3.16 Chemistry of Flavonoid-Based Colors in Plants

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3.16.1 Introduction

Plants exhibit two fundamentally different types of colors, one based upon the physical and optical properties of the plant cell and tissue structures, and the other upon the presence of pigments and their copigments. These two mechanisms for coloration may, and often do, co-exist in the same tissues, and the perceived color will thus depend on the combined effect of the two distinct phenomena. When considering only the number of pigment classes that are commonly recognized in plants, it is found to be fairly modest.[1,2] This does not mean that the main classes answer for all natural plant pigments, but they do include the majority of all known pigments of general occurrence. Chlorophylls can easily be recognized by their characteristic green color, similar to many carotenoids with their deep yellow to orange-red hues. These latter colors are produced by the flavonoid groups, chalcones, and aurones (**Figure 1**) in a restricted number of plants. Even more pronounced are the anthocyanins (**Figure 1**, **Table 5**), a special class of flavonoids, which are responsible for the often intense, orange to blue colors of most flowers, leaves, and fruits (see Chapter 6.18). The betacyanins (**Figure 1**) with restricted distribution mainly in Caryophylalles, show superficial color similarities to anthocyanins, although, their occurrence seem to be mutually exclusive. Less noticeable are the flavones and flavonols (**Figure 1**), which provide rather pale yellow colors, often masked by other pigments and often seen only by the insect eye. This chapter is concerned with plant pigmentation, which is based upon anthocyanins, chalcones, and aurones. Strack and Wray[3] have described anthocyanins as 'the most important group of watersoluble plant pigments visible to the human eye'. Anthocyanin pigmentation is the major part of this chapter, while colors of chalcones and aurones are mainly treated in Section 3.16.4.

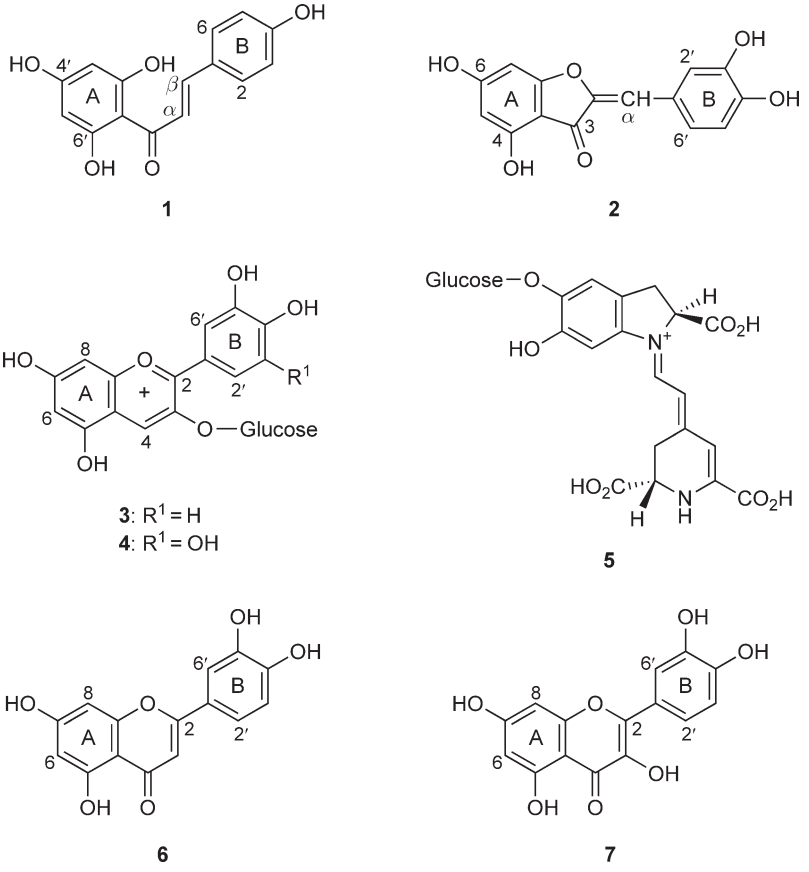


Figure 1 Examples of the colored flavonoid groups, chalcone: isosalipurpol (**1**), aurone: aureusidin (**2**), anthocyanins: cyanidin 3-glucopyranoside (**3**) and delphinidin 3-glucopyranoside (**4**), flavone: luteolin (**6**) and flavonol: quercetin (**7**), and the betalain: betacyanin (**5**).

In nature, anthocyanins are known for providing colors and patterns in flowers, fruits, and seeds to attract or repel pollinators and seed dispersers, thereby enhancing the survival of plants (Sections 3.16.6.1 and 3.16.6.2). The exact functions of anthocyanins and other flavonoid pigments in leaves, seedlings, roots, and stems are far from completely known, although their protective roles against various abiotic stresses and active defensive functions against pathogens, insects, and herbivores have been discussed in many papers (Section 3.16.6.3), especially in recent years. Once anthocyanins are formed in a given organ they operate through evolution to increase the number of individuals producing these compounds, which may explain the range of species-specific anthocyanins, which are found in petals, fruits, and leaves of various genera of higher plants.[4] Up to August 2008 a total of 644 anthocyanins isolated from plants have been identified appropriately;[4–7] however, only a minor fraction have been subjected to further analysis at the molecular level besides their structure elucidation. In general, the anthocyanins are treated as a homogeneous group of pigments. In the European Union's directive for a list of colorants permitted in food, anthocyanins (and grape color extract and grape skin extract) have been given just one common code (E163), in contrast to carotenoids and carotenoid containing sources. The importance of specific anthocyanin structures for various functions under *in vivo* conditions is reflected in the following example.

When compiling the reported anthocyanin content of blue flowers (**Table 1**), it is clear that nearly all anthocyanins are based on just one anthocyanidin, delphinidin (**Table 5**). The majority of these pigments contain aromatic acyl group(s) (**Figure 8**), and those without are reported together with copigments. When considering flowers of all colors, delphinidin derivatives constitute just around 22% of the different anthocyanins, which have been identified.

In recent years there has been worldwide interest in the extended use of anthocyanins as color additives as a consequence of perceived consumer preferences as well as legislative action, which has continued the delisting of approved artificial dyes. The main disadvantages of these pigments seem to be their relative low tinctorial strength and stability, which varies considerably between individual anthocyanins.

Over the past two decades considerable evidences reported that adequate fruit and vegetable consumption has a role in maintaining health and preventing various diseases. Some of these protective effects seem to be caused by the content of anthocyanins and other flavonoids, or their degradation products.[39–44] Certain studies concerned with the absorption of anthocyanins in humans indicate, however, that anthocyanins are only partially bioavailable, in nanomolar concentrations observed in plasma and urinary yields, commonly less than 0.1% of the oral dose.[45,46] Therefore, *if* intake of anthocyanins has a positive health effect, and *if* the various anthocyanins have different effects or properties (bioavailability, stability, etc.) of vital importance, then of course both the qualitative and quantitative content of various fruits and vegetables should be more closely considered. The range of anthocyanin structures found in the human diet (**Tables 2** and **3**) constitute only 20% of all the various anthocyanins, which have been isolated from plants (mainly flowers).[5] More than 55% of the different anthocyanins that occur in vegetables are acylated with aromatic acyl groups, while the corresponding number in fruits is only 21%. Among the commonly eaten fruits, only some grape and gooseberry cultivars have been reported to contain anthocyanins with aromatic acylation as major pigments,[112,124] while the majority of the vegetables contain considerable amounts of such pigments (**Tables 2** and **3**).

Continual improvements in methods and instrumentation (e.g., HPLC, LC–MS, and NMR) used for separation and structure elucidation of anthocyanins have made it easier to use smaller quantities of material, and to achieve results at increasing levels of precision (see Chapters 9.02, 9.06, and 9.11). Discovery of new anthocyanins regularly turn up in plant sources, which already have been well investigated before. When nearly no anthocyanins were reported to be acylated with malonic acid (**Figure 8**) two decades ago, malonyl units are now the most common acylation agent of anthocyanins occurring in 158 different anthocyanins. This chapter makes no attempts to cover methods used for analysis of anthocyanins and other flavonoids, however, the most recent techniques used for extraction, separation, identification, and quantification of these compounds have been treated thoroughly in several recent reviews.[125–133] A complete observation of anthocyanin color should include the molecular structure as well as the environment. Gonnet[134] has specified that an adequate description of anthocyanin color variation caused by pH differences requires that spectral variations considered should be those affecting the entire spectral curve (not only visible λ_{max}), that three color attributes (hue, saturation, and lightness) should be used to describe color (e.g., CIELAB parameters), and that these should refer to the light source and the condition of the observer. Recently, the influence of concentration, pH, and solvent on the

Table 1 Anthocyanin content in blue flowers

Family	*Species*	*Anthocyanin*[a]	*Reference(s)*
Monocotyledoneae			
Hyacinthaceae	*Hyacinthus orientalis* cv.	Dp3-[6-(cum)glc]-5-[6-(mal)glc]	8
Iridaceae	*Crocus antalyensis*[b]	Dp3-glc-5-[6-(mal)glc], Dp3-glc-7-glc, Pt3-glc-7-glc	9
Liliaceae	*Dianella nigra, D. tasmanica*[a]	Dp3-[6-(cum)glc]-7-[6-(cum)glc]-3′-[6-(cum)glc]-5′-[6-(cum)glc], Dp3-glc-7-[6-(cum)glc]-3′-[6-(cum)glc]-5′-[6-(cum)glc], Dp3-glc-7-glc-3′-[6-(cum)glc]-5′-[6-(cum)glc], 2-ace-1,5-di-OH-3-Me-8-[6-(xyl)glc]-naphthalene	10
	Muscari armeniacum	Dp3-[6-(cum)glc]-5-[4-(rha)-6-(mal)glc]	11
	Ophiopogon jaburan[c]	Pt3-[2-(glc)-6-(rha)glc]-3′-glc, Ka3-gal-4′-glc, Ka3,4′-di-glc	12
	Triteleia bridgesii[d]	Dp3-[6-(cum)glc]-5-glc, Dp3-[6-(cum)glc]-5-[6-(mal)glc], Dp3-[6-(4-(glc)cum)glc]-5-[6-(mal)glc]	13
Dicotyledoneae			
Campanulaceae	*Campanula medium*	Dp3-[6-(rha)glc]-7-[6-(4-(6-(4-(glc)hba)glc)hba)glc]	14
Compositae	*Cichorium intybus*	Dp3-[6-(mal)glc]-5-[6-(mal)glc], Dp3-[6(mal)glc], Dp3-[6-(mal)glc]-5-glc, Dp3-glc-5-glc, 3-cum quinic acid	15,16
	Felicia amelloides	Dp3-[2-(rha)glc]-7-[6-(mal)glc], 7-O-MeAp6-*C*-[2-(rha)glc]-4′-glc (ratio 1:18),7-*O*-methylisovitexin	17
	Senecio cruentus	Dp3-[6-(mal)glc)]-7-[6-(4-(6-(caf)glc)caf)glc]-3′-[6-(caf)glc]	18
Convolvulaceae	*Evolvulus pilosus*	Dp3-[6-(4-(6-(4-(glc)caf)glc)caf)glc]-5-[6-(mal)glc], Dp3 -[6-(4-(6-(4-(glc)caf)glc)caf)glc]-5-glc	19
	Ipomoea tricolor	Pn3-[6-(4-(6-(3-(glc)caf)glc)caf)-2-(6-(3-(glc)caf)glc)glc]-5-glc	20
Cornaceae	*Cornus alba* cv.[e]	Dp3-gal-3′-glc-5′-glc, Dp3-gal-3′-glc, Cy3-gal-3′-glc	21
Gentianaceae	*Gentiana* cv.	Cy3-glc-5-[6(caf)glc], Dp3-glc-5-[6-(caf)glc]-3′-glc, Dp3-glc-5-[6-(cum)glc]-3′-glc, Dp3-glc-5-[6-(cum)glc], Dp3-glc-5-[6-(caf)glc]-3′-[6-(cum)glc], Dp3-glc-5-[6-(cum)glc]-3′-[6-(caf)glc], Dp3-glc-5-[6-(caf)glc]-3′-[6-(caf)glc]	22
Goodeniaceae	*Leschenaultia* cv.	Dp3-[6-(mal)glc]-7-[6-(4-(6-(4-(glc)caf)glc)caf)glc]	23
Hydrophyllaceae	*Phacelia campanularia*	Dp3-[6-(4-(6-(4-(glc)caf)glc)caf)glc]-5-[6-(mal)glc]	19
Labiatae	*Salvia patens*	Dp3-[6-(cum)glc]-5-[6-(mal)glc], Ap7,4′-di-glc	24
	Salvia uliginosa	Dp3-[6-(cum)glc]-5-[4-(ace)-6-(mal)glc] Ap7-[4-(glc)glc], Ap7-[4-(glc)glc]-4′-glc	25
Leguminosae	*Clitoria ternatea*	Dp3,3′,5′-trigly (16 acylated ternatins)	26–30
	Lupinus cv.	Dp3-[6-(mal)glc], Ap7-[6-(mal)glc]	31
	Vicia villosa[d]	Dp3-rha-5-glc, Mv3-rha-5-glc, Pt3-rha-5-glc	32
Nymphaeaceae	*Nymphaea caerulea*	Dp3′-[2-(gao)-6-(ace)gal], Dp3′-[2-(gao)gal]	33

Ranunculaceae	*Aconitum chinense*[d]	Dp3-[6-(rha)glc]-7-[6-(4-(6-(hba)glc)hba)glc]	34
	Anemone coronaria[d]	Dp3-[2-(2-(caf)glc)-6-(mal)gal]-7-[6-(caf)glc]-3′-glu, Dp3-[2-(2-(caf)glc)gal]-7-[6-(caf)glc]-3′-glu, Dp3-[2-(2-(caf)glc)-6-(3-(2-(tar)mal)gal]-7-[6-(caf)glc]-3′-glu, Dp3-[2-(2-(caf)glc)-6-(3-(2-(tar)mal)gal]-7-[6-(caf)glc], Cy3-[2-(2-(caf)glc)-6-(3-(2-(tar)mal)gal]-7-[6-(caf)glc]-3′-glu	35
	Delphinium hybridum	Dp3-[6-(rha)glc]-7-[3-(3-(6-(4-(6-(hba)glc)hba)glc)glc)-6-(4-(6-(hba)glc)hba)glc]	36
Rhamnaceae	*Ceanothus papillosus*	Dp3-[6-(rha)glc]-7-[6-(cum)glc]-3′-[6-(cum)glc], Dp3-[6-(rha)glc]-7-[6-(cum)glc]-3′-glc, Ka3-[2-(xyl)rha]	37
Solanaceae	*Browallia speciosa* cv.[d]	Dp3-[6-(4-(caf)rha)glc]-5-[2-(cum)glc]	38

[a] In some cases copigments also identified.
[b] Blue perianth segments.
[c] Seed coats.
[d] Purple-blue.
[e] Fruits.

See **Table 9** for anthocyanin-flavonoid conjugates and **Table 10** for metalloanthocyanins.

Cy, cyanidin; Dp, delphinidin; Hi, hirsutidin; Mv, malvidin; Pg, pelargonidin; Pn, peonidin; Pt, petunidin; CCy, 5-carboxypyranocyanidin; CPg, 5-carboxypyranopelargonidin; CMv, 5-carboxypyranomalvidin; Ap, apigenin; Ka, kaempferol; ace, acetic acid; caf, caffeic acid; cum, *p*-coumaric acid; fer, ferulic acid; mal, malonic acid; gao, gallic (tri-OH-benzoyl) acid; hba, *p*-OH-benzoic acid; sin, sinapic acid; tar, tartaric acid; ara, arabinose; gal, galactose; glc, glucose; glu, glucuronic acid; gly, glycoside; rhamnose, rha; xyl, xylose.

Table 2 Qualitative and quantitative anthocyanin content of selected vegetables used in the human diet

Vegetables	*Major*[a] anthocyanins[b]	*Content (mg 100 g^{-1})*		*Reference(s)*
		FW	*DW*	
Bean, black (*Phaseolus vulgaris*)	Dp-, Mv-, Pt3-glc	24–45	214–278	47–50
Bean, red (*Phaseolus vulgaris*)	Cy3-glc, Cy3-[2-(xyl)glc], Pg3-glc	7	27–74	47,48,51
Cabbage, red (*Brassica oleracea*)	Cy3,5-di-glc, Cy3-[2-(glc)glc]-5-glc, Cy3-[2-(2-(sin)glc)glc]-5-glc, Cy3-[6-(sin)-2-(2-(sin)glc)glc]-5-glc	6–363		47,52–54
Carrot, black (*Daucus carrota*)	Cy3-[2-(xyl)gal], Cy3-[2-(xyl)-6-(glc)gal], Cy3-[2-(xyl)-6-(6-(sin)glc)gal], Cy3-[2-(xyl)-6-(6-(fer)glc)gal], Cy3-[2-(xyl)-6-(6-(cum)glc)gal]	44	4–1799	55–57
Chicory (*Cichorium intybus*)	Cy3-glc, Cy3-[6-(mal)glc], Dp3-[6-(mal)glc]	126–590		58–60
Corn (*Zea mays*)	Cy3-glc, Cy3-[6-(mal)glc], Cy3-[3,6-di-(mal)glc], Pg3-glc, Pn3-glc	54–1734	1680–1878	61,62
Eggplant (*Solanum melongena*)	Dp3-[6-(rha)glc], Dp3-[6-(rha)glc]-5-glc, Dp3-[6-(4-(*Z*/*E*-cum)rha)glc]-5-glc	8–86		47,52,63
Lentil (*Lens culinaris*)	Dp3-[2-(glc)ara]			64
Lettuce, red leaf (*Lactuca sativa*)	Cy3-[6-(mal)glc]	2–5		47,51,52,65
Onions (*Allium cepa*)	Cy3-glc, Cy3-[3-(glc)glc], Cy3-[6-(mal)glc], Cy3-[6-(mal)-3-(glc)glc]	15–49		47,52,66,67
Potatoes (*Solanum* spp.)	12 Pt-, Mv-, Pn-, Pg- and Dp3-[6-(rha)glc]-5-glc monoacylated with cum, fer or caf	2–40		52,68–70
Radish, red (*Raphanus sativus*)	Pg3-[2-(glc)-6-(cum)glc]-5-[6-(mal)glc], Pg3-[2-(glc)-6-(fer)glc]-5-[6-(mal)glc]	32–100		47,51,52,71
Rice, black (*Oryza sativa*)	Cy3-glc	10–493		72
Rhubarb (*Rheum rhabarbaru*)	Cy3-glc, Cy3-[6-(rha)glc]	4		52,73
Shamrock, purple (*Oxalis triangularis*)	Mv3-[6-(rha)glc]-5-glc, Mv3-[6-(4-(mal)rha)glc]-5-glc	195		74,75
Soybean, black (*Glycine* spp.)	Cy3-glc, Dp3-glc		158–2040	76,77
Sweet potato (*Ipomoea batatas*)	10 Cy- and Pn3-[2-(glc)glc]-5-glc mono- or diacylated with fer, caf, cum, or hba	180–184	611–625	62

[a] Major: Compounds estimated to occur in relative anthocyanin amounts higher than 10%. In some papers there exist no discrimination between major and minor compounds.
[b] See **Table 1** for abbreviations.
FW, fresh weight; DW, dry weight.

Table 3 Qualitative and quantitative anthocyanin content of selected fruits used in the human diet

Fruits	*Major*[a] anthocyanin[b]	*Content (mg 100 g^{-1})*		*Reference(s)*
		FW	*DW*	
Acai, jussara (*Euterpe* sp.)	Cy3-glc, Cy3-[6-(rha)glc]	30–293	730–2956	42,78–80
Acerola (*Malpighia* sp.)	Cy3-rha, Pg3-rha	4–60	261–528	79–84
Apple (*Malus sylvestris* spp.)	Cy3-gal	1–50	3–4	47,52,85–91
Baguacu (*Eugenia umbelliflora*)	Cy-, Dp-, Mv-, Pn- and Pt3-glc	342		91
Black currant (*Ribes nigrum*)	Cy3-glc, Cy3-[6-(rha)glc], Dp3-glc, Dp3-[6-(rha)glc]	236–587	744–1072	90,92–94
Black raspberry (*Rubus occidentalis*)	Cy3-glc, Cy3-[6-(rha)glc], Cy3-[2-(xyl)glc]	18–687	87–973	47,95
Blackberry(*Rubus fruticosus*)	Cy3-glc	70–300		47,96,97
Blood orange (*Citrus sinensis*)	Cy3-glc, Cy3-[6-(mal)glc], Dp3-glc	18–84		98–101
Blueberries (*Vaccinium* spp.)	Cy-, Dp-, Mv-, Pn-, Pt3-glc, 3-gal and 3-ara	110–823	2221–3146	47,90,94,97,102,103
Cherries (*Prunus* spp.)	Cy3-glc, Cy3-[6-(rha)glc]	66–144		47,52,104
Chokeberry(*Aronia* sp.)	Cy3-ara, Cy3-gal	410–1480	177–1052	53,90,93,94,102,105
Cowberry/lingonberry (*Vaccinium vitis-idaea*)	Cy3-ara, Cy3-gal	49–174	225–355	52,90,92,94,106–108
Cranberries (*Vaccinium oxycoccos*)	Cy3-glc, Cy3-ara, Cy3-gal, Pn3-ara, Pn3-gal	112–169	395–399	47,90,94,109
Crowberry (*Empetrum* sp.)	Cy-, Dp-, Mv-, Pn-, Pt3-gal and 3-ara	360	2379–4180	52,90,110
Elderberries (*Sambucus* spp.)	Cy3-glc, Cy3-[2-(xyl)glc], Cy3-[2-(xyl)glc]-5-glc, Cy3-[2-(xyl)-6(-Z/E-cum)glc]-5-glc	280–1005		93,94,111
Gooseberry (*Ribes uva-crispa*)	Cy3-xyl, Cy3-[6-(rha)glc], Cy3-[6-(cum)glc], Cy3-[6-(caf)glc], Pn3-glc	3–46	81–85	52,90,112,113
Grapefruit (*Citrus paradisis*)	Cy3-glc	6		52
Grapes *Vitis* spp.	Cy-, Dp-, Mv-, Pn- and Pt3-glc,	16–790	113	47,52,97,114,115
Litchi (*Litchi chinensis*)	Cy3-[6-(rha)glc],	48–177		116,117
Mango (*Mangifera indica*)	7-MeCy3-gal	$(0.2–3.8)\times10^{-4}$		118,119
Nectarine (*Prunus persica* var. *nucipersica*)	Cy3-glc	2–8		47,52
Passion fruits (*Passiflora* spp.)	Cy3-glc, Cy3-[6-(mal)glc], Dp3-glc			120
Peach (*Prunus persica*)	Cy3-glc	4–50		47,52,121
Pear (*Pyrus* spp.)	Cy3-gal	7		122
Plum (*Prunus domestica*)	Cy3-glc, Cy3-[6-(rha)glc], Pn3-glc, Pn3-[6-(rha)glc]	5–1833		47,52,121,123
Red currant (*Ribes rubrum*)	Cy3-[6-(rha)glc], Cy3[2(-xyl)glc], Cy3[2-(xyl)-6-(rha)glc],	1–21	108–118	52,90,93,94,
Red raspberry (*Rubus idaeus*)	Cy3-glc, Cy3-[6-(rha)glc]	2–109	3–594	47,52,90,95,102
Strawberry (*Fragaria* × *ananassa*)	Pg3-glc	18–52	184–235	47,52,90,102

[a] Major: Compounds estimated to occur in relative anthocyanin amounts higher than 10%. In some papers there exist no discrimination between major and minor compounds.
[b] See **Table 1** for abbreviations.
FW, fresh weight; DW, dry weight.

colors analyzed *in vitro* by CIELAB parameters,[131] and the molar absorptivities and visible λ_{max} values of various anthocyanins have been compiled.[135] Some of the drawbacks with respect to standardization of color analysis of pigments like anthocyanins are reflected in the variation, sometimes inconsistent, between the data shown within both of these compilations. Therefore, although the perception of the final anthocyanin pigmentation in plants depends on various factors, the reality is that UV–visible absorption spectra (visible λ_{max} values in particular) have been used as the common tool in most papers to describe and compare anthocyanin colors, as exemplified throughout this chapter. **Table 4** presents a compilation of molar absorption values of various anthocyanins reported after 1990 useful for quantitative determinations. Values reported before 1990 seems to be elevated in discrepancy.[138] On the basis of **Table 4**, we recommend for general use in measurements of anthocyanin concentration (antioxidant effects, etc.) a molar absorptivity value of 22 000 for

Table 4 Molar absorptivity values and visible absorption maxima of selected anthocyanins reported after 1990

Pigment[a]	*Molar absorptivity (ε)*	*$\lambda_{vis\text{-}max}$ (nm)*	*Solvent*	*Reference*
Pelargonidin (Pg)				
Pg	18 420	505	A-aq., pH 1.0	136
	19 780	524	MeOH, 0.1% HCl	136
Pg3-glc	15 600	496	A-aq., pH 1.0	136
	14 300	498	B-aq., pH 1.0	137
	21 021	497	B-aq. pH 1.0	138
	17 330	508	MeOH, 0.1% HCl	136
	23 800	502	MeOH, 0.01 v/v HCl	138
Pg3-(di-caf-glc)-[2-(glc)glc]-5glc	28 000	512	aq., pH 0.8	139
Pg3-[6-(rha)glc]-5-glc+[cum]	32 080	504	A-aq., pH 1.0	136
	39 591	511	MeOH, 0.1% HCl	136
Pg3-[2-(glc)glc]-5-glc	19 000	498	aq., pH 0.8	139
	25 370	497	A-aq., pH 1.0	136
	30 690	506	MeOH, 0.1% HCl	136
Pg3-[2-(glc)glc]-5-glc+[fer]	24 140	506	A-aq., pH 1.0	136
	29 636	507	MeOH, 0.1% HCl	136
Pg3-[2-(glc)glc]-5-glc-caf	19 000	498	aq., pH 0.8	139
Pg3-[2-(glc)glc]-5-glc+[cum]	28 720	506	A-aq., pH 1.0	136
	34 889	508	MeOH, 0.1% HCl	136
Pg3-[2-(glc)glc]-5-glc+[cum]+[mal	33 010	508	A-aq., pH 1.0	136
	39 785	508	MeOH, 0.1% HCl	136
Pg3-[2-(glc)glc]-5-glc+[fer]+[mal]	31 090	508	A-aq., pH 1.0	136
	39 384	508	MeOH, 0.1% HCl	136
CPg3-glc	21 500	495	MeOH, 0.01 v/v HCl	140
Cyanidin (Cy)				
Cy3-glc	18 800	512	10% EtOH, pH 1.5	141
	20 000	510	B-aq. pH 1.0	142
	16 520		B-aq. pH 1.1	143
	20 000	510	B-aq. pH 1.0	138
Cy3-[2-(glc)glc]-5-glc	19 260		B-aq. pH 1.1	143
Cy3-[2-(2-(sin)glc)-6-(sin)glc]-5-glc	23 460		B-aq. pH 1.1	143
Cy3-gal	23 450	508	B-aq. pH 1.0	138
	21 630	519	MeOH, 0.01 v/v HCl	138
CCy3-gal	20 840	506	MeOH, 0.01 v/v HCl	138
Peonidin (Pn)				
Pn3-glc	15 100	510	B-aq. pH 1.0	137
	14 100	512	10% EtOH, pH 1.5	141
Delphinidin (Dp)				
Dp3-glc	23 700	520	10% EtOH, pH 1.5	141
Petunidin (Pt)				
Pt3-glc	21 300	515	B-aq. pH 1.0	137
	18 900	520	10% EtOH, pH 1.5	141
	23 370	527	MeOH, 0.01 v/v HCl	138

(*Continued*)

Table 4 (Continued)

Pigment[a]	*Molar absorptivity (ε)*	$\lambda_{vis\text{-}max}$ *(nm)*	*Solvent*	*Reference*
Malvidin (Mv)				
Mv	16 000	538	MeOH, 0.01% HCl	144
Mv3-glc	23 400	517	B-aq. pH 1.0	137
	25 150	529	MeOH, 0.01 v/v HCl	138
	20 200	520	10% EtOH, pH 1.5	141
CMv3-glc	12 900	532	MeOH, 0.01% HCl	144

[a] See **Table 1** for abbreviations.
A-aq., aqueous buffer 0.025 mol l^{-1} KCl; B-aq., aqueous buffer 0.2 mol l^{-1} KCl – 0.2 mol l^{-1} HCl.

simple nonacylated anthocyanins dissolved in methanolic solutions containing 0.1% conc. hydrochloric acid. With respect to acylated anthocyanins, the values are considerably higher and depend largely on the structure.

A rather detailed description of the various structural elements influencing anthocyanin colors is presented in Section 3.16.2 under various headings. In Section 3.16.2.8, we have summarized copigmentation mechanisms. The primary anthocyanin structure, copigmentation, and pH are shown to be the most important factors influencing anthocyanin colors and stability, however, the exact mechanisms involved are poorly understood. Many isolated anthocyanins, which are nearly colorless in slightly acidic aqueous solvents, express their colors in plant vacuoles, which are indeed slightly acidic. Over the past decades, the question of how blue colors can be produced in flowers has been raised. Our understanding today is that anthocyanin monomers contain multiple structural features, which in combinations contribute to the formation of different supramolecular complexes. Anthocyanins are within the cells most often found dissolved uniformly in vacuolar solutions (see Section 3.16.3). Some anthocyanins have been reported in intensively colored intravascular bodies recently called AVIs (anthocyanic vacuolar inclusions). Although it is generally accepted that anthocyanins as other flavonoids are synthesized on the cytoplasmic surface of the endoplasmic reticulum membrane, the mechanisms for transportation and anthocyanin accumulation in the cells are more indecisive – even the structures of the AVIs are partly unknown.

Anthocyanin pigmentation has been very useful in genetic experiments, including the well-known studies of Gregor Mendel on inheritance of genes responsible for pea seed coat colors. Nowadays, the flavonoid biosynthetic pathway has been almost completely elucidated (Section 3.16.5). Since flower colors are among the key determinants influencing consumer choices, new varieties are of high commercial value. In recent years the intense search for a blue rose and other new anthocyanin flower colors has demanded the need for molecular bioengineering. By introducing new genes in plants encoding for novel enzyme activities, transcription factors, or inactivation of endogenous genes used in anthocyanin biosynthesis, several new varieties with modified flower colors and plant coloration have been created. The interest in and demand for natural food colorants and pharmacologically interesting natural compounds have also encouraged new research initiatives aimed at the development of more efficient means of harvesting anthocyanins. The production of anthocyanins in plant tissue cultures and by microorganisms is treated separately in Sections 3.16.7.1 and 3.16.7.2.

