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IDENTIFICATION AND DISTRIBUTION OF MALONATED ANTHOCYANINS IN PLANTS OF THE COMPOSITAE

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Key Word Index—*Cichorium*; *Dahlia*; *Helenium*; Compositae; acylated anthocyanins; monomalonates; dimalonates; cyanidin 3-malonylglucuronosylglucoside; FAB-MS.

Abstract—A survey of 31 species from 28 genera in the Compositae showed the presence of zwitterionic anthocyanins in petals or stems of 27 species. Detailed investigations, including the use of FAB-MS, showed that mono- and dimalonated esters of pelargonin and cyanin occurred in *Dahlia variabilis* cultivars. The corresponding delphin mono- and dimalonates occur in blue flowers of *Cichorium intybus*. A cyanidin 3-dimalonylglucoside was identified in stems of *Coleostephus myconis* while pelargonidin 3-(6″-malonylglucoside) was found in *Callistephus* petals. A further malonated cyanidin derivative in flowers of *Helenium* cv. Bruno was found to be the 3-glucuronosylglucoside; this is the first report of an anthocyanin with glucuronic acid. Overall, the results confirm that malonated anthocyanins are widespread in the family and that many pigments previously reported in the Compositae as being unacylated probably contain these labile organic acid attachments.

INTRODUCTION

A recent electrophoretic survey of anthocyanins from flowers of 81 species representing 22 families of the angiosperms indicated that malonated or similarly substituted zwitterionic anthocyanins occurred in half the sample [1]. In particular, 18 of 20 species in the Compositae proved to contain pigments that were zwitterionic. Such acyl groups are labile, are lost in pigments extracted with methanolic HCl and may well have been overlooked during earlier investigations. Indeed, our preliminary reinvestigation of the pigments of *Dahlia variabilis*, previously reported as being pelargonin and cyanin [2, 3], showed them to be the 3-(6″-malonylglucoside)-5-glucosides [1]; these two structures have been confirmed during the present work. The occurrence of anthocyanins with organic acid attachment has also been reported in four other genera in the family: cyanidin 3-(6″-malonylglucoside) in leaves of *Cichorium intybus* [4], malonated cyanidin and delphinidin glycosides in flowers of *Senecio cruentus* [5], malonated pelargonidin and cyanidin 3-glucosides in flowers of *Gerbera* [6], and cyanidin 3-(succinyl glucoside)-5-glucoside in flowers of *Centaurea cyanus* [7, 8] and six other *Centaurea* species [9].

As a consequence of the above findings, the anthocyanins of this family were re-examined in order to confirm the widespread occurrence of organic acid acylation and to determine the nature of the acylating acids. We therefore report here a more extended survey of the family for zwitterionic anthocyanins and the identification of mono- and dimalonated pigments in species of *Callistephus*, *Cichorium*, *Coleostephus*, *Dahlia* and *Helenium*. Eight new zwitterionic anthocyanins are described.

RESULTS

Electrophoretic survey

The results of a survey of 31 species representing 28 genera of the Compositae for zwitterionic anthocyanins are shown in Table 1. This includes data from our earlier survey [1]. As will be seen, zwitterionic anthocyanins appear to be widely distributed, since they were found in all but four species. In the case of positive species, all the pigment moved away from the origin during electrophoresis, indicating that all the anthocyanin was present in acylated form.

The survey was reasonably representative at the tribal level in that members of 12 of the 13 tribes of the Compositae were sampled. The member of the Calenduleae examined was unacylated but since this was the only species surveyed, it does not necessarily mean that the tribe as a whole lacks zwitterionic pigments. Clearly further sampling in many genera is desirable. For example, in our survey *Senecio coccinaeflorus* was negative and yet the garden cinerarea *Senecio cruentus* is known to have malonated pigments [5]. Thus further species need to be examined before it can be known whether or not the majority of *Senecio* species accumulate zwitterionic anthocyanins.

Variation is clearly possible within species, especially in those that have been cultivated, since acylation is almost certainly controlled by a single dominant gene. Indeed, among *Zinnia* cultivars, one form 'Persian Carpet' was positive, but two others were negative. This was exceptional. In other cases, e.g. *Dahlia variabilis*, all the cultivars examined proved to be positive.

