

Cyanidin 3-Oxalylglucoside in Orchids

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A new acylated anthocyanin has been isolated from flowers of *Nigritella* and *Orchis* and identified as cyanidin 3-0- β -(6''-0-oxalyl)-glucoside on the basis of TLC, HPLC, FAB MS, ^1H and ^{13}C NMR. This pigment was found to be present in various members of the Orchidaceae.

Introduction

In the last few years increasing evidence has been presented that acylated flavonoids are widespread in higher plants [1–3]. Besides the well known phenolic acids (hydroxybenzoates, hydroxycinnamates), aliphatic acids have only recently been extensively detected as acyl moieties of anthocyanins or glycosides of (iso)flavones and flavonols. To date, acetic, butyric, malonic, 2-methylbutyric, succinic and tiglic acids have been identified, among which malonic acid appears to be the most commonly occurring one. As a consequence of the great lability of aliphatic acid esters, such natural products might have been frequently overlooked in earlier studies [3, 4].

Although there is only fragmentary knowledge of orchid anthocyanins [5], it is known that complex mixtures of acylated and non-acylated anthocyanins cause a broad spectrum of orchid flower colouration. In a survey of European orchids, Uphoff [6] described the widespread occurrence of cyanidin 3-monoglucoside, 3-diglucoside and 3,5-diglucoside together with complex acylated anthocyanins, the nature of which needed further investigation.

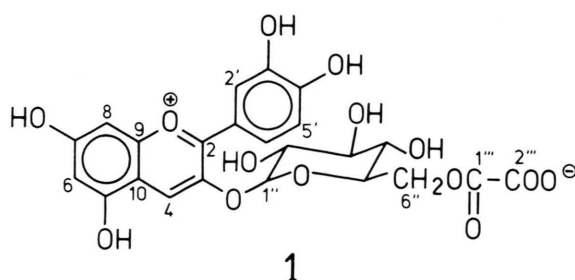
As first part of a comprehensive investigation of phenylpropanoids in flowers of European orchids, we report here on one of their major acylated antho-

cyanins, *i.e.* cyanidin 3-0- β -(6''-0-oxalyl)-glucoside, which is a new natural compound, present in large quantities in flowers of various species of *Orchis* and *Nigritella* as well as in *Ophrys insectifera*.

Results and Discussion

Chromatography (TLC on SS1 and HPLC) of anthocyanin extracts from flowers of some *Nigritella* and *Orchis* species gave complex mixtures of cyanidin glucosides, composed of the 3-mono, 3,5-diglucoside (data not shown) and several unknown components, among which cyanidin 3-oxalylglucoside (**1**) was the major pigment. The latter was isolated (5–6 mg from approx. 1.5 g dry wt.) by two successive column chromatography steps, (i) on polyamide with 50% aq. methanol containing 0.01% HCl (compare ref. [7]) and (ii) on Sephadex LH-20 with water containing 0.01% HCl.

The UV/Vis spectrum of **1** in methanol (0.01% HCl) showed a visible max. at 526 nm with $E_{440}/E_{\text{vis. max}}$ of 21%. The addition of a few drops of 5% AlCl_3 (in MeOH) resulted in a bathochromic shift of 20 nm. Thus the spectral data indicated the presence of a catechol nucleus and 3-glycoside.



Abbreviations: FAB MS, fast atom bombardment mass spectroscopy; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

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Co-chromatographic analyses of a partially hydrolysed intermediate (3-monoglucoside) and products from complete acid hydrolysis (cyanidin, glucose) indicated that the basic structure of **1** was cyanidin 3-monoglucoside. The great lability of **1** (the 3-monoglucoside was readily obtained as breakdown product) and its behaviour on polyamide column chromatography [7] indicated the presence of an acyl moiety, probably an aliphatic acid since neither a hydroxycinnamic nor a hydroxybenzoic acid could be detected as a part of the molecule. This was substantiated by the fact that treatment with 0.05 M NaOH at room temperature gave within 2 min a complete conversion of **1** to the cyanidin 3-monoglucoside. Thus the nature of the possible aliphatic acid moiety and its linkage to the cyanidin 3-monoglucoside remained to be identified.

