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ANTHOCYANIN PATTERNS IN EUROPEAN ORCHIDS AND THEIR TAXONOMIC AND PHYLOGENETIC RELEVANCE

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Key Word Index—*Dactylorhiza*; *Nigritella*; *Orchis*; *Ophrys*; *Serapias*; Orchidaceae; acyl cyanidin derivatives; oxalic acid; intramolecular copigmentation; anthocyanin-flavonol glycoside complex; chemotaxonomy.

Abstract—Seven anthocyanins have been identified from European Orchidaceae on the basis of UV/Vis spectroscopy, various hydrolytic procedures, chromatography (TLC, HPLC) and electrophoresis. These were the known cyanidin glycosides 3-monoglucoside (chrysanthemin), 3,5-diglucoside (cyanin), 3,7-diglucoside (seranin) and 3-oxalylglucoside (ophrysanin) and the new cyanidin glycosides oxalyl-3,5-diglucoside (orchicyanin II), oxalyl-3,7-diglucoside (serapianin) and the intramolecular copigmented anthocyanin cyanidin oxalyl-3,5-diglucoside-kaempferol 7-glucoside (orchicyanin I). A survey of 79 species from 14 genera of the Orchidaceae showed the widespread occurrence of these pigments and revealed characteristic genus-specific patterns of the oxalic acid esters of cyanidin glycosides in this family. On the basis of these results some taxonomic and phylogenetic conclusions have been drawn.

INTRODUCTION

Recently we described the isolation and identification of a new acylated anthocyanin, cyanidin 3-oxalylglucoside, from some members of the European Orchidaceae [1]. This pigment is of marked interest from two points of view, (i) it extends the number of known aliphatic dicarboxylic acid moieties of zwitterionic anthocyanins, such as those containing malonic, succinic or malic acid [2] and (ii) it might be a useful taxonomic marker for certain members of the Orchidaceae.

It is known that complex mixtures of acylated and non-acylated anthocyanins cause a broad spectrum of orchid flower colouration [3]. Uphoff [4] described the widespread occurrence of the 3-monoglucoside (chrysanthemin), 3-diglucoside (mecocyanin) and 3,5-diglucoside (cyanin) of cyanidin together with two unknown acylated cyanidin 3,5-diglucosides named orchicyanin I and orchicyanin II. In particular orchicyanin I is of considerable interest since it was thought that it contained a flavonol as well as an organic acid as acyl moiety (intramolecular copigmentation) which caused intensive flower colours [5]. The occurrence of these special pigments, together with the previously described cyanidin 3-oxalylglucoside, might be of taxonomic and phylogenetic relevance.

In the present study further orchid anthocyanins were identified and flowers from 79 species of the European orchids were investigated by quantitative-analytical HPLC for the occurrence of these pigments. In conjunction with the known morphological, cytological and ecological characteristics some taxonomic and phylogenetic implications of these chemical results are discussed.

RESULTS AND DISCUSSION

Structure elucidation

The following seven anthocyanins (proposed new trivial names indicated by quotation marks) have been isolated from various members of European orchids and their structures elucidated as described below: *chrysanthemin* = cyanidin 3-monoglucoside, *cyanin* = cyanidin 3,5-diglucoside, '*seranin*' = cyanidin 3,7-diglucoside, '*ophrysanin*' = cyanidin 3-oxalylglucoside, *orchicyanin II* = cyanidin oxalyl-3,5-diglucoside, '*serapianin*' = cyanidin oxalyl-3,7-diglucoside, and *orchicyanin I* = cyanidin oxalyl-3,5-diglucoside-kaempferol 7-glucoside.

Chromatographic analyses (TLC in S1 and S2, HPLC) of methanolic extracts of flowers from the European orchids showed complex mixtures of cyanidin conjugates. In addition to the known chrysanthemin, cyanin [1, 4] and ophrysanin [1] four other major components have been isolated; the unacylated components have been isolated by two successive TLC steps and the acylated ones by two successive column chromatography steps. Their structures have been identified by classical techniques, such as UV/Vis spectroscopy, various hydrolytic procedures and chromatographic characteristics and co-chromatography with reference compounds in TLC and HPLC. The results are summarized in Table 1.

In all compounds, total acid hydrolysis gave cyanidin (co-TLC with cyanidin in S1, R_f 0.68, and S2, R_f 0.45) and glucose (co-TLC with glucose in S4, R_f 0.25, and S7, R_f 0.50). UV/Vis spectroscopy showed the presence of a catechol nucleus in each case (bathochromic shift of band I upon addition of $AlCl_3$) and substitution at C-3 and a free hydroxyl group at C-5 ($E_{440}/E_{vis.\ max} \times 100 = 21$–$28$) for one group of pigments and substitution at both C-3 and C-5 ($E_{440}/E_{vis.\ max} \times 100 = 15$–$18$) for certain others.

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Table 1. Summary of identification data of the cyanidin

Treatment and characteristics	Chrysanthemin	Cyanin	Seranin
Total acid hydrolysis:			
Cy	+	+	+
Glc	+	+	+
Peroxide hydrolysis:			
Glc	+	+	+
Controlled acid hydrolysis			
3-Glc		+	+
5-Glc		+	
7-Glc			+
3,5-Diglc			
Oxalyl-3,5-diglc			
Alkaline hydrolysis:			
Oxalic acid			
3-Glc			
3,5-Diglc			
3,7-Diglc			
Kae 7-glc§			
UV/Vis Spectroscopy:			
$\lambda_{\text{vis. max}}$ (nm, HCl-MeOH)	528	524	525
Bathochromic shift with $AlCl_3$	+	+	+
$E_{440}/E_{\text{vis. max}} \times 100$	24	15	22
TLC:			
$R_f \times 100$ in S1	24‖	27¶	20**
$R_f \times 100$ in S2	20‖	39¶	43**
Colour in day light	red	red	red
Orange-red fluorescence (360 nm)	—	+	—
Thin-layer electrophoresis:			
Anodic mobility‡‡ (cm at pH)	—	—	—
HPLC:			
Elution time(min)§§	25.4	21.0	14.0

*Analysed by NMR spectroscopy and MS as well as co-chromatographed

†Positive glyoxylate test [6].

‡Co-chromatographed with reference oxalic acid according to ref. [1].

§Small amounts of quercetin 7-glucoside were also found, most likely derived

‖Co-chromatographed (TLC, HPLC) with reference chrysanthemin (controlled chrysanthemin [1] of 7 in S2 is a misprint.

¶Co-chromatographed (TLC, HPLC) with cyanin from *Rosa* sp. [44].

**Co-chromatographed (TLC, HPLC) with seranin from red-coloured *Petunia*

††Data in parantheses from Uphoff [4].