That acylation with organic acids is a major structural

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Table 1. The presence/absence of zwitterionic anthocyanins in members of the Compositae

Tribe	Genus and species*	Presence/ absence of mobile pigment
Vernonieae	*Stokesia laevis* Hill	+
Eupatorieae	*Ageratum* cv. Ocean	+
	Liatris spicata Willd.	+
Astereae	*Aster* various cvs.	+
	Bellis perennis L.	+
	Callistephus chinensis Cass. cvs†	+
	Erigeron tillingii Voroch	+
Inuleae	*Helichrysum* cv. Swiss Giant	+
	Helipterum roseum Benth.	+
Heliantheae	*Bidens* sp.	−
	Cosmos atrosanguineus Hook.	−
	Dahlia variabilis L. various cvs‡	+
	Gaillardia grandiflora Hort.	+
	Helenium cv. Bruno	+
	Helianthus annuus L. cv.	+
	Zinnia cv. Persian Carpet	+
Senecioneae	*Ligularia przewalski* cv. The Rocket (stem)	+
	Senecio coccinaeflorus Rowl.	−
Anthemideae	*Coleostephus myconis* (L.) Reichenb. fil. (stem)	+
	Tanacetum coccineum Willd.	+
Arctoteae	*Arctotis* sp.	+
Calenduleae	*Osteospermum jucundum* (Phillips) Nordlindh	−
Cynareae	*Centaurea montana* L.	+
	C. nigra L.	+
	Cirsium arvense (L.) Scop.	+
	C. dissectum (L.) Hill	+
	Cynara scolymus L. cv.	+
Mutiseae	*Gerbera jamesonii* Bolus	+
Lactuceae	*Cicerbita plumieri* (L.) Kirschleger	+
	Cichorium endivia L.	+
	C. intybus L.	+

*Unless otherwise indicated, ligules or rays were analysed for anthocyanin.

†Garden asters analysed were the cvs Princess Early Celebration, Professor A. Kippenberg and King George.

‡Monomalonated pigments were recorded in cvs Jill Doe, Alltami Cherry, Biddenham Rust, Shandy and Gay Princess; mono- and dimalonated pigments in cvs David Howard, Bullseye, Willows Violet, Cathy, Alltami Corsair, Rocquencourt, Chimborazo, Ascot Julie and Biddenham Strawberry.

feature of anthocyanins in this family is now clear. As described below, detailed investigation of eight anthocyanins from five selected plants showed that malonylation is very common.

Identification of eight new malonated anthocyanins

Anthocyanins were isolated from fresh plant material (Table 2) by extraction into methanol–acetic acid–water (10:1:9) and solvents containing mineral acid were avoided at all stages. Most pigments were initially chromatographed on Sephadex LH20 to remove non-anthocyanin impurities and were then purified by PC in solvents butanol–acetic acid–water (4:1:5) and 15% acetic acid. The presence of dimalonylation in crude extracts was clear from the initial electrophoresis, since dimalonates moved twice as far towards the anode (*ca* 6 cm) as did the monomalonates (*ca* 3 cm) when electrophoresed in acetate buffer pH 4.4 at 40 V/cm for 2 hr. The purified pigments were monitored for homogeneity by electrophoresis, HPLC and TLC before analysis.

The characterization of malonic acid as the sole acylating group in all eight pigments was based primarily on fast atom bombardment/mass spectrometry (FAB-MS) in glycerol (Table 3), whereby pigments **1**, **2**, **5** and **6** were established as monomalonates and pigments **3**, **4**, **7** and **8** and dimalonates (Table 2). Fragmentation in FAB-MS also indicated clearly the masses of the aglycone and the sugar substituents. The nature of the unacylated moiety in all pigments except **5** was readily confirmed by saponification and direct spectral and chromatographic comparison of the products with authentic markers. Furthermore, the presence of unacylated glucose residues

Table 2. Malonated anthocyanins identified in the Compositae

Structure	Pigment	Plant source
1	Pelargonidin 3-(6″-malonylglucoside)	*Callistephus chinensis* cv Princess Early Celebration—ray flowers
2	Pelargonidin 3-(6″-malonylglucoside)-5-glucoside	*Dahlia variabilis* cv Biddenham Strawberry—rays
3	Perlargonidin 3-(6″-malonylglucoside-5-malonylglucoside	
4	Cyanidin 3-dimalonylglucoside	*Coleostephus myconis*—stems
5	Cyanidin 3-malonylglucuronosylglucoside	*Helenium* cv Bruno—rays
6	Cyanidin 3-(6″-malonylglucoside)-5-glucoside	*Dahlia variabilis* cv Shandy—rays
7	Cyanidin 3-(6″-malonylglucoside)-5-malonylglucoside	
8	Delphinidin 3-(6″-malonylglucoside)-5-malonylglucoside	*Cichorium intybus*—ligules

