# The Widespread Occurrence in Nature of Anthocyanins as Zwitterions

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Z. Naturforsch. **40c,** 305–308 (1985); received January 16, 1985

Angiosperms, Flower Pigments, Malonated Anthocyanins, Zwitterions

An electrophoretic survey of anthocyanins in flowers of 81 species belonging to 27 plant families indicated that malonated or similarly substituted zwitterionic anthocyanins occur in half the sample. In particular, almost all species surveyed in the Compositae and the Labiatae were positive. The same results were also indicated by HPLC separations. Detailed examination of the anthocyanins of *Dahlia variabilis* and *Plectranthus argentatus* confirmed the presence of malonylation through glucose. It appears that anthocyanins substituted by aliphatic dicarboxylic acids may be widespread in the angiosperms.

## Introduction

Until recently, anthocyanins containing acyl substitution were recorded in a small number of plant families and the acyl group present was usually an aromatic organic acid, such as *p*-coumaric, or acetic acid [1, 2]. However, there have been several recent reports of malonated anthocyanins, *e.g.* of pelargonidin 3-malonylsophoroside in flowers of *Papaver nudicaule* [3] and of cyanidin 3-(6-malonylglucoside) in leaves of *Cichorium intybus* [4]. More significantly, Japanese workers investigating anthocyanins responsible for blue flower colour in *Commelina communis* and *Centaurea cyanus,* discovered the pigments to have malonic and succinic acids respectively, where previous investigations had failed to show such acylation to be present [5, 6]. Such acyl groups are labile in solutions containing mineral acid, so that in the standard procedures for extracting anthocyanins from plant tissues which use methanolic HCl, these substituents are likely to be lost. Only by using alcoholic solvents where the HCl is replaced by acetic or formic acids is it possible to isolate the pigments with the dicarboxylic acid substituents intact.

Malonated derivatives have been described among other flavonoids; *e.g.* malonated flavone glycosides occur in *Petroselinum crispum* plant and cell culture extracts [7] and malonated isoflavone glycosides have recently been reported in *Cicer arietinum* roots and stems [8]. In almost all cases, malonic acid is linked through sugar and commonly is attached to the 6-position of glucose. In the case of flavones and isoflavones, the attachment of malonic acid confers a negative charge on the molecule and this may be important to the way the compound is stored in the vacuole or in protecting the glycoside from enzymic hydrolysis by glycosidases. In the case of anthocyanins, malonylation balances the cationic charge on the flavylium ion, so that the malonated anthocyanin is effectively a zwitterion. Thus, the need to assume that anthocyanins occur in the cell vacuole in loose ionic association with organic acids, as suggested in the earlier literature, is removed for such pigments.

Because of their lability, such zwitterionic anthocyanins might well have been overlooked in earlier studies. We therefore set out to investigate whether malonylation or similar acylation might not occur quite widely among the anthocyanins of the angiosperms.

## Results

The observation of Cornuz *et al.* [3] that the malonated pelargonidin 3-sophoroside of *Papaver nudicaule* could be distinguished from the related nonacylated pigment by its mobility on paper electrophoresis at pH 4.4 provided us with a simple general screening procedure for such pigments. We found that at this pH anthocyanin colours tend to fade rapidly, so that after development, we dipped the papers briefly in 1% HCl in order to stabilize the colour. Using an extraction medium based on aqueous methanolic acetic acid, we surveyed the flower tissues of some 81 species and obtained positive results in 40 samples (Table I).

These results considerably extend earlier records of malonated anthocyanins in plants. Of the 27 angiosperm families surveyed, positive records were made in 10. In fact, the number of families contain-

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/85/0500–0305 $ 01.30/0

Table I. Electrophoretic survey of angiosperm flowers for zwitterionic anthocyanins.