‡‡See also Harborne [7]: anodic mobility of *Dactylorhiza elata* pigments.

§§See Fig. 2.

This was substantiated by the results from controlled acid hydrolysis revealing the identity of chrysanthemin (no intermediates) and cyanin (two intermediates, the 3-glucoside as the minor and the 5-glucoside as the major intermediate). These proposed structures were proved by cochromatography with reference compounds. A third compound gave no hydrolytic intermediates but showed a higher retention time in HPLC and higher R_f's in TLC compared to the 3-monoglucoside indicating acylation. Alkali treatment gave cyanidin 3-monoglucoside. This conjugates in flowers of European Orchidaceae

Cyanidin conjugate			
Ophrysanin	Orchicyanin II	Serapianin	Orchicyanin I
+	+	+	+
+	+	+	+
+	+	+	+
	+	+	+
	+		+
		+	
			+
			+
+*	+†	+‡	+†
+			
	+		+
		+	
			+
528	525	524	535
+	+	+	+
21	18	28	16
35	30(30)††	26	3(6)††
24	55(58)††	53	38(39)††
red	red	red	violet
—	+	—	+
3 at 7.5	3 at 4.4	2 at 4.4	—
36.9	27.4	24.2	33.2

(TLC) with reference oxalic acid [1].

from an analogous pigment present as a contaminant at a low concentration.
acid hydrolysis product from cyanin), the previously published R_f value (× 100) of

hybrida [8].

compound was the previously identified cyanidin 3-oxalylglucoside [1], named ophrysanin.

Four other pigments have been isolated and identified on the basis of the results described below and in Table 1. The identity of oxalic acid in the acyl anthocyanins has been determined in the case of serapianin cochromatographically as described previously [1] and in the case of the orchicyanins chemically by the glyoxylate test [6]. The position of oxalic acid in these anthocyanins has not been determined, but is possibly at C-6 of the 3-glucose as

Table 2. Distribution of anthocyanins in flowers of European Orchidaceae

Plant species* (% anthocyanin of petal dry wt)†	Anthocyanin conjugate (% of total anthocyanin content)‡							
	Chrysanthemin	Cyanin	Seranin	Ophrysanin	Orchicyanin II	Serapianin	Orchicyanin I	Unknowns
Anacamptis pyramidalis (2.3)†	1.0	11.5	0.9	4.5	2.3	0.7	1.7	77.4
Barlia metlesicsiana (0.4)	5.7	5.2	10.8	4.2	10.3	18.3	14.0	31.5
B. robertiana (Greece) (0.9)	4.6	9.5	12.4	2.3	27.7	22.9	tr	20.5
B. robertiana (France) (0.9)	3.4	2.4	8.6	3.6	10.8	20.9	18.6	31.7
Cephalanthera kurdica (0.6)	tr	2.3	tr	4.4	tr	3.7	1.4	88.2
C. rubra	1.7	1.2	0.3	3.4	1.6	0.8	1.3	89.7
Dactylorhiza baumanniana (1.7)	1.1	17.5	3.6	1.3	17.1	4.5	17.1	37.8
D. coccinea (1.4)	32.7	1.1	7.7	44.1	1.0	9.1	1.7	2.6
D. foliosa (1.4)	2.1	7.6	2.1	tr	24.0	3.2	43.6	17.4
D. fuchsii (0.5)	1.5	4.5	3.0	1.0	31.5	4.0	37.0	17.5
D. iberica (0.2)	tr	11.9	5.3	—	21.8	16.8	25.3	18.9
D. incarnata (1.9)	tr	3.0	3.0	0.1	9.0	18.5	38.5	27.9
D. incarnata subsp. *pulchella* (3.2)	2.4	10.5	5.9	tr	14.4	6.4	27.1	33.3
D. kalopissii (0.9)	tr	10.3	3.4	0.6	10.5	5.9	34.2	35.1
D. majalis (2.7)	2.0	5.5	1.0	0.5	28.5	4.5	33.5	24.5
pollinia (1.4)	1.0	1.0	4.0	1.3	9.4	38.0	24.5	20.8
D. osmanica (2.8)	tr	8.0	tr	0.9	14.1	4.6	50.7	21.7
D. purpurella (2.1)	—	4.0	2.0	0.5	10.5	11.5	42.0	29.5
D. romana (1.8)	tr	30.5	12.9	tr	9.5	6.5	7.2	33.4
D. saccifera (2.3)	tr	20.1	4.0	tr	16.4	4.0	26.1	29.4
D. sambucina (2.8)	—	6.0	6.5	0.5	21.0	13.5	30.5	22.0
D. sphagnicola (0.6)	—	5.0	1.0	5.0	11.0	4.0	57.5	16.5
D. traunsteineri (2.9)	0.5	7.0	1.0	0.5	14.5	4.5	53.5	18.5
D. umbrosa (3.2)	1.7	9.5	2.4	—	22.8	8.1	30.6	24.9
Epipactis atrorubens	1.7	0.3	3.2	16.0	1.6	0.3	6.2	70.7
Gymnadenia conopsea (0.8)	2.0	4.0	2.5	1.0	26.0	9.5	37.5	17.5
G. odoratissima (0.8)	2.5	5.5	4.5	1.5	23.0	11.5	22.0	29.5
Himantoglossum adriaticum (0.6)	2.2	5.5	15.5	1.6	7.2	20.4	3.7	43.9
Limodorum abortivum	2.8	*tr*	1.2	30.1	tr	0.8	0.9	64.2