The presence of an aliphatic acid was also suggested by thin-layer electrophoresis, which in addition made malonic acid as a possible acyl moiety unlikely. **1** remained at the origin at pH 4.4 at which malonylated anthocyanins showed movements of 3–4 cm towards the anode by the method described in ref. [3]. However, **1** moved 3.3 cm at pH 7.5. These different, pH-dependent mobilities of zwitterionic anthocyanins could be useful indicators

for the nature of aliphatic (dicarboxylic) acids in acylated pigments (hemiesters).

Search by means of co-chromatography (SS4, SS5, SS6) with reference material of a possible aliphatic acid as a deacylation product of **1**, readily excluded malonic acid, but unexpectedly showed identity with oxalic acid.

This was unambiguously proven by ^1H - and ^{13}C NMR spectroscopy and the structure of **1** was found to be cyanidin 3-O- β -(6"-O-oxalyl)-glucoside (see scheme). A two dimensional ^1H COSY spectrum gave directly the through-bond connectivities, while the chemical shifts and proton-proton coupling constants were determined from the normal one dimensional ^1H spectrum. The vicinal coupling constant data were characteristic of a β -glucoside while the ^1H shifts indicated the presence of the cyanidin moiety. The low field shifts of H-6"A and H-6"B showed the presence of a substituent at C-6". Confirmation of the structure was obtained by comparison of the ^1H - and ^{13}C data with recent literature [8] for cyanidin and chrysanthemin (cyanidin 3-O- β -glucoside). In particular, the present ^{13}C data were identical with the latter apart from the shifts of C-5" and C-6" which were indicative of acylation at C-6". The presence of an oxalyl moiety was deduced from the appearance

Table I. Distribution of **1** in flowers of some members of the Orchidaceae; + = detected; ++ = dominant and/or in high concentration; (+) = in low concentration; tr. = trace^a; – = not detected.

Plant species	Occurrence ^b	Plant species	Occurrence
<i>Anacamptis pyramidalis</i>	+	<i>N. l. ssp. corneliana</i>	++
<i>Cephalanthera rubra</i>	–	<i>N. l. ssp. lithopolitana</i>	(+)
<i>Dactylorhiza fuchsii</i>	tr.	<i>Neottianthe cucullata</i>	tr.
<i>D. majalis</i>	(+)	<i>Ophrys insectifera</i> ^c	++
<i>D. traunsteineri</i>	tr.	<i>Orchis mascula</i>	+
<i>Epipactis atrorubens</i>	tr.	<i>O. militaris</i>	+
<i>Gymnadenia conopsea</i>	(+)	<i>O. morio</i>	(+)
<i>G. odoratissima</i>	(+)	<i>O. purpurea</i> ^d	++
<i>Nigritella miniata</i>	++	<i>O. tridentata</i>	+
<i>N. nigra</i>	++	<i>O. ustulata</i>	+
<i>N. n. x G. conopsea</i>	(+)	<i>Traunsteinera globosa</i>	tr.

^a Tentative result; detected only by HPLC.

^b In *Dactylorhiza* species **1** was exclusively found in the pollinia, in *Orchis* species dominant in the pollinia and as a minor constituent in the other parts of the flowers. *N. nigra* and *N. miniata*, harvested in Austria and Italy and in Austria and Switzerland, respectively, did not show any significant habitat-dependent differences in their anthocyanin patterns.

^c 70–75% in the labellum and

^d approx. 55% of the total anthocyanin contents in the pollinia and 30% in the other flower parts.

of two extra carbonyl signals in the ^{13}C spectrum and the absence of any further signals in both the ^{13}C - and ^1H spectra that could be attributed to the substituent.

Finally, the above was confirmed by the negative ion FAB mass spectroscopy. The measurement of this zwitterion spectrum proved difficult and was only successful when a pure sample of **1** was available. The deprotonated molecular ion was observed at m/z 519 and this was confirmed by the observation of a chlorine atom adduct at m/z 554, derived from the HCl always present from the chromatographic purification. Ions corresponding to the sequential loss of oxalate and the sugar moiety were also detected.