Table 3. FAB-MS of malonated anthocyanins

Pigment	$[M]^+$	loss of malonic $[M-86]^+$	loss of glucose $[M-162]^+$	loss of malonylglucose $[M-248]^+$	loss of 2 malonyl $[M-172]^+$	Aglycone $[M]^+$
1	519	433	—	—	—	271
2	681	595	519	433	—	271
3	767	681	—	519	595	271
4	621	535	—	—	—	287
5	711	625	535*	—	—	287
6	697	611	535	449	—	287
7	783	697	—	535	611	287
8	799	713	—	551	—	303

*Loss of glucuronic acid $[M-176]^+$.

is clear from the FAB mass spectra (Table 3). Pigments **2** and **6** lose glucose (162) directly from the molecular ion.

The presence of aliphatic acylation in pigments **1**–**8** was also clear from comparison of the relative R_f values and retention times on HPLC (Table 4). Malonylation characteristically increases the retention times of anthocyanins on a spherisorb-hexyl column, usually doubling the elution time over the free anthocyanin. Malonic acid itself was also readily detected as a saponification product of all eight pigments. Additionally, hydrogen peroxide oxidation of all pigments except **4** and **5** yielded 6-malonylglucose, identified by co-chromatography and co-electrophoresis with authentic material prepared by similar oxidation of cyanidin 3-(6″-malonylglucoside) earlier characterized as such by ^{13}C NMR spectroscopy [4]. This established the position of malonyl attachment to the 3-glucose moiety in most cases. With pigment **4**, hydrogen peroxide oxidation gave a dimalonyl-glucose, which could not be characterized as regards the positions of malonylation because of the lack of authentic materials for comparison. However, the dimalonylglucose was well separated from 6-malonylglucose by its significantly lower R_G values on paper and its greater mobility on electrophoresis (see Table 5 and Experimental). Our failure to detect a dimalonylglucose from the other dimalonated pigments (i.e. **3**, **7** and **8**) indicated that in these cases, the second malonyl group must be attached to the glucose in the 5-position. Thus, they have a symmetrical structure with malonylglucose substituents in both the 3- and 5-positions. The FAB-MS results (Table 3) also support these symmetrical structures, because no direct loss of glucose occurs in these cases.

Only in the case of *Helenium* compound **5** was an unexpected pigment formed on saponification. The original **5** was unusually mobile on electrophoresis, behaving like a dimalonate, which after saponification to remove the malonyl substituent, was still electrophoretically mobile and zwitterionic. Its structure became clear when acid hydrolysis yielded both glucose and glucuronic acid. From the results of partial acid hydrolysis and from the FAB-MS fragmentation pattern (Table 3), it is clear that the glucose moiety is linked directly to cyanidin in the 3-position and that glucuronic acid is the terminal sugar. Confirmation of the structure of the deacylated pigment came from FAB-MS (molecular ion 625, $C_{27}H_{29}O_{17}$ requires 625) and from H_2O_2 oxidation, which yielded the expected disaccharide. Partial acid hydrolysis gave the same disaccharide and cyanidin 3-glucoside in low yield. The resistance of the 3-diglycoside to acid hydrolysis is in keeping with the assignment of the glucuronic acid residue to the terminal position. The presence of glucuronic acid substitution was also evident when acid treatment in a methanolic rather than an aqueous solvent led to the esterification of the free carboxyl of the glucuronic acid substituent. The position of malonylation and the nature of the interglycosidic link remain to be determined, but the FAB mass spectrum shows a loss of glucuronic acid (176) directly from the molecular ion suggesting that it is the glucose residue that is malonated.

This pigment is unique in being the first anthocyanin to be reported with glucuronic acid as one of its sugars. It is clearly zwitterionic, even without the additional malonyl substituent. Disaccharides containing glucose and glucuronic acid have been reported in both the flavone and

Table 4. R_f values, electrophoretic mobilities and retention times of Compositae anthocyanins