| Plant species | Mobile Pigment Present* |
|---|---|
| Boraginaceae | |
| *Borago officinalis* L. | – |
| *Echium vulgare* L. | – |
| Bromeliaceae | |
| *Billbergia decora* Poepp. & Endl. | – |
| Campanulaceae | |
| *Campanula cervicaria* L. | – |
| *Codonopsis cardiophylla* Diels ex Kom. | – |
| Cannaceae | |
| *Canna edulis* Ker-Gawl | – |
| Caryophyllaceae | |
| *Dianthus deltoides* L. | + |
| *Dianthus sylvestris* Wulf. | + |
| *Lychnis flos-jovis* Desr. | + |
| Commelinaceae | |
| *Tradescantia navicularis* Ortgies | – |
| *Tradescantia* cv. Leonora | – |
| Compositae | |
| *Ageratum* cv. Ocean | + |
| *Arctotis* sp. | + |
| *Bellis perennis* L. | + |
| *Bidens* sp. | – |
| *Callistephus hortensis* Cass. | + |
| *Catananche caerulea* L. | + |
| *Centaurea montana* L. | + |
| *C. nigra* L. | + |
| *Cicerbita plumieri* (L.) Kirschleger | + |
| *Cichorium endivia* L. | + |
| *C. intybus* L. | + |
| *Cirsium* sp. | + |
| *Dahlia variabilis* Desf. | + |
| *Helenium* cv. Bruno | + |
| *Helianthus annuus* L. cv. | + |
| *Liatris spicata* Willd. | + |
| *Senecio coccinaeflorus* Rowl. | – |
| *Stokesia laevis* Hill | + |
| *Tanacetum coccineum* Willd. | + |
| *Zinnia* cv. Persian Carpet | + |
| Convolvulaceae | |
| *Convolvulus arvensis* L. | – |
| *Convolvulus* cv. | + |
| Crassulaceae | |
| *Crassula schmidtii* Regel | – |
| Dipsacaceae | |
| *Knautia arvensis* (L.) Coult. | – |
| Ericaceae | |
| *Daboecia cantabrica* (Hudson) C. Koch | – |
| Geraniaceae | |
| *Geranium maderense* Yeo | – |
| *G. pratense* L. | – |
| *G. himalayense* Klotzsch. | – |
| Gesneriaceae | |
| *Streptocarpus* cv. | + |
| *S. saxorum* Engl. | – |
| Iridaceae | |
| *Iris cristata* Soland. | – |
| *Schizostylis coccinea* Backh. & Harv. | – |
| Labiatae | |
| *Hyssopus officinalis* L. | + |
| *Lavandula stoechas* L. | + |
| *Monarda didyma* L. | + |
| *Plectranthus argentatus* Blake | + |
| *Prunella vulgaris* L. | + |
| *Salvia nemorosa* L. | + |
| *S. verticillata* L. | + |
| *S. virgata* Ait. | + |
| *Stachys macrantha* | + |
| *S. sylvatica* L. | + |
| *Thymus praecox* Opiz ssp. *arcticus* | + |
| *T. pulegioides* L. | + |
| Leguminosae | |
| *Lathyrus latifolius* L. | – |
| *L. odoratus* L. | – |
| *Ononis spinosa* L. | – |
| *Vicia sativa* L. | – |
| Lobeliaceae | |
| *Lobelia* cv. Mrs. Clibran | + |
| Malvaceae | |
| *Abutilon hybridum* Hort. | – |
| *Hibiscus rosa-sinensis* L. | – |
| Onagraceae | |
| *Epilobium angustifolium* L. | – |
| *Fuchsia fulgens* DC. | – |
| Papaveraceae | |
| *Eschsholtzia* cv. | – |
| *Papaver orientale* L. | – |
| Polemoniaceae | |
| *Phlox drummondii* L. | + |
| *Polemonium caeruleum* L. | – |
| Punicaceae | |
| *Punica granatum* L. | – |
| Ranunculaceae | |
| *Aconitum napellus* L. | – |
| *Clematis integrifolia* L. | + |
| Rosaceae | |
| *Potentilla atrosanguinea* Loddiges ex D. Don | – |
| *P. nepalensis* Hook | – |
| *Rosa multiflora* cv. Crimson Rambler | – |
| Scrophulariaceae | |
| *Digitalis purpurea* L. | – |
| *Hebe* cv. | + |
| *Penstemon* cv. Skyline | – |
| Solanaceae | |
| *Atropa belladonna* L. | – |
| *Browallia demissa* L. | + |
| *Nicandra physalodes* (L.) Gaertn. | – |
| Valerianaceae | |
| *Centranthus ruber* (L.) DC. | – |

* + = Pigment moves ca 3 cm on electrophoresis in acetate buffer, pH 4.4, at 40 V/cm for 2 h.
– = Pigment remains at origin.

ing malonated anthocyanins is higher than this, since such pigments have been recorded in individual species of the Commelinaceae [5], Leguminosae [9] and Papaveraceae [3]. In some families such as the Leguminosae (see Table I) malonated anthocyanins may be relatively rare, but in others, such as the Compositae and Labiatae, malonated anthocyanins appear to be almost universal. The regular occurrence (Table I) of malonated pigments in the Labiatae is a little surprising since the anthocyanins in this family are usually acylated with hydroxycinnamic acids; clearly malonylation can occur irrespective of any other type of acylation present.