Neottianthe cucullata (1.0)	0.8	2.2	1.1	3.2	11.8	2.1	31.1	47.7
Nigritella corneliana (1.6)	47.5	—	0.5	38.5	0.5	2.5	7.5	3.0
N. nigra (Austria) (6.5)	45.0	0.5	1.0	32.5	1.0	6.5	9.5	4.0
N. nigra (Italy) (6.4)	46.0	0.5	0.5	35.0	1.0	1.5	11.5	4.0
N. n. × *Gymnadenia conopsea* (3.8)	1.8	11.8	4.4	1.0	16.1	4.2	11.0	49.7
N. n × *Gymnadenia odoratissima* (3.6)	2.2	4.6	7.2	—	34.5	25.0	4.4	22.1
N. rubra (Austria) (2.4)	15.5	0.5	1.5	24.0	37.0	7.5	5.5	8.5
N. rubra (Switzerland) (2.2)	13.0	0.5	2.0	27.5	41.0	6.0	5.0	5.0
N. lithopolitanica (1.5)	4.0	4.5	2.5	3.5	55.0	6.0	10.0	14.5
Ophrys ciliata (Greece)								
petals/sepals (0.9)	26.6	—	tr	71.4	tr	tr	tr	2.0
labellum (4.1)	28.8	—	tr	66.5	tr	2.0	—	2.7
Oph. ferrum-equinum (3.7)	64.4	—	3.5	23.6	—	1.5	—	7.0
Oph. fusca (0.9)	54.3	—	19.8	13.0	—	5.4	—	7.5
Oph. holoserica (Switzerland)								
petals/sepals (0.8)	9.0	3.0	3.0	1.5	36.0	5.5	25.5	16.5
labellum (6.4)	7.5	—	1.0	70.5	1.0	14.5	tr	5.5
Oph. holoserica (Italy) (0.7)	3.6	—	tr	50.8	—	12.1	—	33.5
petals/sepals (Turkey) (0.9)§	—	—	—	—	—	tr	80.7	19.3
Oph. insectifera								
labellum (1.3)	45.3	0.4	0.7	10.2	tr	tr	0.8	42.6
Oph. lacaitae (0.2)	39.9	—	4.9	46.3	—	8.9	—	0.0
Oph. lutea subsp. *galilaea* (0.3)	17.0	—	1.4	66.9	—	6.7	—	8.0
Oph. morisii								
petals/sepals (0.5)	3.5	tr	14.4	10.9	10.5	48.1	3.3	9.3
labellum (1.1)	28.8	—	4.4	51.2	tr	8.8	—	6.8
Oph. phrygia								
petals/sepals (0.9)§	—	—	—	—	—	—	95.0	5.0
labellum (1.0)	14.1	—	tr	53.2	—	32.7	tr	0.0
Oph. scolopax subsp. *cornuta* (0.5)	41.3	tr	31.9	9.5	—	12.4	—	4.9
Oph. sphegodes (1.2)	59.7	—	9.2	25.6	—	5.5	—	0.0
labellum (1.9)	tr	—	—	74.0	2.5	—	—	23.5
Oph. straussii								
petals/sepals (1.6)§	—	—	—	—	—	—	65.4	34.6
labellum (2.4)§	17.3	—	—	82.7	—	—	—	0.0
Oph. tenthredinifera								
petals/sepals (0.5)	28.2	8.9	—	—	30.0	7.2	—	25.7
labellum (0.7)	28.0	tr	tr	42.8	tr	29.1	—	0.1

Table 2. *Continued*

Plant species* (% anthocyanin of petal dry wt)†	Anthocyanin conjugate (% of total anthocyanin content)‡							
	Chrysanthemin	Cyanin	Seranin	Ophrysanin	Orchicyanin II	Serapianin	Orchicyanin I	Unknowns
Orchis anatolica (1.9)	tr	tr	tr	tr	tr	tr	11.1	88.9
Or. champagneuxii (0.9)	tr	—	—	tr	1.1	—	17.5	81.4
Or. collina (Kreta) (1.0)	1.5	0.6	8.7	1.8	1.2	10.9	3.6	71.7
Or. collina (Lesbos) (3.1)	1.2	1.3	4.3	1.8	2.1	5.8	7.0	76.5
Or. coriophora (Austria) (0.8)	27.3	0.3	0.3	62.7	0.8	0.5	0.2	7.9
Or. coriophora (Italy) (0.8)	46.5	tr	tr	46.9	tr	tr	—	6.6
Or. coriophora (Greece) (0.2)	71.6	tr	tr	20.3	tr	tr	—	8.1
Or. coriophora subsp. *fragrans* (1.9)	48.9	—	tr	51.3	tr	tr	tr	0.0
Or. langei (2.3)	0.9	tr	3.2	4.7	2.0	11.9	6.9	70.4
Or. laxiflora (1.3)	0.7	0.5	0.9	3.5	1.3	0.6	11.0	81.5
Or. mascula								
petals/sepals (1.4)	1.1	13.6	3.8	17.5	1.0	1.2	3.2	58.6
labellum (1.5)	0.3	2.4	1.0	8.2	0.7	1.3	4.9	81.2
Or. militaris								
petals/sepals (0.9)	0.9	7.4	tr	5.7	6.5	1.0	4.8	73.7
pollinia (1.3)	10.7	26.7	2.5	3.1	22.5	tr	12.5	22.0
Or. m. × *Or. simia*								
petals/sepals (0.6)	1.2	3.1	0.9	1.6	8.1	2.0	13.7	69.4
labellum (0.7)	tr	1.7	1.2	1.8	2.9	1.4	21.0	70.0
pollinia (2.0)	4.6	10.2	6.1	5.8	5.3	5.9	15.5	46.6
Or. morio (1.2)	0.3	3.4	1.3	7.3	2.8	0.5	4.7	79.7
petals/sepals	0.6	0.7	0.6	5.3	6.0	0.2	18.2	68.4
Or. olbiensis (France) (1.2)	tr	tr	4.7	2.6	9.6	9.2	4.1	69.8
Or. palustris (0.7)	0.1	0.6	0.9	3.5	0.4	0.5	11.3	82.7
Or. papilionacea (Rhodos) (1.2)	tr	tr	19.8	tr	tr	29.9	4.4	45.9
Or. papilionacea (Lesbos) (0.3)	1.1	1.2	1.3	2.0	4.4	8.4	5.2	76.4
Or. papilionacea								
ssp. *grandiflora* (2.2)	tr	tr	tr	0.5	0.9	1.0	3.8	93.8
Or. patens (1.0)	1.0	1.5	tr	5.3	7.4	1.1	7.1	76.6
Or. prisca (1.8)	tr	3.5	2.6	0.9	3.6	1.6	10.8	77.0
Or. pseudolaxiflora (Turkey) (1.2)	tr	tr	tr	tr	1.4	tr	16.6	82.0
Or. purpurea								
petals/sepals (3.0)	13.4	20.0	6.0	2.0	1.2	1.0	35.0	21.4
labellum (0.8)	5.7	17.6	4.6	tr	0.8	0.9	25.0	45.4
pollinia (1.9)	7.0	tr	5.2	56.0	5.5	7.5	14.0	4.8