Pigment	R_f (×100) in*				Mobility† (cm)	RR_t†
	BAW	BuHCl	1% HCl	HOAc–HCl		
Pelargonidin glycosides						
3-Glc	46	33	09	30	0.0	1.00
3-malonyl Glc (1)	48	45	11	39	2.1	2.11
3,5-di Glc	39	16	23	49	0.0	1.00
3-malonyl Glc-5-Glc (2)	38	28	35	67	3.6	1.87
3-malonyl Glc-5-malonyl Glc (3)	38	34			7.1	2.67
Cyanidin glycosides						
3-Glc	29	20	04	19	0.0	1.00
3-malonyl Glc	36	35	07	24	1.3	2.14
3-dimalonyl Glc (4)	36	47	08	30	3.4	2.96
3-malonyl Glc Glur (5)	22	26	54	74	4.5	2.70
3-Glc Glur	21	21	46	65	1.6	—
3,5-di Glc	23	06	13	34	0.0	1.0
3-malonyl Glc-5-Glc (6)	27	20	26	53	3.7	1.76
3-malonyl Glc-5-malonyl Glc (7)	27	24			7.0	2.35
Delphinidin derivatives						
3,5-di Glc	10	02	07	20	0.0	1.00
3-malonyl Glc-5-Glc	22	11	22	46	3.5	1.73
3-malonyl Glc-5-malonyl Glc (8)	22	31			7.0	3.55

*R_fs were measured on microcrystalline cellulose after ascending TLC.

†Mobilities after electrophoresis on Whatman No. 3 paper at pH 4.4 at 40 V/cm and 1 mA/cm for 1.5 hr.

‡Retention times were measured in sec and are related to the times of the corresponding unacylated anthocyanin, e.g. 1 (978 sec) in relation to Pg 3-Glc (463 sec), 2 (433 sec) and 3 (619 sec) in relation to Pg 3,5-di Glc (232 sec), etc. For HPLC conditions, see Experimental.

Table 5. R_G and E_S values for acylated glucoses

Acylated sugar	R_G value in*				E_S†
	BAW	BTPW	BEW	PhOH	
6-Malonylglucose	1.07	0.19	0.26	0.37	0.54
Dimalonylglucose	1.26	0.05	0.09	0.20	0.93
Malonylglucuronosylglucose	0.29	0.02	0.06	0.04	0.85
Succinylglucose‡	1.81	0.30	0.41	1.03	0.28
Malylglucose‡	0.80	0.17	0.19	2.28	0.40

*Measured on PC in *n*-BuOH–toluene–pyridine–H_2O (5:1:3:3), *n*-BuOH–EtOH–H_2O (4:1:2:2) and PhOH (PhOH–H_2O, 3:1).

†Mobility relative to salicylic acid on Whatman No. 1 paper at pH 4.4 and 40 V/cm and 0.5 mA/cm.

‡Obtained respectively from *Centaurea* and *Dianthus* anthocyanins, shown for comparative purposes.

flavonol glycoside series, but without complete characterization. It is therefore not possible at present to say whether they are the same or different from this disaccharide in *Helenium*.

DISCUSSION

As a result of these investigations, the number of zwitterionic anthocyanins known in plants has been significantly expanded. Of the eight new pigments, four might well be expected to be of fairly common occurrence in plant families where zwitterionic anthocyanins are common [cf. 1]. These are the monomalonates of pelargonidin 3-glucoside, pelargonin, cyanin and delphin. Indeed, the 3-(6″-malonylglucoside) of pelargonidin may be identical to the partially characterized 3-malonylglucoside of *Gerbera* flowers [6]. The pigment has also provisionally been detected in *Verbena* flowers [J. B. Harborne, unpublished results].

From electrophoretic surveys, it appears that dimalonates are much less common than monomalonates. So far, they have only been detected in *Cichorium*,

Coleostephus and *Dahlia*, where they are accompanied by the corresponding monomalonates. Two types of dimalonate are apparent: those with two malonic residues attached to the same sugar as in **4**; and those with malonic acid attached separately to different sugars, as in **3**, **7** and **8**.

In all the plants examined in detail, malonic acid was the only organic acid attached to the anthocyanins in spite of the earlier report of acylation by succinic acid in the *Centaurea* anthocyanin. So malonic acid is probably the more usual acyl substituent in the Compositae, since representatives of five different tribes have now been shown to contain malonated pigments. It is possible that the replacement of malonic by succinic is confined either to the genus *Centaurea* or to the tribe Cynareae to which it belongs.

As already mentioned, the discovery of zwitterionic anthocyanins as being almost universally present in the Compositae means that earlier investigations of anthocyanins, where precautions against deacylation were not taken, will have to be revised. This is true in the case of the cornflower *Centaurea cyanus*, the pigment of which was reported in classical times as cyanin but which has been revised as the 6″-succinate [7, 8]. In our work, we have found petals of 14 named cultivars of *Dahlia variabilis* to contain 6″-malonates of perlargonin and cyanin, whereas the unacylated pigments were reported earlier [2, 3]. Additionally nine of the 14 cultivars contain dimalonates (see Table 1). Similarly, earlier investigation of the anthocyanin in blue chicory flowers indicated the presence of delphin [10] while re-examination now shows a mixture of mono- and dimalonate.