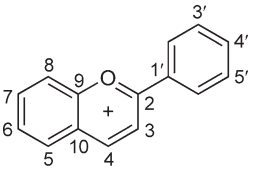
3.16.2 Color Variation Owing to Anthocyanin Structure

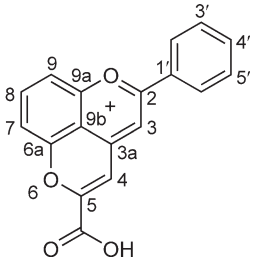
The anthocyanins are responsible for cyanic colors ranging from salmon pink through red and violet to dark blue in most flowers, fruits, and leaves of angiosperms. They are sometimes present in other plant tissues such as roots, tubers, stems, bulbils, and are also found in various gymnosperms, ferns, and some bryophytes. The term anthocyanin was initially coined to designate the substance responsible for the color of the cornflower (from the Greek words *anthos* (flower) and *kyanos* (blue). At present the actual number of anthocyanins reported with complete structure elucidation is 644.[4–7] The anthocyanins differ with respect to their aglycone (anthocyanidin), nature of glycosyl and potential aliphatic and aromatic acyl moieties, and their substitution positions.[4] During the last 15 years one new methylated anthocyanidin (7-*O*-methylcyanidin from mango, *Mangifera indica*), seven new deoxyanthocyanidins, and a novel type of anthocyanidins called pyranoanthocyanidins have been reported

(**Table 5**). In addition, new types of anthocyanins called flavanol–anthocyanidin heterodimers and anthocyanin–flavonoid conjugates as well as some new metalloanthocyanins have been identified.

In 1962, Hayashi summarized the major factors that caused the wide range of flower colors as a result of the presence of anthocyanin pigments: (1) The co-existence of several anthocyanins, (2) variation in the cellular concentration of anthocyanins, (3) the pH of the cell, (4) the phenomenon of copigmentation, (5) the colloidal condition of the cell sap, and (6) association of anthocyanins with metals.[145] Considerable efforts have later been made to improve explanations for the color variations expressed by anthocyanins.[4,146] Today various assets with the anthocyanin structure including (1) nature and concentration of the anthocyanidins, (2) anthocyanin

Table 5 Structures of naturally occurring anthocyanidins





	Substitution pattern						
Anthocyanidins[a]	*3*	*5*	*6*	*7*	*3′*	*4′*	*5′*
Common anthocyanidins							
Pelargonidin (Pg)	OH	OH	H	OH	H	OH	H
Cyanidin (Cy)	OH	OH	H	OH	H	OH	H
Delphinidin (Dp)	OH	OH	H	OH	OH	OH	OH
Peonidin (Pn)	OH	OH	H	OH	OMe	OH	H
Petunidin (Pt)	OH	OH	H	OH	OMe	OH	OH
Malvidin (Mv)	OH	OH	H	OH	OMe	OH	OMe
Rare methylated anthocyanidins							
5-MethylCy	OH	OMe	H	OH	OH	OH	H
7-MethylCy	OH	OH	H	OMe	OH	OH	H
7-MethylPn (Rosinidin)	OH	OH	H	OMe	OMe	OH	H
5-MethylDp (Pulchellidin)	OH	OMe	H	OH	OH	OH	OH
5-MethylPt (Europinidin)	OH	OMe	H	OH	OMe	OH	OH
5-MethylMv (Capensinidin)	OH	OMe	H	OH	OMe	OH	OMe
7-MethylMv (Hirsutidin)	OH	OH	H	OMe	OMe	OH	OMe
6-Hydroxylated anthocyanidins							
6-HydroxyPg	OH	OH	OH	OH	H	OH	H
6-HydroxyCy	OH	OH	OH	OH	OH	OH	H
6-HydroxyDp	OH	OH	OH	OH	OH	OH	OH
3-Deoxyanthocyanidins							
Apigeninidin (Ap)	H	OH	H	OH	H	OH	H
Luteolinidin (Lt)	H	OH	H	OH	OH	OH	H
Tricetinidin (Tr)	H	OH	H	OH	OH	OH	OH
7-MethylAp	H	OH	H	OMe	H	OH	H
5-MethylLt	H	OMe	H	OH	OH	OH	H
5-Methyl-6-hydroxyAp (Carajurone)	H	OMe	OH	OH	H	OH	H
5,4′-Dimethyl-6-hydroxyAp (Carajurin)	H	OMe	OH	OH	H	OMe	H
5-Methyl-6-hydroxyLt	H	OMe	OH	OH	OH	OH	H
5,4′-Dimethyl-6-hydroxyLt	H	OMe	OH	OH	OH	OMe	H
Pyranoanthocyanidins		*6a*	*7*	*8*			
5-CarboxypyranoPg (CPg)	OH	O-	H	OH	H	OH	H
5-CarboxypyranoCy (CCy)	OH	O-	H	OH	OH	OH	H

[a] See **Figure 2** for riccionidins A and B, sphagnorubins A–C, and rosacyanins A1, A2, and B.
The numbering of the structures on the left and right is used for anthocyanins and pyranoanthocyanins, respectively.

glycosidation and acylation, (3) flavanol–anthocyanidin heterodimers, (4) anthocyanin–flavonoid conjugates, (5) metal complexes, (6) anthocyanidin secondary structures (equilibrium forms), (7) nature and concentration of copigmentation including intra- and/or inter-molecular association mechanisms, (8) tertiary organization in the so-called AVIs (anthocyanic vacuolar inclusions) have been examined for their impact on anthocyanin coloration. In addition, external factors like pH, salts, temperature, involvement by pigment matrix/solvents, and so on, have been found to influence anthocyanin colors. Inter- and intramolecular copigmentation is supposed to be the most common mechanism in anthocyanin stabilization *in vivo*, and in the formation of most blue flower colors.[4,146–148] The following section describes various factors influencing anthocyanin colors.

3.16.2.1 Anthocyanidin Skeleton

The anthocyanidins (anthocyanin aglycones) are derivatives of 2-phenylbenzopyrylium (flavylium cation). The numbering of the left structure in **Table 5** is used for most anthocyanins, including anthocyanidins having the classical C_{15} skeleton. The pyranoanthocyanins, which have at least one additional C_3 unit, are based on the skeleton represented by the structure on the right in **Table 5**. While thirty-two naturally occurring monomeric anthocyanidins have been properly identified (**Table 5**), most of the identified anthocyanins are based on cyanidin (31%), delphinidin (22%), and pelargonidin (18%), respectively,[5] which only differ by the hydroxylation pattern of their B-rings. The other three common anthocyanidins (peonidin, malvidin, and petunidin), which contain methoxyl group(s) on their B-rings, constitute together the aglycones of 21% of the reported anthocyanins. This means that the rest of the anthocyanins, which have been identified (8%), are based on as many as 24 different anthocyanin aglycones. Although anthocyanin aglycones have been reported to occur *in vivo*, these findings have normally been treated as artifacts formed during the extraction and isolation stages. Recently, the natural presence of cyanidin, peonidin, and pelargonidin in extracts of beans has been suggested after careful consideration of the process of extraction and purification followed by LC–MS for identification purposes.[149] Otherwise, the 3-deoxyanthocyanidins found in *Sorghum bicolor*, spagnorubins in peat moss (*Sphagnum* spp.), and rosacyanins from petals of *Rosa hybrida* are the only anthocyanidins found in their nonglycosidated forms in plants (**Figure 2**).[7]

Although the perception of the final flower color based on anthocyanins depends on various factors, an UV–visible absorption spectrum of an anthocyanin gives a fair idea about its color. A typical anthocyanin exhibits a broad absorption maximum in the visible spectral region and has one less intense maximum in the UV region at about 275 nm (**Figure 3**). However, spectroscopic properties of anthocyanidins and anthocyanins are highly influenced by substituents and changes made to solvent and pH. This latter effect is shown by apigeninidin (**Table 5**), for which the absorption maximum (λ_{max}) is reported at wavelengths from 468 to 547 nm.[150,151] To understand the effect of a specific hydroxyl or methoxyl substituent on the color of an anthocyanidin, we have compiled from literature a standardized set of spectroscopic absorption data obtained at room temperature and with 0.01% (or 0.1%) hydrochloric acid in methanol as the solvent (**Table 6**). The interpretations (**Figure 4**) are based on the calculated shift difference in observed λ_{max} values between anthocyanidins differing at exactly one specific position. For instance, the difference in λ_{max} of 7-hydroxyflavylium (441 nm) and 3,7-dihydroxyflavylium (488 nm) is 47 nm caused by the hydroxyl group in the 3-position, while the difference between similar values of 4′-hydroxyflavylium (453 nm) and 3,4′-dihydroxyflavylium (484 nm) is 31 nm again caused by the 3-hydroxyl group. From **Table 6** we thus are able to obtain altogether seven $\Delta\lambda_{max}$ values caused by the effect of the 3-hydroxyl group, which have an average value of $\Delta\lambda_{max} = 37$ nm (**Figure 4**). Although the data behind the $\Delta\lambda_{max}$ values in **Figure 4** of the various anthocyanidin OH-substituents are somewhat scarce with respect to some positions, the following general conclusions given in Sections 3.16.2.1–3.16.2.3 may be drawn. As seen in **Table 6**, the replacement of an −OH moiety for an −OMe group leads to only minor hypsochromic effects on λ_{max}, meaning minute reddening effect on the color. This effect is just a couple of nanometers for the common anthocyanidins (**Table 5**), peonidin (3′-*O*-methylation), petunidin (3′-*O*-methylation), and malvidin (3′,5′-di-*O*-methylation). Anthocyanidins with 5-, 7-, or 4′-*O*-methylation are very rare.[4] The hypsochromic effect of methylation in such compounds seems to be from 5 to 10 nm. Toki *et al.*[13] have for the series cyanidin (3,5,7,3′,4′-pentahydroxyflavylium), peonidin (3,5,7,4′-tetrahydroxy-3′-methoxyflavylium), 7-*O*-methylcyanidin (3,5,3′,4′-tetrahydroxy-7-methoxyflavylium), and rosinidin (3,5,4′-trihydroxy-7,3′-dimethoxyflavylium) reported $\lambda_{vis\text{-}max} = 538$, 537, 532, and 530 nm, respectively, in accordance with these trends. When several substitutions on the aglycone skeleton exist, synergetic effects between substituents in the various positions have to be considered.

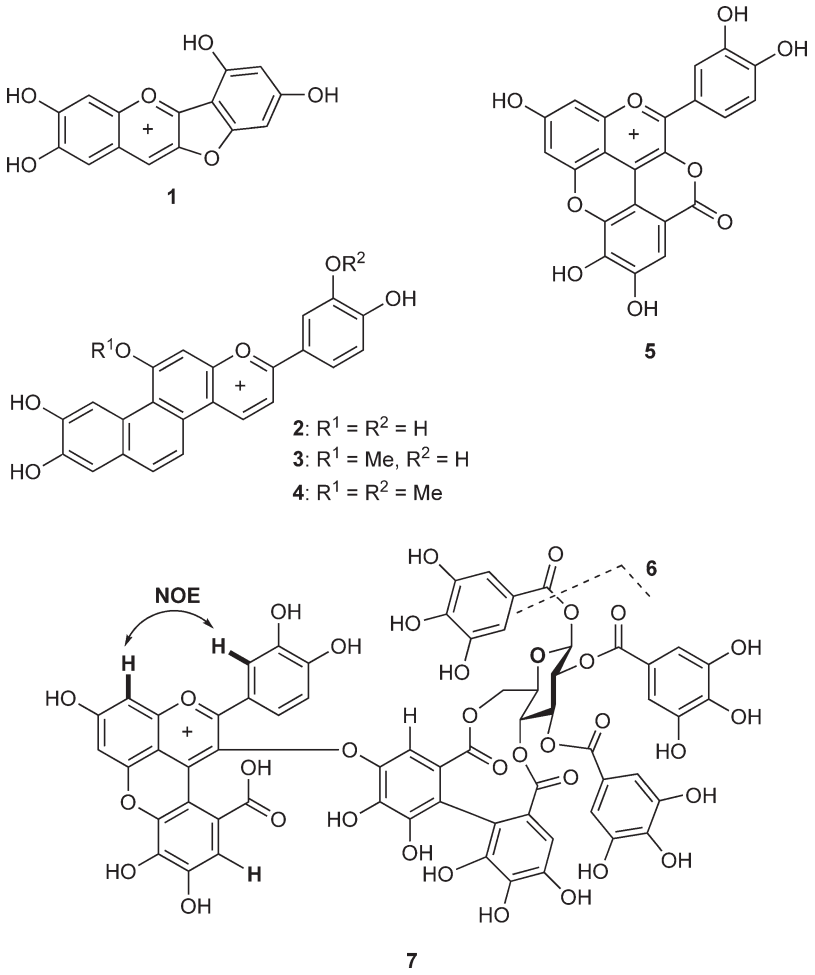


Figure 2 Structures of some rare anthocyanidins. Riccionidin A (**1**), sphagnorubins A-C (**2–4**), rosacyanin B (**5**), rosacyanin A1 (**6**), and A2 (**7**). In structure **7** the NOE between H-2′ and H-8 in the NMR spectrum is highlighted. Other anthocyanidin structures are found in **Table 5**.

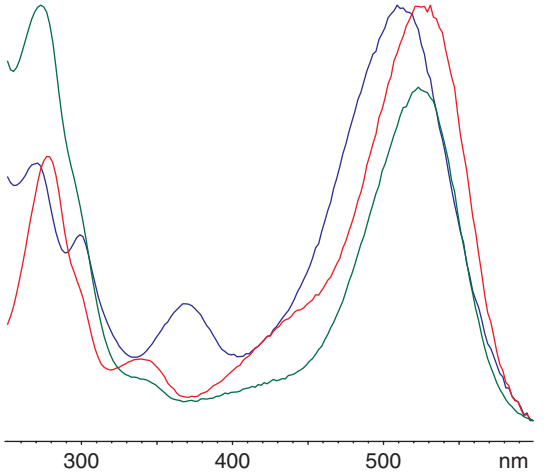


Figure 3 UV–visible spectra recorded on-line during HPLC analysis for petunidin 3-glucoside (red), petunidin 3,5-diglucoside (green), and 5-carboxypetunidin 3-*O*-β-glucoside (blue). See **Table 5** for anthocyanidin structures. See Jordheim *et al.*[138] for experimental conditions.

Table 6 Colors and absorption maxima of selected anthocyanidins dissolved in 0.01% (or 0.1%) conc. HCl in MeOH

Anthocyanidin (trivial name)	*Substitution pattern*	*Color*	$\lambda_{UV\text{-}max}$ *(nm)*	$\lambda_{vis\text{-}max}$ *(nm)*	*Reference(s)*
One O-substituent					
6-Hydroxyflavylium	6-OH	Light-yellow		388	152
7-Hydroxyflavylium	7-OH	Yellow		441	152
4′-Hydroxyflavylium	4′-OH	Yellow		453	152
Two O-substituents					
5,7-Dihydroxyflavylium	5,7-diOH	Yellow		461	152
7,4′-Dihydroxyflavylium	7,4′-diOH	Yellow		476	152
3′,4′-Dihydroxyflavylium	3′,4′-diOH	Orange		480	152
3,4′-Dihydroxyflavylium	3,4′-diOH	Orange		484	152
3,7-Dihydroxyflavylium	3,7-diOH	Orange		488	152
Three O-substituents					
3,4′-Dihydroxy-8-methoxyflavylium	3,4′-diOH; 8-OMe	Yellow		467	152
7-*O*-Methylapigeninidin[a]	5,4′-diOH; 7-OMe	Yellow	279	476	153
7,3′-Dihydroxy-4′-methoxyflavylium	7,3′-diOH; 4′-OMe	Yellow		477	152
Apigenidin[a]	5,7,4′-triOH	Orange		486	152
3,7,4′-Trihydroxyflavylium	3,7,4′-triOH	Orange-red		508	152
Four O-substituents					
Carajurin[a]	6,7-diOH; 5,4′-diOMe	Yellow	285	469	154
Carajuron[a]	6,7,4′-triOH; 5-OMe	Yellow	295	475	154
3,8,3′,4′-Tetrahydroxyflavylium	3,8,3′,4′-tetraOH	Orange-red		500	152
Luteolinidin[a]	5,7,3′,4′-tetraOH	Orange-red		502	152
Pelargonidin[a]	3,5,7,4′-tetraOH	Red	270	520	150,151
Fisetinidin	3,7,3′,4′-tetraOH	Red		526	151,152
Five O-substituents					
5-*O*-Methyl-6-hydroxyluteolinidin[a]	6,7,3′,4′-tetraOH; 5-OMe	Orange	302	492	154
Aurantinidin[a]	3,5,6,7,4′-pentaOH	Orange-red	286	499	150
Tricetinidin[a]	5,7,3′,4′,5′-pentaOH	Orange-red	281	513	150
Rosinidin[a]	3,5,4′-triOH; 7,3′-diOMe	Magenta	276	524[b]	155
Peonidin[a]	3,5,7,4′-tetraOH; 3′-OMe	Magenta	277	532[b]	150
7-*O*-Methylcyanidin[a]	3,5,3′,4′-tetraOH; 7-OMe	Magenta	273	533[b]	150
Cyanidin[a]	3,5,7,3′,4′-pentaOH	Magenta	277	535[b]	150,152
Six O-substituents					
Hirsutidin[a]	3,5,4′-triOH; 7,3′,5′-triOMe	Magenta		536	150
Capensinidin[a]	3,7,4′-triOH; 5,3′,5′-triOMe	Magenta	273	538	150
Europinidin[a]	3,5,7,4′-tetraOH; 3′,5′-diOMe	Purple	270	542	150
Malvidin[a]	3,5,7,4′-tetraOH; 3′,5′-diOMe	Purple	275	542	150
Petunidin[a]	3,5,7,4′,5′-pentaOH; 3′-OMe	Purple	276	543	150,151
Pulchellidin[a]	3,7,3′,4′,5′-pentaOH; 5-OMe	Purple	278	543	150
Delphinidin[a]	3,5,7,3′,4′,5′-hexaOH	Purple	277	546	150,151

[a] Naturally occurring.
[b] In the series rosinidin, 7-*O*-methylcyanidin, peonidin, and cyanidin, Toki *et al.*[29] have reported $\lambda_{vis\text{-}max}$ = 530, 532, 537, and 538 nm, respectively.

3.16.2.1.1 3-Deoksyanthocyanidins – lack of 3-hydroxyl on the anthocyanidin C-ring

A hydroxyl substituent in position 3 on the C-ring of the flavylium cation strongly favors shift of the absorption maximum to longer wavelengths (bathochromic shift) (**Figure 4**). This indicates that the 3-deoxyanthocyanidins (**Table 5**), which lack this 3-hydroxyl group, have a large hypsochromic shift (around

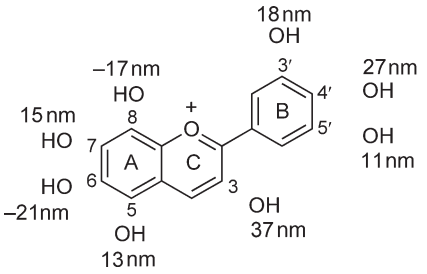


Figure 4 The numbers represent calculated shift differences (nm) between visible λ_{max} values in absorption spectra of anthocyanidins differing at exactly one specific position. For example, the introduction of an OH-group in the 7-position gives on average a bathochromic effect of 15 nm in the absorption spectrum, while an OH-group in the 6-position gives on average a hypsochromic shift of −21 nm.

37 nm) giving yellow, orange, and bright red plant colors. The 3-deoxyanthocyanidins constitute the few anthocyanins of ferns and bryophytes,[156–159] and have been found rarely in a few diverse angiosperm taxa; including some species belonging to Poaceae, *Arrabidaea chica* (Bignoniaceae), and abundantly in New World species of Gesneriaceae (e.g., the ornamental *Sinningia cardinalis)*. In fact, the 3-deoxyanthocyanidins occurred in 18 out of 21 species studied in the subfamily Gesnerioideae (Gesneriaceae) on the American continent, and in none of the 25 cyanic species in the subfamily Cyrtandroideae (Gesneriaceae) of the Old World.[160] The high frequency of 3-deoxyanthocyanidins in the Gesnerioideae has been linked with the pattern of ornithophily in this group (see Section 3.16.6.1). The bright orange-red colors produced by 3-deoxyanthocyanins are effective as bird-attracting colors, and production of these compounds is therefore believed to have evolved separately in the subfamily Gesnerioideae.

In recent years, a series of new 3-deoxyanthocyanidins have been reported (**Table 5**). 7-*O*-Methylapigeninidin, has been isolated in low concentration from grains and leaf sheaths of *Sorghum caudatum* (Poaceae).[153] A similar 3-deoxyanthocyanidin has been detected in grains of *S. bicolor* after incubation with the fungus *Colletotrichum sublineolum*.[161] In addition to plasma desorption mass spectrometry data, bathochromic shift analyses indicated that the structure of the compound was consistent with that of 5-*O*-methylluteolinidin. The spectrum of this phytoalexin, which showed greater fungitoxicity than luteolinidin, has its absorption maximum at 495 nm in pure methanol. Although the synthesis of the deoxyanthocyanidin carajurin, 6,7-dihydroxy-5,4′-dimethoxy-flavylium, isolated from leaves of *A. chica* was published in 1953,[162] the structure of this pigment was considered to be only partially described.[163,164] Later two groups nearly simultaneously confirmed the structure of carajurin – even by presenting a crystal structure.[154,165] The structure of carajurone was revised to be 6,7,4′-trihydroxy-5-methoxy-flavylium.[165] Additionally, the two new 3-deoxyanthocyanidins, 6,7,3′-trihydroxy-5,4′-dimethoxy-flavylium and 6,7,3′,4′-tetrahydroxy-5-methoxy-flavylium were isolated from the leaves,[154,165] which are traditionally used by some indigenous populations of South America for body painting and for dyeing fibers. The 3-deoxyanthocyanidins are relative stable toward pH changes,[166,167] and some 3-deoxyanthocyanidins have recently been demonstrated to be more cytotoxic to cancer cells than their anthocyanidin analogues.[168]

3.16.2.1.2 O-Substituents on the anthocyanidin B-ring

According to **Figure 4**, hydroxyl substituents on the anthocyanidin B-ring give comparable effects on the visible absorption maximum as a 3-OH substituent, though to a lesser extent. $\Delta\lambda_{max}$ for 4′-OH, 3′-OH, and 5′-OH is 27, 18, and 11 nm, respectively. Some representative studies reporting the distribution pattern of anthocyanins in various genera ensuing this correlation between substitution pattern on the anthocyanidin B-ring and flower color follows.

The qualitative and relative quantitative anthocyanin content of petal-like tepals of 17 different tulip (*Tulipa*) species and 25 cultivars have been analyzed as a background for carrying out breeding programs directed in particular toward flower colors.[169] Correlations between colors described by CIELab coordinates and anthocyanin content of individual samples were performed by multivariate analysis. Altogether five anthocyanins were identified as the 3-rutinosides of delphinidin, cyanidin, and pelargonidin, and the 3-[2‴-acetylrutinosides] of

cyanidin and pelargonidin. All tepals classified with hue angles described as 'blue nuances' were from the cultivars. They contained delphinidin 3-rutinoside (3 OH-substituents on the B-ring) as the major anthocyanin, and no or just traces of pelargonidin derivatives. The species and cultivars having 'magenta nuances' showed similar anthocyanin content with increased relative proportions of cyanidin 3-rutinoside (2 OH-substituents on the B-ring) at the expense of delphinidin 3-rutinoside. Orange-colored tepals were to a large extent correlated with high relative proportions of the pelargonidin derivatives (1 OH-substituent on the B-ring). Acetylation of anthocyanins furnished a weak color effect opposite to the blueing effect previously reported for anthocyanins with aromatic acyl groups.[170]

The impact of pigment structure, composition and concentration, pigment to copigment ratio, and pH on colors of *Pelargonium* flowers was investigated as background for any attempt to modify flower color via genetic manipulation.[171] The major factors responsible for color variation were shown to be the *types* and *relative levels* of pigments present. Variations in pH and copigment levels were not found to contribute significantly. Flowers with colors ranging from cream and pink to deep purple, including salmon, orange, and red, were studied. While either flavonols or carotenoids were responsible for cream/yellow coloration, all other colors resulted from anthocyanin mixtures. The major anthocyanins of various *Pelargonium* species and cultivars were identified as the 3,5-diglucosides and 3-glucoside-5-[6-(acetyl)glucosides] of the six common anthocyanidins.

Approximately twenty similar anthocyanidin 3,5-diglucosides with a cinnamic acid (**Table 5**, **Figure 8**) derivative located on the 6-position of the 3-sugar and possible malonyl or acetyl units (**Figure 8**) connected to the 5-sugar, have been isolated from flowers of *Hyacinthus orientalis*.[172–174] A survey of the anthocyanins in the floral organs (perianth, anthers, and ovaries) revealed that the dominant anthocyanin was delphinidin derivatives in four cultivars with blue flowers and cyanidin- or pelargonidin derivatives in cultivars with red or pink flowers.[8] Different patterns of anthocyanins were observed in each floral organ.

Around 35 different anthocyanins have been reported to occur in one or more species in the family Ranunculaceae.[4] Flowers of species in the genera *Delphinium* (blue),[36,175] *Consolida* (blue-violet), and *Aconitum* (purplish-blue) contain similar anthocyanins with polyacyl substitution based on *p*-hydroxybenzoylglucose residues at the 7-hydroxyl of delphinidin, in addition to a simpler glycosyl moiety at the 3-position.[34,176] Red flowers of *Delphinium hybridum* share a similar 3,7-disubstitution pattern based on pelargonidin instead of delphinidin.[177,178]

In *Salvia* and other genera belonging to Labiatae the red, scarlet, and pink-colored flower varieties contained pelargonidin derivatives, the blue ones delphinidin derivatives, while the amethyst- and grape-violet-colored ones were based on cyanidin derivatives.[24,25,179,180]

3.16.2.1.3 *O*-Substituents on the anthocyanidin A-ring – 6-hydroxyanthocyanidins

Regarding the anthocyanidin A-ring, the situation with respect to color effects of hydroxyl groups in the various positions is more intricate. While an OH-substituent in position 7 or 5 induces shifts to longer wavelengths ($\Delta\lambda_{max} = 15$ and 13 nm, respectively), an OH-substituent in position 6 or 8 implies even larger shifts to shorter wavelengths (hypsochromic shifts) with $\Delta\lambda_{max} = -21$ and -17 nm, respectively (**Figure 4**). All natural anthocyanins have $-$OH, $-$OMe, or $-O$-glycoside in their 5- and 7-positions. However, natural anthocyanins with 6-OH have very limited distribution, mainly within the genus *Alstroemeria*. In addition, aurantinidin has been reported to occur in *Impatiens aurantiaca* (Balsaminaceae),[181] however, this report has not been confirmed. The flower color, hue, and color intensity of fresh tepals of 28 Chilean *Alstroemeria* species and 183 interspecific hybrids have been described by parameters of CIELab.[182] Compared with flowers containing exclusively cyanidin 3-glycosides (**Figure 1**), the hues of flowers with 6-hydroxycyanidin 3-glycosides (**Table 5**) were more reddish. The relationship between flower color and anthocyanin content in 45 *Alstroemeria* cultivars showed that the major anthocyanins of outer perianths were cyanidin 3-rutinoside and 6-hydroxycyanidin 3-rutinoside in cultivars with red flowers, 6-hydroxydelphinidin 3-rutinoside in those that were red-purple, and delphinidin 3-rutinoside in purple ones.[183] The same group has also isolated the 3-(glucoside) and 3-[6-(rhamnosyl)glucoside] of 6-hydroxypelargonidin (aurantinidin) from extracts of the orange-red flowers of the *Alstroemeria* cultivars 'Oreiju', 'Mayprista,' and 'Spotty-red.'[184] The position of the 6-hydroxyl of 6-hydroxyanthocyanins has been unambiguously assigned by homo- and heteronuclear NMR techniques.[185]

3.16.2.1.4 Pyranoanthocyanidins

The group of pyranoanthocyanins (**Table 5**) has gained much attention during the last 10 years, mostly because of their color evolution in wine during maturation [186] (see Chapter 3.26). There are also some reports on the identification of pyranoanthocyanins from juices and other processed foodstuff.[187–193] Only the reports of rosacyanins from *R. hybrida* petals,[194,195] 5-carboxypyranopelargonidin 3-glucoside from strawberry fruits and 5-carboxypyranocyanidin 3-glucosides from outer scales of red onion (*Allium cepa*) are from fresh plant material (see **Figure 2** and **Table 5**).[140,196] The additional ring unit of the pyranoanthocyanins linking C-4 and the C-5 hydroxyl group of the flavylium nucleus, influences the various chromophores, and both bathochromic and hypsochromic effects have been observed.

