Polyphenol–Anthocyanin Copigmentation

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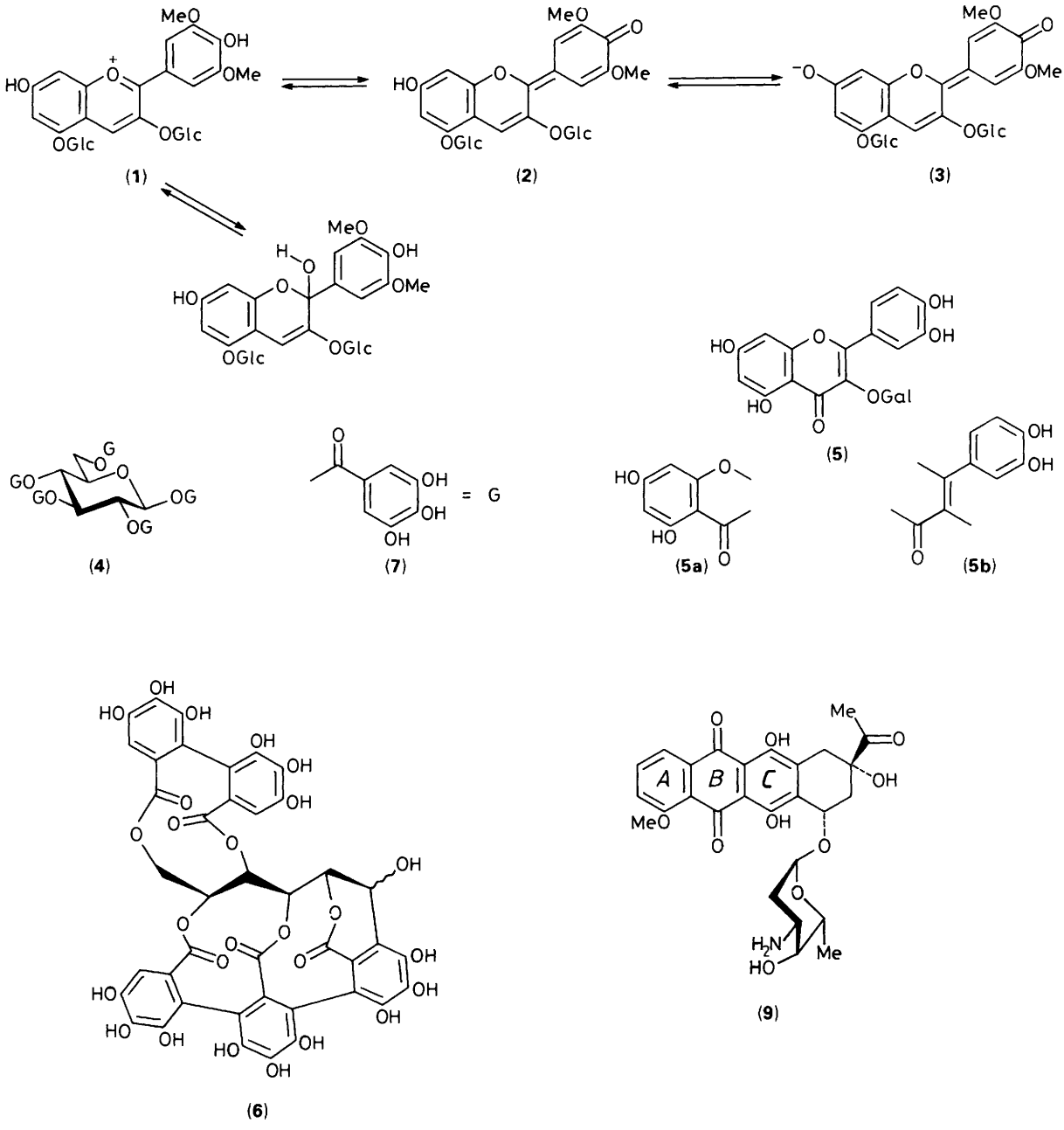
The complexation of natural polyphenols with malvin chloride has been examined; a model for the preferred mode of chelation of β-1,2,3,4,6-penta-*O*-galloyl-D-glucose (**4**) with various aromatic substrates (the flavylium ion, caffeine, methylene blue, and daunomycin) is proposed.

Anthocyanins are stable in strongly acidic media in the form of the flavylium ion (**1**) but as the pH is raised this is successively deprotonated to give the anhydrobase (**2**) and its anion (**3**) with corresponding changes in colour from red to blue.[1] As flower cell sap is normally weakly acidic, anthocyanins cannot produce stable colours, such as blue, unless other mechanisms exist for colour variation and stabilisation.[2,3] The observation that the colour of isolated anthocyanins could be varied by the presence of other substances (copigmentation) was first made by Willstatter and Zollinger[4] and Robinson and Robinson.[5] Both groups noted that 'tannin' induced a strong bathochromic shift in the visible absorption of the anthocyanin and



Robinson[6] further suggested that polysaccharides and polypeptides might have similar effects.