Again, early investigation of the pigments in the Chinese aster, *Callistephus chinensis*, indicated the presence of cyanidin and pelargonidin 3-glucoside [11]. More recently, Forkmann [12] reported unidentified aliphatic acid acylation in the anthocyanins of this plant. Our present report of pelargonidin 3-(6″-malonylglucoside) in a scarlet red colour form suggests that other malonated anthocyanins will be found in the petals of this very variable ornamental plant. Further investigation of the anthocyanins of this and other composite genera are in progress.

EXPERIMENTAL

Plant material. Most of the plants sampled were in living collections of the University of Reading botanic garden or of the Department of Horticulture at Shinfield Grange. *Coleostephus myconis*, *Cichorium endivia* and *C. intybus* were grown from authenticated seed. Species of *Gaillardia*, *Helichrysum*, *Helipterum*, *Liatris* and *Osteospermum* were plants of horticultural origin.

Isolation and purification. Pigments were extracted from freshly picked flowers with MeOH–HOAc–H_2O (10:1:9, MAW) and the filtered extracts taken to dryness *in vacuo* at 30°. The residue, after dissolution in MAW, was passed through a Sephadex LH 20 column (1.0 × 20 cm) in the same solvent. The pigment fraction was again taken to dryness and further purified by prep. PC in the solvents *n*-BuOH–HOAc–H_2O (4:1:5, top) and 15% HOAc. Finally, the pigment was once more purified on Sephadex LH 20 in MAW. Since malonated pigments decompose slowly in soln, they were routinely stored at 0° as solids, after evaporation to dryness.

Pigment identifications. The pure pigments were subjected to absorption spectrophotometry, FAB-MS (Table 3), TLC on microcrystalline cellulose against standards in four solvents (Table 4), electrophoresis at pH 4.4 (Table 4) and HPLC (see below). Acid hydrolyses and H_2O_2 oxidations were carried out by standard procedures and the products identified in the usual way. R_G and E_S values for the acylated sugars obtained by H_2O_2 oxidation are given in Table 5.

Mass spectrometry. Positive ion FAB mass spectra were recorded on UV-sensitive paper using the Kratos MS9/50TC mass spectrometer operated at 8 kV accelerating voltage and equipped with an Ion-Tech II NF atom gun. A beam of fast atoms of xenon (9 kV nominal) was used to desorb ions of the sample dissolved in glycerol. The data reported in Table 3 represent the major ions attributed to the sample, the matrix background having been subtracted.

High performance liquid chromatography. HPLC was carried out on a reversed phase Spherisorb-hexyl (5 μm) column (1.5 × 10 cm) at 35°, gradient eluted with solvent A, 0.6% aq. $HClO_4$ and solvent B, MeOH, with detection at 520 nm and a flow rate of 1 ml min^{-1}. For pigment **4** (Table 4), the gradient was based on increasing the amount of B in A, i.e. 20% B for 15 min, 30% B for 5 min, 40% B for 8 min, followed by 98% B. For all the other pigments, the gradient changed from 22% to 33% B in 11 min, 33% B for 10 min, changing from 33% to 40% in 14 min, followed by 95% B.

Detection of malonic acid. After saponification of each acylated pigment and acidification, the hydrolysate was allowed to evaporate to dryness in air current at room temp. The organic acid was dissolved into Et_2O and this extract was chromatographed on microcrystalline cellulose against authentic markers in four solvents, with detection with glucose–aniline. The solvents were BAW, *n*-BuOH–HCO_2H–H_2O (4:1:5), EtOAc–HOAc–H_2O (3:1:1) and EtOH–H_2O–NH_4OH (16:3:1). In all cases, malonic acid was the only acid detected.

Helenium anthocyanin. This pigment had $\lambda_{max}^{MeOH-HCl}$ at 284, 335 and 532 nm, the E_{440}/E_{max} ratio being 21%. The saponified pigment (cyanidin 3-glucuronosylglucoside) gave a disaccharide on H_2O_2 oxidation. On controlled acid hydrolysis in 2 N HCl at 85°, it was very resistant (some unchanged pigment after 2 hr) but was slowly degraded to cyanidin, only traces of cyanidin 3-glucoside were formed as intermediate. Controlled acid hydrolysis in 2 N HCl containing 70% MeOH rapidly led to the formation of the methyl ester and this gradually degraded to cyanidin.

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