The first pyranoanthocyanidin (rosacyanin B) found to occur in intact plants, was isolated in small amounts together with red cyanidin 3,5-diglucoside from the mauve petals of *R. hybrida* cv. 'M'me Violet.'[194] Rosacyanin B, which contained no sugar, however a galloyl moiety linked to the 5-OH and 4-position of cyanidin, was reported to be very stable in acidic alcoholic solutions. Under neutral or weakly acidic aqueous conditions it was precipitated before forming the colorless hemiketal form. Recently, Fukui *et al.*[195] showed that rosacyanin B in fact was connected in the 3-position to ellagitannins in two pigments named rosacyanin A1 and A2 (**Figure 2**). In comparison to cyanidin ($\lambda_{vis\text{-}max}$ at 531 nm in 0.1% conc. HCI in methanol), rosacyanin A1 and A2 possess bathochromic shifts with corresponding $\lambda_{vis\text{-}max}$ values at 585 nm giving more blue-colored solutions. The authors suggested that these colors were due to horizontal or vertical stacking. However, no nuclear Overhauser effects (NOEs) was observed between signals of the tellimagrandin 1 moiety and the cyanidin nuclei in rosacyanin A1. NOE effects were observed between cyanidin A-8 and B-ring protons (**Figure 2**) supporting a longer distance between the protons of the cyanidin nucleus and tellimagrandin 1 than between A-8 and B-2′/B-6′ of the cyanidin nucleus. Rosacyanin B was not very stable under neutral conditions, but the rosacyanin A's were blue or violet in a wide pH range (pH 1–7) (**Table 7**). Fukui and co-workers have indicated the possibility of preparing a blue rose based on the accumulation of large amounts of rosacyanins in the petals. Similar to the rosacyanins, the Port wine created portisins showed bathochromic shifts ($\lambda_{vis\text{-}max}$ values around 575 nm) compared to the spectra of their mother anthocyanins.[197]

Other types of pyranoanthocyanins created during wine maturation, including vitisins, hydroxyphenyl-pyranoanthocyanins, and vinylflavanol-pyranoanthocyanins, showed hypsochromic shifts compared to the absorption spectra of their mother anthocyanins. This results in more orange coloration. Similar hypsochromic shifts ($\lambda_{vis\text{-}max}$ values around 507 nm in 0.1% conc. HCl in methanol) have also been observed for 5-carboxypyranocyanidin 3-glucosides (vitisin A-type) isolated from acidified, methanolic extracts of the edible scales as well as from the dry outer scales of red onion (*A. cepa*),[196] and for 5-carboxypyranopelargonidin 3-glucoside isolated in small amounts from strawberries (*Fragaria* × *ananassa*) (**Figure 2** and **Table 5**).[140] By comparing UV–visible absorption spectra, 5-carboxypyranopelargonidin 3-glucoside showed in contrast to ordinary

Table 7 Visible absorption maxima of pelargonidin 3-*O*-β-glucopyranoside (Pg3-glc), 5-carboxypyranopelargonidin 3-*O*-β-glucopyranoside (CPg3-glc),[140] and rosacyanin A1[195] at various pH values

pH	*Pg3-glc*[a] λ_{max} *(nm)*	*CPg3-glc*[a] λ_{max} *(nm)*	*Rosacyanin A1*[b] λ_{max} *(nm)*
1	496.5	484.0	567.0
2			565.5
3	502.5	480.0	557.5
4			554.5
5	510.0	490.5	555.0
6	521.5	493.5	557.0
7	540.0	503.5	564.0
8	549.5	533.0	573.0
9	553.0	549.5	

[a] See **Table 5** for structure.
[b] See **Figure 1** for structure.

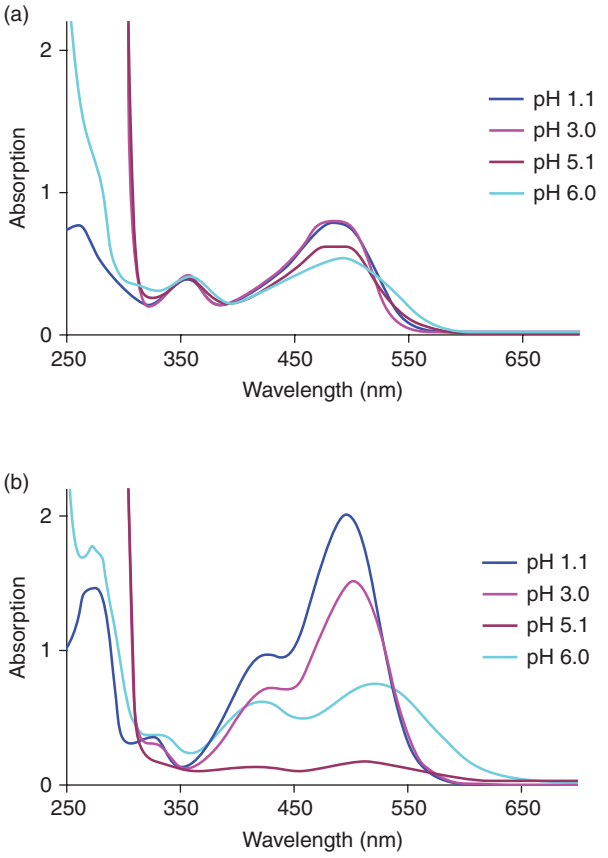


Figure 5 UV–visible absorption spectra of 5-carboxypyranopelargonidin 3-glucoside (0.10 mmol l^{-1}) (a) and pelargonidin 3-glucoside (0.10 mmol l^{-1}) (b) in four buffered solutions with pH ranging from 1.1 to 6.0.[140] While the main pigment of strawberries (b) is nearly colorless at pH 6.1, the 5-carboxypyrano-analogue retains most of its color at this pH.

pelargonidin 3-glucoside, a characteristic local absorption peak around 360 nm, a hypsochromic shift (8 nm) of the visible absorption maximum, and lack of a distinct UV absorption peak around 280 nm. This hypsochromic effect is shown in **Figure 3**. The similarities between the absorption spectra of 5-carboxypyranopelargonidin 3-glucoside in various acidic and neutral buffer solutions implied restricted formation of the unstable colorless equilibrium forms (**Table 7**, **Figure 5**), which are typical for most anthocyanins in weakly acidic solutions.[140,198] This is because the substitution at position 4 of the flavylium cation affects the distribution of the charge throughout the molecule. As a result, positions 2 and 4 become less reactive toward nucleophilic attack (hydration), which increases the stability of this type of anthocyanins in weakly acidic and neutral aqueous solutions.[199] Another consequence of the existence of colored flavylium cations of 5-carboxypyranopelargonidin 3-glucoside in a broad pH range is that the molar absorptivity of this pigment varied little with pH, contrary to similar values obtained for pelargonidin 3-glucoside.[140] At pH 5.1, the ϵ-value of 5-carboxypyranopelargonidin 3-glucoside (6250) was nearly four times the corresponding value of pelargonidin 3-glucoside (1720), which indicated that 5-carboxypyranopelargonidin derivatives may be beneficial as colorants of solutions with pH around 5. Similarly, Vivar-Quintana *et al.*[187] have reported that vitisin-like pigments made the major contribution to the color of wine at pH 4.

3.16.2.2 Anthocyanin Glycosides

Anthocyanins bear glycosyl units in the anthocyanidin 3-, 5-, 7-, 3′-, 4′-, or 5′-position. With exemption of the 3-deoxyanthocyanins, nearly all anthocyanins have a sugar located at the 3-position. The only reported exceptions are the 3′-[2-(galloyl)galactoside] and 3′-[2-(galloyl)-6-(acetyl)galactoside] of delphinidin

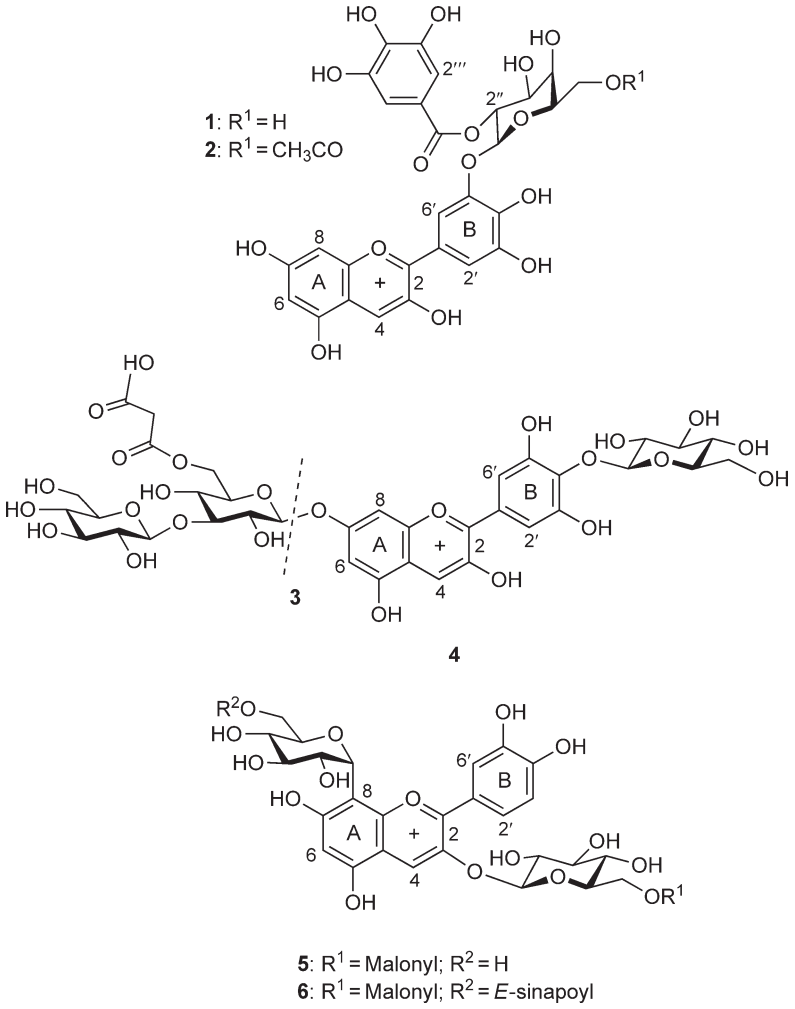


Figure 6 Structures of delphinidin 3′-[2-(galloyl)galactoside] (**1**) and delphinidin 3′-[2-(galloyl)-6-(acetyl)galactoside] (**2**) isolated from blue flowers of the African water lily *Nymphaea caerulea*,[33] cyanidin 4′-glucoside (**3**), and cyanidin 7-[3-(glucosyl)-6-(malonyl)glucoside]-4′-glucoside (**4**) from red onion (*Allium cepa*),[200] and cyanidin 3-*O*-[6-*O*-(malonyl)glucoside]-8-*C*-glucoside (**5**), and cyanidin 3-*O*-[6-*O*-(malonyl)-glucoside]-8-*C*-[6-*O*-(*trans*-sinapoyl)-glucoside] (**6**) isolated from the purple flowers of *Tricyrtis formosana*.[202,203]

(**Figure 6**) isolated from blue flowers of the African water lily *Nymphaea caerulea*,[33] and the 4′-glucoside and 7-[3-(glucosyl)-6-(malonyl)glucoside]-4′-glucoside of cyanidin (**Figure 6**) from red onion (*A. cepa*).[200] Several anthocyanidin 5-monoglycosides and anthocyanidin 7-monoglycosides without sugar in their 3-positions have been reported to occur naturally,[201] however, they may be classified as tentative structures due to limited experimental data for exact identification of the linkage positions of the sugar groups. The sugar(s) is normally connected to the anthocyanidin through an *O*-linkage. However, both cyanidin 3-*O*-[6-*O*-(malonyl)-*β*-glucopyranoside]-8-*C*-*β*-glucopyranoside and cyanidin 3-*O*-[6-*O*-(malonyl)-*β*-glucopyranoside]-8-*C*-[6-*O*-(*trans*-sinapoyl)-*β*-glucopyranoside] (**Figure 6**) have been isolated from the purple flowers of *Tricyrtis formosana* cultivar Fujimusume (Liliaceae) together with four known cyanidin derivatives.[202,203] Eight 3-deoxyanthocyanidin *C*-glycosides have recently been made from their respective flavone 6-*C*-glycosides.[204] Apigeninidin 6,8-di-*C*-*β*-glucoside with two *C*–*C* linkages between the sugar moieties and the aglycone, was found to be far more stable toward acid hydrolysis than pelargonidin 3-*O*-glucoside, which has the common anthocyanidin *C*–*O* linkage between the aglycone and the sugar.

The monosaccharide units found in anthocyanins are represented by glucose, galactose, rhamnose, arabinose, xylose, and glucuronic acid. Glucosyl moieties have been identified in more than 90% of the various anthocyanins, while the most unusual glycosyl moiety in anthocyanins, glucuronosyl, is limited to 11 anthocyanins.[5] Most anthocyanins contain one, two, or three monosaccharide units, however, as much as seven units have been found in ternatin A1 (*Clitoria ternatea*) and cyanodelphin (*D. hybridum*).[28,36] Altogether 287 different anthocyanins contain one or more disaccharides out of 12 different disaccharides.[5] The most common disaccharides, sophorosyl and rutinosyl, have been found in 84 and 76 anthocyanins, respectively. Only 20 different anthocyanins contain a trisaccharide among the 8 trisaccharides, which have been reported.[5] No tetrasaccharide has yet been found in an anthocyanin. See Andersen and Jordheim[4] for distribution of the various anthocyanin glycosyl moieties.

The addition of a sugar residue to the anthocyanidin 3-position produces in general a hypsochromic effect of 10–14 nm in the visible region of the absorption spectra, depending on the solvent and nature of the aglycone. The nature of the glycosyl unit has no effect as long as it is not acylated. The addition of a second sugar residue in a new aglycone position of anthocyanidin 3-glycosides, produces with one exemption, the 5-position, a hypsochromic effect of 8–12 nm in the visible region of the absorption spectra (**Figure 7**). UV–visible spectra have been recorded on-line during HPLC for delphinidin 3-galactoside-3′,5′-diglucoside, delphinidin 3-galactoside-3′-glucoside and cyanidin 3-galactoside-3′-glucoside isolated from bluish white berries of Siberian dogwood, *Cornus alba* 'Sibirica.'[21] When the spectra of delphinidin 3-galactoside-3′-glucoside and cyanidin 3-galactoside-3′-glucoside were compared with analogous spectra of the corresponding anthocyanidin 3-galactosides, hypsochromic shifts (about 8–10 nm), and increased $A_{440}/A_{\text{Vis.max}}$ ratios were observed. The corresponding hypsochromic shift for delphinidin 3-galactoside-3′,5′-diglucoside was 16 nm. The UV–visible data for cyanidin 3-galactoside-3′-glucoside are quite similar to that of cyanidin 3,4′-diglucoside,[200] cyanidin 3,5,3′-triglucoside,[205] and cyanidin 3,7,3′-triglucoside.[206] Thus, whether the glucosyl is located either in the anthocyanidin 3′-, 4′-, or 5′-position, it seems to have the same characteristic hypsochromic shift effect on the UV–visible maxima and diagnostic hyperchromic effect on the absorbances around 440 nm.

Compared with spectra of cyanidin 3-glucoside, cyanidin 4′-glucosides from red onions showed hypsochromic shifts (12 nm) of the visible $\lambda_{\max}$, and hyperchromic effects on wavelengths around 440 nm, similar to pelargonidin 3-glycosides.[200] These spectra characteristics were nearly identical for cyanidin 4′-glucoside, cyanidin 3,4′-diglucoside, cyanidin 3-[3-(glucosyl)-6-(malonyl)glucoside]-4′-glucoside, and cyanidin 7-[3-(glucosyl)-6-(malonyl)glucoside]-4′-glucoside, showing that an extra sugar residue in the 3- or 7-position has really no effect when there also is a sugar residue in the 4′-position. As indicated above, when the sugar moiety is added to the 5-position, the visible $\lambda_{\max}$ shows hypsochromic shifts by only a couple of nanometers, if at all. However, the two most common classes of anthocyanins, the 3- and 3,5-diglucosides, have differences in intensity around 440 nm, which is of diagnostic value (Harborne;[207] **Figure 3**). Thus will the anthocyanidin 3,5-diglycosides have only about 50% of the absorbance measured for anthocyanidin 3-glycosides at this wavelength.

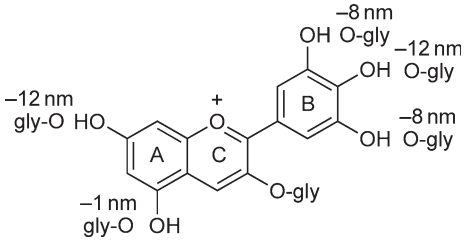


Figure 7 The numbers represent observed shift differences (nm) of visible $\lambda_{\max}$ values in absorption spectra of anthocyanidin glycosides obtained after addition of a second sugar residue in a new aglycone position of the corresponding anthocyanidin 3-glycoside. For example, cyanidin 3,7-diglucoside will have its visible $\lambda_{\max}$ at 12 nm shorter wavelength compared to similar absorption spectrum of cyanidin 3-glucoside.

3.16.2.3 Anthocyanidin Acylglycosides

More than 66% of the reported anthocyanins with well characterized structures have one or more acyl moieties linked to their sugar unit(s).[5] The colors of these pigments in plants are highly affected by the nature, number, and linkage positions of the acyl groups. As many as 319 different anthocyanins have aromatic acylation, which include various hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, sinapic, and 3,5-dihydroxycinnamic acids) and two hydroxybenzoic acids (*p*-hydroxybenzoic and gallic acids) (**Figure 8**). These acyl groups may participate in intramolecular copigmentation of the anthocyanidin nucleus with huge impact on the colors revealed by the plants, especially in flowers (see Section 3.16.2.8). The structural variation between the various anthocyanins found in fruits and vegetative tissues are limited compared to the variation found among flowers. Considering the anthocyanins eaten in a typical European diet (**Tables 2** and **3**), around 55% of the different anthocyanins in vegetables contain aromatic acyl groups, while the corresponding number in fruits is only around 20%. The different distribution of anthocyanins acylated with aromatic acyl groups may reflect the different functions of this type of anthocyanins in fruits and flowers.

Malonic acid, which is identified in 25% of the various anthocyanins, is the most frequently occurring acyl moiety of anthocyanins. This acyl unit constitutes the aliphatic acyl moieties together with acetic, malic, oxalic, succinic, and tartaric acids (**Figure 8**), which have been identified in altogether 205 anthocyanins.[5] Tartaric acid has the most limited distribution among the acylation agents, identified in only four anthocyanins isolated from flowers of *Anemone coronaria* (Ranunculaceae).[35,208] The only anthocyanins found conjugated with sulfate, malvidin 3-glucoside-5-[2-(sulfato)glucoside] and malvidin 3-glucoside-5-[2-(sulfato)-6-(malonyl)glucoside], have been isolated from violet flowers of *Babiana stricta* (Iridaceae).[209] As many as four different acyl groups located at four different glycosyl moieties have been identified in Lobelinin B isolated from flowers of *Lobelia erinus* (Lobeliaceae).[210]

More than 86% of the acylated anthocyanins have one or more acyl moieties located to the 6-position(s) on the monosaccharide(s), while 13 and 11% of the anthocyanins have an acyl group in the 2- and 4-position, respectively. The location of the acyl group to the 3-position is only found in five anthocyanins, either in family Gramineae,[211] Alliaceae,[211,212] Liliaceae,[213] Aceraceae,[214] or Compositae.[215] The location of the acyl group to the sugar 5-position is even more restricted including three different anthocyanins in either family Gramineae or Commelinaceae.[216–218] In these latter cases the sugar is an α-L-arabinofuranosyl. Restricted distribution of

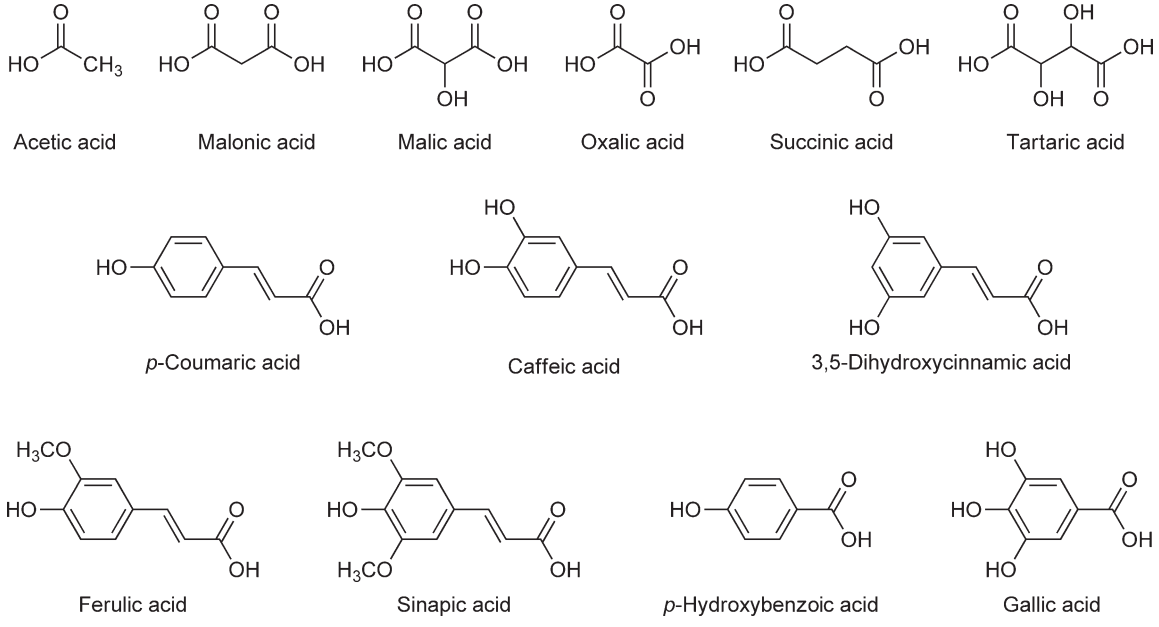


Figure 8 Structures of the aromatic and aliphatic acyl units, which have been found connected to a glycosyl moiety of acylated anthocyanins.

any sugar or acyl unit of anthocyanins, and rare linkage positions, might have chemotaxonomic relevance. Details regarding distribution of acyl and sugar moieties of anthocyanins, including some chemotaxonomic considerations, have been treated elsewhere.[4,219]

3.16.2.4 Anthocyanidin Equilibrium Forms and Stability

Anthocyanins are outstanding in the way each anthocyanidin may be involved in a series of equilibria giving rise to different forms (secondary structures), which exhibit their own properties including color expression.[198,220–228] The secondary structures have been examined/proposed using pH-jump methods, UV–visible, and fluorescence spectroscopy, and NMR spectroscopy. The experimental proofs for accurate structural assignments of other aglycone forms than the flavylium cation, have been incomplete for most anthocyanins. The knowledge about distribution of the individual aglycone secondary structures is limited for most anthocyanins both under *in vitro* and *in vivo* conditions. The color and distribution of the various secondary structures is highly linked to the stability of the various anthocyanin molecules.[143,229]

When a common anthocyanidin mono- or diglycoside is dissolved in water, secondary structures (**Figure 9**) are formed according to different acid–base, hydration and tautomeric reactions. **Figure 9** shows some possible anthocyanin transformations in aqueous solution, however, other reactions may be involved. **Table 8** containing visible λ_{max}-values of the six common anthocyanidin 3-glucosides in buffered aqueous solutions at different pH values recorded after 1 h, reflects the impact of variation of secondary structures in the pH range of 1–11. The flavylium cation (**Figure 9**, **1**) with reddish nuances is the predominant form in relative strong acidic aqueous solutions (below pH 2). Under more mildly acidic pH conditions, the anthocyanin solution is typically only slightly colored. The amount of colored forms drops down to 10% or less for the six common anthocyanidin 3-glucosides based on comparison of their molar absorptivities at visible λ_{max} at pH 5 and 1.[229] This is caused by displacement of the hydration equilibrium of the flavylium cation toward colorless hydroxy adducts (called carbinol bases, pseudobases, hemiacetals, or hemiketals) formed by a nucleophilic reaction with water mainly in the 2-position (**Figure 9**, **8**). The presence of a 4-adduct has also been presented (**Figure 9**, **9**). The hemiketal will to some extent be rapidly converted into its open-chain isomer, *cis*-retrochalcone (**Figure 9**, ***Z*-10**),[230] and finally *trans*-retrochalcone (**Figure 9**, ***E*-10**), which also are nearly colorless forms. For malvidin 3,5-diglucoside the ratio between the hemiketal and *cis*-retrochalcone forms is 4:1 at room temperature in weakly acidic aqueous solutions. A further pH increase to 6 leads to uncharged tautomeric quinonoidal bases (**Figure 9**, **2–4**) (anhydrobases) with purple colors derived from the flavylium cation by deprotonation, and finally to anionic structures with bluish nuances (**Figure 9**, **5–7**).

Color stability of nonacylated anthocyanins has been found to vary tremendously in aqueous solutions depending on pH.[229] Although initially detected after 1 h in aqueous solutions, no color was observed for instance for malvidin 3-glucoside after one day storage at pH 6 and 6.5. Opening of the pyrylium ring and chalcone formation have been postulated as the first degradation step of anthocyanins;[231,232] however, hydrolysis of the glycosidic moiety and aglycon formation has also been proposed as the initial reaction.[233] In a recent study of heat-treated elderberry and strawberry pigment isolates, the presence of chalcone glycosides and the absence of aglycones at pH 3.5 demonstrated pH-dependent degradation pathways of the anthocyanins.[234] Supposedly, the first step of thermal degradation at pH 3.5 was not anthocyanin deglycosylation, but opening of the pyrylium ring and chalcone glycoside formation. Recently, the hemiketal forms of the 3-glucosides of delphinidin, petunidin, and malvidin and cyanidin 3-galactoside dissolved in deuterated methanolic solutions were characterized as two epimeric 2-hydroxy-hemiketals on the basis of assignments of both proton and carbon NMR signals together with chemical shift considerations.[198] No 4-hydroxy-hemiacetal form was detected for any of the pigments. For each anthocyanin dissolved in deuterated methanol, the equilibrium between each of the two epimeric hemiketals and the corresponding flavylium cation was confirmed by the observed positive exchange cross-peaks in the 2D ^{1}H NOESY spectra. The molar proportions of the flavylium cation and the two hemiketal forms of the four pigments in deuterated methanol were very similar (70:30) for all pigments, even during storage for weeks. No other secondary structures were observed in this study. The reason for the stability of the anthocyanin pigments in the NMR solvent (deuterated methanol) might be the lack of conversion of hemiketals into chalcone forms. The same supposed mechanism might be the reason for high color stability of even simple anthocyanidin mono- and disaccharides under *in vivo* conditions in plants.

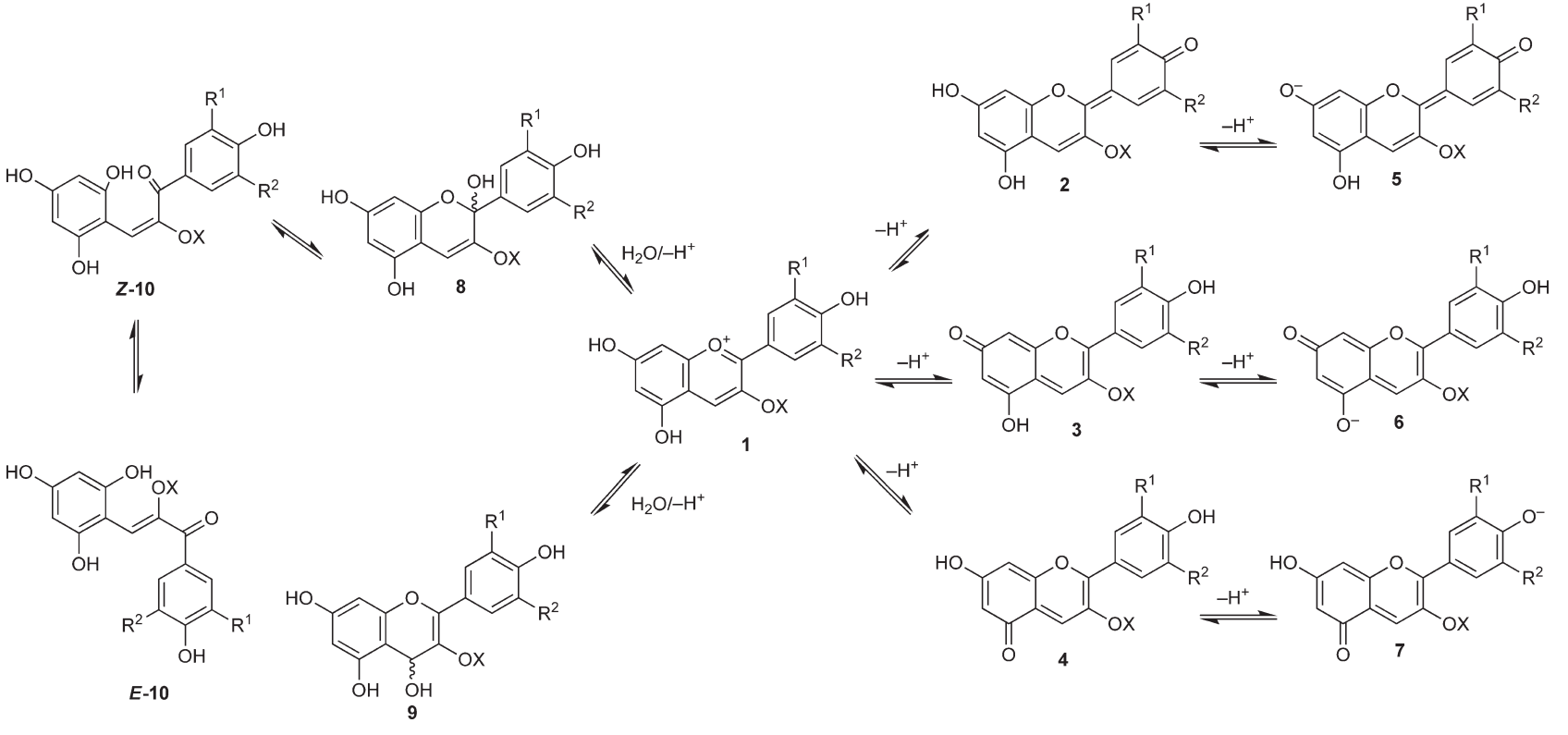


Figure 9 The scheme shows some possible anthocyanin transformations in aqueous solution. X = glycoside, R^1 and R^2 can be hydroxyl and/or methoxyl groups, depending on the type of aglycone. Other transformations may be involved.

