermodynamic constant ($K_a$) of the **AH/A** proton-transfer equilibrium was obtained from pH-jump experiments and curve fitting of the plot of the apparent rate constant of hydration (first-order) *vs.* final pH. Both procedures were recently published with details [4].

*Copigmentation Equilibria.* For the spectral measurements under the most acidic conditions, a determined amount of pigment (typically a few mg) was dissolved in 1 ml of MeOH and diluted to 100 ml upon addition of aq. 0.2M HCl/0.3M NaCl. After dilution, the pigment concentration was typically $5 \cdot 10^{-5}$ M. The soln. was divided in two, and to one part, a carefully weighed amount of copigment corresponding to the largest concentration studied (typically, 0.2M for chlorogenic acid (**7**) and 0.5M for caffeine (**8**)) was added. The two solns. were then mixed in determined proportions so as to cover the intermediate range of copigment concentration. The same procedure was repeated for the measurements in weakly acidic conditions, a formate buffer (obtained by mixing aq. 0.5M $HCO_2$/0.5M NaCl and 0.5M NaOH) replacing the 0.2M HCl/0.3M NaCl. The pH of the buffer ranged from 2.7 to 3.7 depending on the flavylium ion investigated. In that case, the solns. were left for 2 h before recording the spectra to ensure complete equilibrium.

*Materials.* Malvin (**6**), chlorogenic acid (**7**), and caffeine (**8**) were purchased from *Roth*, Germany, and oenin (**5**) was extracted from grapes according to [23]. Their purity was checked by reversed-phase HPLC (UV/VIS detection at multiple wavelengths), revealing a contamination of **5** with *ca.* 5% (based on peak integration) of the corresponding non-glycosyloxylated pigment. No attempt was made to further purify the sample.

*2′,4′-Dihydroxy-4-methoxychalcone* ( = *1-(2,4-Dihydroxyphenyl)-3-(4-methoxyphenyl)prop-2-enone*; **15**) was synthesized by aldol condensation of anisaldehyde and 2,4-dihydroxyacetophenone in 50% aq. NaOH soln./EtOH 1:1 (55°, 20 h). The chalcone was precipitated upon acidification by cold 6M HCl, washed with hexane, and dried under vacuum. Its purity was checked by reversed-phase HPLC and $^1$H-NMR.

*2-(4-Hydroxyphenyl)-2,3-dimethoxy-2*H*-1-benzopyran-7-ol* (**16**). Flavylium chloride **1** (0.2 mmol; see below) was dissolved in 0.01M NaOH/MeOH (20 ml). The initially deeply coloured soln. turned pale-purple because of the slow and almost complete conversion of the quinonoid compounds initially formed into the colourless hemiacetals. The soln. was then evaporated and the residue twice co-evaporated with $CCl_4$ before dissolution in $CD_3OD/D_2O$ for $^1$H-NMR measurements.

*4′-Acetoxy-2-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyloxy)acetophenone* (**12**). After purification on silica gel *60* (particle size: 60–200 μm, *Bio-Rad*, $Et_2O$/hexane 5:3), 4′-acetoxy-2-hydroxyacetophenone (**11**; 3.4 mmol) was dissolved in toluene (20 ml, distilled over $CaH_2$), under Ar and anh. $CaSO_4$ (0.8 g), activated 4-Å molecular sieves (0.8 g; *Aldrich*), and $Hg(CN)_2$ (6.8 mmol) were added. A soln. of 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (4.4 mmol) in toluene (10 ml) was then added dropwise under stirring and the mixture brought to reflux for 2 h, then cooled down, and filtered on *Celite*. Toluene was distilled off, the residue dissolved in $CH_2Cl_2$, and the soln. washed with $H_2O$ (×3), dried ($MgSO_4$), and evaporated. Purification by column chromatography (silica gel *60*, $CH_2Cl_2/Et_2O$ 95:5) gave pure **12**. Yield 40%. White solid. $^1$H-NMR (200 MHz, $CDCl_3$): 7.96 (*d*, *J* = 8.7, H–C(2′),H–C(6′)); 7.21 (*d*, *J* = 8.7, H–C(3′), H–C(5′)); 5.3–5.0 (*m*, H–C(3)(Glc), H–C(2)(Glc), H–C(4)(Glc)); 4.94 (*d*, *J* = 16.2, 1 H–C(2)); 4.83 (*d*, *J* = 16.2, 1 H–C(2)); 4.68 (*d*, *J* = 7.6, H–C(1)(Glc)); 4.23 (*dd*, *J* = 12.3, 4.4, 1 H–C(6)(Glc)); 4.12 (*dd*, *J* = 12.3, 2.4, 1 H–C(6)(Glc)); 3.69 (*m*, H–C(5)(Glc)); 2.33 (*s*, AcO–C(4′)); 2.10–1.95 (4*s*, 4 Ac(Glc)).