In order to confirm these findings, detailed studies were made on the anthocyanins in a representative species of the Compositae, in *Dahlia variabilis,* and of the Labiatae, in *Plectranthus argentatus.* In the case of *Dahlia,* earlier investigations have established the presence of pelargonidin 3,5-diglucoside (pelargonin) and cyanidin 3,5-diglucoside variously in garden forms [1, 2]. Extraction under mild conditions of the major pelargonidin derivative from petals of a scarlet form of *D. variabilis* gave an acylated pigment, different in Rf from pelargonin (see Experimental). On mild hydrolysis, it gave pelargonin while on complete hydrolysis, it gave pelargonidin and glucose. Confirmation of the presence of a malonyl group was obtained by $H_2O_2$ oxidation, which gave a malonylglucose, liberated from the 3-position, which co-chromatographed in 4 solvents with authentic 6-malonylglucose, prepared similarly from the cyanidin 3-malonylglucoside of chicory leaves. The original *Dahlia* pigment can thus be formulated as pelargonidin 3-(6-malonylglucoside)-5-glucoside, which has not been described before. Similar studies on the zwitterionic leaf pigment of *Plectranthus argentatus* showed the presence of peonidin 3,5-diglucoside, substituted with both malonic and *p*-coumaric acids.

HPLC was also used to confirm the acylated nature of these pigments. Using a $C_8$ column and gradient elution with aqueous methanolic acetic acid mixtures, malonated pigments consistently eluted later than the corresponding unacylated anthocyanins (Table II). Attempts to use HPLC for direct screening of plant extracts for these pigments have not so far been successful, but the use of ion-exchange columns may make this possible. It was of interest that a flower extract containing a malonated pigment, when placed on an ion exchange column (Amberlite IRC 50), was not retained in the usual way [11], but passed straight through.

Table II. HPLC separation of Anthocyanins from their malonyl derivates.

| Compound | $t_R$ [min]* |
|---|---|
| Cyanidin 3-glucoside | 10.77 |
| Malonate from *Cichorium intybus* leaf | 15.49 |
| Delphinidin 3,5-diglucoside | 5.14 |
| Dimalonate (?) from *Cichorium intybus* petals | 12.23 |
| Pelargonidin 3,5-diglucoside | 7.37 |
| Malonate from *Dahlia variabilis* petals | 10.33 |
| Peonidin 3,5-diglucoside | 6.76 |
| *p*-coumaryl malonate from *Plectranthus* leaf | 21.11 |

* On a Partisil 5 CCS $C_8$ column (25 cm × 4 mm) eluted with A, 5% aqu HOAc; B, MeOH-$H_2O$-HOAc (18:1:1), initial concentration 20% B in A, increasing by 2% B per min, flow rate 1.7 ml $min^{-1}$, detection at 546 nm.

## Discussion

These results together indicate that anthocyanins substituted with aliphatic dicarboxylic acids may be quite widespread in nature, particularly among the more highly evolved angiosperm families. Of the known organic acids, malonic would seem to be the most common, since it has been reported most frequently. However, succinic acid has been reported as the acylating acid in the *Centaurea* anthocyanin [6] and it is possible that other aliphatic dicarboxylic acids will be found as well.

The present studies were conducted mainly on flower pigments but there is little doubt that malonated pigments occur in other parts of the plant as well. Where pigmentation is found in both leaf and flower, it is likely that malonated anthocyanins will be found in both parts. This is true in the chicory, *Cichorium intybus,* where cyanidin 3-(6-malonylglucoside) has already been identified in varieties with reddish leaves [4]. Our preliminary examination of the delphinidin derivative of the blue flowers indicates that it is a mixture of mono- and dimalonated delphinidin 3,5-diglucoside and not delphin, as previously recorded [10].

The present data do now indicate the need to check for the presence of malonyl groups in any modern investigation of plant anthocyanins. This can be done using a weak acid extraction medium and electrophoresis or HPLC. Clearly, further work is needed to substantiate the findings reported here and more detailed investigations of the anthocyanins in the Compositae and Labiatae are in progress.