To the best of our knowledge, compound **1** is a new naturally occurring acylated anthocyanin. Results of a survey (TLC on SS1, HPLC) of flower pigmentation in some orchids from various European countries (see Experimental and Table I) indicated that **1** might be a marker pigment, characteristic for species of *Nigritella*, *Orchis* and possibly also *Ophrys*. It appears also to be present as a minor constituent in some species listed in Table I. This finding could be relevant to the systematics of orchids (compare refs. [5, 6]; see also a survey of leaf flavonoids in ref. [9]). A representative survey of the complete patterns of anthocyanins in orchid flowers and their possible usefulness in studies on European orchids systematics is presently in progress in our laboratories (E. B., doctoral thesis). The previously published suggestion of Uphoff [6] for the existence of "orchicyanins", which are cyanidin 3,5-diglucosides with substituents of both an organic acid and a flavonol and described to occur in various members of the Orchidaceae, as well as in *Dactylorhiza*, *Gymnadenia* and *Orchis*, could not yet be verified.

In general, this paper supports the assumption that acylating aliphatic acids are more widespread in anthocyanin structures than known to date [3, 10] and they may play a prominent role in plant pigmentation.

Experimental

Plant material

The following orchids, harvested at their habitats from May to July 1983, were investigated: *Anacamptis pyramidalis* L. C. Rich. var. *tanayensis* Chenev. (Miex, Switzerland), *Dactylorhiza fuchsii* (Druce)

Soo (Miex, Switzerland), *D. majalis* (Rchb.) Hunt & Summerh. (Lütmarsen, FRG), *D. traunsteineri* (Saut ex Rchb.) Soo (Waterford, Ireland); *Cephalanthera rubra* L. C. Rich. (Stahle/Höxter, FRG), *Epipactis atrorubens* (Hoffm. ex Bernh.) Besser (Salzhemmendorf, FRG), *Gymnadenia conopsea* (L.) R. Br. (Nieheim, FRG), *G. odoratissima* (L.) L. C. Rich. (Sexten-Moos, Italy); *Neottianthe cucullata* (L.) R. Schltr. (Augustow, Poland), *Nigritella lithopolitana* Ravník ssp. *corneliana* (Beauv.) Teppner & Klein (Sestriere, Italy), *N. lithopolitana* Ravník ssp. *lithopolitana* (Petzen, Austria), *N. miniata* (Crantz) Janchen (Bernina-Paß, Switzerland; Schneeberg, Austria), *N. nigra* (L.) Rchb. fil. (Schneeberg, Austria; Puflatsch, Italy), *N. nigra* x *Gymnadenia conopsea* (Puflatsch, Italy); *Ophrys insectifera* L. (Fredesloh, FRG); *Orchis mascula* (L.) L. (Brenkhausen/Höxter, FRG; Fredesloh, FRG), *O. militaris* L. (Amelunxen, FRG), *O. morio* L. (Stadt Kyll, FRG), *O. purpurea* Huds. (Bevern/Holzminden, FRG; Einbeck, FRG), *O. tridentata* Scop. (Ottbergen, FRG), *O. ustulata* L. (Miex, Switzerland), *Traunsteinera globosa* (L.) Rchb. (Miex, Switzerland).

Extraction and isolation

Flowers freshly picked in their habitats were immediately submerged into 90% MeOH–1% citric acid (v/v) in H_2O in brown bottles. Once in the laboratory, these were stored at -20°C until they were processed as follows. After filtration the extraction of the remaining flower material was completed by several washes with MeOH. The filtrates were concentrated under vacuum at 30°C to a small volume (2–5 ml). Compound **1** was isolated from combined extracts of flowers from species of *Nigritella* and *Orchis*. The anthocyanins were fractionated on a polyamide column at room temperature (CC-6, 4×32 cm; Macherey, Nagel & Co., Düren, FRG) using H_2O (2 l), MeOH (2 l) and 0.01% HCl (by volume) in 50% aq. MeOH (1 l) (compare ref. [7]). The latter fraction contained **1** and was evaporated to dryness and the resulting residue redissolved in approx. 4 ml H_2O . This was chromatographed in a cold room (4°C) on a Sephadex LH-20 column (3×60 cm; Pharmacia, Uppsala, Sweden) using approx. 5.5 l of 0.01% HCl in H_2O (v/v) at a flow-rate of 1.0–1.5 ml/min. This method allowed complete elimination of contaminating phenolics and separation of some of the deacylation product, the

cyanidin 3-monoglucoside. Elution was monitored continuously by UV absorption at 254 nm (Uvicord; LKB, Gräfelfing, FRG) for detection of non-pigment phenolics. Elution of **1** was visually controlled.