Or. sancta (Lesbos) (0.6)	28.6	1.1	0.3	49.0	1.0	0.6	—	18.5
Or. spitzelii (France) (0.5)	—	—	7.4	tr	—	40.5	3.8	48.3
Or. spitzelii (Greece) (1.2)	0.8	7.7	4.4	1.0	3.3	3.1	11.1	68.6
Or. tridentata								
petals/sepals (0.5)	1.2	3.0	3.0	1.0	12.1	6.1	41.0	32.6
labellum (0.7)	1.1	3.1	3.9	0.5	7.9	5.4	43.4	34.7
pollinia (0.6)	8.0	6.5	8.5	15.5	9.0	11.5	31.0	10.0
Or. ustulata (3.8)	13.0	—	20.7	15.4	—	39.0	—	11.9
Serapias cordigera (France) (13.3)	3.4	1.2	3.4	3.0	3.3	16.4	2.0	67.3
S. cordigera (Italy (12.8)	4.3	tr	9.7	10.0	4.5	22.8	2.0	46.7
S. cordigera (Greece) (6.3)	14.3	3.4	17.2	2.7	3.0	17.1	1.5	40.8
S. lingua (France) (1.6)	5.0	2.6	6.8	4.4	4.6	22.7	1.2	52.7
S. lingua (Greece) (1.7)	12.4	—	19.9	6.1	2.6	17.4	1.3	40.3
S. neglecta (5.1)	0.8	—	4.7	2.9	1.1	21.7	2.3	66.5
S. nurrica (4.1)	1.0	tr	9.2	5.9	2.3	47.9	0.5	33.2
S. olbia (13.8)	1.9	tr	2.6	15.3	2.4	13.3	2.4	62.1
S. parviflora (France) (2.2)	1.4	tr	5.5	5.8	1.8	23.4	0.6	61.5
S. parviflora (Greece) (1.7)	3.5	1.8	21.1	2.3	3.0	19.9	1.1	47.3
S. vomeracea (6.6)	1.8	—	5.4	4.7	4.6	14.1	3.2	66.2
S. v. subsp. *laxiflora* (Turkey) (3.7)	3.3	tr	19.2	5.8	2.5	38.2	1.0	30.0
S. v. subsp. *laxiflora* (Greece) (4.7)	2.9	tr	18.0	2.8	4.2	29.7	1.6	40.8
S. v. subsp. *orientalis* (Karp.) (4.2)	3.8	tr	14.9	7.3	4.0	24.9	1.9	43.2
Traunsteinera globosa (1.1)	1.5	3.5	3.0	0.5	33.0	2.5	22.0	34.0

*For complete names see Experimental.

†Total anthocyanin content in parentheses, expressed as % of dry wt of extracted petal residues (insoluble material) in terms of cyanidin 3,5-diglycoside in 0.1 M HCl, using an extinction coefficient of log ε 4.48 [43] (data from one of two experiments which gave similar results); not indicated in cases of insufficient amount of petal tissue.

‡Per cent of total absorbance of all detected pigments at 525 nm in HPLC analysis.

§Low amount of material, only major compounds detectable by HPLC.

Key:—= not detected; tr = trace. Not detected anthocyanins in low-concentrated extracts and trace amounts of detected anthocyanins have to be considered as tentative results dependent on the HPLC technique.

shown for ophrysanin [1]. Ophrysanin is likely to be the basic structure for all the other acylated orchid anthocyanins. The relative R_f values (acyl cyanidin glycoside/corresponding non-acyl cyanidin glycoside) gave no indication of di- or polyacyl pigments. In addition, the anodic movement (2–3 cm) of the acyl pigments in electrophoresis is characteristic for monoacylation [7]. Some further specific characteristics of the new pigments are discussed below.

Orchicyanin II [4]. Liberated as degradation product from orchicyanin I. Rechromatography (TLC in S1) and alkali treatment gave one product which showed chromatographic identity (TLC in S1 and S2, HPLC) with cyanin. Controlled acid hydrolysis gave two products, chrysanthemin and the 5-monoglucoside.

Seranin. Isolated from *Orchis ustulata*. Controlled acid hydrolysis gave two products, chrysanthemin and the 7-monoglucoside. Co-chromatography (TLC) of the hydrolysis products with those from cyanin gave identity of one product with chrysanthemin but the second product was chromatographically different from the 5-monoglucoside (fluorescent under UV): in S1, S2 and S3 with respective R_f's of 0.41, 0.37 and 0.58; cyanidin 7-monoglucoside (non-fluorescent under UV): in S1, S2 and S3 with respective R_f's of 0.39, 0.25 and 0.51. These characteristics are in agreement with those of the cyanidin 3,7-diglucoside isolated by Birkofer *et al.* [8] from red-flowering *Petunia hybrida* and by Yoshitama and Abe [9] from *Senecio cruentus*, and clearly differ from those of the 3,5-diglucoside. The more restricted occurrence of the 3,7-substitution has also been described for other flavonoids in orchid flowers, e.g. the flavonol glycosides kaempferol and quercetin 3,7-diglucosides in *Gymnadenia conopsea* [10] or quercetin 3,7-diglucoside in *Dactylorhiza sambucina* [11]. These species showed also appreciable amounts of the 3,7-substituted cyanidins seranin and serapianin (Table 2).

Serapianin. Isolated from *Orchis ustulata*. Rechromatography (TLC in S1) and alkali treatment gave one product which showed chromatographic identity (TLC in S1 and S2; HPLC) with seranin.

Orchicyanin I [4]. Isolated from *Dactylorhiza sphagnicola* and *Gymnadenia conopsea*. This pigment was isolated by two different methods, by open CC and HPLC and gave identical results for both preparations. Rechromatography (TLC in S1) gave several degradation products, the cyanidin oxalyl-3,5-diglucoside (orchicyanin II) and cyanidin 3,5-diglucoside, the latter identified by co-chromatography (TLC in S1 and S2, HPLC) with cyanin, and one major and two minor intense yellow UV-fluorescent components, identified by co-chromatography (TLC, HPLC) with reference compounds (collection of E. Wollenweber) as kaempferol 7-glucoside (major component, R_f's in S5 and S6 of 0.72 and 0.59, respectively), quercetin 7-glucoside (minor component, R_f's in S5 and S6 of 0.50 and 0.52, respectively, most likely derived from an analogous orchicyanin pigment present as a contamination at low concentration) and kaempferol 7-oxalylglucoside (tentative structure, detection of kaempferol 7-glucoside after rechromatography in S1 and S2 with respective R_f's of 0.48 and 0.13). In addition, UV/Vis spectra (±shift reagents) of kaempferol 7-glucoside were identical to those described by Mabry *et al.* [12] for kaempferol with substitution at the 7-OH.

This intramolecular copigmentation of orchicyanin I was readily recognized by UV/Vis spectroscopy (HCl–MeOH as solvent), showing besides absorption band I at 535 nm and band II at 265 nm a third absorption band at 352 nm ($E_{352}/E_{535} \times 100 = 74$; a value which is usually *ca* 10), representing band I of the kaempferol 7-glucoside. The E_{265}/E_{535} value ($\times$ 100) was found to be 120, *ca* double the respective usual value (45–65) of the un-copigmented cyanidin conjugates. In conclusion the results indicate that this unique pigment is cyanidin 3,5-diglucoside covalently bound to kaempferol 7-glucoside with oxalic acid as the linking molecule (oxalyl diester), probably located between the 3-glucose of the cyanidin and the 7-glucose of the kaempferol (see the proposed tentative structure in Fig. 1). It is interesting to note that malonic acid has also been described as a diester with two flavonoid moieties, acacetin 7-glucosides, in *Agastache rugosa* (Lamiaceae) [13].