Table 8 Visible λ_{max} values (nm) for chloride salts of the six common anthocyanidin[a] 3-glucosides (1.0×10^{-4} mol l^{-1})

pH	*Pg3-glc*	*Cy3-glc*	*Pn3-glc*	*Dp3-glc*	*Pt3-glc*	*Mv3-glc*
1.0	498	510	510	514	515	517
2.4	501	512	516	521	521	525
3.1	504	517	518	525	525	528
4.0	507	520	522	528	529	533
5.0	515	523	527	530	531	535
6.0	519	528	532	558	565	537
6.5	525	539	537	567	569	559
7.0	540	554	554	576	584	576
7.3	547	562	568	574	589	586
7.7	551	571	571	577	590	593
8.1	553	570	574	574	588	594
8.6	553	539	571	542	543	595
9.0	555	540	573	547	542	596
9.5	554	542	573	552	543	597
9.8	553	541	573	546	598	
10.6	554	569	575	595		
11.5	588					

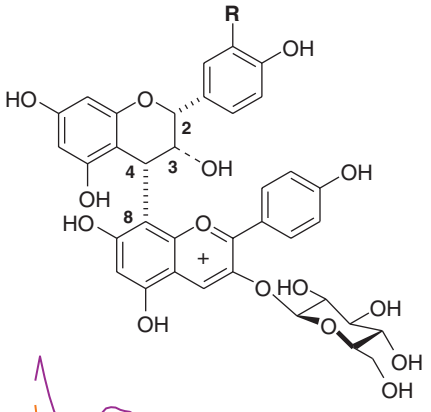
[a] See **Table 5** for structures.
One hour after dissolution in buffered aqueous solutions at various pH values in room temperature.[229]

The pH-dependent reaction from flavylium cation toward colorless hemiketals in slightly acidic aqueous solutions is affected by the type, position, and number of substituent groups attached to the aglycone.[139,235] When the substituent groups are long enough to adopt a folded conformation over the pyrylium ring of the anthocyanidin, the reactive sites (C-2 and C-4) may be protected against nucleophilic water attack, thus favoring the existence of the colored forms. When a covalently linked anthocyanin–flavone *C*-glycoside isolated from purple leaves of *Oxalis triangularis* (Oxalidaceae) dissolved in deuterated methanol and trifluoroacetic acid (95:5) was observed by NMR 45 min after sample preparation, the pigment occurred mainly as flavylium cation (38%) and two equilibrium forms assigned to be quinonoidal bases (54%).[236] More simple anthocyanins are normally considered to be on the flavylium cation form in this acidified deuterated methanolic solvent.[198] The NMR results indicated the presence of vertical π–π stacking between the B-ring of the flavone unit and the A-ring of each of the two quinonoidal bases.[236] It was not possible to discriminate between inter- or intramolecular association mechanisms. Only minor amounts of the two hemiketal forms were present. After five days of storage at 27 °C, the hemiketals (39%) and flavylium cation (38%) constituted the main forms of the pigment. More examples related to the effect of copigmentation on secondary anthocyanidin structures are given in Section 3.16.2.8.

The deep-red color of the Dragon's blood is a natural resin obtained from *Dracaena draco* and *D. cinnabaris* (Dracaenaceae).[237] The resin is known to appear in injured parts of the tree and has been used over the centuries for medicinal and artistic purposes. The compound 7,4′-dihydroxy-5-methoxyflavylium (dracoflavylium) was identified as the major red colorant of this resin. It was concluded that the red color was due to a stable quinonoidal base, which was the major species at pH 4–7. As for the *Oxalis* pigment described above, here we have a second example where the quinonoidal form of the pigment is the major species under slightly acidic conditions. In this latter case the methoxyl group in the 5-position is most probably of significant importance for stabilization of the quinonoidal forms. Similar to the other flavylium compounds, 7,4′-dihydroxy-5--methoxyflavylium was involved in a complex network of chemical reactions in which the different forms can be reversibly interconverted by changing the pH.

3.16.2.5 Flavanol-Anthocyanidin Heterodimers – 'Blueing Effect'

Most reported anthocyanins are monomeric in nature, however, more recently new types of flavonoids consisting of an anthocyanidin moiety covalently linked to another flavonoid unit, have been reported. Anthocyanins resulting from direct condensation between an anthocyanidin unit and a flavanol have been



Compound	On-line HPLC (diode array detection)	
	$\lambda_{Vis\text{-}max}$ (nm)	$A_{440}/A_{Vis\text{-}max}$ (%)
1	518, 438	81
2	518, 433	70
3	516, 434	69
4	520, 432	68
Pg 3-glc	504	43

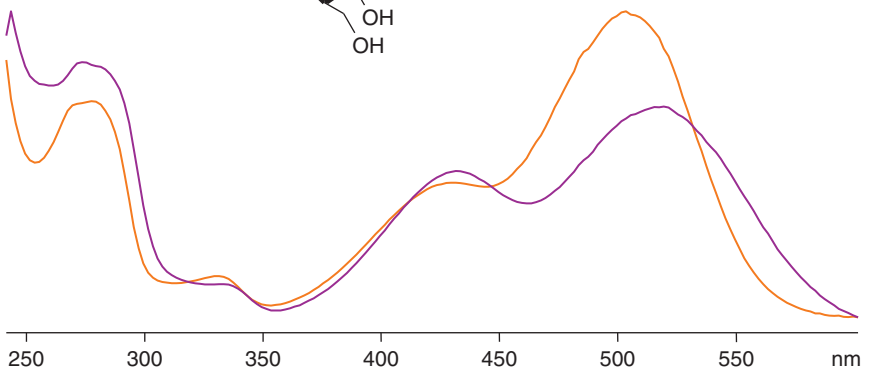


Figure 10 UV–Vis spectroscopy data recorded on-line during HPLC of catechin(4$\alpha \rightarrow$8)pelargonidin 3-glucoside (R = OH), **1**, epicatechin(4$\alpha \rightarrow$8)pelargonidin 3-glucoside (R = OH), **2**, afzelechin(4$\alpha \rightarrow$8)pelargonidin 3-glucoside (R = H), **3**, epiafzelechin(4$\alpha \rightarrow$8)pelargonidin 3-glucoside (R = H), **4**, and pelargonidin 3-glucoside from strawberries.[239] The UV–Vis spectra display epiafzelechin(4$\alpha \rightarrow$8)pelargonidin 3-glucoside (**4**) (purple) and pelargonidin 3-glucoside (orange). The colorless flavan-3-ol derivatives in the dimers provide a substantial bathochromic copigment effect on the anthocyanin.

assumed to be formed exclusively during storage and processing in plant-derived foods including wines.[238] However, this type of pigments seems also to appear naturally, although in small quantities, in extracts of unprocessed plants. In extracts of fresh strawberries four purple-colored pigments (**Figure 10**) were characterized by spectroscopic methods to be catechin (4$\alpha \rightarrow$8) pelargonidin 3-glucoside (**1**), epicatechin (4$\alpha \rightarrow$8) pelargonidin 3-glucoside (**2**), afzelechin (4$\alpha \rightarrow$8) pelargonidin 3-glucoside (**3**), epiafzelechin (4$\alpha \rightarrow$8) pelargonidin 3-glucoside (**4**).[239] The stereochemistry at the 3- and 4-positions of the flavan-3-ols was elucidated after assumption of the *R*-configuration at C-2. Because of rotational hindrance around the linkage between C-8 of the anthocyanidin moiety and C-4 of the flavanol, conformational isomers (two rotamers) of each heterodimer were identified in the NMR solvent.

The UV–visible spectra of the flavanol–anthocyanin heterodimers recorded on-line during HPLC analysis showed two visible absorption maxima at 516–520 nm and 432–438 nm (**Figure 10**). The purple colors of the heterodimers were different from the scarlet color of pelargonidin 3-glucoside, which constitute the monomeric anthocyanin unit of these heterodimers. For comparison, when a hydroxyl group is located at the 8-position of the anthocyanin as in 8-hydroxyanthocyanidin, a shift of the visible absorption maximum to lower wavelengths (red shift) is experienced (see Section 3.16.2.1). However, when a flavanol is linked to the 8-position of the anthocyanidin, as in the flavanol–anthocyanin heterodimers, a shift to longer wavelengths (12–16 nm) (**Figure 10**) is observed. The flavanol–anthocyanin heterodimers represent other types of structures thus enhancing bluish colors in plants.

The same four heterodimers as reported by Fossen *et al.*[239] together with afzelechin-(4 → 8)-pelargonidin 3-rutinoside were tentatively identified in extracts of the strawberry cultivar 'Camarosa.'[240] Similarly, (epi)catechin-cyanidin 3,5-diglucoside has been identified in the extract of purple corn, (epi)catechin-peonidin 3-glucoside and (epi)catechin-malvidin 3-glucoside in extract of grape skin, while (epi)catechin-cyanidin

3-glucoside, (epi)gallocatechin-delphinidin and (epi)catechin linked to cyanidin, petunidin, and peonidin have been reported to occur in extracts of various beans (*Phaseolus coccineus*, *P. coccineus*, and *P. vulgaris*).[49,149,240] Putative flavanol–anthocyanin condensation products have also been detected in a concentrate from black currant (*Ribes nigrum*) fruits and in extracts of the fig (*Ficus carica*).[241,242]

3.16.2.6 Anthocyanin-Flavonoid Conjugates – 'Blueing Effect'

In a few cases anthocyanins have been found to be covalently linked to another flavonoid unit, either flavone- or flavonol-glycoside, through a disubstituted dicarboxylic acid (**Table 9**). When the visible maxima in the UV–visible spectra of the anthocyanin–flavone/flavonol conjugates are compared with similar spectra of the same monomeric anthocyanins, bathochromic shifts (11–28 nm) are observed in all cases (**Table 9**). These bathochromic effects reveal intramolecular (and/or intermolecular) association between the anthocyanidin and flavonol units, which produce 'more bluish' colors than expressed by their monomeric counterparts. It is interesting to note that this effect is pronounced regardless of anthocyanidin type (delphinidin, cyanidin, or malvidin). The various conjugates, which have been reported are explained below.

Two anthocyanin–flavone *O*-glycoside conjugates have been isolated from blue-violet flowers of *Eichhornia crassipes* (Pontederiaceae) by Toki *et al.*[243,244] The major *Eichhornia* anthocyanin A has apigenin 7-glucoside attached with an ester bond to one end of malonic acid, and delphinidin 3-gentiobioside linked with a similar bond to the other end. The minor *Eichhornia* anthocyanin B has a similar structure with apigenin 7-glucoside replaced with luteolin 7-glucoside. The three-dimensional structure of these pigments were suggested from the observation of negative Cotton effects at λ_{max} (535 and 547 nm, respectively). The chromophore (delphinidin) and the copigment (flavone) occupy a folding conformation as a binary complex.[243,244] The existence of intramolecular hydrophobic interactions between the chromophoric skeleton and the flavone group was indicated by reduction in the hydration constant when compared with the parent delphinidin 3-glycoside.[246] *Eichhornia* anthocyanin A exhibited remarkable color stability in aqueous solution at mildly acidic pH values.

Recently, a covalently linked anthocyanin–flavone *C*-glycoside has been isolated from purple leaves of *O. triangularis* (Oxalidaceae).[236] This pigment has an apigenin 6-*C*-sophoroside molecule attached with an ester bond to one end of malonic acid, and malvidin 3-*O*-rutinoside-5-*O*-glucoside linked to the other end (**Table 9**). See more about the distribution of the various equilibrium forms of this pigment in Section 3.16.2.4. The existence of other anthocyanin–flavone conjugates has been indicated in salvia, *Salvia patens*,[24] and the blue flower color of garden lupine Russel hybrids (*Lupinus* sp.) has been proposed to be due to copigmentation of the malonylated glucosides of delphinidin and apigenin – possibly linked *in vivo* covalently through a common malonic acid residue.[31]

Two anthocyanin–flavonol conjugates have been isolated from the pale-purple flowers of chive (*Allium schoenoprasum*).[211] These pigments, which constituted more than 65% of the total anthocyanin content, were based on either cyanidin 3-glucoside or cyanidin 3-[3-(acetyl)glucoside] esterified to one end of malonic acid, and kaempferol 3-[2-(glucosyl)glucoside]-7-glucosiduronic acid connected to the other end. The chemical shifts of the anthocyanidin H-4 in the two complexes were 0.3 ppm upfield compared to the same shifts of the monomeric anthocyanins without connection to a flavonol moiety, indicating intramolecular association between the anthocyanidin and flavonol moieties. Two similar anthocyanin–flavonol pigments have been isolated from the blue *Agapanthus* flowers (Agapanthaceae).[245] In these structures the succinate was involved instead of malonate to connect delphinidin 3-[6-(*p*-coumaloyl)glucoside]-7-glucoside to either kaempferol 3,4′-di-glucoside-7-xyloside or kaempferol 3,7,4′-tri-glucoside. An anthocyanin–flavonol conjugate has also been suggested for orchicyanin I, which has been isolated from several orchids.[247] This pigment has been given a hypothetical structure, cyanidin oxalyl-3,5-diglucoside-kaempferol 7-glucoside.[248]

3.16.2.7 Metalloanthocyanins – 'Blueing Effect'

In a few extraordinary cases anthocyanins and flavones/flavonols in complexation with metal ions have been reported to be efficient in producing blue flower colors (**Table 10**). Previous investigations of most of these complexes (commelinin, protocyanin, protodelphin, and hydrangea blue pigment) have recently been reviewed by Takeda[253], while Ellestad[258] similarly has reviewed experimental results obtained over the past 30 years for

Table 9 Anthocyanin–flavonoid conjugates reported from plants

Anthocyanin-flavonoid conjugate	*λ_{max} in the visible region*[a]	*Plant*	*Reference*
(6″-(delphinidin 3-[6″-(glucosyl)glucoside])) (6″-(apigenin 7-glucoside))malonate[M]	548 (538) nm[b]	*Eichhornia crassipes* (water hyacinth) blue-violet flowers	243
(6″-(delphinidin 3-[6″-(glucosyl)glucoside])) (6″-(luteolin 7-glucoside))malonate[m]	548 (537) nm[b]		244
(4′^{V}-(malvidin 3-[6″-(rhamnosyl)glucoside]-5-glucoside)) (6‴-(apigenin 6-*C*-[2″-(glucosyl)glucoside]))malonate[m]	558 (530) nm[c]	*Oxalis triangularis* (purple shamrock) purple leaves	236
(6″-(cyanidin 3-[3″-(acetyl)glucoside])) (4′^{V}-(kaempferol 3-[2″-(glucosyl)glucoside]-7-glucosiduronic acid))malonate[M]	540 (522) nm[c]	*Allium schoenoprasum* (chive) pale-purple flowers	236
(6″-(cyanidin 3-glucoside)) (4′^{V}-(kaempferol 3-[2-(glucosyl)glucoside]-7-glucosiduronic acid))malonate[m]	538 (522) nm[b]		
(6‴-(delphinidin 3-[6″-(*p*-coumaroyl)glucoside]-7-glucoside)) (6′^{V}-(kaempferol 3,4′-diglucoside-7-xyloside))succinate[M]	548 (526) nm[c]	*Agapanthus praecox* sp. *orientalis* (African lily) blue flowers	245
(6‴-(delphinidin 3-[6″-(*p*-coumaroyl)glucoside]-7-glucoside)) (6′^{V}-(kaempferol 3,7,4′-triglucoside))succinate[M]	548 (526) nm[c]		

[a] Values in brackets correspond to data recorded for the monomeric anthocyanin.
[b] In 0.1% HCl–MeOH.
[c] In on-line HPLC solvent.
[M] Major.
[m] Minor.

Table 10 Metalloanthocyanins from plants producing blue flower colors

Anthocyanin	*Composition*	*Color expression*	*Plant*	*Reference(s)*
Commelinin	Delphinidin 3-[6-(*p*-coumaryl)glucoside]-5-[6-(malonyl)glucoside] (malonylawobanin) $\times$ 6, 7-methoxyapigenin 6-*C*-,4′-*O*-diglucoside (flavocommelin) $\times$ 6, $Mg^{2+} \times 2$.	Self-association between the anthocyanin moieties. The blue flower-color development and the stability of the color were explained by metal complexation of the anthocyanin and intermolecular hydrophobic association.	*Commelina communis* (dayflower)	249–251
Protodelphin	Malonylawobanin $\times$ 6, apigenin 7,4′-diglucoside $\times$ 6, $Mg^{2+} \times 2$.	Restricted chiral and structural recognition controlled the entire self-assembly of the metalloanthocyanin and was responsible for the blue flower color.	*Salvia patens* (blue salvia)	24,252,253
Protocyanin	Cyanidin 3-[6-(succinyl)glucoside]-5-glucoside] $\times$ 6, apigenin 7-glucuronide-4′-[6-(malonyl)glucoside] $\times$ 6, Fe^{2+}, Mg^{2+}, Ca^{2+}.	The blue color is caused by LMCT interaction between succinylcyanin and Fe^{3+}.	*Centaurea cyanus* (cornflower)	253,254,255
Meconopsis metalloanthocyanin complex	Cyanidin derivative, two or more equivalents of kaempferol derivatives, 1/6 equivalents of Fe^{3+} and excess of Mg^{2+}.	Ferric ions essential for blue color development by chelating the *ortho*-dihydroxy group of the cyanidin B-ring. The flavonols might stack on both sides of cyanidin. Final composition is not known.	*Meconopsis grandis*, (Himalayan blue poppy)	256
Hydrangea metalloanthocyanin complex	Delphinidin 3-glucoside, caffeoylquinic acid, or *p*-coumaroylquinic acid, Al^{3+}.	Al^{3+} complexes with the *ortho*-dihydroxy group of the delphinidin B-ring and the carboxyl and α-hydroxyl groups of the quinic acid moiety. Final composition is not known.	*Hydrangea macrophylla* (hydrangea) blue sepals	170,253,257

elucidating the self-assembly of the same metalloanthocyanins. This latter review focuses also on the role of the pendant sugars in directing the observed stacking chirality, and end up with speculation on the biological significance of the stacking chirality of the pigments in flower petals and its importance as to the possibility that insects might be sensitive to reflected circularly polarized light from flowers. A short description of the various metalloanthocyanins, which have been reported is as follows.

An anthocyanin with hydroxyl groups in *ortho*-position to each other on the B-ring of the anthocyanidin forms a metal complex with aluminum ion (Al^{3+}), leading to bathochromic and hyperchromic shift effects in the absorption spectrum. An interesting example here is the flower color of *Hydrangea macrophylla*. When grown in neutral to basic soils, hydrangea has its sepals colored red by the anthocyanin, delphinidin-3-glucoside. However, these sepals can become blue when the shrubs are grown in acidic soil. Here the Al^{3+} ion is soluble and can be absorbed and transported to the sepals, where Al^{3+} complexes with the anthocyanidin resulting in the blue color.[257,259,260] Under alkaline conditions the Al^{3+} ion becomes insoluble and the sepals turn out to be red. Sepal color of hydrangeas is, however, not determined by the acidity of the soil alone. It is also affected by copigments, amounts of Al^{3+}, and vacuolar pH.[253,257,260,261] The metal-complex pigment in hydrangeas is suggested to consist of delphinidin 3-glucoside, copigments (5-*O*-caffeoylquinic acid, and/or 5-*O*-*p*-coumaroylquinic acid), and sufficient Al^{3+} in an aqueous solution around pH 4.0, although neither its structure nor composition is completely known. Complexation of Al^{3+} with various synthetic and natural anthocyanins has been investigated in aqueous solutions within the pH range 2–5.[262,263] Shown by UV–visible spectroscopic data the complexes involved not only the colored forms, but also colorless forms of the pigments. ^{1}H NMR analysis confirmed conversion of anthocyanins (dissolved in deuterated methanol) from the red flavylium form into deep-purple quinonoidal forms upon coordination with Al^{3+}.[262] From relaxation kinetics measurements (pH jump), complexation constants of Al^{3+} and several synthetic and natural anthocyanins have been calculated.[262–264]

Commelinin from blue flowers of *Commelina communis* has been found to consist of six molecules of delphinidin 3-[6-(*p*-coumaryl)glucoside]-5-[6-(malonyl)glucoside] (malonylawobanin) copigmented with six flavone (flavocommelin) molecules complexed with two Mg^{2+} ions (**Figure 11**).[251] Self-association was shown to exist between the anthocyanidin moieties. The blue flower-color development and the stability of the color were explained by

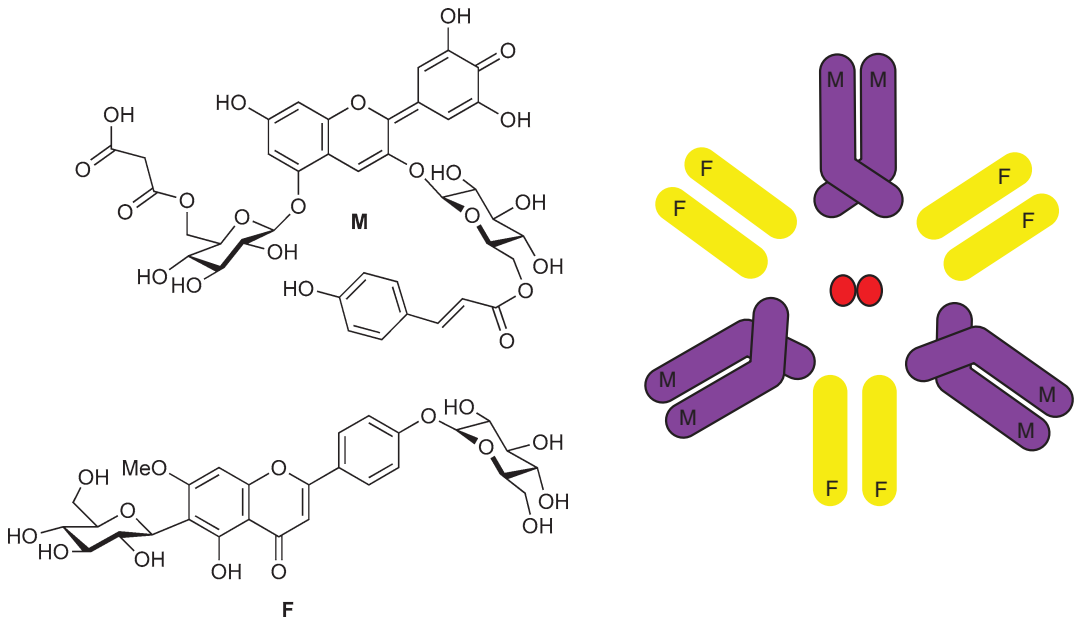


Figure 11 The metalloanthocyanin commelinin responsible for the blue coloration of flowers of *Commelina communis*.[251] Commelinin consists of six molecules of delphinidin 3-[6-(*p*-coumaryl)glucoside]-5-[6-(malonyl)glucoside] (malonylawobanin) (**M**, purple) copigmented with six flavone molecules (**F**, yellow) complexed with two magnesium atoms (red).

metal complexation of the anthocyanidin and intermolecular hydrophobic association. The octaacetate derivative of the flavone part of this molecule has been determined by X-ray diffraction,[265] and in the crystal the flavone molecules were arranged parallel to each other according to the periodicity of the crystal lattice. Intermolecular stacking of the flavone skeletons was, however, not observed, and the hydrophilicity of the glucose moieties was suggested as an important factor governing the self-association of the anthocyanidin moieties.

The structure of protocyanin from cornflower, *Centaurea cyanus*, was suggested to be similar to that of commelinin, composing of six molecules each of apigenin 7-glucuronide-4′-[6-(malonyl)glucoside] and succinylcyanin, complexed with Mg^{2+} and Fe^{3+} ions.[254,255,266,267] It has been proposed that the molecular stacking of the aromatic units in protocyanin prevent hydration of the anthocyanidin nucleus.[268] The blue color of protocyanin was found to be caused by ligand to metal charge transfer (LMCT) interaction between succinylcyanin and Fe^{3+}, which is a different mechanism from that known to operate for commelinin. Recently it has been shown that the additional presence of two Ca^{2+} ions was essential for the formation of protocyanin.[269,270]

Protodelphin, which also is similar to commelinin, has been isolated from flowers of *S. patens*.[24,252] Protodelphin includes six molecules malonylawobanin, two Mg^{2+} ions, and six molecules of another flavone than commelinin, apigenin 7,4′-diglucosides. Takeda *et al.*[24] resynthesized the natural blue pigment *in vitro* by adding the three components together. Mg^{2+} could be substituted *in vitro* by other divalent metal cations (e.g., Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+}).

The blue petal color of the Himalayan blue poppy, *Meconopsis grandis*, has been proposed to be based on a new type of anthocyanin complex containing a cyanidin derivative, two or more equivalents of flavonol (kaempferol) derivatives, 1/6 equivalents of Fe^{3+} and excess of Mg^{2+} ions.[256] The ferric ions chelated the *ortho*-dihydroxy group of the B-ring of the anthocyanidin and were essential for blue color development. The flavonols might be stacked on both sides of cyanidin and stabilized by a copigmentation effect. The experiments indicated that the malonyl group of the anthocyanin was not required for blue color development. The full structure of this complex has not yet been solved, however, it was suggested that it may represent a new type of metal pigment complex similar to that responsible for the blue flower color of hydrangea.[256]

Finally with respect to reports on metalloanthocyanins, the blue petals of *Phacelia campanularia* may be developed by intra- and inter-molecular stackings and the existence of a very small amount of metal ions.[19] The involvement of copigments seems not to be vital in this complex.

3.16.2.8 Copigmentation 'Blueing Effect'

Copigmentation of anthocyanins is one of the most important factors for producing anthocyanin coloration in plants. In this chapter the term *copigment* is used broadly to cover any molecule influencing the anthocyanin chromophore, including self-association of several anthocyanidin nucleus. The exact mechanisms for copigmentation of anthocyanins are poorly understood, as indicated with some examples below. Several models for copigmentation of anthocyanins have been proposed, however, their complex nature demand improved experimental basis in most cases. It is difficult to separate between intra- and intermolecular association (including self-association phenomenon), and the exact orientation of the copigment in relation to the anthocyanin chromophore in the associated complexes is only rarely measured experimentally. In **Figure 12** we have sketched the main associations of the various models, which have been proposed for copigmentation between nonacylated anthocyanins, monoacylated anthocyanins, di-, and polyacylated anthocyanins as well between anthocyanins and other aromatic molecules (intermolecular copigmentation). Copigmentation complexes involving metal ions have been described in Section 3.16.2.7. Structural elements of anthocyanins described in Sections 3.16.2.3–3.16.2.6 have, of course, relevance for the copigmentation phenomenon. The research carried out on copigmentation of anthocyanins by the groups of Professors Tadeo Kondo, Kumi Yoshida, and late Toshio Goto at Nagoya University, Japan has really been outstanding.

3.16.2.8.1 Nature of copigmentation of anthocyanins

The colorless and weakly colored hemiketal and chalcone forms are the prevalent forms of most nonacylated and monoacylated anthocyanins in aqueous solutions in the pH range 2–6. Since this also includes the pH range of most plant vacuoles, plants should expose, based on this fact alone, rather faint anthocyanin coloration in many situations in which this certainly is not the case. Therefore, in plants the colored forms of these

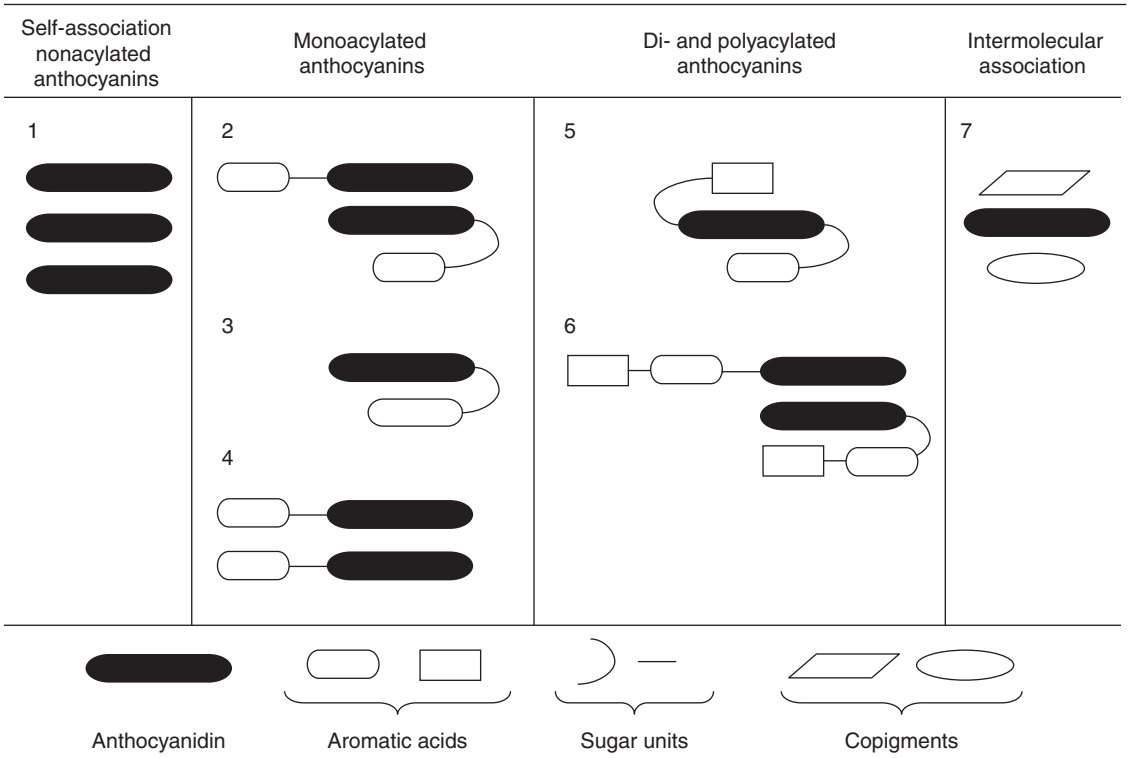


Figure 12 Model sketches showing the main molecular interactions, which have been proposed for copigmentation between nonacylated anthocyanins (self-association) (1), monoacylated anthocyanins (2–4), di- and polyacylated anthocyanins (5–6), and between anthocyanins and other aromatic molecules (intermolecular copigmentation) (7). Both intra- and intermolecular associations as well as self-association contribute to the models presented for monoacylated and di- and polyacylated anthocyanins, respectively.

anthocyanins (flavylium cation and/or quinonoidal bases) must be stabilized to some extent in the cells. When it comes to anthocyanins, which are diacylated or polyacylated with aromatic acids, it has been shown that these do not readily undergo loss of color even at pH > 5.[28,30,271] This suggested a specific role for the aromatic acyl units in stabilization of this type of anthocyanins.