As a part of a comprehensive series of studies of the intermolecular complexation of polyphenols (*syn* vegetable tannins)[7—9] the copigmentation of malvin chloride with natural polyphenols has been investigated. At pH 3.65 (0.2 M acetate buffer) and 22 °C natural flavan-3-ols and related proanthocyanidins[10] display very small copigmentation effects but galloyl esters induce not only a significant bathochromic shift in the visible absorption ($\lambda_{max.}$) of malvin chloride but also an increase in the absorptivity, which is enhanced in the presence of magnesium chloride (0.25 M). The copigmentation phenomenon was reduced by increases in temperature. The extent of these effects is broadly related (Table 1) to the number of 'galloyl ester-like groups' in the polyphenol and, as in related complexations,[7—9] is directly related to the conformational flexibility of the polyphenol, *c.f.* the minimal copigmentation by ellagitannins observed in rose petals.[11] None of the galloyl esters examined however was as effective a copigment as the flavonol glycosides[12,13] *e.g.* quercetin-3-galactoside (**5**) (Table 1). Gelatin, pectin, and polyvinylpyrrolidone (PVP, $M \sim 10000$) had little effect on solutions of malvin chloride (pH 3.65) but the addition of either gelatin or PVP to solutions of malvin chloride containing β-1,2,3,4,6-penta-*O*-galloyl-D-glucose (**4**) rapidly reduced the copigmentation due to (**4**)—presumably by preferential complexation with the polyphenol. Pectin gave a modest enhancement (~15%) in the copigment extinction coefficient.

In aqueous trifluoroacetic acid solution (1.1% v/v) malvin chloride exists almost exclusively in the flavylium ion form (**1**)

Table 1. Malvin chloride–polyphenol copigmentation.[a]

Polyphenol	$\frac{A - A_0}{A_0} \times 100\%$	$\lambda - \lambda_0$/nm
(−)-Epicatechin	18	0.8
(−)-Epigallocatechin	21	1.6
(−)-Epigallocatechin-3-gallate[b]	44	2.3
Procyanidin B-2 : [(−)-epicatechin]$_2$	15	—
Procyanidin C-1 : [(−)-epicatechin]$_3$	9	—
Sorghum polymer:[c] (+)-catechin[(−)-epicatechin]$_5$	14	—
Quercetin 3-galactoside (**5**)	173	18.9
Methyl gallate	18	2.4
Aesculin	14	0.5
2,6-Digalloyl-1,5-anydro-D-glucitol[d]	10	2.0
β-1,3,6-Tri-*O*-galloyl-D-glucopyranose[e]	50	3.2
β-1,2,4,6-Tetra-*O*-galloyl-D-glucopyranose[e]	60	4.8
β-1,2,3,4,6-Penta-*O*-galloyl-D-glucopyranose[e] (**4**)	121	12.0
β-1,2,3,4,6-Penta-*O*-galloyl-D-glucopyranose–$MgCl_2$ (0.25 M)	488	12.0
Vescalagin, castalagin (**6**)	7	0.5
Sanguin H-6[f]	115	8.0

[a] Malvin chloride (1.0×10^{-4} M), polyphenol (2.0×10^{-4} M), in 0.02 M acetate buffer pH 3.65. A_0 = absorption of malvin chloride, A = absorption of anthocyanin plus polyphenol at 22 °C. [b] Ref. 24. [c] Ref. 25. [d] Ref. 26. [e] Ref. 27. [f] Refs. 20 and 28.

and the visible absorption at $\lambda_{max.}$ 520.1 nm is at a maximum. Addition of polyphenols to such solutions produces no increase in absorption but only the appropriate change in the visible $\lambda_{max.}$ (Table 1). This observation strongly supports the view that, in solutions of intermediate pH, copigmentation results from complexation of the polyphenol with the flavylium ion (**1**) and displacement of the anthocyanin equilibria towards this form of the pigment (**1**).