With the results described above the previously published suggestion of Uphoff [4] for the existence of acylated cyanidin 3,5-diglucosides with substituents of both an organic acid and a flavonol found in various members of the Orchidaceae, which we could not verify in our previous communication [1], is now substantiated. Uphoff's orchicyanins I and II are most likely identical with those described in the present study. However, their proposed structures, particularly that of orchicyanin I possessing a possible intramolecular copigmented structure for its *in vivo* stabilization [14], await final proof.

To search for the minimum conformation with regard to copigmentation, a computer-assisted structure modelling (not documented) of orchicyanin I was carried out using the CHEMLAB II suite of programmes (MDL, San Leandro, California, U.S.A.). It was found that there was no intramolecular stacking of the two flavonoid nuclei as found in other types of copigmentation or in some structures of metalloanthocyanins [15]; however, the computed results indicated that the bis-hydroxyphenyl ring of the cyanin portion is located over the kaempferol aromatic system (folded conformation), suggesting some form of charge-transfer mechanism which could explain the observed bathochromic shift of the visible absorption band of orchicyanin I relative to orchicyanin II (Table 1).

Spectroscopic work on orchicyanin I is in progress. Preliminary positive ion fast atom bombardment mass spectra (data not documented) were compatible with the proposed structure. Although a molecular ion was not yet observed, fragments possibly corresponding to cyanidin 3,5-diglucoside (*m/z* 611) and kaempferol 7-oxalylglucoside (*m/z* 503) were observed.

Taxonomic and phylogenetic conclusions

Flowers from 79 European orchids were surveyed by HPLC for the distribution of the seven cyanidin conjugates identified. Traces from representative HPLC

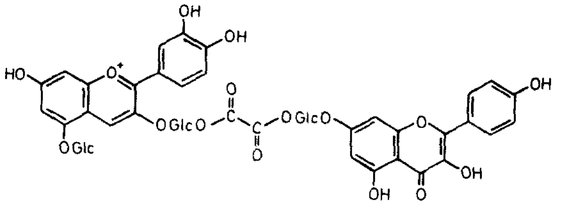

Fig. 1. Hypothetical structure of orchicyanin I.

analyses for one member each of the genera *Dactylorhiza*, *Ophrys*, *Orchis* and *Serapias* are shown in Fig. 2 and Table 2 lists the complete results from these quantitative-analytical measurements. The identity of the anthocyanins in this survey was checked by co-HPLC and co-TLC (S1 and S2) with a standard mixture of the isolated identified pigments. In those cases where low amounts or traces of compounds were present, the identities were checked only by HPLC and have to be taken as being tentative.

The data shown in Table 2 illustrate (i) the widespread occurrence of the cyanidin glycosides and, particularly interesting, of their oxalic acid esters in the orchids investigated and (ii) distinct quantitative patterns of these anthocyanins in species of the different genera. The following oxalyl conjugates occur as major constituents and show characteristic distributions: orchicyanin I and II in *Dactylorhiza* and *Gymnadenia* species which agrees with Uphoff's results [4], ophrysanin in *Nigritella* and *Ophrys* species, and serapianin in *Serapias* species. This clearly shows that the different cyanidin oxalylglucosides are taxonomic markers for some of the European orchids. Special attention should also be paid to the sum of the percentage values of the unknowns as they indicate that the anthocyanin patterns of some species show several unknown components in high concentrations (see e.g. 'serapidin' from *Serapias lingua* in Fig. 2) and much more identification work on these orchids has to be done.

For the other species investigated it is interesting to note that the anthocyanin patterns of *Barlia* and *Himantoglossum* are similar to that of *Serapias*, that of *Neottianthe* and *Traunsteinera* similar to that of *Dactylorhiza* and *Gymnadenia*, and that of *Limodorum* similar to that of *Ophrys*. It should be noted that *Cephalanthera* and some species of *Orchis* show the described pigments as minor components whereas major unknown components

