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STRUCTURE AND PROPERTIES OF THE ACYLATED ANTHOCYANINS FROM *VITIS* SPECIES

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Abstract—The acylated anthocyanins of Ives grapes have been isolated using column chromatography on polyamide and polyvinylpyrrolidone Controlled hydrolysis with Dowex 50W-X8 ion exchange resin, KOH, peroxide oxidation and spectroscopic characterization revealed their structure as the 3-(6-*O*-*p*-coumarylglucoside)-5-glucosides of cyanidin, peonidin, delphinidin, petunidin, and malvidin and the 3-(6-*O*-*p*-coumarylglucoside)s of delphinidin, petunidin and malvidin On cellulose TLCs in the five solvent systems used, no clear-cut separation of these pigments could be obtained without their preliminary separation on polyamide and polyvinylpyrrolidone columns

INTRODUCTION

IN RECENT years a number of acylated anthocyanins have been reported in the family of Vitaceae [1–13] The most commonly found acyl groups are *p*-coumaric[1–3,5,6,8,10,12–14] and caffeic[3,8,12,14] acids These are attached, similarly to other identified acylated anthocyanins of the Plant Kingdom,[15] to the 3-glucose of the five common *Vitis* anthocyanidins (e g cyanidin, peonidin, delphinidin, petunidin and malvidin) Only in two cases in the above family have acyl groups other than *p*-coumaric and caffeic acid been reported [7,11]

Few reports have appeared on the occurrence[2,9] and identification[14] of acylated anthocyanidin-3,5-diglucosides in the genus *Vitis* The position of the acyl group on the glucose has not been determined

In this paper we wish to report the isolation of the acylated anthocyanins from Ives (*Vitis labrusca* × *V aestivalis*?)[16] grapes and the position of acylation on these pigments

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RESULTS AND DISCUSSION

Column chromatography of the Ives anthocyanins on polyamide with gradient aqueous EtOH separated three pigment fractions (*A*, *B* and *C*) Fraction *A*, which was eluted with 900 ml solvent, contained the residual 3,5-diglucosides of cyanidin, peonidin, delphinidin, petunidin and malvidin, identified with authentic reference compounds[17] Fraction *B*, eluted between 1500 and 2700 ml showed 5 pigment components on cellulose TLC in solvents 1, 2 and 3, with R_f values higher than the anthocyanidin-3, and 3,5-glucosides Preliminary experiments showed that the pigments of this fraction were strongly adsorbed to the polyvinylpyrrolidone (PVP), and the use of one column for the separation and elution of the individual pigments would have been time and solvent consuming, with consequent loss in the yield Therefore, the separation was carried out using two PVP-columns (2, 5 × 15 and 5 × 35 cm) After elution of the 3 faster-migrating pigment bands (B_1–B_3) from the first column onto the second, the columns were disconnected and eluted separately Bands B_1 and B_3 were obtained pure, bands B_2, B_4 and B_5 required additional chromatography to obtain them in pure state

Fraction *C* of the polyamide column appeared on the chromatogram as a diffuse spot with the highest R_f value (× 100) (70, 69, 68 in solvents 1, 2 and 3) This pigment fraction was separated into 3 components (C_1–C_3) on a PVP column (2 × 20 cm) using 50% aq, EtOH as eluent C_1, present only in a small amount, was concentrated and chromatographed on cellulose TLC (solvent 1) to remove breakdown products of the pigment formed during evaporation Pigments C_2 and C_3 were obtained pure

TABLE 1 SPECTRAL AND CHROMATOGRAPHIC PROPERTIES OF THE ACYLATED ANTHOCYANINS FROM IVES GRAPES

Anthocyanin	λ_{max} nm (ε) MeOH	MeOH + $AlCl_3$	R_f × 100 Solvent 1	2	3	4	5
B_1 Malvidin 3 (6 *O p* coumaryl glucoside)-5 glucoside	540 (32 800) 308 (21 700) 303 (21 600) 281 5 (20 900)		61	63	50	9	45
B_2 Peonidin 3 (6 *O p* coumaryl glucoside)-5 glucoside	527 (31 800) 308 sh (17 600) 294 (21 000) 281 (23 000)		63	65	54	12	50
B_3 Petunidin 3-(6 *O p* coumaryl-glucoside) 5 glucoside	539 (37 200) 302 (19 000) 281 5 (20 800)	585 314 282 5	58	60	44	6	37
B_4 Cyanidin 3 (6 *O-p* coumaryl glucoside)-5 glucoside	531 310 298 281 5	546 310 298 282	59	61	48	8	43
B_5 Delphinidin 3 (6 *O-p*-coumaryl glucoside)-5-glucoside	538 (21 600) 308 (13 300) 302 (13 300) 281 5 (14 100)	585 314 283	57	58	41	5	33
C_1 Malvidin 3 (6-*O p* coumaryl glucoside)	540 310 sh 300 sh 283 5		74	73	70	3	22
C_2 Petunidin 3-(6-*O p*-coumaryl glucoside)	540 (20 700) 300 sh (15 200) 283 5 (17 100)	573 312 284 5	70	69	68	3	20
C_3 Delphinidin 3 (6 *O p*-coumaryl glucoside)	542 5 (33 000) 308 sh (22 200) 300 sh (23 100) 283 5 (26 200)	567 314 286 5	65	68	61	2	13