Many anthocyanins are indeed proposed or found to be associated noncovalently with auxiliary molecules (copigments), which both modify their color expression and increase their stability. The copigmentation phenomenon is observed as a bathochromic shift (blueing effect) since the absorption wavelengths around visible λ_{max} are shifted to longer wavelengths compared to similar absorptions of the anthocyanin without copigment. In most cases the color is also intensified (hyperchromic effect). The magnitude of the copigmentation effects has been shown to be influenced by the nature of the anthocyanidin and the copigment, the concentration of the anthocyanin, the copigment:anthocyanin molar ratio, as well as pH and temperature.[147,272–276] Organic acids like benzoic and cinnamic acids, other flavonoid types and anthocyanins themselves, alkaloids, primary metabolites like polysaccharides, peptides and nucleotides, and metals, have all been found capable of inducing copigmentation effects.[277–279] According to Asen[280] the anthocyanin concentration requires to be above 3.5×10^{-5} mol l^{-1} before copigmentation reactions are possible, however, the significance of the concentration depends most probably on the nature of the copigment–anthocyanin complex. The copigmentation complexes are disrupted by dilution, which can be used to distinguish copigmentation phenomenon. Copigmentation of malvidin 3,5-diglucoside appears to be an exothermic process with unfavorable entropy change in the case of cinnamic acids, chlorogenic acid, and (+)-catechin, and a temperature increase will thus favor dissociation of these copigmentation complexes resulting in loss of color.[275,281] At relative low pH values, where the flavylium cation dominates, copigmentation reactions are normally weaker than at pH values where also the quinonoidal equilibrium forms exist.[282]

3.16.2.8.2 Proposed mechanisms

Although strong attractive interactions between π-systems have been known for almost a century, they still do not have a clear explanation.[283] They control such diverse phenomena as the vertical base–base interactions which stabilize the double helical structure of DNA, the tertiary structures of proteins, complexation in porphyrin aggregations, and so forth, and in our case most probably copigmentation of anthocyanins. Several theories have been proposed as mechanisms for copigmentation of anthocyanins. The theory of horizontal stacking, which is based on hydrogen bonding of the hydroxyl and carbonyl groups on the aromatic nuclei (and sugar moieties),[272,282,284] has especially been used to describe intermolecular interactions. In more recent papers this theory has generally been replaced by the proposal of vertical stacking between the anthocyanidin nucleus and copigment(s) (e.g., de Freitas and Mateus; Dangles and Brouillard; Goto *et al.*).[193,281,285] However, the nature of this vertical stacking is still under discussion. Mori *et al.*[19] have recently described the proposed vertical stacking structures and mechanisms of intramolecular charge-transfer suggested for many polyacylated anthocyanins as obscure.

It is generally accepted that vertical associations between copigments and anthocyanins in slightly acidic to neutral solvents or vacuoles protect the anthocyanidin nucleus from hydration, especially in position 2, making the percentage of colorless forms of the anthocyanidins smaller than expected according to the pH. However further details here lead to various models and sometimes opposing proposals. Brouillard and Dangles[146] have discussed copigmentation of anthocyanins in detail. In their review they express that hydrophobic contributions in addition to dispersion forces (especially π–π overlap) between pigment and copigment, provide the major driving force for copigmentation. Da Silva *et al.*[286] has opposed this and have instead proposed the generality of charge transfer (strictly a charge-shift), from the copigment to the flavylium cation, as a major driving force for the stabilization of anthocyanin–copigment complexes. Thus, polyphenols with lower ionization potential (e.g., the flavonol rutin) should serve as stronger copigments than those with higher ionization potentials (e.g., benzoic acids). However, Hunter and Sanders[283] have previously in a more general context reported that π–π interactions are not due to any attractive electronic interaction between the two π-systems, but occur when the attractive interactions between π-electrons and the σ-framework outweigh unfavorable contributions such as π-electron repulsion. Their model implies that the donor–acceptor concept can be misleading when used to describe π–π interactions: It is the properties of the atoms in the regions of molecular contact that control the strength and geometry of interactions, rather than the overall molecular oxidation or reduction potentials.

3.16.2.8.2(i) Nonacylated anthocyanins Nonacylated anthocyanins are normally not related to copigmentation effects. However, anthocyanidin-3,5-diglucosides in their quinonoidal forms at pH 7 have been suggested as being vertically stacked with the A rings on top of each other in a left or right-handed screw axis, supported by data obtained by circular dichroism (CD) and NMR measurements.[285,287–290] This association mechanism, which is called *self-association* (**Figure 12**), is relatively weak in nature. The CD data for the 3,5-diglucosides of cyanidin and pelargonidin did show aberrant properties compared to the other anthocyanidin-3,5-diglucosides examined.[287,291] The vertical stacking mechanism has also been suggested for flavylium cations, however, the CD intensities of the flavylium cations of all six common anthocyanidin-3,5-diglucosides were small compared with those of the quinonoidal bases.[289] After analyses of the 3-glucosides of malvidin, delphinidin, and peonidin in wine-like solutions (12% ethanol, pH 3.6), the existence of anthocyanin self-association and its influence on the apparent hydration constant of the anthocyanins with subsequent modification in the color of the solutions was recognized in all cases.[279] The authors observed that the greater the degree of methoxylation of the anthocyanin B-ring, the greater was the magnitude of the self-association. For malvidin 3-glucosides it has been suggested that the flavylium cation can be stabilized by self-aggregation or by complexation with the chalcone *Z*-form in moderate acidic environment.[292] On the basis of studies on temperature and concentration dependencies of proton chemical shifts of cyanidin 3-[2-(xylosyl)-6-(glucosyl)galactoside] it has been shown that not all nonacylated anthocyanins were protected by self-association.[293]

3.16.2.8.2(ii) Monoacylated anthocyanins Although the effect is normally not as strong as for anthocyanins with several aromatic acyl groups, the presence of one aromatic acyl group hinders hydrolysis of the red flavylium cationic form to colorless hemiketal forms, allowing preferential formation of the blue quinonoidal bases, thereby resulting in pigments remaining colored in mildly acidic or neutral media. Altogether 179

different anthocyanins have been reported to be monoacylated with an aromatic acyl unit, and although just a few of them have been examined with respect to their association mechanisms, three different mechanisms (**Figure 12**) have been proposed related to monoacylated anthocyanins.

The most common mechanism used to explain copigmentation effects of monoacylated anthocyanins with aromatic acyl groups includes an intramolecular copigmentation process bringing together the chromophoric part (anthocyanidin) and the aromatic acyl group which belong to the same anthocyanin in a folded conformation.[293,294] This has been demonstrated by the observation of long-range NOEs in NMR spectra. In some cases the chemical shifts of for instance the cinnamic acid protons, which lie markedly upfield with respect to the analogous methyl cinnamate, have been taken as evidence for copigmentation.

Some monoacylated anthocyanins are more stable than others in neutral aqueous solutions. Yoshida *et al.*[295] have reported that the monoacylated anthocyanin, cyanidin 3-[6-(6-(sinapoyl)glucosyl)glucoside], isolated from the tuber of purple yam *Dioscorea alata*, is unusually stable even in neutral aqueous solutions. The stability is ascribed both to the intramolecular stacking of the sinapoyl unit and the chiral self-association of the anthocyanidin nuclei. The position of the acyl group on the anthocyanin, the position of the sugar moiety, and the length of the sugar spacer were reckoned as relevant factors for good stacking. Two processes of association were also observed for four monoacylated anthocyanins isolated from cell cultures of the wild carrot (*Daucus carota* ssp. *carota*). The formation of strong intramolecular π-complexes that involved both the double bond and aromatic ring of sinapic acid, and intermolecular association of these π-complexes into larger aggregates upon decreasing the temperature and/or increasing the concentration.[293] These aggregates dissociated upon diluting the solution, while the intramolecular π-complexes were disrupted only upon increasing the temperature above 30 °C.

The third mechanism is explained by intermolecular association of two anthocyanins as a dimer.[296,297] The two anthocyanidin nuclei and the two aromatic acyl groups are associated in a type of self-association. Nuclear Overhauser enhancement (NOESY) NMR was used for analyses of petanin (petunidin 3-[6-(4-*E*-*p*-coumaroyl)rhamnosyl)glucoside]-5-glucoside) from blue potatoes in acidified methanolic solutions. Intra- and inter-molecular NOESY cross-peaks were observed, and the corresponding proton–proton distance bounds were used in distance geometry calculations to determine distances between the units of the complex. The orientation of two self-associated petanin aglycones was found to be head-to-tail along both the long and the short aglycone axis, while the two associated coumaroyl groups were found to be head-to-tail along the long coumaroyl group axis. Lack of observed NOESY cross-peaks between protons of the coumaroyl group and the aglycone indicated absence of the intramolecular coumaroyl group–aglycone association, which has been suggested for other acylated anthocyanins. Noncoplanarity between the planes of the benzopyrylium and the phenyl rings was also shown. It was suggested that the dimer might protect the aglycone from hydration, and thereby prevent formation of hemiketals and chalcones. It was indicated that the dimer could be part of a tetramer. Some of the measured associations disappeared when the temperature was increased.

3.16.2.8.2(iii) Di- and polyacylated anthocyanins Since Goto *et al.*[298] in 1982 reported the structure of gentiodelphin from the blue petals of *Gentiana makinoi*, 144 more anthocyanins have been identified containing two or more aromatic acyl units, including several being responsible, at least partly, for blue coloration of petals (**Table 1**). Most di- and polyacylated anthocyanins are remarkably stable in neutral or weakly acidic aqueous solutions,[28,30,271] and both the shifts to more bluish colors and increased anthocyanin stability have been ascribed to two different mechanisms (**Figure 12**). The most common model describes intramolecular copigmentation involving a sandwich-type complex in which two aromatic acyl moieties stack above and below the anthocyanidin nucleus, thus providing protection against nucleophilic water attack.[146,147,219] The second model is based on studies of the dicaffeoyl anthocyanin, phacelianin, isolated from blue petals of *P. campanularia*.[19] It was suggested that the pigment chromophores of phacelianin might stack intermolecularly in an anticlockwise manner in the blue-colored vacuoles. At the same time the caffeoyl residues were suggested to stack intramolecularly in the anthocyanidin nucleus. The authors also indicated that small amounts of metal ions might be involved in the blue coloration of *Phacelia* petals.

In a detailed review by Honda and Saito[219] progress in the chemistry of polyacylated anthocyanins as flower color pigments has been outlined. It was recognized that both the blueing effect and stabilization of flower

colors depended on the number of aromatic acids presented in the polyacylated anthocyanins. After classification of the polyacylated anthocyanins into seven types by the substitution pattern of the acyl functions, it was concluded that anthocyanins with the aromatic acyl groups in glycosyls in both the 7- and 3′-positions were considered to make the most stable colors in the flowers. This conclusion was also supported through studies of the diacylated anthocyanin gentiodelphin, a pigment from the blue flower of *G. makinoi*, and its two mono-deacyl derivatives.[299] The acyl residue in the 3′-position on the B-ring contributed more to blue color development than the acyl residue in the 7-position on the A-ring.

Red-purple colors in the flowers of orchids have been shown to be derived from altogether 15 cyanidin and peonidin glycosides, with aromatic acylated sugars attached both at the 7- and 3′-positions.[300–307] Intramolecular associations of these planar molecules provided stable colors without the need for any copigment or metal cation.[308] Figueiredo *et al.*[308] proposed that the glycosyl-acyl 'side chains' attached to both positions 3′ and 7 of the chromophore favored a better overlap and stronger interaction with the π-system of the central chromophore, than what was observed for other acylated anthocyanins. They supported the assessment by molecular calculations, which gave minimum energy conformation for a 'sandwich' type with the 3′-chain folded 'over' and the 7-chain folded 'under' the chromophore. Similar acylation of glycosyls in anthocyanidin 7- and 3′-positions has also been reported for anthocyanins in Commelinaceae,[218] Compositae,[309] Liliaceae,[310] and Rhamnaceae.[37] The final example in this context concerns three acylated delphinidin 3,7,3′,5′-tetraglucosides from berries of two *Dianella* species (Liliaceae). These pigments showed exceptional blueness at *in vivo* pH values due to effective intramolecular copigmentation involving *p*-coumaryl-glucose units (GC) at the aglycone 7-, 3′-, and 5′-positions.[10] Evidences showed that the effectiveness of the copigmentation could be ranked as 3′,5′-GC > 7-GC > 3-GC.

The Morning Glory flowers (*Ipomoea/Pharbitis nil*) exist in a wide range of color forms. There is a good correlation between scarlet flower color and the occurrence of pelargonidin derivatives.[176,302] Lu *et al.*[311] have shown that the flower color of *P. nil* gradually shifts to more bluish colors with increasing numbers of caffeic acid residues in the polyacylated pelargonidin glycosides. Blue flower colors, attractive to bee pollinators, are generally based on delphinidin (**Table 1**). However, some exceptional cases are found, for instance in *I. tricolor* and *P. nil*, where the blue flower colors are caused by the 'Heavenly Blue Anthocyanin,' HBA, pigment.[208,307,312] HBA, a peonidin 3-sophoroside-5-glucoside with three caffeylglucosyl residues,[20] is among the largest monomeric anthocyanins, which has been isolated. Yoshida *et al.*[313] have shown that the color change of *I. tricolor*, while flowering, was due to vacuolar pH changes from 6.6 to 7.7, at which the quinonoidal base anion of HBA was formed and stabilized by intramolecular stacking. HBA was actually found to be more stable at physiological pH (pH 7.5) than in strong acidic or weakly acidic solutions.[137] Anthocyanins are normally considered to be more stable in strong acidic than neutral aqueous media. It has also been reported that polyacylated anthocyanins like HBA are more tolerant to UV-B than nonacylated anthocyanins.[314] These results suggest that petal anthocyanins might play some biological role in protecting petal tissues from solar radiation.

3.16.2.8.2(iv) Intermolecular associations Intermolecular copigmentation describes the interaction between the anthocyanidin nucleus and another colorless molecule (copigment), which is not bound covalently to the anthocyanin molecule.[315] This mechanism is proposed to play a major role in the stabilization of anthocyanins lacking acyl moieties. When considering the anthocyanin content in fruits and berries in **Table 3**, it is clear that very few of them contain anthocyanins with aromatic acylation. In these cases intermolecular interaction is the most probable means of copigmentation. An electronic delocalization on a planar system seems to be required for a molecule to act as a copigment. No evidence of the existence of interactions taking place between a copigment and the colorless forms of anthocyanins has been reported, which suggests π–π overlap (vertical stacking) between aromatic residues in the intermolecular associations.[146] Intermolecular copigmentation interactions are specific in nature, and by varying the copigment a variety of colors may be produced. Some examples involving intermolecular copigmentation in flowers are explained below. Intermolecular copigmentation is very important for the metalloanthocyanin complexes, which have been reported (Section 3.16.2.7)

The blue flower color of *Ceanothus papillosus* (Rhamnaceae) has been proposed to arise from a supramolecular complex of high stoichiometry including anthocyanins and the flavonol kaempferol 3-[2-(xylosyl)rhamnoside] (Bloor,[37] Tabell 1). This copigmentation effect appeared to be quite specific, and did not occur to the same

extent with other more common flavonols. An extraordinary, long wavelength visible absorption maximum at 680 nm was produced, which conferred additional blueness. The blue color of the petals of the blue marguerite daisy (*Felicia amelloides*) has been found to arise from copigmentation between delphinidin 3-[2-(rhamnosyl)glucoside]-7-[6-(malonyl)glucoside] and the flavone *C*-glycoside swertisin 2″-*O*-rhamnoside-4′-*O*-glucoside (Bloor,[17] Tabell 1). The visible spectrum of the upper epidermal peel showed the characteristic triple maxima shape of many violet or blue flowers with specific absorption maxima at 550, 585, and 632 nm. The flavones were present at high concentration in the petal; the molar ratio of flavone to anthocyanin was estimated to be at least 18:1, and the anthocyanin concentration in the petal sap was *c.* 1.8 mmol l^{-1}.

3.16.2.8.2(v) Cis (Z)- and trans (E)-configuration of cinnamic acids Around 20 anthocyanins acylated with hydroxycinnamic acids have been reported to occur in both the *cis* (*Z*)- and *trans* (*E*)-configuration, however, this number is most probably somewhat underestimated due to lack of proper determination of this configuration during structure elucidation of the cinnamic acids. George *et al.*[316] have compared the pairs of 3-[6-(*E*/Z-*p*-coumaryl)glucoside]-5-[6-(malonyl)glucosides] of malvidin and delphinidin. They observed that the *cis* isomers exhibited ϵ values about 1.5 times greater than the *trans* isomers, in both pairs. It was calculated that the *cis* forms were less prone to undergo hydration reactions forming the colorless anthocyanin forms. On the basis of computed structures the more co-planar arrangement allowed by the *cis* isomers was postulated as the rationale supporting the enhanced color stability.[316] When considering the color effect of this type of intramolecular copigmentation *in vivo*, one should bear in mind that the *trans* isomer seems to predominate, and that the conversion between the two isomers is rare. When Yoshida *et al.*[314] studied the *E,Z*-isomerization reaction and stability of several types of acylated anthocyanins under the influence of UV irradiation, their interest was focused on the reason why isomerization reaction of some acyl residues was prevented in living plant cells. They concluded that the stability of anthocyanins under irradiation highly depended on molecular stacking. They proposed that light energy absorbed by cinnamoyl residues might be transferred to the anthocyanidin nucleus and released without any isomerization reaction or degradation of pigments. Thus, the flower color may be stable for a long time under strong solar radiation.

3.16.2.8.2(vi) Sugar moieties The anthocyanins contain sugar(s) that contribute to hydrogen bondings, which constrain the possibilities for orientations of the anthocyanin–copigment complex. The crucial role of the hydrogen bondings of the sugar moieties in studies of anthocyanin copigmentation is mostly overlooked due to experimental limitations. The different effects of D- and L-glucose in experiments related to the metalloanthocyanin, protodelphin are highlighted in Section 3.16.2.7. This blue pigment consists of the anthocyanin malonylawobanin (M), the flavone apigenin 7,4′-di-*O*-β-D-glucoside (F), and Mg^{2+} ions; $M_6F_6Mg_2$.[252] Mixing of malonylawobanin, with synthetic apigenin 7,4′-di-*O*-β-D-glucoside and apigenin 7,4′-di-*O*–β-L-glucoside yielded protodelphin containing only the D-glucosyl, while the L-glucosyl was completely excluded. Three flavone molecules in protodelphin were associated to form a helical structure (minus form), similar to a propeller with three blades. They were bound at the pivot point by a strong hydrogen-bonding network among the hydroxyl groups at C-2 and C-3 of the 4′-*O*-β-D-glucosides. Two sets of this helical flavone structure fit closely into the vacant space formed from the metal complex of six molecules of M and two Mg^{2+} ions. Replacement of D- by L-glucosyl at the 4′-OH position of apigenin inverted the helical structure of the three associated flavones (plus form), with the consequence that it did not fit into the vacant space. The authors concluded that restricted chiral and structural recognition controlled the entire self-assembly of the metalloanthocyanin, and was responsible for the blue flower color.

3.16.3 Anthocyanin Localization in Plant Tissue

Several decades ago microscopic examinations have shown a compartmentalized and sharply delimited location of anthocyanins and other flavonoids.[317] With bi-colored roses for instance, anthocyanins are invariably concentrated on the inner and carotenoids on the outer side of the petal. In many flowers, flavonoid colors are enriched in epidermal cells while adjacent sub-epidermal cells are colorless. However, the shoot meristems of many angiosperms consist of three layers of cells, designated L1, L2, and L3 cells.[318] The L1 cells give rise to

the epidermal layer, the L2 cells to the sub-epidermis, and the L3 cells to the internal tissues. Each of the cell layers in petals generally originates from one of these three layers, and the layers of anthocyanin-producing cells differ among species; L2 cells are used in *Petunia* and *Antirrhinum*, and all three of them (L1–L3 cells) in *Pharbites*.[319] In leaves, anthocyanins may be found in the upper epidermis, lower epidermis, palisade mesophyll, spongy mesophyll, and trichomes, either in one cell type or in almost any combination of them.[320]

It is generally accepted that anthocyanins as other flavonoids are synthesized on the cytoplasmic surface of the endoplasmic reticulum membrane.[321,322] Although the biosynthetic pathways for flavonoids and their regulation have been closely studied (see Section 3.16.5.1 and references therein), the mechanisms for anthocyanin accumulation in the cells are more indecisive.

3.16.3.1 From *Anthocyanoplasts* to *Anthocyanic Vacuolar Inclusions*

Inside cells, the anthocyanins are most often found dissolved uniformly in vacuolar solutions. However, Pecket and Small[323] listed 26 dicotyledon and 7 monocotyledon families in which the presence of pigmented bodies, which they called *anthocyanoplasts*, had been noted. These spherical bodies were described as membrane-bound organelles that provide intense coloration in the vacuoles of mature plant cells. Such pigmented bodies have been described as 'blue spherules' in epidermal rose petal cells,[324] 'blue crystals' in *Consolida ambigua* petals,[325] 'crystals,' and 'ball-like structures' in *Matthiola incana* petals,[326] 'red crystals' in mung bean hypocotyl,[327] and as 'intravacuolar spherical bodies' in *Polygonum cuspidatum* seedlings.[328] Similar structures were found to occur in the leaves of various Brassicaceae,[329,330] in grapes,[331] and in the tubers of *Ipomoea batatas*.[332] It was then indicated that these globular inclusions may be protein matrices,[332,333] and that they possess neither a membrane boundary nor an internal structure.[333–335] Recent anatomical observations of anthocyanin-rich cells in apple skin carried out by light and electron microscopy showed that the skin with fully developed red color had more layers of anthocyanin-containing epidermal cells than those of green skin.[336] The anthocyanins were frequently found in clusters or in agglomerations that were round in shape in the epidermal cells of the red skin. There was no distinct envelope membrane on the anthocyanin granule in the vacuoles. The anthocyanins seemed to be synthesized around the tonoplast and condensed on the inward side of the vacuole.

However, not much was documented about the chemical nature and the functional significance of these inclusions in petal cells before Markham *et al.*[337] reported intensively colored intravascular bodies in petals of lisianthus (*Eustoma grandiflorum*) and blue-gray carnations (*Dianthus caryophyllus*), which they named AVIs. The AVIs occurred predominantly in the adaxial epidermal cells, and their presence was shown to have major influence on flower color by enhancing both intensity and blueness. This latter effect was especially dramatic in blue-gray carnations where the normally pink 3,5-diglucoside and 3-glucoside of pelargonidin produced a blue-gray coloration. In contrast, epidermal cells of pink carnation petals lacked AVIs but contained vacuoles that were homogeneously pigmented pink with the same pelargonidin derivatives. The absolute level of anthocyanins in the blue-gray tissue as measured spectrophotometrically, was four times that in the pink tissue. This much higher level of anthocyanins in the blue-gray petals was associated almost entirely with the AVIs as little color was seen in the surrounding vacuolar solution. The presence of AVIs thus appeared to be the predominant factor that accounted for the observed color difference.

In lisianthus, the presence of large AVIs produced marked color intensification in the inner zone of the petal by concentrating anthocyanins above levels that would be possible in vacuolar solutions.[337] The electron microscopy studies on lisianthus epidermal tissue failed to detect a membrane boundary in AVI bodies, and the isolated AVIs were shown to have a protein matrix. Bound to this matrix were four cyanidin- and delphinidin acylated 3,5-diglycosides, which were relatively minor anthocyanins in the whole petal extracts where acylated delphinidin triglycosides predominated. Flavonol glycosides were not found to be bound to the AVIs. The specificity of this 'anthocyanin trapping' was confirmed by the presence in the surrounding vacuolar solution of only delphinidin triglycosides, accompanied by the full range of flavonol glycosides. 'Trapped anthocyanins' were shown to differ from solution anthocyanins only in that they lack a terminal rhamnose on the 3-linked galactose. On a closer look by light and electron microscopy on the epidermal cells of different regions of the lisianthus petal, Zhang *et al.*[338] observed that the AVIs occurred on three different forms: vesicle-like, rod-like, and irregular shaped. Again no membrane encompassing the AVIs was observed, however, the AVI itself consisted of membranous and thread structures throughout. The results strongly suggested the existence of

mass transport for anthocyanins from biosynthetic sites in the cytoplasm to the central vacuole. The anthocyanins were found to accumulate first as vesicle-like bodies in the cytoplasm, which themselves were contained in prevacuolar compartments (PVCs). The vesicle-like bodies seemed to be transported into the central vacuole through the merging of the PVCs and the central vacuole in the epidermal cells.[338]

Analogous 'anthocyanin trapping' as reported for lisianthus has also been reported by Conn *et al.*,[339] who found that AVIs in two lines of grapevine (*Vitis vinifera*) cell suspension culture appeared as dark red-to-purple spheres of various sizes in vacuoles due to their interaction with anthocyanins. Compared with the total anthocyanin profile, the profile of the AVI-bound anthocyanins showed an increase of approximately 28–29% in acylated (*p*-coumarylated) anthocyanins in both lines. At the subcellular level in maize (*Zea mays*) it has recently been found that light induces an alteration in the way the anthocyanins were distributed within vacuolar compartments.[340] In sorghum (*S. bicolor*) 3-deoxyanthocyanidins accumulate as inclusions in leaf cells under fungal attack, and function as phytoalexins by inhibiting infection in a site-specific response.[341–343] The cytological response commences when colorless 3-deoxyanthocyanidin inclusions (0.1 mm diameter) accumulate exclusively in those leaf cells, which are under fungal attack. These inclusions become orange to red in color and accumulate at sites of physical contact between host and pathogen. Dark red inclusions of up to 20 mm appear by coalescence. The progressive color shift of the 3-deoxyanthocyanidins from faint orange to dark red during defense response is most likely caused by changes in local, subcellular pH. It has been shown that the 3-deoxyanthocyanidin, luteolinidin, when self-organized as pigmented inclusions, mediates disruption of plant and fungal plasma membranes as well as reconstructed bilayer liposomes.

3.16.4 Colors of Aurones and Chalcones

3.16.4.1 Introduction

Carotenoids play the principal role in yellow to orange floral pigmentation.[1,344] Anthocyanins in their natural environment (vacuoles, AVIs) do not provide yellow coloring of plants. Among the flavonoids involved in yellow to orange plant colors are the aurones and chalcones, and to some degree flavonols. The chalcones and aurones have, however, limited distribution in the plant kingdom as colorants. Some striking examples include yellow and red quinochalcones from safflower (*Carthamus tinctorius*, Asteraceae), which have been used as textiles dyes throughout history. Likewise are colored kamalachalcones the pigment basis of kamala, an orange-colored exudate of *Mallotus philippensis* (Euphorbiaceae) fruits used as dye to produce yellow to orange colors on wool, mohair, and silk. It has further been found that the only reported colored plant nectar in nature, which has been revealed in three bird/gecko-pollinated plant species in Mauritius, are based on a red-colored aurone (see Section 3.16.6). Chalcones and aurones are nevertheless best known for providing yellow flower colors to some popular ornamental plants in family Asteraceae and snapdragon (*Antirrhinum majus*, Scrophulariaceae). They are also found in the bark, wood, leaves, and seedlings of a variety of plants.[201]

The chalcones, and the closely related dihyrochalcones, are unique among the flavonoids by lacking a central heterocyclic C-ring (**Figure 13**). Furthermore, their nomenclature is based on a unique numbering system having the primed positions on the A-ring, as opposed to the B-ring in all cyclic flavonoids. Altogether, around 700 different chalcone structures have been reported, including aglycones, glycosides, chalcone conjugates, quinochalcones, chalcone dimers, and oligomers, as well as chalcone Diels–Alder adducts.[6,345] In addition nearly 250 dihydrochalcones have been identified. Both numbers of structures and structural complexity of new chalcone and dihydrochalcone aglycones have advanced considerably during the last decade. However, the occurrence of complicated glycosidic patterns among the chalcones are lacking compared to those of other flavonoid groups such as flavonols and flavones.[346] Most chalcone monoglycosides are *β*-glucopyranosides, and only a few disaccharides are encountered with any frequency. The majority of the chalcone glucosides found in nature are based on just a few aglycones such as isoliquiritigenin (4,2′,4′-trihydroxychalcone), chalconaringenin (4,2′,4′,6′-tetrahydroxychalcone) and okanin (3,4,2′,3′,4′-pentahydroxychalcone). Around 25 chalcone glycosides are acylated with either aromatic or aliphatic acyl groups.

The name 'aurone' comes indeed from the Latin word 'aurum' (= gold) because of the golden-yellow colors.[347] The systematic name of the skeleton is 2-benzylidene-3(2*H*)-benzofuran-3-one, also called

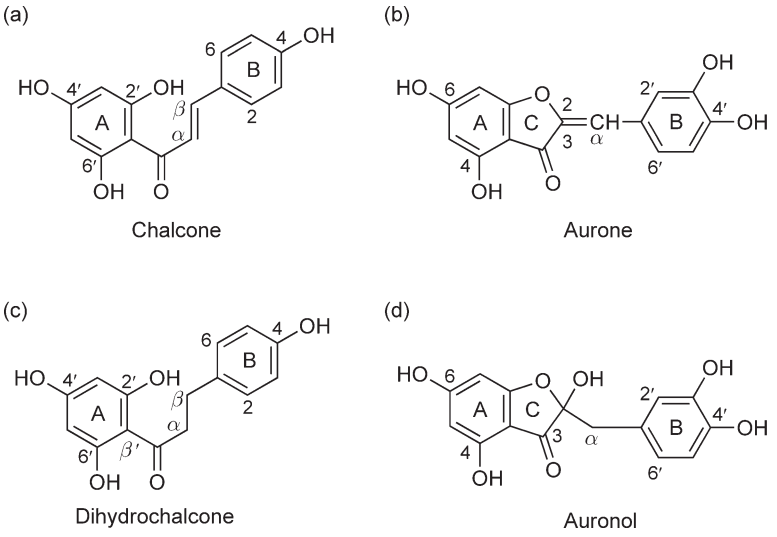


Figure 13 Structure examples, ring labeling, and atom numbering of chalcones (a), aurones (b), dihydrochalcones (c), and auronols (d).

2-benzylidenecoumaran-3-one. The compounds in the subgroup, auronols, are based on the 2-hydroxy-2-benzylcoumaran-3-one skeleton (**Figure 13**). Positions in the aurones are identified using the 'normal' flavonoid nomenclature, however, the 4-position in aurones is biosynthetically equivalent to the 5-position in 'normal' flavonoids. There are two possible geometric isomers of aurones with respect to the C2–Cα double bond. The aurones comprise the smallest group in the flavonoid family including just above 100 different structures as aglycones, aglycone dimers, and glycosides.[6,345] The majority of the aurone glycosides are β-glucopyranosides or α-rhamnopyranosides, and acylation has just been found in some maritimetin 6-*O*-glucosides.

The chalcones and aurones often occur together in plants. They have been referred to as the 'anthochlor' pigments because of their alkali-induced bathochromic shifts.[346] These specific shifts are and have been important tools in their structural elucidation.[347,348] Chalcones can be converted into aurones in the presence of weak base and atmospheric oxygen. Conversion of chalcones into aurones by enzyme extracts from plant tissue has also been demonstrated.[349] The main focus on chalcones and aurones in this section will be on their role as plant pigments, and examples of their natural presence will be given. Some examples of their UV-absorbing character in pollination is presented in Section 3.16.6.1.