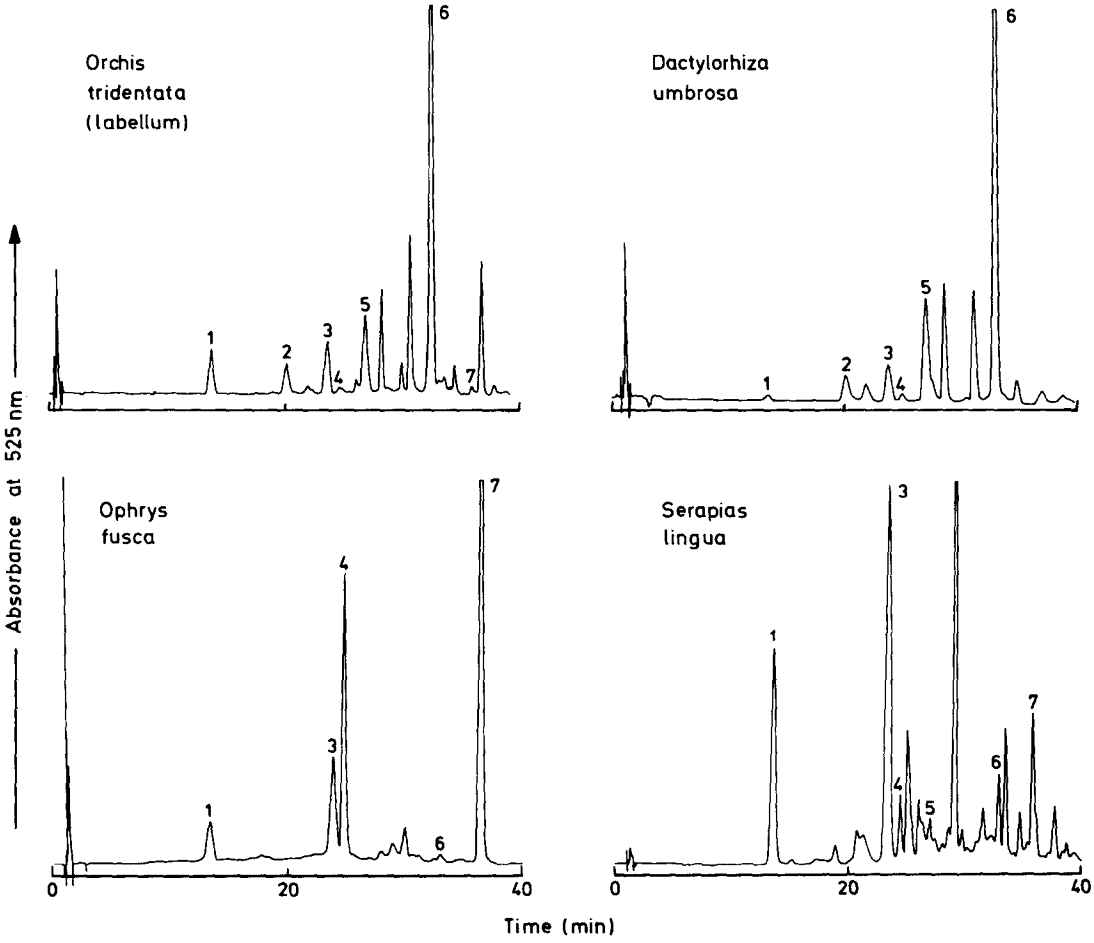

Fig. 2. Selected traces from HPLC analyses of orchid anthocyanins. Chromatographic conditions: Nucleosil-C$_{18}$ column (5 μm, 250 mm × 4 mm i.d.; Macherey–Nagel, Düren); at a flow rate of 1.5 ml/min in 35 min from 10 to 40% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) followed by 5 min at 40% solvent B in solvent A (1.5% H_3PO_4 in H_2O); detection at 525 nm at 0.064 absorbance unit full scale. Peak identification: 1 = seranin (cyanidin 3,7-diglucoside); 2 = cyanin (cyanidin 3,5-diglucoside); 3 = serapianin (cyanidin oxalyl-3,7-diglucoside); 4 = chrysanthemin (cyanidin 3-glucoside); 5 = orchicyanin II (cyanidin oxalyl-3,5-diglucoside); 6 = orchicyanin I (cyanidin oxalyl-3,5-diglucoside-kaempferol 7-glucoside); 7 = ophrysanin (cyanidin 3-oxalylglucoside). *Serapias lingua* shows a characteristic major unidentified peak at 27 min ('serapidin') which could be observed in all *Serapias* species investigated.
]

amount to more than 80% of the total anthocyanin content.

In conjunction with other known characteristics of the orchids our chemical results allow the following further conclusions:

Dactylorhiza

Two of the *Dactylorhiza* species (*D. coccinea* and *D. romana*) show marked differences in their anthocyanin patterns compared to the other members of this genus, especially with regard to the low concentration of the genus-specific orchicyanin I. *Dactylorhiza coccinea* shows instead high concentrations of chrysanthemin and its oxalyl conjugate ophrysanin and *D. romana* high amounts of cyanin and seranin. Both species are ecologically separated.

Dactylorhiza coccinea grows mainly in coastal dune slacks in Ireland, Scotland and western England and differs markedly morphologically from *D. incarnata* and its subspecies [16, 17]. Also there are no hybrids with other *Dactylorhiza* species known. Thus the exceptional anthocyanin pattern and the morphological characteristics of *D. coccinea* supports the suggestion by Averyanov [18] in 1984 to define this plant as a new species, formerly known as *D. incarnata* subsp. *coccinea* (Pugsl.) Soó.

The results of the *Dactylorhiza* anthocyanin analyses are in contrast to the floral pigmentation scheme of Bateman and Denholm [19] suggesting a similar anthocyanin pattern with the presence of cyanin and orchicyanin II as the sole components in *D. coccinea* and *D. incarnata*.

Dactylorhiza romana (red-violet and yellow flowering) grows in Italy, Greece and western Turkey. This plant belongs to the *Dactylorhiza* section *Sambucinae* although it is clearly ecologically and morphologically distinguishable from *D. sambucina*, although morphologically similar to *D. flavescens* (red-violet and yellow flowering) and *D. markusii* (yellow flowering). It is also remarkable that the species of the *D. romana*-group (*romana, flavescens, markusii*) are ecologically different from *D. sambucina*. The former grow in chestnut, pine and cedar woods within the shade in dry, acidic soils whereas the latter grows in the open in sunny, moist meadows. Based on these characteristics we propose, in conjunction with the present chemical results on the anthocyanin pattern, to define a new *Dactylorhiza* section, '*Dactylorhiza Sectio Romanae*', as follows:

Dactylorhiza Sectio Romanae E. Klein & D. Strack *sect. nov.* Typus: Dactylorhiza romana (Sebastiani) Soó; *Nom. nov. gen. Dactylorh.*: 3, 1962. *Tuberibus maturis brevedigitatis. Foliis basalibus rosulatis. Calcare sursum flexuoso.* Species: 1. *Dactylorhiza romana* (Sebastiani) Soó; 2. *Dactylorhiza flavescens* (C. Koch) Holub; 3. *Dactylorhiza markusii* (Tineo) H. Baumann & Künkele.

Nigritella and Gymnadenia

The systematic relationship of *Nigritella* and *Gymnadenia* has been discussed at length [20–23] and it is not clear yet whether these genera should be placed in one genus or kept separate. According to our results the genera should be separated. This is clearly shown by their markedly different anthocyanin patterns. *Gymnadenia*, which is the evolutionary more advanced genus, is characterized by the presence of orchicyanin I and II as main components, indicating a closer relationship to *Dactylorhiza*, whereas *Nigritella* shows predominantly the possibly more original chrysanthemin and ophrysanin.

An exceptional anthocyanin pattern was observed in flowers of the relict orchid *Nigritella lithopolitanica* (exclusively found in the Steiner Alpen and Karwanken), resembling the patterns of *Gymnadenia* species. The quite similar anthocyanin patterns of *Nigritella corneliana* and *N. nigra* is notable. The former occurs exclusively in the western alps whereas the latter grows at panalpine habitats. It is interesting to note that the bigeneric hybrids *Gymnigritella* exhibit the more complex, evolutionary younger anthocyanins, indicating the possible genetic dominance of *Gymnadenia* in this hybrid.

Ophrys

The flowers of *Ophrys* are specialized to attract pollinators by their morphology and specific scents [24–27]. It is known that volatile compounds from the flowers form an important chemical basis for the relationship between *Ophrys* orchids and their pollinators [28–31]. Therefore it is not surprising that the anthocyanins of the intense coloured labella show almost exclusively the simpler structures chrysanthemin and ophrysanin. On the other hand, *Ophrys* species with red or red violet petals and sepals also show the more complex pigments. This indicates that these species are able to synthesize these pigments in general, although there is a wider range of strategy for survival including attraction through flower morphology and volatile attractants. Therefore the results of these anthocyanin analyses cannot be used for possible taxonomic interpretations for the species in this genus.