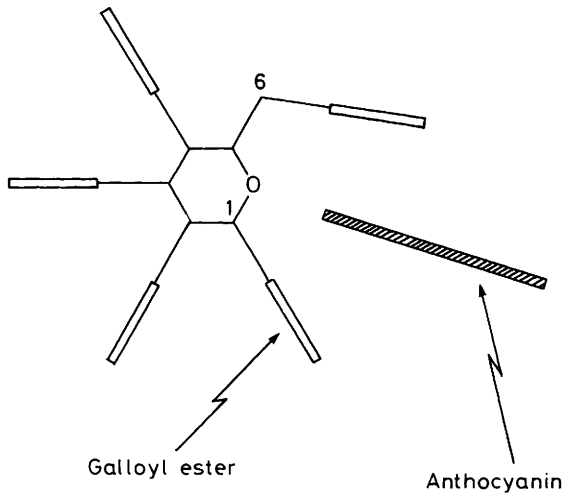
Polyphenol : Flavylium ion (**1**)

⇅

Carbinol-pseudobase ⇋ Flavylium ion ⇋ Anhydrobase
(**1**) (**2**)

High resolution (400 MHz) ^{1}H NMR analysis of solutions (D_2O) of malvin chloride containing β-1,2,3,4,6-penta-*O*-galloyl-D-glucose (**4**) showed that significant magnetic anisotropically induced chemical shift changes occurred only in the protons of the flavylium ion form (**1**) of the anthocyanin, thus confirming the view that copigmentation derives primarily, if not exclusively, from the association of (**4**) with (**1**). Measurement of the chemical shift changes ($\Delta\delta$) induced by the addition of increasing aliquots of (**4**) to malvin chloride in D_2O–CF_3CO_2H (1.1% v/v) and application of a modified Benesi–Hildebrand[15] equation to the data gave a value for the association constant for the formation of a 1 : 1 complex between (**1**) and (**4**) at 45 °C of 508(±8) mol^{-1} dm^3. Determination of association constants from UV data (in H_2O containing 0.22% v/v CF_3CO_2H) by measurements of absorption at two wavelengths gave values of 987(±37) and 1686(±58) mol^{-1} dm^3 at 22 °C for the formation of 1 : 1 complexes between (**1**) and (**4**) (measured at λ_1 520.1 and λ_2 539.8 nm) and (**1**) and (**5**) (measured at λ_1 520.1 and λ_2 564.6 nm), respectively.

Although flavonoid–anthocyanin copigmentation has been ascribed[13,16] to hydrogen bonding Goto[2,3] has suggested that the phenomenon is due to 'hydrophobic stacking' of the aromatic nuclei of the anthocyanin and the cosubstrate. The present observations strongly favour this interpretation. Thus whilst (**4**) displays substantial effects, both vescalagin and castalagin[17,18] (**6**) epimeric at C-1, [formally 6 hydrogen atoms less than (**4**)] which have the same number of potential sites for hydrogen bonding but whose 'galloyl ester like groups' are all constrained to a rigid, inflexible, sterically hindered conformation exhibit a very small influence on the visible absorption of (**1**), Table 1.



(**8**)

These results are very similar in form to those from studies of polyphenol complexation with caffeine and with methylene blue.[7–9,19,20] They support the view that effective intermolecular recognition of substrates containing galloyl (and hydroxycinnamoyl) esters derives from the development (induced polarisation) in the aroyl ester of characteristics which complement those of the cosubstrate.[21] Good anthocyanin copigments thus contain part structures such as (**7**), (**5a**), and (**5b**). ^{1}H NMR (400 MHz, D_2O–CF_3CO_2H, 1.1% v/v) analysis of the complexation of malvin chloride with β-1,2,3,4,6-penta-*O*-galloyl-D-glucose (**4**) in which the chemical shift changes of the galloyl ester protons were monitored as a function of increasing anthocyanin concentration showed that complexation takes place overwhelmingly at or near the galloyl ester groups at positions 1 and 6 on the D-glucopyranose ring.[20] This suggests a model such as (**8**) for the formation of a 1 : 1 complex in which the two galloyl ester groups at C-1 and C-6 are maintained in the optimum position and separation for 'hydrophobic stacking' with the anthocyanin by the buttressing effect of the remaining galloyl ester groups on the D-glucopyranose ring.

This model is valid for the association of the polyphenol (**4**) with a range of other aromatic substrates *e.g.*, caffeine, methylene blue, and the carcinostatic antibiotic daunomycin (**9**). Polyphenols can strongly inhibit the action of some direct acting mutagens[22,23] and in the context of such studies the complexation of (**9**) with (**4**) has been examined (in collaboration with Dr. R. M. McGrath, Ms. H. Grimmer, Department of Biochemistry, University of the Witwatersrand, and Dr. C. M. Spencer). The antibiotic is analogously preferentially chelated between the galloyl ester groups at C-1 and C-6 of (**4**), with rings *A*, *B*, and *C* inserted into the 'sandwich'. An association constant K = 366 (±7) mol^{-1} l at 60 °C was measured (^{1}H NMR) for the 1 : 1 complex.

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