## Experimental

Floral material was freshly collected either from the University of Reading botanic gardens or from wild plants collected locally. Pigments were extracted into MeOH-HOAc-$H_2O$ (19:2:19), filtered and the solution concentrated in vacuo at 30°. Electrophoresis was conducted on Whatman No. 3 paper in acetate buffer pH 4.4 for 2 h at 40 V/cm. The papers were immediately removed, dipped briefly in 1% MeOH-HCl and then dried. Cationic anthocyanins remained close to the origin, whereas zwitterionic anthocyanins were clearly distinguished by their movement (3–4 cm) towards the anode.

The malonated pigments in individual species were isolated from similar crude extracts by prep. paper chromatography using the solvents BAW (*n*-BuOH-HOAc-$H_2O$, 4:1:5, top) and 15% HOAc, repeating where necessary to remove impurities. $R_f$ measurements were determined in these two solvents, as well as in BuHCl (*n*-BuOH-2 N HCl, 1:1, top) and HOAc-HCl (HOAc-HCl-$H_2O$, 15:3:82). Details of HPLC separations are given in Table II.

Pelargonidin 3-malonylglucoside-5-glucoside isolated from scarlet blooms of *Dahlia variabilis* had the following $R_f$ (×100) (pelargonin values in parentheses) in BAW 41 (36), in BuHCl 55 (36), in 1% HCL 40 [26] and in HOAc-HCl 66 [53]. The spectral properties were identical to those of pelargonin. On electrophoresis at pH 4.4 for 2 h, it moved 3.4 cm, but some pelargonin (0.8 cm) was formed as a result of hydrolysis. On $H_2O_2$ oxidation, it gave a malonylglucose, identical in 4 solvents with 6-malonylglucose prepared from cyanidin 3-(6-malonylglucoside) from chicory leaves. $R_G$ values were 1.2 in BAW, 0.17 in *n*-BuOH-toluene-pyridine-$H_2O$ (5:1:3:3), 0.34 in *n*-BuOH-EtOH-$H_2O$ (4:1:2:2, top) and 0.36 in PhOH-$H_2O$ (3:1). These agree with lit. values [3].

The peonidin 3,5-diglucoside acylated with both *p*-coumaric and malonic acids from red leaves of *Plectranthus argentatus* had the following Rfs (×100) (di-*p*-coumaroylpeonidin 3,5-diglucoside values in parentheses) in BAW 55 (63), in BuHCl 47 (43), in 1% HCl 12 (12) and in HOAc-HCl 58 (59). The spectral properties, $\lambda_{max}^{MeOH\text{-}HCl}$ 282, 315 ($E_{315}/E_{max}$ 84%), 440 ($E_{440}/E_{max}$ 15%), 528 nm indicated disubstitution with *p*-coumaric acid and on acid hydrolysis, *p*-coumaric, acid, peonidin and glucose were readily detected. Deacylation with alkali gave peonidin 3,5-diglucoside. $H_2O_2$ oxidation gave 6-malonylglucose, identified as above. These results suggest that the *p*-coumaroyl groups are attached to the 5-glucose residue, while the malonyl group is present on the 3-glucose.

The authors thank Mrs. Margaret Meakin for experimental assistance.

[1] J.B. Harborne, Comparative Biochemistry of the Flavonoids, Academic Press, London 1967.

[2] G. Hrazdina, The Flavonoids: Advances in Research (J.B. Harborne and T. Mabry, eds.) pp. 135–188. Chapman & Hall, London 1982.

[3] G. Cornuz, H. Wyler, and J. Lauterwein, Phytochemistry **20,** 1461 (1981).

[4] P. Bridle, R.S.T. Loeffler, C.F. Timberlake, and R. Self, Phytochemistry **23,** 2968 (1984).

[5] T. Goto, T. Kondo, H. Tamura, and S. Takase, Tetrahedron Lett. **24,** 4863 (1983).

[6] H. Tamura, T. Kondo, Y. Kato, and T. Goto, Tetrahedron Lett. **24,** 5749 (1983).

[7] F. Kreuzaler and K. Hahlbrock, Phytochemistry **12,** 1149 (1973).

[8] J. Köster, D. Strack, and W. Barz, Planta Med. **48,** 131 (1983).

[9] N. Saito, K. Abe, T. Honda, C.F. Timberlake, and P. Bridle, Phytochemistry **24** in press (1985).

[10] J.B. Harborne, The Biology and Chemistry of the Compositae (V.H. Heywood, J.B. Harborne and B.L. Turner, eds.) pp. 359–384. Academic Press, London 1977.

[11] S.J. Jarman and R.K. Crowden, Phytochemistry **12,** 171 (1973).