Thin-layer chromatography

On microcrystalline cellulose ("Avicel"; Macherey, Nagel): (SS1) acetic acid–HCl–H₂O (15:3:82, by volume), (SS2) acetic acid–HCl–H₂O (30:3:10, by volume), (SS3) *n*-butanol–acetic acid–water (4:1:5, by volume, upper phase), (SS4) MeOH–acetone–acetic acid–water (2:4:1:1, by volume), (SS5) pentanol–formic acid–water (6.1:6.1:0.3, by volume); on polyamide (TLC-6; Macherey, Nagel): (SS6) acetonitrile–ethyl acetate–formic acid (81.8:9.1:9.1); on silica gel (Merck, Darmstadt, FRG) according to ref. [11], impregnated with a 0.5 M NaH₂PO₄ solution before use: (SS7) 0.1 M lactic acid–isopropanol–acetone (1:2:2, by volume), development of sugar spots by spraying with aniline–diphenylamine–acetone–80% phosphoric acid (2:2:100:15, ml/g/ml/ml) and heating for 15 min at 100 °C (glucose gave blue colour). Carboxylic acids (SS4, SS5, SS6) were visualized by spraying the dried chromatogram with bromocresol green in alkaline (NaOH) ethanol. *R_f* values (× 100): **1** on SS1 = 27; cyanidin 3-glucoside on SS1 = 7, SS2 = 48, SS3 = 65; oxalic acid on SS4 = 61, SS5 = 44, SS6 = 11; glucose on SS7 = 42. Co-chromatography with cyanidin 3-glucoside (*Petunia hybrida*) [12] and cyanidin (hydrolysis product from an extract from *Sambucus nigra*) [13] from the institute collection (Cologne), with glucose and oxalic acid from commercial sources.

Thin-layer electrophoresis

This was performed on Avicel plates in acetate buffer for 2 h at 40 V/cm (compare ref. [3]). Whereas there was no movement at pH 4.4, at pH 7.5 compound **1** moved 3.3 cm towards the anode (no movement of cyanidin 3-glucoside).

High performance liquid chromatography

The HPLC apparatus (LKB) and the data processor (Shimadzu) are described elsewhere [14]. The chromatographic column (250 mm long, 4.6 mm inner diameter) was prepacked with Shandon ODS-Hypersil (5 µm) (Bischoff Analysentechnik und -geräte GmbH, Leonberg, FRG); detection at

525 nm; elution system: linear gradient elution within 50 min from 5 to 40% solvent B (1.5% phosphoric acid, 20% acetic acid, 25% acetonitrile in water) in solvent A (1.5% phosphoric acid in water); flow-rate at 1 ml/min (cyanidin 3-glucoside = *R_t* 24.4 min, **1** = *R_t* 28.9 min).

Hydrolysis

For complete hydrolysis **1** was heated in 25% aq. HCl (v/v) for 5–10 min at 100 °C. The aglycone was extracted into a small volume of amyl alcohol followed by TLC on SS2 and SS3. The aq. phase was kept for sugar identification (TLC on SS5). For partial hydrolysis and HPLC of its products see ref. [15]. Deacylation (liberation of oxalate) was accomplished by treatment with 0.05 M NaOH in water at room temperature: 50% deacylation after 20 s, completed after 2 min (HPLC analysis). The latter was neutralized by means of cation exchange column chromatography (Dowex 50 W × 8, Serva, H⁺ form).

UV/Vis spectroscopy was performed according to ref. [16].

¹H NMR spectra (400 MHz) and ¹³C NMR spectra (100 MHz) were recorded at ambient temperature on a Bruker WM-400 NMR spectrometer locked to the deuterium resonance of the solvent, CD₃OD. A two dimensional COSY ¹H spectrum was recorded with a 90°-t₁-90°-FID(t₂) pulse sequence. The spectral width was F2 3144.6 Hz and F1 ± 1572.3 Hz with 1K data points in t₂ and 512 data points in t₁. Eight pulses were taken for each t₁-increment with a relaxation delay of 0.4 s between pulse sequences to give a total accumulation of 1.2 h. The data were multiplied by sine-bell functions and one level of zero-filling was used for both t₁ and t₂. All one and two dimensional spectra were recorded using the standard Bruker software package. Chemical shifts are recorded in ppm relative to TMS and coupling constants in Hz.