*3-(β-*D*-Glycopyranosyloxy)-4′,5,7-trihydroxyflavylium Chloride* (= *3-(β-*D*-Glucopyranosyloxy)-5,7-dihydroxy-2-(4-hydroxyphenyl)-1-benzopyrylium Chloride*; **4**). Equimolar amounts (0.26 mmol) of **12** and 2,4-diacetoxy-6-hydroxybenzaldehyde (from 2,4,6-trihydroxybenzaldehyde, $Ac_2O$ (2 equiv.), and 4-(dimethylamino)pyridine in refluxing AcOEt; yield 60%) were dissolved in dry AcOEt (7 ml) and cooled to −10°. HCl (generated by action of 98% $H_2SO_4$ on solid NaCl) was gently bubbled through soln. for *ca.* 0.5 h. The deep-red soln. of **14** was then allowed to stay at −20° for 2 days. HPLC showed that partial deacetylation of the phenolic groups had occurred during the condensation, thus giving a mixture of glycosyloxylated flavylium ions. No purification was undertaken at this stage. AcOEt was distilled off, the red powder obtained dissolved in MeOH (3 ml) and $H_2O$ (3 ml), and KOH (0.2 g) added. After 3 h at r.t., the soln. was carefully acidified to pH 1 with 1M HCl, left overnight at 4° and then evaporated. The residue was triturated in MeOH and KCl partially filtered off. After evaporation, the crude powder containing **4**, residual KCl, and small amounts of colourless org. compounds resulting from uncomplete conversion during the condensation was dissolved in $H_2O$/AcOH 9:1 and the soln. eluted on a small column of reversed-phase silica gel (*LiChroprep RP-18*, particle size 40–63 μm, *E. Merck*, Darmstadt, Germany) to completely remove KCl. Finally, cellulose microcrystalline column chromatography (*E. Merck*, Darmstadt, Germany) with $H_2O$/AcOH 9:1 afforded pure **4**. Overall yield for condensation, deacetylation, and two-step purification: *ca.* 40%. The purity of **4** was carefully checked by reversed-phase HPLC. UV/VIS (0.2M HCl): 496 (28700 $M^{-1}cm^{-1}$; similar to ε's of naturally occurring 3-(β-D-glucopyranosyloxy)flavylium ions [24]). $^1$H-NMR (400 MHz, 1D- and 2D-DQF-COSY, $CD_3OD/CF_3CO_2D$ 98:2): 9.07 (*s*, H–C(4)); 8.59 (*d*, *J* = 9.1, H–C(2′), H–C(6′)); 7.04 (*d*, *J* = 9.1, H–C(3′), H–C(5′)); 6.92 (*s*, H–C(8)); 6.66 (*s*, H–C(6)); 5.27 (*d*, *J* = 7.7, H–C(1)(Glc)); 3.90 (*dd*, *J* = 12.5, 2.0, 1 H–C(6)(Glc)); 3.69 (*dd*, *J* = 12.5, 5.8, 1 H–C(6)(Glc)); 3.63 (*dd*, '*t*', *J* = 9.0, 7.7, H–C(2)(Glc)); 3.52 (*t*, *J* = 9.0, H–C(3)(Glc); *m*, H–C(5)(Glc)); 3.42 (br. *d*, *J* = 9.0, H–C(4)(Glc)). FAB-MS (pos. mode): 433.1.