Orchis

Whereas *Orchis coriophora*, *Or. coriophora* subsp. *fragrans* and *Or. sancta* exhibit relatively simple anthocyanin patterns with chrysanthemin and ophrysanin as the major components, all the other *Orchis* species investigated show widely varying patterns and high amounts of unidentified components. The more 'primitive' anthocyanin patterns of the three species is in agreement with other results such as from hybridization [32], morphological [33] and cytological studies [34] which clearly separates these, possibly evolutionary older species, from all the other ones within this genus. On the basis of these results we propose to define a new orchid genus, '*Anteriorchis*', as follows:

Anteriorchis E. Klein & D. Strack *gen. nov.*; *basionym*:, *Orchis sect. Coriophorae Parlat., Parlatore*, F., Flora Italiana, III, p. 468, Firenze 1848. *Diagnosis*: *Herba perennis*; *tuberibus subglobosis, breviter stipitatis*; *caule folioso*; *foliis basilaribus fere rosulatis caulem basi amplectentibus, lanceolatis*; *foliis superioribus vaginaliformibus*; *bracteis membranaceis*; *sepalibus et petalibus acuminatis, in galeam lanceolatam arctius cohaerentibus*; *labello tribolo*; *calcare incurvo, nectarifero*; *pigmento antho-*

cyanum floris valde pristino. Typus: *Anteriorchis coriophora* (L.) E. Klein & D. Strack = *Orchis coriophora* L.; Spec. Pl. (1753) p. 940. *Species*: 1. *Anteriorchis coriophora* (L.) E. Klein & D. Strack; *basionym*: *Orchis coriophora* L., Spec. Pl. (1753) p. 940; *synonym*: *Orchis fragrans Pollini*, Elm. bot. 2, 155 (1811). 2. *Anteriorchis sancta* (L.) E. Klein & D. Strack; *basionym*: *Orchis sancta* L., Spec. Pl. ed. 2 (1763) p. 1330.

Serapias

Species of the genus *Serapias* showed remarkable uniform anthocyanin patterns with serapianin and an unknown second pigment, named 'serapidin' (see Fig. 2), as the major components throughout. This result is in agreement with the fact that this taxon represents a homogenous, evolutionary older group of species which might have the same phylogenetic root as the genus *Orchis*; formation of *Orchis* × *Serapias* has occasionally been observed.

EXPERIMENTAL

Plant material. The following orchids, harvested at their habitats from 1983 to 1988, were investigated: *Anacamptis pyramidalis* (L.) L. C. M. Rich. var. *tanayensis* Chenevard (Wallis, Switzerland), *Barlia metlesicsiana* Teschner (Teneriffa), *B. robertiana* (Loisel.) W. Greuter (Rhodos, Greece and southern France) *Cephalanthera kurdica* Bornm. (eastern Turkey), *C. rubra* L. C. M. Rich. (Weserbergland, F.R.G.), *Dactylorhiza baumanniana* Hölzinger and Künkele (northern Greece), *D. coccinea* (Pugsl.) Averyanov (Ireland), *D. foliosa* (Vermeulen) Soó (Madeira), *D. fuchsii* (Druce) Soó (Wallis, Switzerland), *D. iberica* (Willd.) Soó (northern Greece), *D. incarnata* (L.) Soó (Austria), *D. incarnata* subsp. *pulchella* (Druce) Soó (Ireland), *D. kalopissii* E. Nelson (northern Greece), *D. majalis* (Reichenb.) P. F. Hunt and Summerhayes (Weserbergland, F.R.G.), *D. osmanica* (Klinge) Soó (eastern Turkey), *D. purpurella* (T. and T. A. Stephenson) Soó (Denmark), *D. romana* (Sebastiani) Soó (Sardinia), *D. saccifera* (Brongn.) Soó (Italy), *D. sambucina* (L.) (Austria), *D. sphagnicola* (Höppner) Soó (Lüneburger Heide, F.R.G.), *D. traunsteineri* (Saut ex Rchb.) Soó (Ireland), *D. umbrosa* (Kar. and Kir.) Nevski (Eastern Turkey), *Epipactis atrorubens* (Hoffm. ex Bernh.) Besser (Weserbergland, F.R.G.), *Gymnadenia conopsea* (L.) R. Br. (Weserbergland, F.R.G.), *G. odoratissima* (L.) L. C. M. Richard (Austria), *Himantoglossum adriaticum* H. Baumann (Austria), *Limodorum abortivum* (L.) Sw. (Kreta), *Neottianthe cucullata* (L.) R. Schlechter (Poland), *Nigritella corneliana* (Beauverd) Gölz and Reinhard (Italy), *N. lithopolitanica* Ravnik (Austria), *N. nigra* (L.) Reichenb. fil. (Switzerland and Austria), *N. rubra* (Wettst.) K. Richter (Switzerland and Austria), × *Gymnigritella suaveolens* G. Camus (= *N. nigra* × *Gymnadenia conopsea*) (Italy), × *Gymnigritella heufleri* G. Camus (= *N. nigra* × *Gymnadenia odoratissima*) (Austria), *Ophyrs ciliata* Biv. (Poloponnes, Greece), *Oph. ferrum equinum* Desf. (Rhodos, Greece), *Oph. fusca* Link (Lesbos, Greece), *Oph. holoserica* (Burm. fil.) W. Greuter (Italy, Switzerland, Turkey), *Oph. lacaitae* Lojac. (Italy), *Oph. lutea* subsp. *galilaea* (H. Fleischm. and Bornm.) Soó (Lesbos, Greece), *Oph. morisii* (U. Martelli) Ciferri and Giacomini (Sardinia), *Oph. phrygia* H. Fleischm. and Bornm. (eastern Turkey), *Oph. scolopax* subsp. *cornuta* (Steven) G. Camus (northern Greece), *Oph. sphegodes* Miller (southern France), *Oph. straussii* H. Fleischm. and Bornm. (eastern Turkey), *Oph. tenthredinifera* Willd. (Rhodos, Greece), *Orchis anatolica* Boiss. (Kreta, Greece), *Or. champagneuxii* Bernéoud (southern France), *Or. collina* Banks and Soland. ex Russel (Kreta and Lesbos, Greece), *Or. coriphora* L. (Austria, Greece, Italy), *Or. coriophora* subsp. *fragrans* (Pollini) K. Richter (northern Greece), *Or. langei* K. Richter (Spain), *Or. laxiflora* Lam. (southern France), *Or. mascula* L. Brenkhausen/Höxter, F.R.G.; Fredesloh, F.R.G.), *Or. militaris* L. (Weserbergland, F.R.G.), *Or. m.* × *Or. simia* (= *Or.* × *beyrichii* Kern.) (southern F.R.G.), *O. morio* L. (Stadt Kyll, F.R.G.), *Or. olbiensis* Reuter ex Barla (southern France), *Or. palustris* Jacq. (Austria), *Or. papilionacea* L. (Rhodos and Lesbos, Greece), *Or. patens* Desf. (Ligurien, Italy), *Or. prisca* Hautzinger (Kreta, Greece), *Or. pseudolaxiflora* Czerniak. (eastern turkey), *Or. purpurea* Huds. (Weserbergland, F.R.G.), *Or. sancta* L. (Lesbos, Greece), *Or. spitzelii* Koch (Greece, southern France), *Or. tridentata* Scop. (Weserbergland, F.R.G.), *Or. ustulata* L. (Miex, Switzerland), *Serapias cordigera* L. (Greece, Italy, southern France), *S. lingua* L. (Greece, southern France), *S. neglecta* De Not. (southern France), *S. nurrica* Corrias (Sardinia), *S. olbia* Verguin (southern France), *S. parviflora* Parl. (southern France, Greece, Italy), *S. vomeracea* (Brum. fil.) Briq. (Gardasee, Italy), *S. vomeracea* subsp. *laxiflora* (Soó) Gölz and Reinhard (northern Greece, eastern Turkey), *S. vomeracea* subsp. *orientalis* W. Greuter (Karpathos), *Traunsteinera globosa* (L.) Rchb. (Miex, Switzerland).