3.16.4.2 Occurrences and Colors

The UV–visible spectra of chalcones and aurones are characterized by intense Band I and diminished Band II absorptions.[347] For chalcones the most intense band usually occurs in the range of 340–390 in methanolic solutions, although chalcones lacking B-ring oxygenation may have their Band I absorptions at considerably shorter wavelengths. Band II is usually a minor peak in the 220–270 nm region. As with flavones and flavonols, increased oxygenation of both the A- and B-rings usually results predominantly in bathochromic shifts of Band I (**Table 11**). Going from 2′,4′-dihydroxychalcone ($\lambda_{max} = 345$ nm) via 4,2′,4′-trihydroxychalcone ($\lambda_{max} = 369$ nm) to 3,4,2′,4′-tetrahydroxy ($\lambda_{max} = 379$ nm), considerable bathochromic shifts caused by extra hydroxyl groups on the B-ring are experienced. The same effect is revealed for the A-ring when comparing absorption spectra of 4,4′-trihydroxychalcone ($\lambda_{max} = 348$ nm) and 4,2′,4′-trihydroxychalcone ($\lambda_{max} = 369$ nm). With respect to the A-ring, an interesting effect is observed when comparing absorption spectra of 3,4,2′,4′-tetrahydroxychalcone ($\lambda_{max} = 379$ nm) with those of 3,4,2′,4′,6′-pentahydroxychalcone ($\lambda_{max} = 378$ nm) and 3,4,2′,3′,4′- pentahydroxychalcone ($\lambda_{max} = 384$ nm). The former with the phloroglucinol pattern (2′,4′,6′-trihydroxy-) shows no effect on the wavelength of the absorption maximum, while the latter with the

Table 11 Visible λ_{max} values in absorption spectra of selected chalcones dissolved in methanol or ethanol

Compound	λ_{max} *(nm)*	*Reference*
Chalcone		
2′,4′-dihydroxy	345[a]	350
2′,4′-dihydroxy-4-methoxy	362[a]	350
4′,4′-dihydroxy	348[a]	350
4,2′,4′-trihydroxy (isoliquiritegenin)	369	351
4,2′,4′,6′-tetrahydroxy (chalconaringenin, isosalipurpol)	369	351
4′-*O*-glucoside	368	351
3,4,2′,4′-tetrahydroxy (butein)	379	352
4′-*O*-glucoside	380	353
4′-*O*-malonylglucoside	380	353
4′-*O*-sophoroside	377	353
4′-*O*-malonylsophoroside	379	353
3,4,2′,3′,4′-pentahydroxy (okanin)	384[b]	354
4′-*O*-[2″-(caffeoyl)-6″-(acetyl)glucoside]	380	355
4′-*O*-[2″-(caffeoyl)-6″-(coumaroyl)glucoside]	380	355
3,2′,3′,4′-tetrahydroxy-4-methoxy (methylokanin)		
4′-*O*-[6″-(coumaroyl)glucoside]	373	355
4′-*O*-[6″-(acetyl)glucoside]	372	355
4′-*O*-[2″-(caffeoyl)-6″-(coumaroyl)glucoside]	360	355
3,4,2′,4′,6′-pentahydroxy	378	351
4′-*O*-glucoside	378	351
Chalcone dimer		
Kamalachalcone A	344	356
Kamalachalcone B	345	356
Quinochalcone		
2,2,6-tri-isoprenyl-cyclohex-5-ene-1,3-dione (munchiwarin)	422	357
Quinochalcone dimer		
Precarthamin	406	358
Anhydrosafflor B	410	359
Carthamin	519	360
Dihydrochalcone		
4,2′,4′,6′-Tetrahydroxy-4,3′-dimethoxy	284	361

[a] In EtOH.
[b] In 98% EtOH.

pyrogallol pattern (2′,3′,4′-trihydroxy-) has a small bathochromic shift effect compared to the absorption spectrum of 3,4,2′,4′-tetrahydroxychalcone. Glycosyl substitution on the aglycones shows no or very weak hypsochromic shift effects on the spectra.

The aurones produce 'stronger' yellow colors than chalcones due to their absorbances at longer wavelengths. The majority of aurones show four absorption maxima.[362] Two (sometimes one) of these absorption bands are usually found in the 370–430 nm region due to resonance contribution of the carbonyl group with the different conjugated systems in the aurone molecules, although some of the simpler aurones absorbs at much shorter wavelengths (**Table 12**). The effect of hydroxyl- and methoxyl groups of aurones on UV–visible absorption spectra have been described in detail by Geissmann and Harborne.[362] The following hydroxyl groups give bathochromic effects: 7-OH, 2′-OH, 4′-OH in the presence of 6-OH, and 3′-OH in the presence of 4′-OH. While the presence of a 4-OH or 3′-OH, or a 5-OH in a 6-hydroxyaurone, does not change the spectra appreciably, a 6-OH has a pronounced hypsochromic effect. An *O*-glycosyl in the 6-position causes a small bathochromic effect (**Table 12**) compared to the spectra of analogous 6-hydroxyaurones. Introduction of *O*-glycosyls in other hydroxyl positions of aurones, has only minute effects on the spectra.

Table 12 Visible λ_{max} values in absorption spectra of selected aurones dissolved in methanol or ethanol

Compound	*λ_{max} (nm)*	*Reference*
Aurone		
4-Hydroxy	389[a]	362
4-Methoxy	387[a]	362
6-Hydroxy	344[a]	362
2′-Hydroxy	402[a]	362
3′-Hydroxy	381[a]	362
4′-Hydroxy	405[a]	362
5,6-Dihydroxy	347[a]	362
6,4′-Dihydroxy (hispidol)	388	347
4,6,4′-Trihydroxy	393	351
6,3′,4′-Trihydroxy (sulfuretin)	399[a]	351
6-*O*-Glucoside	404[a]	351
6-di-*O*-Glucoside	402	363
4,6,3′,4′-Tetrahydroxy (aureusidin)	398	364
4-*O*-Glucoside (cernuoside)	404	364
6-*O*-glucoside (auresin)	407	364
6-*O*-rhamnoside	404	365
4,6-di-*O*-glucoside	411	364
5,6,3′,4′-tetrahydroxy	395[a]	362
6,7,3′,4′-tetrahydroxy (maritimetin)	412	347
6-*O*-glucoside (maritimein)	419[a]	362
7-*O*-glucoside	404	366
6-*O*-[6-(coumaroyl)glucoside]	412	366
6-*O*-[6-(acetyl)glucoside]	411	366
6,3′,4′-dihydroxy-7-methoxy	406[a]	362
6-*O*-glucoside (letopsin)	411[a]	362
7,3′,4′-trihydroxy-6-methoxy	413[a]	362
6,7,3′,4′-tetramethoxy	404[a]	362
6-hydroxy-7,3′,4′-trimethoxy	401[a]	362
7-hydroxy-6,3′,4′-trimethoxy	411[a]	362
4,6,3′,4′,5′-pentahydroxy (bracetin)	403[a]	350
4-*O*-glucoside	409[a]	350
6-*O*-glucoside	408[a]	350
Aurone dimer		
2×(4,6,3′,4′-Tetrahydroxy)(C5′ → C5)(aulacomniumbiaureudsidin)	411	367
4,6,3,4-Tetrahydroxy(C5′ → C6)5,7,3,4-tetrahydroxyflavanone(capylopusaurone)	402	368
Auronol		
2,4,6,3′,4′,5′-Hexahydroxy (amaronol A)	333	369
2,4,6,3′,5′-Hexahydroxy-4′-methoxy (amaronol B)	335	369

[a] In EtOH.

Owing to some loss of conjugation, the dihydrochalcones and auronols have as expected absorbances at shorter wavelengths than corresponding chalcones and aurones. Lusianin (4,2′,4′,6′-tetrahydroxy-4,3′-dimethoxydihydrochalcone) isolated from the orchid *Lusia volucris*, shows UV absorption peaks at 205, 215, and 284 nm in methanol,[361] while the pale yellow amaronols A and B (2,4,6,3′,4′,5′-hexahydroxyauronol and 2,4,6,3′,5′-pentahydroxy-4′-methoxyauronol) isolated from the bark of *Pseudolarix amabilis*, have similar UV spectra with absorption peaks at 212, 288, and 333/335 nm in methanol.[369]

The real *in vivo* colors based on chalcones and aurones are of course influenced by the matrix of these pigments, including intermolecular associations with solvent and other molecules in their surroundings, as well as physical parameters. However, the impact of these factors has hardly been treated in papers reporting anthochlor colors. Some examples where plant colors are related to chalcone or aurone structures are as follows.

3.16.4.2.1 Chalcone and aurone monomers

Chalcones and aurones are best known for their provision of yellow flower colors to some popular ornamental plants such as *Dahlia*, *Coreopsis*, *Cosmos* (Asteraceae) and snapdragon (*A. majus*, Scrophulariaceae). Yellow coloration of *Dahlia variabilis* flowers is mainly due to the presence of 4′-malonylglucosides of the 6′-deoxychalcones isoliquiritigenin and butein (3,4,2′,4′-tetrahydroxychalcone),[370–372] while accumulation of butein 4′-glucoside and the aurone sulfuretin 6-glucoside are responsible for the yellow petal color of some *Cosmos* species.[373,374] In 1957 Shimokoriyama isolated two chalcones, okanin and okanin-4′-glucoside, from flowers of *Coreopsis tinctoria*.[354] Chalcones were indeed found to occur in floral tissue of all the 46 *Coreopsis* species of North America.[375] Recently, altogether 11 flavonoids, including several chalcones, flavanones, and flavonols, were reported to occur in flower extracts of *C. tinctoria*.[376] The yellow snapdragon is one of the best-known sources for aurones. Small amounts of the 4′-*O*-glucosides of chalconaringenin and 3,4,2′,4′,6′-pentahydroxychalcone serve as direct precursors of the 6-glucosides of the aurones aureusidin (4,6,3′,4′-tetrahydroxyaurone) and bracteatin (4,6,3′,4′,5′-pentahydroxyaurone), which are the main pigments responsible for the yellow flower color.[377–383] Yellow snapdragon has become the model species for the study of aurone biosynthesis.[379,384] Aureusidin 6-*O*-glucoside is also the main yellow pigment in the orange petals of *Mussaenda hirsutissima* (Rubiaceae),[364] where it co-exists with aureusidin 4,6-di-*O*-glucoside and aureusidin 4-*O*-glucoside (cernuoside). Okanin derivatives are in general typical for species in the genus *Bidens* (Asteraceae), where additionally butein, sulfuretin (6,3′,4′-trihydroxyaurone) and maritimetin (6,7,3′,4′-tetrahydroxyaurone) derivatives are reported.[355,366,385,386] Isoliquiritigenin glycosides generate yellow flower colors in Leguminosae, however not exclusively.[387,388] The glycosidic patterns of these chalcones are rather simple compared to the glycosyl moieties of other flavonoid groups found in this family.

3.16.4.2.2 Chalcone and aurone dimers

Dimeric and oligomeric structures of chalcones are most commonly found in family Ochnaceae, and in particular from species in genera *Lophira* and *Ochna*, but they are also represented in Anacardiaceae.[345] Two chalcone dimers with unusual structures, kamalachalcone A and B (**Figure 14**) have among other compounds been isolated from kamala,[356] an orange-colored exudate from grandular trichomes on the surface of the fruits of *M. philippensis* (Euphorbiaceae).

Kamala has been used as a dye to produce yellow to orange colors on wool, mohair, and silk. Kamalachalcone A has been described as a yellow powder, while kamalachalcone B has been described as an orange powder,[356] however, they have approximately the same maximum wavelengths, 344 and 345 nm, respectively, measured in methanolic solutions. In kamalachalcone B an acetophenone was connected with the chalcone moiety through a methylene group. We may speculate in that the orange color of kamalachalcone B powder is due to intramolecular association between the acetophenone with the dimeric structure, or alternatively that the methylene–acetophenone group improves the chromophore by increasing the planarity

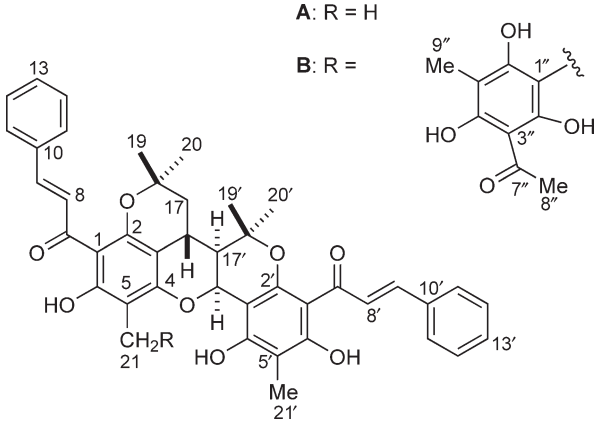


Figure 14 Structures of kamalachalcone **A** and **B** isolated from exudate of the fruits of *Mallotus phillippensis*.[356]

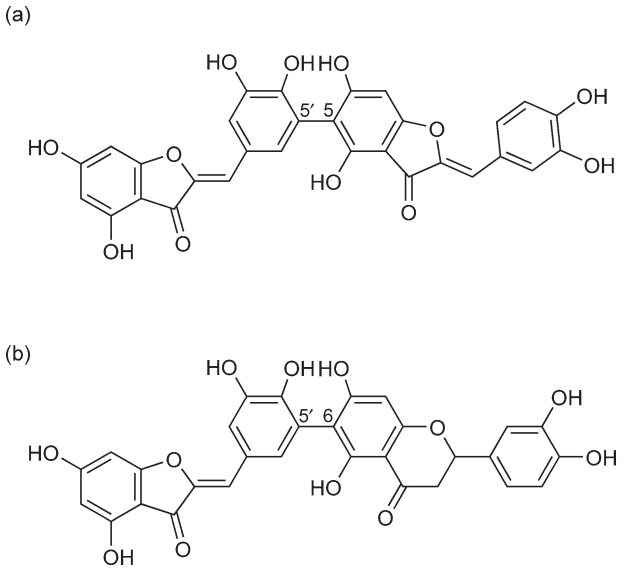


Figure 15 Structures of aulacomniumbiaureusidin (a) (biaurone) isolated from *Aulacomnium* species and campylopusaurone (b) (auroneflavanone biflavonoid) isolated from the mosses *Campylopus clavatus* and *Campylopus holomitrium*.[367,368]

within the dimeric structure. More recently acetone extracts of kamala, yielded two intense yellow kamalachalcones (C and D), which are characterized by fused benzopyran rings.[389]

The first biaurone found in nature was isolated from the gametophytes of the mosses *Aulacomnium androgynum* and *A. palustre*.[367] The dimer of two aureusidin (4,6,3′,4′-tetrahydroxyaurone) molecules with a C–C bond from C-5′ → C-5 constitutes the bright-green pigment named aulacomniumbiaureusidin (**Figure 15**(**a**)). The chromophore of this dimer ($\lambda_{max} = 411$ nm) in methanol was improved compared to the chromophore of its monomeric units (both aureusidin), which had λ_{max} at 398 nm in methanol. In comparison, the bright-yellow aurone heterodimer (**Figure 15**(**b**)) isolated from the moss *Campylopus* sp., gave rise to an absorption maximum at 402 nm in methanol.[368] In this aureusidin–eridodictyol heterodimer the flavanone moiety (eriodictyol with $\lambda_{max} = 324$ nm in methanol) did not influence the absorption maximum of the aureusidin moiety at all.

3.16.4.2.3 Quinochalcones

Quinochalcones is a small group consisting of eight aglycones and ten *C*-glycosides (both monomers and dimers).[345] In the field of plant colors they have a pronounced position as major pigments in the flowers of safflower (*C. tinctorius*, Asteraceae). The botanical genus name *Carthamus* derives from the Arabic verb *qurtum* 'dye,' in reference to the usage of safflower flowers for textile dyeing, while the botanical species name *tinctorius* is an adjective corresponding to the noun *tinctor* 'dyer.' The flowers has been used for coloring textiles in ancient times in Egypt, Persia, India, and China, while the use of this dye in cotton textiles started in Europe in the eighteenth century. In food the flowers sometimes serve as a color substitute for saffron, and recently the dye from the extract has been used in cosmetics. The flowers are used for treatment of various diseases, especially in Chinese medicine.

The flowers of safflower (*C. tinctorius*, Asteraceae) are yellow just after flowering and changes gradually to red within some days. Altogether 11 different quinochalcones have been identified in the flowers.[359,390] The color transition is mainly due to the enzymatic conversion of yellow quinochalcones (precarthamin and anhydrosafflor yellow B) into a red quinochalcone, carthamin, which accumulates in mature petals.[359,391–393] The conversion from yellow precarthamin ($\lambda_{max} = 406$ nm) and anhydrosafflor yellow B ($\lambda_{max} = 410$ nm) into red carthamin ($\lambda_{max} = 519$ nm) involves the removal of a carboxyl or a glucosyl moiety, respectively

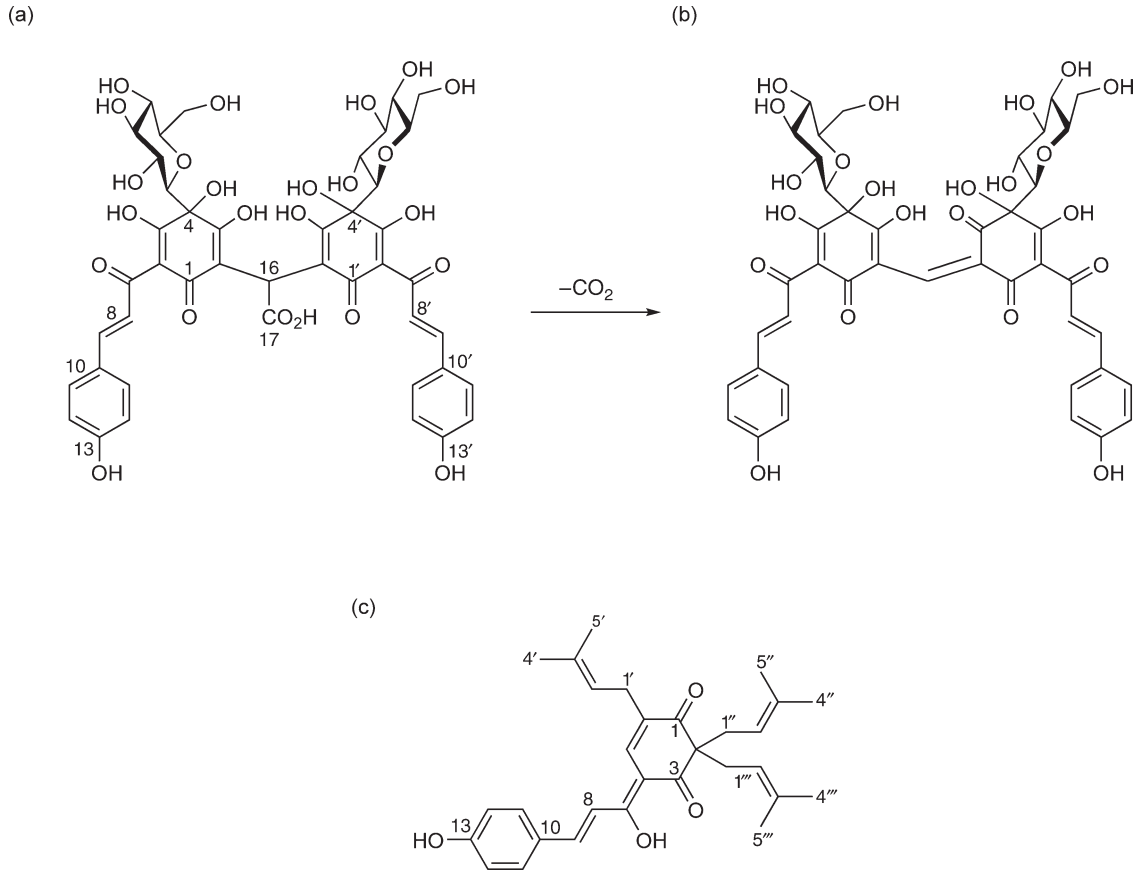


Figure 16 Structures of precarthamin (a) and carthamin (b) isolated from flowers of safflower.[359,391] When the carboxyl group is enzymatically removed from (a), the pigment changes color from yellow to red. (c) Represents the extraordinary planar structure of the orange chalcone, munchiwarin, isolated from roots of *Crotalaria trifoliastrum* (Leguminosae).[390]

(**Figure 16**). The red color of carthamin is caused by the double bond created at the bridging carbon between the two quinochalcone monomers, which increases the chromophore.

Another interesting colored quinochalcones include munchiwarin (**Figure 16**) isolated from roots of *Crotalaria trifoliastrum* (Leguminosae).[357] This orange pigment ($\lambda_{max} = 422$ nm in methanol) is the only known natural product possessing a 2,2,6-tri-isoprenyl-cyclohex-5-ene-1,3-dione ring system. The crystal structure shows a long conjugated system from the phenol to a keto-stabilized resorcinol group with three isopentenyl units attached. The conjugated unit is rather planar with a mean deviation from the best plane of only 0.09 Å; hence the orange color of the substance. The planarity of the structure is additionally supported by a hydrogen bond between the hydroxy group at the C-7 position and the carbonyl at position 3.

3.16.5 Biosynthesis of Flavonoids

The biosynthesis of flavonoids is most probably the best characterized pathway leading to any group of secondary metabolites (see Chapter 6.18). Floral pigmentation including anthocyanins has been used to help elucidation of fundamental genetic principles since the days of Mendel, and knowledge acquired through understanding of the various steps in flavonoid biosynthesis is used today in genetic engineering to expand the floriculture gene pool. Flower colors are among the key determinants influencing consumer choices, and new varieties have commercial value. A brief overview of the biosynthetic pathway leading to chalcones, aurones, anthocyanins, and

3-deoxyanthocyanidins (**Figure 17**), including a few examples of modern molecular bioengineering in the field, will be given in this section. An excellent detailed description of the various steps in biosynthesis of flavonoids, and advances in molecular biology and biotechnology of flavonoids, have been given by Davies and Schwinn.[394] Other relevant reviews covering biosynthesis of anthocyanins and other plant pigments[322,395–400] and manipulation of flower colors[401–403] expose important progress made within these fields in recent years.

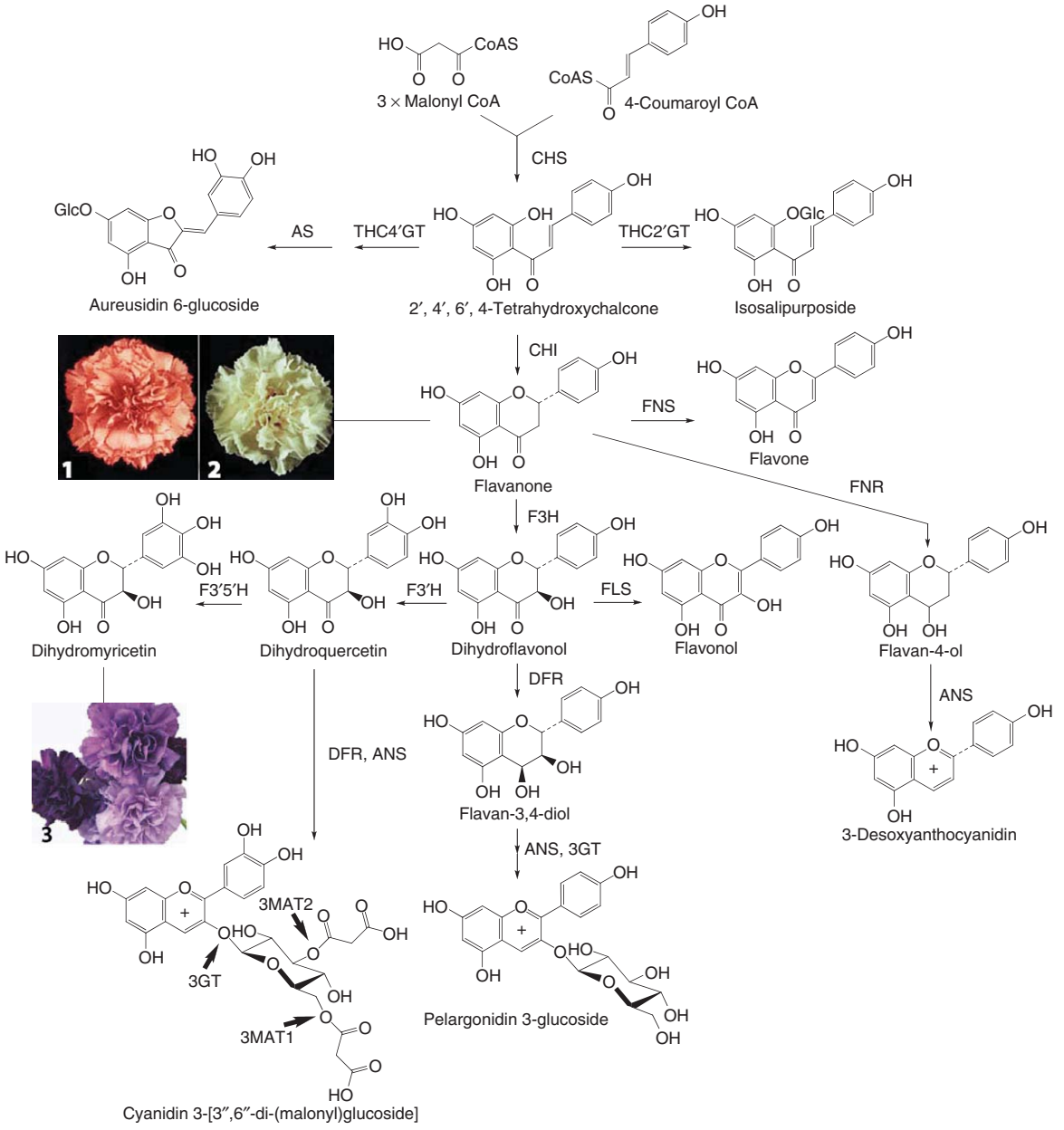


Figure 17 General biosynthesis scheme, which leads to most of the flavonoid classes. The pictures of red **(1)** and **(2)** white carnations show the difference in color achieved when the activity of the flavanone 3β-hydroxylase (F3H) has been inhibited. Photos courtesy: A. Zuker; T. Tzfira; H. Ben-Meir; M. Ovadis; E. Shklarman; H. Itzhaki; G. Forkman; S. Martens; I. Neta-Sharir; D. Weiss; A. Vainstein, *Mol. Breed.* **2002**, *9*, 33–41. Overexpression of the flavonoid 3′,5′-hydroxylase (F3′5′H enzyme) produced purple to violet transgenic flower colors due to the induction of the synthesis of delphinidin derivatives **(3)**. Photos courtesy: Y. Tanaka; A. Ohmiya, *Curr. Opin. Biotechnol.* **2008**, *19*, 190–197. See text in Section 3.16.5.1 for explanations of the abbreviations used for the enzymes involved in the various steps.

3.16.5.1 Biosynthetic Steps Leading to Chalcones, Aurones, Anthocyanins, and 3-Deoxyanthocyanins

It is generally accepted that flavonoids are synthesized in the cytosol, and the involved enzymes are connected to the membrane of the endoplasmic reticulum.[321,322] The pathway starts with formation of the C_{15} backbone by chalcone synthase (CHS), which catalyzes synthesis of 4,2′,4′,6′-tetrahydroxychalcone (THC) from one molecule of coumaroyl-CoA and three molecules of malonyl CoA (**Figure 17**). This polyketide synthase displays high flexibility with respect to various starters.[397]

Chalcones are precursor in biosynthesis of aurones (**Figure 17**), which is catalyzed by a homologue of plant polyphenol oxidase.[379] The final biosynthetic mechanism for forming aurones from chalcones has recently been clarified.[382,404] It has been revealed that the chalcones in snapdragon (*A. majus*) flowers are 4′-*O*-glucosylated in the cytoplasm by chalcone 4′-*O*-glucosyltransferase and then transported to the vacuole. Within the vacuoles they are enzymatically converted into aurone 6-*O*-glucosides by an aurone synthase (AS), which in snapdragon has the name aureusidin synthase (AUS). This metabolic pathway is unique, because for all other flavonoids the carbon backbone is completed before transport to the vacuole.

In the biosynthesis of anthocyanins (and other flavonoid groups) the unstable chalcone THC, is converted stereospecifically into the flavanone, (2*S*)-naringenin, by chalcone isomerase (CHI) (**Figure 17**). This was the first enzyme involved in flavonoid biosynthesis to be described,[405] and is today one of the best-characterized enzymes involved in plant secondary metabolism. In the absence of CHI the isomerization of THC occurs spontaneously, yielding a racemic mixture of (2*R*/2*S*)-naringenin.[395] (2*S*)-flavanones are *in vivo* the exclusive substrates of the downstream enzymes of the flavonoid pathway, and thus, CHI guarantees the efficient formation of biologically active (2*S*)-flavonoid isomers. Mutants lacking CHI activity accumulate only trace amounts of flavonoids.[406] Flavanones are converted into dihydroflavonols by hydroxylation in position 3 catalyzed by flavanone 3β-hydroxylase (*F3H* or *FHT*). This enzyme is classified as a soluble 2-oxoglutarate-dependent dioxygenase according to its requirement of the co-factors 2-oxyoglutarate, molecular oxygen, ferrous iron (Fe(II)), and ascorbate. Dihydroflavonols (dihydrokaempferol, dihydroquercetin, and dihydromyricetin) are reduced to flavan-3,4-diols/leucoanthocyanidins (leucopelargonidin, leucocyanidin, or leucodelphinidin, respectively) by dihydroflavonol 4-reductase (DFR) in the course of anthocyanidin and/or catechin biosynthesis. The various DFR has different substrate specificity, which finally affects the type of anthocyanidins produced by each species (more about substrate specificity of petunia DFR in Section 3.16.5.2). Anthocyanidin synthase (ANS) catalyzes the final oxidation of a colorless flavan-3,4-diol (leucoanthocyanidin) to an anthocyanidin. Similar to F3Hs and flavonol synthases (FLSs), ANSs belong also to the 2-oxoglutarate-dependent oxygenases. The formed anthocyanidin is relatively unstable. It is readily glucosylated by glucosyltransferase (GT), and in some cases acylated by aromatic/aliphatic acyltransferase (AT) and/or methylated by methyltransferase (MT).