*3-(β-*D*-Glycopyranosyloxy)-4′,7-dihydroxyflavylium Chloride* (= *3-(β-*D*-Glucoypranosyloxy)-7-hydroxy-2-(4-hydroxyphenyl)-1-benzopyrylium Chloride*; **3**). As described for **4**, 2,4-dihydroxybenzaldehyde replacing 2,4-diacetoxy-6-hydroxybenzaldehyde. Precipitation of **13** occurred during the condensation, thus facilitating the purification (washings with AcOEt). After deacetylation, reacidification, and removal of KCl by elution on reversed-phase silica gel, **3** was HPLC-pure. UV/VIS (0.2M HCl): 486. $^1$H-NMR (400 MHz, 1D- and 2D-DQF-COSY, $D_2O/CF_3CO_2D$ 98:2): 8.60 (*s*, H–C(4)); 8.17 (*d*, *J* = 8.9, H–C(2′), H–C(6′)); 7.81 (*d*, *J* = 9.1, H–C(5)); 7.23 (*dd*, *J* = 9.1, 2.3, H–C(6)); 7.11 (*s*, H–C(8)); 6.72 (*d*, *J* = 8.9, H–C(3′), H–C(5′)); 5.24 (*d*, *J* = 7.4, H–C(1)(Glc)); 3.96 (*dd*, *J* = 12.5, 1.8, 1 H–C(6)(Glc)); 3.78 (*dd*, *J* = 12.5, 5.8, 1 H–C(6)(Glc)); 3.73–3.49 (*m*, H–C(2)(Glc), H–C(3)(Glc), H–C(4)(Glc), H–C(5)(Glc)). FAB-MS (pos. mode): 417.1.

*4′,7-Dihydroxy-3-methoxyflavylium Chloride* (= *7-Hydroxy-2-(4-hydroxyphenyl)-3-methoxy-1-benzopyrylium Chloride*; **1**). After purification on silica gel *60* ($Et_2O$/hexane 5:3), **10** was condensed with 2,4-dihydroxybenzaldehyde in dry AcOEt at 0° in the presence of HCl. Precipitation of **1** occurred and was completed by keeping the mixture at −20° for 3 days. The solid was then filtered, thoroughly washed by AcOEt and dried under vacuum: **1**, HPLC-pure. UV/VIS (0.2M HCl): 486. $^1$H-NMR (200 MHz, $CD_3OD$/DCl 98:2): 8.87 (*s*, H–C(4)); 8.61 (*d*, *J* = 9.1, H–C(2′), H–C(6′)); 8.04 (*d*, *J* = 8.5, H–C(5)); 7.44 (*s*, H–C(8)); 7.38 (*d*, *J* = 8.5, H–C(6)); 7.06 (*d*, *J* = 9.1, H–C(3′), H–C(5′)); 4.22 (*s*, Me). FAB-MS (pos. mode): 269.0.

*4′,5,6-Trihydroxy-3-methoxyflavylium Chloride* (= *5,7-Dihydroxy-2-(4-hydroxyphenyl)-3-methoxy-1-benzopyrylium Chloride*; **2**). At 0°, **10** was condensed with 2,4,6-dihydroxybenzaldehyde in HCOOH in the presence of HCl. Workup as described for **1** gave HPLC-pure **2**. UV/VIS (0.2M HCl): 508. $^1$H-NMR (200 MHz, $CD_3OD$/DCl 98:2): 8.80 (*s*, H–C(4)); 8.51 (*d*, *J* = 9.1, H–C(2′), H–C(6′)); 7.04 (*d*, *J* = 9.1, H–C(3′), H–C(5′)); 6.91 (*s*, H–C(8)); 6.67 (*s*, H–C(6)); 4.19 (*s*, Me). FAB-MS (pos. mode): 285.0.

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