Preparation of extracts. Flowers, freshly picked at their habitats, were submerged in 90% aq. MeOH–1% citric acid (v/w) in brown bottles on the spot. This solvent has been successfully applied for extractions in the field and longer-time transportation in the analysis of cyanidin 3-oxalylglucoside [1] (protective effects of citric acid [cf. ref. 35], avoidance of possible acylation with weak volatile acids). Once in the laboratory, they were stored at −20° until extract preparation. After filtration the extraction of the remaining flower materials was completed by several washes with MeOH. The filtrates were dried *in vacuo* at 30° and the residues redissolved in a small vol. of 50% aq. MeOH, 1–5 ml depending on the anthocyanin concentration (monitor visually).

TLC. On microcrystalline cellulose ('Avicel'): in S1, *n*-BuOH–HOAc–H_2O (4:1:5, upper phase); in S2, HOAc–HCl–H_2O (15:3:82), in S3, *n*-BuOH–HCl–H_2O (7:2:5, upper phase), in S4, *n*-BuOH–HOAc–H_2O (3:1:1); on polyamide ('DC 11'): in S5, $CHCl_3$–MeOH–MeCOEt (5:2:1); on silica gel: in S6, EtOAc–H_2O–HCO_2H (67:26:7); on silica gel impregnated with 0.5 M NaH_2PO_4 in H_2O–MeOH, 3:1: in S7, *iso*-PrOH–Me_2CO–0.1 M lactic acid (2:2:1) [36].

Thin-layer electrophoresis. On 'Avicel' in Ac buffer (pH's 4.4 and 6.0) or Pi buffer (pH 7.5) for 2 hr at 40 V/cm [37, 38].

Open CC. On MN-polyamide (CC 6, perlon; Mecherey–Nagel, Düren): 35 × 4 cm i.d.; stepwise gradient elution with H_2O, MeOH, 20% aq. MeOH (containing 0.01% HCl, by vol.), and 50% aq. MeOH (containing 0.01% HCl, by vol.); on Sephadex LH-20 (Sigma, Deisenhofen): 75 × 3.5 cm i.d.; elution with H_2O (containing 0.01% HCl, by vol.).

HPLC. The liquid chromatographic system and the data processor (Chromatopac C-R3A) for quantitative calculations used were from Pharmacia-LKB (Freiburg) and Shimadzu (Kyoto, Japan), respectively. Injections were performed via a Rheodyne rotary valve (Cotati, CA, U.S.A.) with a 20 μl loop. For details of the chromatographic conditions see Fig. 2.

Hydrolysis and separation of its products. Total acid hydrolysis: in 4 M aq. HCl for 20 min at 100°; sepn of aglycones from conjugation moieties by extraction with amyl alcohol (aglycones and sugars in the alcohol and H_2O phases, respectively). Controlled acid hydrolysis: in 2 M aq. HCl for 2.5, 5, 10, 20, and 40 min at 100°; separation and identification of intermediate hydrolysis products by HPLC [39] and TLC in S2. Alkaline hydrolysis: in 0.05 M aq. NaOH for 5–10 min; neutralization by treatment with Dowex 50 × 8 (Sigma, Deisenhofen). Peroxide hydrolysis: in 6% H_2O_2 (30% H_2O_2 mixed with the respective aq. anthocyanin solution) and allowed to stand until pigment bleaching followed by treatments (i) with Pd and (ii) with NH_4OH for a few min at 100° [40].

UV/Vis spectroscopy was performed according to refs [12, 41].

Isolation of individual anthocyanins. The non-acylated anthocyanins were isolated by two successive TLC chromatographic steps: (i) crude extracts in S2 and (ii) the isolated anthocyanin bands (elution with 50% aq. MeOH and concentration *in vacuo* at 30°) in S1 (chrysanthemin from combined extracts of some *Nigritella* species, cyanin and seranin from some *Dactylorhiza* species and *Orchis ustulata*, respectively). Before use, the cellulose plates were developed twice in the respective solvents. The acylated anthocyanins were isolated by two successive column chromatographic steps: serapianin from *Orchis ustulata* and orchicyanin I from *Dactylorhiza sphagnicola* and *Gymnadenia conopsea* on polyamide with 20 and 30% aq. MeOH, respectively, containing 0.01% HCl (concn *in vacuo* at 30°) after elutions with H_2O and MeOH. They were purified by a following chromatography on Sephadex LH-20 with H_2O containing 0.01% HCl. Alternatively orchicyanin I was isolated by HPLC (Waters/Millipore GmbH, System 600) under the following conditions: prepacked Multosorb-C_{18} column (10 μm, 250 mm × 20 mm i.d.) from CS-Chromatographie Service, Eschweiler; detection at 525 nm. Elution system: in 40 min from solvent A (0.2% aq. HCO_2H) to 30% solvent B (0.2% HCO_2H in MeCN in (A + B) and subsequent to 60 min at 30% and to 100 min at 60%; flow rate of 5 ml/min (concentration *in vacuo* at 30°, followed by chromatography on Sephadex LH-20).

Quantification of anthocyanins. Aliquots from each extract were diluted to give three different concentrations between 0.1 and 0.4 extinction units at λ_{max}. In each case the linearity of the Vis spectroscopic measurements was checked [cf. ref. 42]. Anthocyanin contents were calculated in terms of cyanin in 0.1 M HCl, using an extinction coefficient of log ε 4.48 [43]. The amounts of the individual anthocyanins in each of the extracts are given as per cent of total absorbance of all detected pigments at 525 nm in HPLC analysis.

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