The biosynthesis of 3-deoxyanthocyanins is on the other hand thought to occur through the formation of flavan-4-ols by the activity of flavanone 4-reductase (FNR) and finally through the action of ANS (**Figure 17**). Recent studies on FNR of recombinant *S. cardinalis* showed that this enzyme both has DFR and FNR activity,[407] which is in accordance with the ability of the recombinant DFR enzymes of *Malus domestica*, *Pyrus communis*, and *Z. mays* to produce 3-deoxyflavonoids.[408,409]

3.16.5.2 New Anthocyanin Flower Colors by Molecular Bioengineering

Classical breeding methods including continuous crossing/selection and in some cases mutations, have been used to develop new cultivars with flowers varying in both colors and patterns. However, most species lack a particular color due to the absence of a biosynthetic gene or because of the substrate specificity of an enzyme in the pathway. The search for the blue rose is just one example. Over the past two decades knowledge about flower coloration at the biochemical and molecular level has made it possible to achieve new varieties by genetic engineering. Today virtually all the genes that encode the enzymes of anthocyanin biosynthesis have been isolated. By introducing new genes in plants encoding for novel enzyme activities and transcription factors or inactivation of endogenous genes used in anthocyanin biosynthesis, new varieties with modified flower colors and plant coloration have been created. A few examples are depicted below.

Pelargonidin glycosides are not found in petunias (see cross references in Andersen and Jordheim[4]), which is the main reason for the absence of orange- to nearly scarlet-colored *Petunia* species in nature. The enzyme DFR in *Petunia* has strict substrate specificity, and is unable to convert dihydrokaempferol into the substrate for pelargonidin, namely leucopelargonidin. An orange petunia was, however, created two decades ago,[410] and represents the first product of successful manipulation of flower color by gene technology. This was achieved by producing the maize DFR enzyme, which was able to convert dihydrokaempferol in a white petunia variety accumulating this substrate.

Flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H), which are members of the cytochrome P450 family, play key roles in the determination of the substitution pattern of the B-rings of the anthocyanidins. These enzymes have generally broad substrate specificity, and are able to catalyze hydroxylation of flavanones, dihydroflavonols, flavonols, and flavones. F3′H is necessary for the synthesis of 3′-hydroxylated anthocyanidins (e.g., cyanidin), while F3′5′H participates in the synthesis of 3′5′-hydroxylated anthocyanidins (e.g., delphinidin). Thus, will the development of blue roses, carnations, chrysanthemums, or tulips by molecular breeding, include introduction of F3′5′H activity for production of delphinidin derivatives in the petals, which are not produced in native flowers. Florigene Ltd. (Australia) and Suntory Ltd. (Japan) have successfully developed transgenic violet carnations by introduction of petunia F3′5′H and DFR genes into a DFR-deficient white carnation.[411] The petals of these carnations predominantly contained delphinidin derivatives. Under the name Moondust they were the first transgenic floricultural crop to be sold. However, blue to violet flower colors are known to depend on more factors than just their content of delphinidin derivatives (see Section 3.16.2). After a closer look on Moondust, Fukui *et al.*[412] concluded that the following reasons accounted for the bluish hue of the transgenic carnation flowers: (1) accumulation of the delphinidin-type anthocyanins as a result of flavonoid 3′,5′-hydroxylase gene expression, (2) the presence of a flavone derivative as a strong copigment, and (3) an estimated relatively high vacuolar pH of 5.5.

3.16.6 Functions of Flavonoid Pigments in Plants

In nature, flavonoid pigments are involved in a wide range of known and most probably unknown functions. They are integrated into the plant's strategies for survival by providing pigmentation for flowers, fruits, and seeds to attract pollinators and seed dispersers (Sections 3.16.6.1 and 3.16.6.2), serving protective roles as shields against abiotic stresses like UV–B radiation, temperature variation, mineral stress, and so on, and active defensive roles against pathogens, insects, and herbivores (Section 3.16.6.3) (see Chapter 4.08). The functions of flavonoid pigments in leaves, seedlings, roots, and stems are, however, less obvious than those reported for flowers and fruits. Understandably, most plant physiologists and ecologists are more inclined to consider the physiological and ecological roles of the pigments than to concern themselves with their chemical nature, as a number of excellent reviews and papers in this field attest (e.g., Chalker-Scott; Harborne and Grayer; Simmonds; Gould; Gould and Lister; and references therein).[413–417] In a recent paper data have been reported which suggest that specific polyacylated anthocyanins in flower petals can screen harmful UV–B efficiently.[418] See more about the mechanism in Section 3.16.2.8.

3.16.6.1 Flavonoid Pigments in Pollination

The importance of flavonoid pigments in flowers for attracting bees, butterflies, birds, and other animals to ensure pollination is well established.[414] The pollination syndrome hypothesis (e.g., Vogel, Faegri and van der Pijl, Fenster *et al.*)[419–421] has provided an important conceptual framework for how plants and pollinators interact. It has been assumed that pollinators are the primary selective agents influencing factors like flower color, while transitions to different colors represent adaptation to different suites of pollinators. However, in recent years alternative interpretations have also been suggested, including the possibilities that flower color transitions are nonadaptive, or reflect natural selection on pleiotropic effects of genetic variants that affect flower color.[422]

Bird pollination (ornithophily) appears to have evolved independently in a variety of plant genera, usually from bee pollination.[423,424] Ornithophilous flowers, which are typically red or orange, have elongated floral tubes, reduced floral limbs, exserted stigmas, and copious dilute nectar. Some phenotypic convergences in plants with this pollination syndrome have recently been reviewed by Cronk and Ojeda.[425] Thus far, only one gene, flavonoid-3′-hydroxylase (F3′H) in morning glories (*Ipomoea*/*Pharbitis*) has been linked with shifts to

ornithophily.[426] The ancestral color in *Ipomoea* is blue or purple based on cyanidin and peonidin glycosides (see cross references in Andersen and Jordheim[4]), and together with other traits this indicates an adaptation to bee pollination.[427] Blue and mauve flower colors, attractive to bee pollinators, are generally based on delphinidin, petunidin, or malvidin, however, the blue or purple colors of the peonidin and cyanidin derivatives of *Ipomoea* spp. are most probably caused by the intramolecular association with caffeic acid residues in these polyacylated molecules. In one clade including *I. quamoclit* and five other species, there has been a shift to red flowers containing pelargonidin derivatives implying hummingbird pollination. The F3′H gene, which is required for the production of cyanidin rather than pelargonidin, has been downregulated in the *I. quamoclit* lineage.

In the genus *Mimulus* (monkeyflowers) two closely related species, *M. lewisii* and *M. cardinalis* display great differences in floral characteristics. The former is pollinated mainly by bumblebees and has pink flowers, higher proportion of pelargonidin derivatives, nectar guides, and the dominant allele *YUP*, which prevents carotenoid deposition. The latter is associated with hummingbird pollination, red flowers, higher proportion of pelargonidin derivatives and the recessive allele *yup*, which allows carotenoid deposition.[415,428,429] When the *yup* allele of *M. cardinalis* is introgressed into the *M. lewisii* background, hummingbird visitation increases dramatically, whereas bee visitation is considerably lowered.[430] This suggests that an adaptive divergence in pollination syndrome can be initiated by a major change in flower color alone.[425] However, a recent study indicates that the evolutionarily recent appearance of red-pigmented flowers in the 'yellow monkeyflower' section of *Mimulus* was not associated with a transition to 'red-flower' pollinators such as hummingbirds.[431]

Although floral traits including color have been associated with particular pollination mechanisms as far back as in the work of the Neapolitan botanist Federico Delpino (1833–1905), the following example may illustrate some difficulties in the process of revealing exact pollination mechanisms, even today. In 1998 Olesen *et al.*[432] published an article entitled *Mauritian Red Nectar Remains a Mystery*. They reported that the unique presence of scarlet-red nectar in three bird-pollinated plant species in Mauritius was based on an aurone (**Figure 18**, **1**). The authors stated that the three endogenous species, *Nesocodon mauritianus* (Campanulaceae),

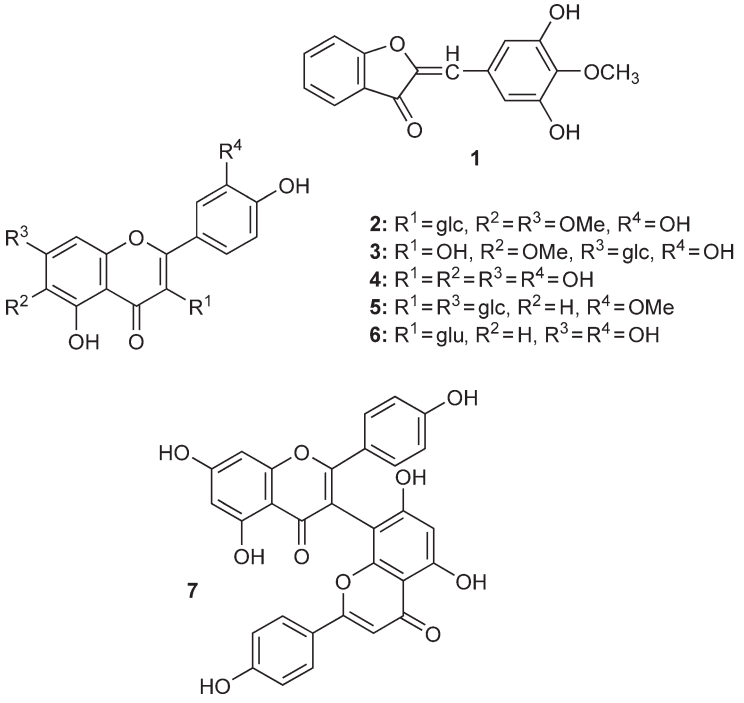


Figure 18 Structures of 3′,5′-dihydroxy-4′-methoxyaurone (**1**), 6,7-dimethoxy-3′,4′,5-trihydroxyflavone-3-*O*-glucoside (**2**), 6-methoxy-3′,4′,3,5-tetrahydroxyflavone 7-*O*-glucoside (**3**), 3,5,6,7,3′,4′-hexahydroxyflavone (**4**), isorhamnetin (5,3′-dihydroxy-4′-methoxyflavone) 3,7-diglucoside (**5**), quercetin (5,7,3′,4′-tetrahydroxyflavone) 3-*O*-glucuronide (**6**), and biapigenin (dimeric flavone) (**7**).

Trochetia boutoniana, and *T. blackburniana* (Malvaceae), were the only ones in the world that produce a colored nectar. They envisaged three explanations for the evolution of the unique coloration: (1) the pigment was an attractant for an endemic recently extinct original pollinator; (2) the red color was an honest signal to pollinators, thereby improving their foraging efficiency and consequently providing an advantage to the red nectar containing plant species; and (3) the red pigment was associated with a deterrent against nectar robbers. Olesen *et al.*[432] considered explanations (2) and (3) as unlikely.

A year later, by using knowledge-based computational structure–activity relationship models, explanation (3) was on the other hand supported.[433] In this paper it was hypothesized that the aurone responsible for the uniquely red nectar functions as a repellant of nectar-robbing or herbivorous mammalian species. Recently, a new dimension was brought into this mystery.[434] It was reported that at least two of the three red nectar-producing species were visited and pollinated by endemic lizards (**Figure 19**). Experimental evidence reports that *Phelsuma* geckos preferred colored over clear nectar in artificial flowers. Hansen *et al.* expressed that colored nectar could additionally function as an honest signal that allows pollinators to assert the presence and judge the size of a reward prior to flower visitation, and to adjust their behavior accordingly, leading to increased pollinator efficiency according to explanation (2). It was reported by Olesen *et al.*[432] that the nectar's pH was as high as 9.2 (the known pH range of all species is 3–10), and when placed in acid, the red nectar turned yellow. No other chemical data were supplied with the pigment. The red pigment of the nectar is a tri-*O*-substituted aurone with a substitution pattern, which has not been reported for any aurone before (**Figure 18**, **1**). The author's suggest that the red color of the pigment is due to the anionic form of the aurone. We suggest that this form is achieved by deprotonation of the phenolic groups under the relative basic conditions in the nectar, and will thus have an increased chromophore giving red color instead of the yellow color of the aurone under acidic conditions.

3.16.6.1.1 Nectar guides

Many flowers contain visible dots, stripes, and patterns. The foxglove (*Digitalis purpurea*), for instance, has a pink bell-shaped corolla pigmented with cyanidin and peonidin 3,5-diglucosides. Higher concentrations of the same pigments inside the bell makes patterns, called nectar guides or honey guides, which helps pollinating insects to the stigma and style. Not surprisingly, the nectar guides in general are displayed predominately on the exposed 'facial' surface of the flower, where the pollinator makes its landing. Other flowers have UV patterns invisible to humans but visible to insects, again with the purpose of guiding pollinating insects. In radial flowers, the UV-absorbing pigments responsible for the UV demarcation are often concentrated in the center of the flower. The petals of the black-eyed Susan (*Rudbeckia hirta*, Compositae) was found to contain three flavonols

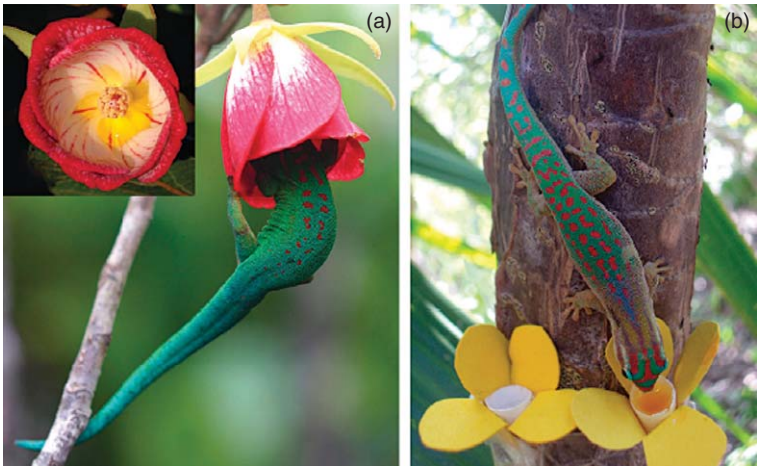


Figure 19 *Phelsuma* geckos and colored nectar. (a) *Phelsuma cepediana* nectar-feeding at *Trochetia blackburniana*. (b) *Phelsuma ornate* choosing between clear and colored nectar at experimental flowers. Photos courtesy: D. M. Hansen; K. Beer; C. B. Muller, *Biol. Lett.* **2006**, *2*, 165–168.

(6,7-dimethoxy-3′,4′,5-trihydroxyflavone-3-*O*-glucoside, 6-methoxy-3′,4′,3,5-tetrahydroxyflavone 7-*O*-glucoside, and 3,5,6,7,3′,4′-hexahydroxyflavone) (**Figure 18**, **2**–**4**) with restricted distribution to the petal bases.[435] These compounds, which showed intense spectral absorptions from 340 to 380 nm, created petal zones of orientation value to the pollinating insect. This was the first time ultraviolet (UV) absorption in a nectar guide was interpreted in chemical terms. The first reports in the field of chemical basis for nectar guides have been reviewed thoroughly by Harborne and Grayer.[414] More recently it has been reported that the corolla of *Brassica rapa* (Cruciferae) has an UV-absorbing zone in its center, containing isorhamnetin 3,7-diglucoside (**Figure 18**, **5**).[436] This flavonol is present at 13-fold greater amounts in the basal parts of the petals than in the apical regions, which is presumed to contribute to the visual attractiveness of *B. rapa* flowers to insect pollinators. The flower of *Hypericum calycinum*, which appears uniformly yellow to humans, bears a UV pattern, presumably visible to insects. Two categories of pigments, flavonoids (the flavonol quercetin 3-*O*-glucuronide and the dimeric flavone, biapigenin) (**Figure 18**, **6**–**7**) and smaller amounts of dearomatized isoprenylated phloroglucinols, were responsible for the UV demarcations of this flower.[437]

The chalcones and aurones, as other flavonoids, absorb strong UV-light giving pattern in petals, which would otherwise be seen as dull or translucent by insect eyes. In wild-type *A. majus*, aurones are produced only in the inner epidermis, accumulating in the hinge (face) region of the petal lobe and in two stripes within the throat. This yellow region is surrounded by magenta anthocyanins, which provide the majority of the color in the petal but are usually absent from the two aurone-producing regions.[438] The pattern of aurone and anthocyanin pigmentation is thought to provide a nectar guide for pollinating bumblebees, and their biosynthesis has evolutionary importance concerning plant–pollinator interaction. Several studies have reported that the loci regulates yellow flower coloration of *A. majus*.[382,438–440] In *Helianthus* (Asteraceae) honey guides in some species resulted from UV absorbance by the chalcone coreopsin and the aurone sulfurein, whereas in *H. annuus* the honey guides resulted from absorbance by quercetin 3- and 7-glucosides.[441] However, the occurrence of yellow flavonoids in other flowers may not be directly correlated with the presence of UV nectar guides. A detailed study of the distribution of chalcones in *Coreopsis bigelovii* flowers revealed that these pigments were present in epidermal cells on both upper and lower surfaces.[441]

In general in plants, nectar guides are prominent in those flowers, which are pollinated by bees. It has also been suggested that carnivore plants use contrasting stripes or UV marks on their pitchers to lure insects. However, after recent experiments with visual signaling it was emphasized that insect traps did not need to sport contrasting colors to be attractive.[442] It might be sufficient that the pitchers are just different from their background.

The chemical basis of UV–visible absorptions in nectar guides has remained remarkably unexplained in many plants. One reason for this is related to analytical difficulties when small amount of material is available. However, the field of nectar guides and pollination may be seen from other more complex angles. Insects and vertebrates have been shown to have multiple classes of photoreceptors that contribute to vision, for example, the honeybee has trichromatic vision based on UV, blue, and green photoreceptors.[443] Perception of color will thus require the integration of information from all the primary receptors, however, the UV receptors have often been singled out for special consideration. It has also been postulated, although related to carotenoids, that insects may sense patterns of polarized light as reflected from the flowers and, in fact, use this as a signal for pollination of a given plant.[444] Finally we will draw attention to AVIs, which are discussed in Section 3.16.3.1. The occurrence of AVIs in many flowers is most probably of vital importance for the presence of nectar guides.

3.16.6.2 Flavonoid Pigments in Seed Dispersal

Herbivorous and frugivorous animals rely on color for identification of edible tissues and for judgment of vegetable ripeness. A gardener will thus experience that yellow- or amber-colored mutants of red raspberries mostly are ignored by birds. The distinctive colors of many fruits and ‘fruit-similar’ structures are derived from anthocyanins, which render the fruit attractive for seed dispersing animals. Other classes of flavonoids contribute occasionally to yellow, orange, red, or brown colors in fruits (see Harborne[445]). The anthocyanins may be present throughout the fruit (European bilberries, *Vaccinium myrtillus*), while in other cases it is limited to the skin (lowbush blueberries, *V. angustifolium*) and juice (blood orange, *Citrus sinensis*). Anthocyanin colors are as for other flavonoid colors primarily determined genetically, although environmental factors such as pH,

temperature, light conditions, and availability of nutrition can have effect on pigment composition and on the final hue of the fruit.

The qualitative and quantitative anthocyanin content of most of the common fruits used in the human diet is now determined (**Table 3**), however, some variation in content between different varieties and cultivars are very common. On the basis of principal component analysis of the content of 15 different anthocyanins in 30 samples of bilberries (*V. myrtillus*) of various origins, a clear separation between a group composed of Norwegian and Swedish berries and a group of berries with Italian or Romanian origin was revealed.[446] Cyanidin glycosides were slightly better represented in all the samples of the first group, while delphinidin glycosides were better represented in the latter. Recently, the variation of the content of the same 15 anthocyanins in berries from 179 individual bilberry plants in 20 populations on a south–north axis of about 1000 km in Finland were analyzed.[447] A significantly lower content of the total anthocyanins was observed in the berries of the southern region compared to those in the central and northern regions. Differences in the proportions of anthocyanins were also observed.

Burns and Dalen[448] postulated that red-orange autumn foliage of Canadian shrub species would accentuate the conspicuousness of black-colored fruits to birds. Experimental manipulation of fruit and background foliage colors confirmed that the black-red contrast was indeed an effective enhancer of fruit-removal rates by avian dispersers. Although fruit colors are traditionally viewed as an adaptation to seed dispersers, the selective pressure on fruit coloration are not well understood.[449] Most bird species exhibit inconsistent and transient color choices with high variability within and between individuals. Cazetta *et al.*[449] suggest that fruit colors differ between habitats because fruit colors that have strong chromatic contrasts against background can increase plants' reproductive success, particularly under variable light conditions.

In the Gymnospermae, anthocyanin pigmentation is most commonly observed in the reproductive structures (the strobili or cones),[450] which is quite interesting since anthocyanins are mainly associated with flower color in the Angiospermae. From flowers and cones of species in the Pinaceae, variation between simple 3-glucosides of cyanidin and delphinidin and their methylated analogues, peonidin, petunidin, and malvidin (**Table 5**), have been reported.[451–453] These pigments are the only reports of methylated anthocyanins being found outside of the Angiospermae. For some Pinaceae species (e.g., Norway spruce, *Picea abies*) two types of clones were found.[452,453] One type contained methylated anthocyanidins (peonidin and petunidin), while the other did not, which suggested that the methylating genes have evolved recently. Anthocyanins have been reported to play various roles in protecting plants (Section 3.16.6). Several observations suggest that anthocyanins may lack this protective function in conifer cones. First, the anthocyanins are restricted to the outer cone scales. Second, the anthocyanins are only present for a short period of time early in development and disappear once the pollen and egg cells are formed.[453]

In the Gymnospermae family Podocarpaceae, anthocyanins are as well located mainly in seed-bearing structures, where they have a comparable role to angiosperm fruit pigments. A typical ripe female ovule of white pine (*Dacrycarpus dacrydioides,* Podocarpacaeae) consists of an orange-red receptacle, atop a bluish seed and two dark-blue undeveloped ovules, which must be among the most outstanding anthocyanin-colored structures in nature. It gives the appearance of an angiosperm fruit, and the anthocyanins obviously render the structure more readily detectable and aid in animal dispersion of the seed. While pelargonidin 3-neohesperidoside (2-(rha)glc) was the major pigment in the receptacles, cyanidin 3-glucoside and delphinidin 3-glucoside constituted the major anthocyanins in the seeds and undeveloped ovules.[454] Since the undeveloped ovules are nonmature seeds, it is expected that the anthocyanin content in seeds and ovules are rather similar, however, the relative proportions of these two pigments were different. The receptacles of *Podocarpus* species, which mainly contain cyanidin 3-neohesperidoside are more reddish in color than the receptacles of white pine,[156,455] which contain pelargonidin 3-neohesperidoside, again in accordance with the colors of the receptacles. In fact, anthocyanins containing neohesperidosides are very rare,[4] and the 3-neohesperidosides of cyanidin and pelargonidin have not been found outside the genera *Podocarpus* and *Dacrycarpus*. Finally, we want to highlight the extraordinary color similarities of the receptacles of several *Podocarpus* species, which are mainly located in the Southern hemisphere, and the arils of *Taxus baccata*, Pinaceae, mainly located in the Northern hemisphere. While the receptacles are colored by hydrophilic anthocyanins, the arils are colored by lipophilic carotenoids (rhodoxanthin, etc.).

As the most visible role of anthocyanins is to impart colors, the adaptive significance of anthocyanins in fruits, seeds, and fruit-similar structures is invariably attributed to the attraction of seed dispersers. However, as suggested in Section 3.16.6, anthocyanins in vegetative tissue may also have other functions, for instance in plant defense. Finally, here we include one report related to fruit color polymorphism. This phenomenon occurs in at least 19 plant families;[456–458] however, the ecological and evolutionary dynamics of fruit color polymorphisms remain poorly known because patterns and agents of selection have rarely been identified. *Acacia ligulata* populations are composed of two or three color morphs, producing red, yellow, or (more rarely) orange arillate diaspores.[459] Seed production differences between these morphs were found to be a function of both intrinsic plant characters (fruit production) and predispersal seed predation.[460] Thus, it was suggested that pleiotropic effects might be a common feature of fruit color polymorphisms, and that the most obvious selective agents (i.e., seed dispersers) may not always be the most important.

3.16.6.3 Roles of Anthocyanins in Vegetative Tissue, Mainly Leaves

The functions of red colorants in vegetative tissue have puzzled scientists for more than a century. The presence of colored flavonoids in young leaves, seedlings, roots, and stems has not been looked upon as obvious, as the presence of colored flavonoids in fruits and flowers. Lee and Collins[320] have studied the distribution of anthocyanins (and betacyanins) in leaves (expanding, mature, and senescing) of tropical plants. At both expanding and senescing stages they found anthocyanins primarily in the mesophyll. In their opinion was the presence of anthocyanins in the mesophyll of so many species inconsistent with the hypothesis of protection against UV damage and fungal pathogens. Dominy *et al.*[461] have noted that a common location for most of the anthocyanin in young leaves is just above the lower epidermis and well away from photosynthetic tissue,[145,462] and express that this would appear to offer little benefit for either photoprotection or photoinhibition. Gould and Lister[417] have pointed out that the vacuolar location of the colored forms of the anthocyanins precludes any major role in free-radical scavenging in planta, since almost all free radicals originate from organelles, the plasma membrane, and the apoplasm. Cytoplasmic antioxidants, and the extremely efficient enzyme superoxide dismutase, should be more optimally located to scavenge organelle-derived reactive oxygen.

However, there exist increasing evidences that anthocyanins, particularly when they are located at the upper surface of the leaf or in the epidermal cells, also play roles in the physiological survival of plants. It has been outlined that foliar anthocyanins accumulate in young, expanding foliage, in autumnal foliage of deciduous species, in response to nutrient deficiency, temperature changes, or UV radiation exposure, and in association with damage or defense against browsing herbivores or pathogenic fungal infections. The functions have in this context mainly been hypothesized around the anthocyanins as compatible solutes contributing to osmotic adjustment to drought and frost stress, as antioxidants, and as UV and visible light protectants. Johnson *et al.*[463] placed the function of anthocyanins in leaf, or in their case in stems, into a fundamental punch line in their title 'better red than dead,' which may illuminate the importance of anthocyanin coloration also in vegetative tissue. In this chapter the different responses have been separated under subtitles, although they in many cases may be related to each other. The chapter is far from being exhaustive with respect to literature coverage. Its nature is more introductory with selected examples from the most recent publications in the field. For further reading reviews by Chalker-Scott,[413] Harborne and Williams,[148] Gould and Lee,[464] Dominy,[461] Simmonds,[415] Close and Beadle,[465] Gould and Lister,[417] and Manetas[466] are highly recommended.

3.16.6.3.1 Photoprotection

Historically, the first scientific reference concerning the role of anthocyanins in vegetative tissues is attributed to Haberlandt,[353] who assumed a kind of photoprotective role of the red leaf colorants. According to Karageorgou *et al.*[467] this function is still preferred among physiologists. The anthocyanins are thought to be working either as sunscreens by attenuation of excess visible light, which reduce excitation load in the underlying mesophyll cells, or/and by their detoxification of oxy-radicals produced during photosynthesis. However, the literature is far from consistent here. While laboratory trials indicate that red leaves are less prone to undergo photoinhibition than green leaves,[468–471] field studies have failed to show any actual photoprotective superiority of red leaves.[472–478] Some recent specific results reflecting correlation between anthocyanin content in vegetative tissue and their potential function(s) are discussed.

In 2002 the first report on anthocyanins was published, which proved this type of pigments to function as photoprotectors of light-sensitive defensive compounds in plants.[477] Silver beachwood (*Ambrosia chamissonis*) located along the sunny Pacific coast of North America, contains high amounts of thiarubrines in stems and leaf petioles. Thiarubrines are red plant pigments that decompose easily to colorless thiophenes when exposed to sunlight (**Figure 20**). They are in tissue compartmentalized in laticifers that are surrounded by anthocyanin-containing cells. In leaves and stems of seedlings the anthocyanins were identified as mainly cyanidin 3-*O*-[6″-*O*-(malonyl)glucoside] and cyanidin 3-*O*-glucoside (**Figure 20**), while none of these anthocyanins was detected in roots. To correlate anthocyanin distribution with thiarubrine photoprotection, changes in thiarubrine A and thiophene A levels were measured in seedlings and roots exposed to light. In roots, thiarubrine A levels decreased by 94 and 100% after 30 min and 4 h of irradiation, respectively, with a concomitant threefold increase in thiophene A levels. In leaves and stems, thiarubrine A levels did not change appreciably during light exposure. To confirm the photoprotective function of anthocyanins, solutions of cyanidin 3-*O*-glucoside were used to filter visible light incident on a solution of thiarubrine A. Anthocyanin solutions with concentrations higher than 0.1 mmol l^{-1} completely prevented thiarubrine photoconversion. The conclusion is that when the light-screening sheath of anthocyanins is absent and the laticifers containing red thiarubrines are exposed to light, rapid bleaching of the thiarubrine content occurs. Without a mechanism for photoprotection including anthocyanins, sunlight would rapidly convert the red thiarubrines in *A. chamissonis* into colorless thiophenes.