Negative ion fast atom bombardment (FAB) mass spectra were recorded on a Kratos MS 50 mass spectrometer equipped with a Kratos FAB source; glycerol, initially acidified with acetic acid, was used as matrix.

Cyanidin 3-*O*-β-(6''-*O*-oxalylglucoside) (**1**). ¹H NMR (CD₃OD/trace DCl): δ = 8.949 [s; H-4]; 8.285 [d; H-6'; J(6'-5') 8.2 Hz]; 8.025 [s; H-2']; 7.029 [d;

H-5'); 6.929 [s; H-8]; 6.705 [s; H-6]; 5.313 [d; H-1"; J(1"-2") 7.6]; 4.592 [d; H-6"B; J(6"B-5") 7.2]; 3.854 [d, d; H-5"; J(5"-4") 9.6]; 3.706 [d, d; H-2"; J(2"-3") 8.8]; 3.590 [d, d; H-3"; J(3"-4") 8.9]; 3.452 [d, d; H-4"]. — ¹³C NMR (CD₃OD/trace DCl): δ = 170.43 (s, C-7), 169.95, 168.53 (s × 2, C-1"', C-2'''), 164.50 (s, C-2), 159.03 (s, C-5), 157.72 (s, C-9), 155.81 (s, C-4'), 147.38 (s, C-3'), 145.48 (s, C-3), 137.00 (d, C-4), 128.47 (d, C-6'), 121.17 (s, C-1'), 118.44 (d, C-2'), 117.40 (d, C-5'), 113.24 (s, C-10), 103.71, 103.50 (d × 2, C-1'', C-6), 95.35 (d, C-8), 77.89 (d, C-3''), 75.95 (d, C-5''),

74.65 (d, C-2''), 71.32 (d, C-4''), 65.41 (t, C-6''). — FAB MS *m/z*: 554 [M-H+Cl]⁻, 519 [M-H]⁻, 449 [M-C₂O₃+H]⁻, 287 [M-C₈H₁₀O₈+H]⁻, 286 [M-C₈H₁₀O₈]⁻.

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- [1] J. B. Harborne and C. A. Williams, in: *The Flavonoids: Advances in Research* (J. B. Harborne and T. J. Mabry, eds.), p. 261, Chapman and Hall, London, New York 1982.
- [2] G. Hrazdina, in: *The Flavonoids: Advances in Research* (J. B. Harborne and T. J. Mabry, eds.), p. 135, Chapman and Hall, London, New York 1982.
- [3] J. B. Harborne and M. Boardley, *Z. Naturforsch.* **40c**, 305 (1985).
- [4] J. Köster, D. Strack, and W. Barz, *Planta Med.* **48**, 131 (1983).
- [5] J. Arditti and M. H. Fisch, in: *Orchid Biology – Review and Perspectives, I* (J. Arditti, ed.), p. 118, Comstock Publishing Associates, division of Cornell University Press, Ithaca, London 1977.
- [6] W. Uphoff, *Experientia* **35**, 1013 (1979).
- [7] J. Krause and D. Strack, *Z. Pflanzenphysiol.* **95**, 183 (1979).
- [8] H. Ikuta, T. Fukai, T. Nomura, and J. Uzawa, *Heterocycles* **23**, 121 (1985).
- [9] C. A. Williams, *Phytochemistry* **18**, 803 (1979).
- [10] N. Saito, K. Abe, T. Honda, C. F. Timberlake, and P. Bridle, *Phytochemistry* **24**, 1583 (1985).
- [11] S. A. Hansen, *J. Chromatogr.* **107**, 224 (1975).
- [12] L. Birkofer, C. Kaiser, W. Koch, and H. W. Lange, *Z. Naturforsch.* **18b**, 367 (1963).
- [13] J. B. Harborne, *Arch. Biochem. Biophys.* **96**, 171 (1962).
- [14] D. Strack, R. Ruhoff, and W. Gräwe, *Phytochemistry* **25**, 833 (1986).
- [15] D. Strack, N. Akavia, and H. Reznik, *Z. Naturforsch.* **35c**, 533 (1980).
- [16] J. B. Harborne, *Biochem. J.* **70**, 22 (1958).