The red-to-blue colors of juvenile leaves is most commonly caused by anthocyanins appearing within vacuoles of epidermal and/or mesophyll cells within hours to days during seedling germination. It has been

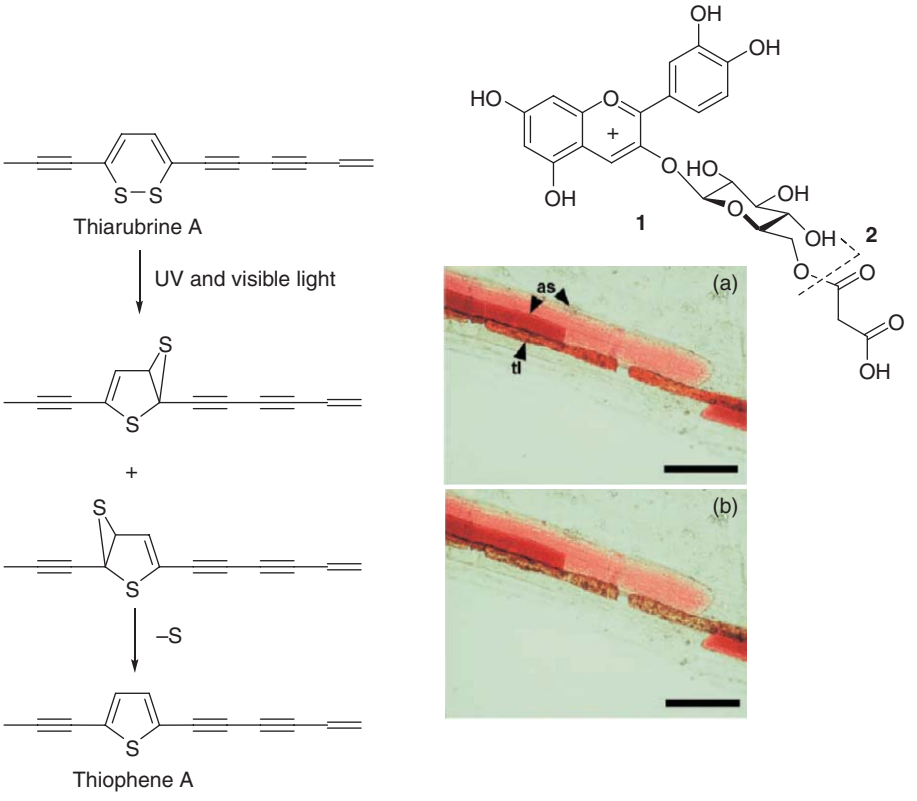


Figure 20 Chemical structures of thiarubrines and anthocyanins occurring in *Ambrosia chamissonis*. *Left*: thiarubrine A is converted into its photoproduct thiophene A by exposure to UV and visible light. *Pictures*: Anatomy of thiarubrine photoprotection in *A. chamissonis*. (a), (b) Thiarubrine laticifers (**tl**) and anthocyanin sheath (**as**) cells before (a) and after (b) 2 min irradiation. The discoloration and granular appearance of the thiarubrine laticifer after light exposure is visible. Bars = 200 μm. Photos courtesy: J. E. Page; G. H. N. Towers, *Can. Planta* **2002**, *215*, 478–484. *Top right*: structures of cyanidin 3-[6-(malonyl)glucoside] (**1**) and cyanidin 3-glucoside (**2**).

argued that juvenile leaves contain anthocyanins to protect themselves in early development stages. In developing leaves, Hughes *et al.*[478] found that anthocyanin disappearance occurred when: *c.*80% of mature leaf thickness had been attained, *c.*50% of mature photopigment concentrations was developed, and after differentiation of the mesophyll into palisade and spongy layers. The loss of anthocyanins during leaf development may thus correspond to a decreased need for photoprotection, as photosynthetic maturation allows leaves to utilize higher light intensities. Gould *et al.*[479] on the other hand surveyed 1000 leaves from a forest population of *Quintinia serrata*, which displayed natural polymorphism in leaf color. Red leaves contained cyanidin 3-glucoside and cyanidin 3-galactoside, while green leaves lacked anthocyanins, but had otherwise similar pigment profiles. The anthocyanins were most commonly located in the vacuoles of photosynthetic cells, and most abundant in older leaves on trees found at the uppermost level of a mature forest with south-facing gaps. It was therefore indicated that anthocyanins most probably were associated with photosynthesis. However, the anthocyanins did not serve any auxiliary phytoprotective role. Their function was to protect shade-adapted chloroplasts from brief exposure to high-intensity sunflecks.

Leaf color in some individuals of *Cistus creticus* turns transiently to red during winter, while neighboring individuals occupying the same site remain green. Kytridis *et al.*[480] have analyzed the accumulation of leaf anthocyanins in the two phenotypes. The frequency of red individuals was considerably higher in fully exposed sites, pointing to a photoprotective function of leaf anthocyanins. Red leaves were among other factors also characterized by lower nitrogen contents at all sampling dates throughout the year. The nitrogen content of leaves is strongly correlated with photosynthetic capacity,[481] and a link between the lower nitrogen levels and the lower linear electron transport rates in the red phenotype of *C. creticus* was assumed.[480] Lower nitrogen levels may leave the red phenotype more vulnerable to photoinhibition and oxidative stress, due to lack of inadequate photochemical and nonphotochemical sinks for excess excitation energy. On the basis of correlative evidences it was thus assumed that the anthocyanins in red leaves were an adaptation to compensate for this deficiency, with the aim of reducing the risk of photodamage.

3.16.6.3.2 Antioxidant activity

Pure anthocyanins and purified anthocyanin extracts have been shown to have strong antioxidant activity in many *in vitro* assays. Anthocyanins, as other flavonoids, have been shown to act as scavengers of various oxidizing species, that is, superoxide anion, hydroxyl radical, or peroxy radicals. They may act as quenchers of singlet oxygen or they may react with metal ions and thereby indirectly decrease hydroxyl radical production. Anthocyanins do not react specifically with a single species, and so a number of different evaluation methods (assays) have been developed. This makes comparison of the various studies very problematic, and the antioxidant-related effects difficult to interpret for *in vivo* conditions. Regarding anthocyanins in living vegetative cells, the purpose whether they scavenge or quench reactive oxygen species is inadequately known. A growing body of results indicates that anthocyanins contribute to control the levels of reactive oxygen in plant cells.[417,482–485] However, not all results are of the same kind: While Gould *et al.*[486] have proposed that cytosolic and organelle-bound antioxidants, rather than the vacuolar anthocyanins, may offer the first line of defense against oxidative stress in leaves, Kytridis and Manetas[484] have concluded that leaf vacuolar anthocyanins may afford a detoxifying sink for some reactive oxygen species when the chloroplastic, the first line of antioxidative defense, is surpassed. Although not optimally located in relation to the chloroplastic source of oxy-radical production, this latter function is more possible for anthocyanins located in mesophyll than in epidermal vacuoles. Here are some of the more recent results in the field.

Red leaf lettuce (*Lactuca sativa*) (Lollo Rosso) has been grown under three types of plastic films that varied in transparency to UV radiation.[487] Exposure to increased levels of UV radiation during cultivation caused the leaves to redden and considerably increased concentrations of cyanidin glycosides and other phenolics. Red coloration was found mainly in the outer leaves and toward the extremities of the inner leaves, where the leaves were exposed to most light. Neil and Gould[488] have examined the potential of anthocyanins to extenuate photooxidative injury in a similar type of leaves, both by shielding chloroplasts from excess high-energy quanta, and by scavenging reactive oxygen species. To distinguish between the impacts of these two putative mechanisms, superoxide ($O_2^{\bullet -}$) concentration and chlorophyll oxidation were measured for chloroplast suspensions under various light and antioxidant-supplemented environments. A red cellulose filter, which had optical properties approximated that of anthocyanins, was used to shield irradiated chloroplasts. The outcome was a

33% decline in rate of $O_2^{\bullet -}$ generation and 37% reduction in chlorophyll bleaching. Colorless and blue tautomers of cyanidin 3-*O*-[6″-*O*-(malonyl)glucoside] at pH 7 removed up to 17% of $O_2^{\bullet -}$ generated by chloroplasts, indicating that cytosolic anthocyanins can serve as effective antioxidants. Red flavylium cation forms, typical of vacuolar anthocyanins at lower pH values, also showed strong reducing potentials as indicated by cyclic voltammetry potentials, which declined by 40% after 15 min exposure to $O_2^{\bullet -}$.

Shao *et al.*[485] have looked at antioxidant capability, among other factors, in leaves of the wild type *Arabidopsis thaliana* L. and tree mutants deficient in anthocyanin biosynthesis during treatment with temperatures ranging from 25 to 45 °C (see Chapter 3.28). High temperatures are harmful to plant development, and influence the formation and functions of the photosynthetic apparatus in plants. In comparison to the wild type, the mutants lacking anthocyanins had lower activities of superoxide dismutase, ascorbate peroxidase, and inferior scavenging capability to DPPH (1,1-diphenyl-2-picrylhydrazyl) radical under heat stress. In addition H_2O_2 accumulated in the leaf vein and mesophyll cells of the mutants at 40 °C. The same group has also investigated antioxidative capability within the same type of leaves under photooxidation stress induced by methyl viologen (5 μm) in light.[489] In comparison with the wild-type plant, photooxidation resulted in significant decreases in the contents of total phenolics and flavonoids, total antioxidative capability, and chlorophyll fluorescence parameters, and to increase in cell-membrane leakiness in the three mutants, which were deficient in anthocyanin biosynthesis.

3.16.6.3.3 Antiherbivory activity

It is generally accepted that flavonoids, along with other plant polyphenols, play a role in protecting plants from both insect and mammalian herbivory (see Chapter 4.08). Among the flavonoids, attention has been mainly centered on polymeric flavolans or proanthocyanidins but some research has been concerned with monomeric flavones, flavonols, and isoflavones [148] (see Chapter 6.18). The roles of colored anthocyanins are in this context still under discussion. As physiologists seem to prefer the photoprotecitve role for anthocyanins in leaves, the antiherbivory theory has been championed by ecologists.[461,490,491] The fact that the anthocyanins are located in vacuoles of epidermis and the mesophyll away from the photosynthesis apparatus and the chloroplastic source of oxy-radical production, has among other factors supported antiherbivory hypotheses. Here are more recent hypotheses, which propose that nongreen plant coloration based on anthocyanins has evolved as a defense against herbivores.

Hamilton *et al.*[490] have proposed that leaf colors function to signal the defensive strength of an individual plant to herbivorous insects. It was predicted that tree species suffering greater insect damage would, on an average, invest more in autumn-color signaling than less troubled species. Protective anthocyanin coloration promotes handicap signals, which indicate plant fitness. Karageorgou *et al.*[467] have examined whether the assumed handicap signal is honest and, accordingly, costly, by seeking a correlation between anthocyanin and total phenolic levels in 11 plants exhibiting variation in the expression of the red character, either between individuals or between modules on the same individual. On the basis of the results they concluded that for senescing leaves the redness was both honest and costly. They did not find the same results for young, developing leaves, and questioned the handicap signal hypothesis in this case. Young leaf redness fits more to alternative hypotheses that red leaf color is less easily perceived by folivorous insect photoreceptors, or that red leaf color undermines insect camouflage.[467]

Lev-Yadun *et al.*[491] have earlier proposed that the diversity of plant coloration undermines the crypsis of their herbivorous predators. Many color patterns in plants undermine the camouflage of invertebrate herbivores, especially insects, thus exposing them to predation and causing them to avoid plant organs with unsuitable coloration, to the benefit of the plants. In antiherbivory strategy dark colors can camouflage leaves against the exposed soil and litter of forest floors,[492,493] or they can mimic dead leaves.[494] Red leaves might appear dark or dead to a potential herbivore, since most nonmammalian folivores lack red light receptors.[461]

The anthocyanins are in contrast to certain other phenolic compounds reckoned to be nontoxic to higher animal species. However, cyanidin 3-glucoside, which is the most abundant foliar anthocyanin, has been reported to inhibit the growth of larvae of the tobacco budworm, *Heliothis virescens*, an important pest of cotton and other crops.[495] Recently, Johnson *et al.*[496] have examined resistance due to anthocyanins from commercial petunia flowers (*Petunia hybrida*) for insecticide or antifeedant activity against corn earworm (*Helicoverpa zea*) and cabbage looper (*Trichoplusia ni*). The petunia flowers studied contained a star pattern, with colored and white sectors. Corn earworm larvae ate in most cases significantly less colored sectors than white sectors in no-choice bioassays. The studies demonstrated that the colored sectors of these petunia cultivars slowed the

development of the larvae, and indicated that anthocyanins play some part in flower defense in petunia. Herbivory and fungal infection of Chinese cabbage (*B. rapa* ssp. *pekinensis*) leaves have been found to increase the total amount of anthocyanins.[497] However, anthocyanin-rich extracts did not influence the feeding behavior or survival rate of aphids, nor inhibit larval growth of the fruitworm.[498–500]

3.16.6.3.4 Anthocyanin induction caused by different stressors

An assortment of intrinsic and environmental factors has been linked to anthocyanin induction, accumulation, or inhibition in vegetative tissue. Plants are most probably equipped with specific pathways to activate anthocyanin synthesis to cope with different stressors.[413] More recent examples of different stressors which have been studied are deficiencies in phosphorous,[501,502] nitrogen,[503,504] increased level of metals,[505] drought, heat, cold, and salinity,[506–510] wounding,[511] pathogen infection,[512] and fungal elicitors.[512] Temporal variation of anthocyanins may also be related to the severity of induced photoinhibition (see Section 3.16.6.3.1),[513] and oxidative stress injury caused by enhanced solar UV–B radiation (see Section 3.16.6.3.2).[514] Here are some selected illustrations.

Schaefer *et al.*[515] have found that anthocyanins can reduce fungal growth in fruits. They reported that the risk of fruit-rot in grape varieties infected with *Botrytis cinerea* decreased with increasing anthocyanin content. Anthocyanins did also inhibit growth rates of nine fruit-rot fungi on agar plates. Based on the phenomena that different stressors initiate anthocyanin production, Chalker-Scott[413,506] has provided a generalized role for the anthocyanins as osmoregulators in plant cells, since most types of suboptimal environments induce water stress, either directly or indirectly. She indicated that since developing leaves lack cell wall modifications to induce cross-resistance, they must rely on vacuolar substances to modify water relations. The high water solubility of anthocyanins makes them easy to accumulate in vacuoles, and they may in this manner serve to decrease leaf osmotic potential. The resulting depression of leaf water potential might increase water uptake and/or reduce transpirational losses. The often transitory nature of foliar anthocyanin accumulation may in this manner allow plants to respond quickly and temporarily to environmental variability rather than through more permanent anatomical or morphological modifications.

To understand the response of plants to varying nitrogen (N) levels, a growth system has recently been developed where N was the growth-limiting factor.[504] An *Arabidopsis* whole genome microarray was used to evaluate global gene expression under different N conditions. Plants went obviously purple in color under severe N limiting conditions. The genes involved in anthocyanin biosynthesis, such as leucoanthocyanidin dioxygenase and dihydroflavonol reductase, were upregulated just over twofold under mild N stress, but increased to about 14- and 18-fold under severe N stress. CHS, which participates in the early stages of the biosynthetic pathway to all flavonoids was upregulated only under severe N stress.

3.16.7 Anthocyanin Production

The main current methods for producing anthocyanins rely on plant extraction, a process that often is subjected to seasonal variability, low purity, poor yields, and high expenditures. Over the past decade interest in and demand for natural food colorants and pharmacologically interesting natural compounds have encouraged new research initiatives aimed at the development of more efficient means of harvesting anthocyanins. Among these are various attempts to produce anthocyanins from plant cell and tissue cultures. The construction of *Escherichia coli* recombinant strains and the development of fermentation approaches that has allowed relatively high yield anthocyanin production from this microorganism,[516] is very promising. Anthocyanins may also be produced by synthesis, or by hemisynthesis from other types of flavonoids;[4] however, restrictions with respect to legislation limits the applications of these compounds. In the past, the leading techniques employed to elucidate biosynthetic pathways in plants have consisted of feeding experiments with radioactive or isotope-labeled precursors. Isotope-labeling methods lead to selective enhancement of signals from nuclei with low natural abundance. With the development of plant cell culture methodologies, it has become feasible to reveal biosynthetic pathways by isolating and characterizing the participating enzymes. Alternatively, if isotope labeled compounds like anthocyanins are made, their content in tissue and derived metabolites can be measured quantitatively by hetero-nuclear NMR.

3.16.7.1 Production of Anthocyanins in Plant Tissue Cultures

When growth procedures are optimized, cell culture systems have the potential of producing both higher anthocyanin concentrations within reduced time, and another selection of anthocyanins relative to production in whole plants. To improve production of anthocyanins, efforts have mainly been devoted to the optimization of biosynthetic pathways by both process and genetic engineering approaches. The productivity in the cultures is, however, determined by synthetic capacity, storage capacity, and the capacity to metabolize the compounds in the transport and detoxification processes.[517] In a general review, Ramachandra Rao and Ravishankar[517] have dealt with the production of high-value secondary metabolites including anthocyanins through plant cell cultures, shoot cultures, root cultures, and transgenic roots obtained through biotechnological means. In an overview of the status and prospects in the commercial development of plant cell cultures for production of anthocyanin, Zhang and Furusaki[518] have focused on strategies for enhancement of anthocyanin biosynthesis to achieve economically viable technology. The potential of manipulation and optimization of postbiosynthetic events have been reviewed by Zhang *et al.*[519] These events, including chemical and enzymatic modifications, transport, storage or secretion, and catabolism or degradation, were outlined with anthocyanin production in plant cell cultures as case studies.

Production of anthocyanins in plant cell and tissue cultures has been reported for more than 30 species including *D. carota*, *Fragaria* × *ananassa*, *Vaccinium* spp., *Vitis hybrida*, *Solanum tuberosum*, *Malus sylvestris*, *Aralia cordata*, *Perilla frutescens*, *I. batatas*, *Euphorbia millii*, *Strobilanthes dyeriana*, *Hibiscus sabariffa*, *Dioscorea cirrhosa*, and so on(see examples in **Table 13**).[518,551,552] The production has shown to be influenced by a variety of environmental stimuli such as light irradiation, UV light, low temperature, oxygen level, hormones, fungal elicitors, low nutrient levels, and so forth.[517,518,551] Increased level of O_2 supply and light irradiation have, for instance, shown independently positive influence on the production of anthocyanins in suspended cultures of *P. frutescens* cells in a bioreactor.[553] However, a combination of irradiation with a higher oxygen supply reduced the production. In *Vaccinium pahalae* cell cultures, anthocyanin yield was enhanced by increasing sucrose concentration in the liquid suspension medium and by manipulating the initial inoculum density.[546] *Catharanthus roseus* flowers and cell cultures have been shown to accumulate the same type of anthocyanins, however, the differentiated petal cells showed a higher capacity for anthocyanin accumulation than the undifferentiated cell suspension cells.[533] It is also interesting to note that the anthocyanin production within cultures of this species was located to only a fixed percentage of the cells, and that all these cells had about the same concentration of anthocyanins.[554] The anthocyanin production seemed to be ruled by a feedback mechanism giving physiological maximum anthocyanin concentration. Bioreactor-based systems for mass production of anthocyanins from cultured plant cells have been described for several species.[520,553,555–559]

A cell culture system has the potential advantage of facilitating selective production of certain anthocyanins. The nine acylated anthocyanins produced by flowers of *H. orientalis* regenerated *in vitro*, were identical to those of field-grown flowers.[560] However, the concentration of cyanidin 3-[6-(*p*-coumaryl)glucoside]-5-[6-(malonyl)glucoside] was considerably higher in the regenerated flowers. Lower concentration of 2,4-dichlorophenoxyacetic acid in the medium used for strawberry suspension cultures has, for instance, limited cell growth and enhanced both anthocyanin production and anthocyanin methylation.[551] The ratio of peonidin-3-glucoside to the total anthocyanin content increased significantly under these conditions. A methylated anthocyanin like peonidin 3-glucoside is normally not found in intact strawberries, and although the activity of anthocyanin methyltransferase was not measured by Nakamura *et al.*,[551] the results indicated that lower 2,4-dichlorophenoxyacetic acid concentrations enhanced the activity of anthocyanin methyltransferase. Do and Cormier[561] have reported that increased osmotic potential in the medium resulted in a significant intracellular accumulation of peonidin-3-glucoside in *V. vinifera* cells. Similarly, jasmonic acid has been reported to increase the peonidin 3-glucoside content considerably, while the other major anthocyanins only experienced smaller increments.[562]

To improve understanding of the ways in which cinnamic acid groups alter the color retention of anthocyanins, a series of anthocyanins that differed systematically in their acyl group was needed. When cinnamic acids were fed to wild carrot suspension cultures, the proportion of acylated to nonacylated anthocyanins increased.[563] With high relevance for future metabolic studies, *V. vinifera* cells grown in a bioreactor have been used for production of isotopically ^{13}C-labeled phenolic substances such as

Table 13 Qualitative and quantitative anthocyanin content in cell cultures of various plants

Plant species	*Anthocyanins*[a]	*Yield*	*Reference(s)*
Aralia cordata	Cy3-[2-(xyl)gal], Pn3-[2-(xyl)gal]	7.0–17.2% DW	520–522
Ajuga reptans	Cy3,5-di-glc, Cy3-[2-(6-(cum)glc)-6-(cum)glc]-5-glc, Cy3-[2-(6-(cum)glc)-6-(cum)glc]-5-[6-(mal)glc], Cy3-[fer-cum(2-(glc)glc)]-5-[mal-glc], Dp3,5-di-glc, Dp3-[di-fer(2glc-glc)]-5-glc, Dp3-[2-(6-(fer)glc)-6-(fer)glc]-5-[6-(mal)glc], Dp3-[2-(6-(fer)glc)-6-(cum)glc]-5-[6-(mal)glc]	1–3% DW	523–528
Catharanthus roseus	Hi3-[6-(cum)glc], Hi3-glc, Mv3-[6-(cum)glc], Mv3-glc, Pt3-[6-(cum)glc], Pt3-glc	0.6–2.8 mmol l^{-1}	529–533
Daucus carota	Cy3-glc, Cy3-[2-(xyl)-6-(glc)gal], Cy3-[2-(xyl)gal], Cy3-[2(xyl)-6-(6(sin)glc)gal], Cy3-[2(xyl)-6-(6(fer)glc)gal], Cy3-[2(xyl)-6-(6(cum)glc)gal], Cy3-[2(xyl)-6-(6(3,4,5-tri-MeOHcin)glc)gal], Cy3-[2(xyl)-6-(6(di-MeOHcin)glc)gal], Cy3-[6-(6(sin)glc)gal], Cy3-gal	5.4–23.7% DW	534–537
Euphorbia millii	NR	64 mg l^{-1} day^{-1} 4% DW	538,539
Fragaria × *ananassa*	Cy3-glc, Pg3-glc, Pg3-[6-(mal)glc], Pn3-glc	0.9 mg g^{-1} FW 30.2 mg l^{-1} day^{-1}	518,540–542
Glehnia littoralis	Cy3-[6-(6-(fer)glc)-2-(xyl)glc]	14% DW	543
Ipomoea batatas	Cy3-[2-(glc)glc]-5-glc, Cy3-[2-(6-(cum)glc)glc]-5-glc,Cy3-[6-(caf)-2-(glc)glc]-5-glc, Pn3-[6-(caf)-2-(glc)glc]-5-glc, Cy3-[2-(6-(hba)glc)-6-(caf)glc]-5-glc, Cy3-[2-(6-(caf)glc)-6-(caf)glc]-5-glc, Cy3-[2-(6-(fer)glc)-6-(caf)glc]-5-glc, Pn3-[2-(6-(fer)glc)-6-(caf)glc]-5-glc, Pn3-[2-(6-(caf)glc)-6-(caf)glc]-5-glc, Pn[2-(6-(hba)glc)-6-(caf)glc]-5-glc	NR	4,544[b]
Perilla frutescens	Cy3-[6(cum)glc]-5-glc, Cy3-[6(cum)glc]-5-[6-(mal)glc], Cy3-[6-(fer)glc]-5-[6-(mal)glc]	24% DW	539,545
Vaccinium spp.	Cy3-ara, Cy3-gal, Pn3-gal,^{14}C-ANC (after feeding medium with ^{14}C-sucrose)	max 70 g l^{-1}	546,547
Vitis spp.	Cy3-glc, Cy3-cum-glc, Mv3,5-di-glc, Pn3-glc, Pn3,5-di-glc, Pn3-ace-glc, Pn 3-caf-glc, Pn 3-[6(cum)glc]	1.0–16% DW	539,540,548,549
Zea mays	Cy3-[3,6-di-(mal)glc], Cy3-[6-(mal)glc], Cy3-glc, Pn3-glc	NR	550[b]

[a] See **Table 1** for abbreviations.
[b] Not reported from cell culture but isolated from the species.
FW, fresh weight; DW, dry weight; NR, not reported.

anthocyanins.[555,564] The enrichment of labeling (between 40 and 65%) obtained for all compounds, should be sufficient to investigate their absorption and metabolism in humans. Similarly, ^{14}C-L-phenylalanine has been incorporated into a range of polyphenolic compounds when fed to cell cultures.[565,566] Experiments with *V. pahalae* berries and *V. vinifera* suspension cultures, using [^{14}C]-sucrose as the carbon source, have demonstrated a 20–23% efficiency of ^{14}C incorporation into the flavonoid-rich fractions.[567] All in all there has, however, been limited success in achieving processes, which are commercially viable, using plant tissue and cell cultures for anthocyanin production – in part because of some unique engineering challenges inherent in mass cultivation of plant cultures.

3.16.7.2 Production of Anthocyanins by Microorganisms

Both prokaryotic and eukaryotic microbes have been used for the expression of genes that are able to convert fed precursors or endogenously produced substrates into valuable end products. The first report of plant-specific anthocyanins produced by a microorganism involved *E. coli* cells.[516] In order to produce stable, glycosylated anthocyanins from colorless flavanones such as naringenin and eriodictyol, a four-step metabolic pathway that contained plant genes from heterologous origins: flavanone 3-β-hydroxylase from *M. domestica*, DFR from *Anthurium andraeanum*, ANS also from *M. domestica*, and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (3GT) from *P. hybrida,* was constructed. Using two rounds of polymerase chain reaction each of the four genes was first placed under control of the *trc* promoter and its own bacterial ribosome-binding site. Then they were cloned sequentially into vector pK184. *E. coli* cells containing the recombinant plant pathway were able to take up either naringenin or eriodictyol and convert these compounds into the corresponding glycosylated anthocyanins, pelargonidin 3-glucoside or cyanidin 3-glucoside. The formed anthocyanins were present at low concentrations.

More recently it was, however, reported that the recombinant *E. coli* cells successfully could achieve milligram level production of the same two anthocyanins, pelargonidin 3-glucoside (1.0 mg l^{-1}) and cyanidin 3-glucoside (2.1 mg l^{-1}) from their respective flavanone precursors.[568] Cyanidin 3-glucoside was produced at even higher yields (16.1 mg l^{-1}) from the flavan-3-ol precursor, (+)-catechin. It was demonstrated that availability of the glucosyl donor, UDP glucose, was the key metabolic limitation, while product instability at normal pH was identified as a barrier. It is known that common anthocyanidin 3-glucosides rapidly will break down in weakly acidic and neutral aqueous solutions,[229] and production optimization of anthocyanidin 3-glucoside from flavan-3-ol precursors in *E. coli* cells was demonstrated by adjusting the pH to mimic the acidic condition of plant vacuole and stabilize the anthocyanin compounds.[568] A translational fusion of ANS and 3GT was created to mimic the enzyme complex, which may exist in the plant cells, to facilitate the transportation of the unstable intermediate anthocyanidins from ANS to 3GT. The metabolic network of the host *E. coli* BL21 was rationally manipulated to channel carbon flux into the UDP-glucose biosynthetic pathway, a key precursor in anthocyanin biosynthesis. As a result, production of as much as 79 mg l^{-1} pelargonidin 3-glucoside and 71 mg l^{-1} cyanidin 3-glucoside was achieved from their precursor (flavan-3-ols) without supplementation with extracellular UDP glucose.

Glossary

afzelechin/epiafzelechin flavan-3-ol (flavonoid) epimers, 5,7-dihydroxy-2-(4-hydroxy)phenyl-3,4-dihydro-2*H*-chromen-3-ols

anthocyanidin anthocyanin aglycone

apigenin flavone (flavonoid), 5,7-dihydroxy-2-(4-hydroxy)phenylchromen-4-one

bathochromic shift change of spectral band position in the absorption spectrum of a molecule to a longer wavelength (lower frequency)

catechin/epicatechin flavan-3-ol (flavonoid) epimers, 5,7-dihydroxy-2-(3,4-dihydroxy)phenyl-3,4-dihydro-2*H*-chromen-3-ols

chemotaxonomy classification of organisms according to demonstrable differences and similarities in their biochemical compositions, here according to anthocyanin content

epimer two epimers are diastereomers, which differ in configuration of only one stereogenic center
flavone flavonoid class based on the backbone of 2-phenylchromen-4-one
flavonol flavonoid class based on the backbone of 3-hydroxy-2-phenylchromen-4-one
HPLC high performance liquid chromatography
hyperchromic effect increase in absorbance of a spectral band in the absorption spectrum of a molecule
hypsochromic shift change of spectral band position in the absorption spectrum of a molecule to a shorter wavelength (higher frequency)
kaempferol a flavonol (flavonoid), 3,5,7,4′-tetrahydroxy-2-phenylchromen-4-one
LC–MS liquid chromatography–mass spectroscopy
NMR nuclear magnetic resonance spectroscopy
NOESY nuclear Overhauser effect spectroscopy, a two-dimensional homo-nuclear magnetic resonance (NMR) technique, which is based upon coupling between protons through space. The method can provide information about the molecular geometry and linkages between anthocyanin sub-units
photoinhibition reduction in a plant's (or other photosynthetic organism's) capacity for photosynthesis caused by exposure to strong light (above the saturation point)

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Biographical Sketches

Øyvind M. Andersen, a full Professor of Chemistry since 1993, has specialized in the chemistry of flavonoids. He received his Dr. Philos. degree in 1988 from University of Bergen. He along with Dr. Ken R. Markham is the editor of the book *Flavonoids: Chemistry, Biochemistry and Applications* and author of over 100 international journal articles, ten invited book chapters and five patents in the field of anthocyanins and other flavonoids. He has supervised over 40 M.Sc. and Ph.D. students in natural product chemistry, and the activities of Dr. Andersen's research group have led to the establishment of several flavonoid-based companies. His research projects concentrate on structure elucidation of new compounds, methodology within NMR spectroscopy and chromatography, and bioprospecting. Some of the latter projects treat comparable behavior and functions of individual anthocyanins with the underlying aim of exploring their pharmaceutical potential and use as colorants in food. Dr. Andersen received the Groupe Polyphenols Award in 2006.

Monica Jordheim born in 1979 has at present a post doctoral position at Department of Chemistry, University of Bergen, Norway. She completed here M.Sc. in 2003 and her Ph.D. in 2007 at the University of Bergen, both in natural product chemistry, with anthocyanins as her main research field. From 2005 she has been teaching organic analytical chemistry at Bachelor and Master levels and supervised M.Sc. students. Her publications focus on structure elucidation of anthocyanins, the stability and reducing capacity of anthocyanins, and the complexity of their purity determinations using various chromatographic techniques and advanced NMR spectroscopy. She has co-authored the chapter along with Dr. Andersen entitled *Anthocyanins* in the book *Flavonoids: Chemistry, Biochemistry and Applications.*