Spectral characteristics of the isolated pigments (Table 1) having a distinct peak or shoulder in the 310 nm region indicated acylation with *p*-coumaric acid[18] Alkaline hydrolysis of the above anthocyanins produced *p*-coumaric acid as the sole acylating agent from all 8 pigments The anthocyanins produced upon saponification of the B_1–B_5 pigment fraction were identified respectively as the 3,5-diglucosides of malvidin, peonidin, petunidin, cyanidin and delphinidin Saponification of the C_1–C_3 fractions gave the 3-monoglucosides of malvidin, petunidin and delphinidin

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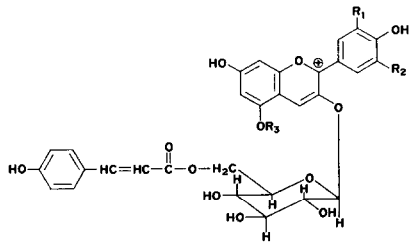
Since the absorbance of the acylated anthocyanidin-3,5-diglucosides (B_1–B_5) in the 310 nm region exceeded that of monardein[18] and was equal or near equal to the 280 nm region absorbance, it was of interest to determine quantitatively the *p*-coumaryl moiety of the pigments The *p*-coumaric acid of both the *B*- and *C*- fraction pigments (5×10^{-5} mol) after alkaline hydrolysis was found to be $3\,7 \times 10^{-5}$ mol *p*-Coumaric acid (5×10^{-5} mol), subjected to the same treatment as the pigments, gave $4\,2 \times 10^{-5}$ mol concentration The differences between the original *p*-coumaric acid concentration and those found after treatment apparently derive from the breakdown of the acid under alkaline conditions Thus, an anthocyanin-*p*-coumaric acid ratio of 1 1 is established

The pigments, when subjected to peroxide oxidation[19] and alkaline hydrolysis, produced 6-*O*-*p*-coumaryl glucose as the sole acylated sugar Mild acidic hydrolysis on Dowex 50W-X8[20,21] produced 6-*O*-*p*-coumaryl glucose, glucose and *p*-coumaric acid, found to be identical with authentic compounds on both TLC and GLC analysis

4-*O*-*p*-Coumaryl glucose could not be detected among the reaction products neither during acidic hydrolysis, nor during H_2O_2-oxidation of the pigments Hence, the identified 6-*O*-*p*-coumaryl glucose is assumed to be genuine and not produced as an artifact by acyl migration [22–24]

Based on the above results, the structure of the acylated Ives pigments have been identified, in order of elution from the PVP column, as the malvidin-3-(6-*O*-*p*-coumaryl-glucoside)-5-glucoside (B_1), peonidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside (B_2), petunidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside (B_3), cyanidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside (B_4), delphinidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside (B_5), malvidin-3-(6-*O*-*p*-coumarylglucoside) (C_1), petunidin-3-(6-*O*-*p*-coumarylglucoside) (C_2) and delphinidin-3-(6-*O*-*p*-coumarylglucoside) (C_3) With the exception of petunidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside, which to our knowledge has not been previously described, all pigments have been found either in the Vitaceae or elsewhere in the Plant Kingdom



Malvidin-3-(6-*O*-*p* coumarylglucoside)-5-glucoside $R_1 = R_2 = OMe$, R_3 = glucose
Peonidin 3-(6 *O p* coumarylglucoside)-5-glucoside R_1 = OMe $R_2 = H$ R_3 = glucose
Petunidin 3-(6-*O p*-coumarylglucoside)-5 glucoside R_1 = OMe, R_2 = OH R_3 = glucose
Cyanidin 3-(6 *O p* coumarylglucoside) 5 glucoside R_1 = OH, $R_2 = H$ R_3 = glucose
Delphinidin 3-(6 *O p* coumarylglucoside) 5 glucoside $R_1 = R_2$ = OH, R_3 = glucose
Malvidin 3 (6-*O p* coumarylglucoside) $R_1 = R_2 = OMe$, $R_3 = H$
Petunidin 3 (6 *O p* coumarylglucoside) R_1 = OMe, R_2 = OH, $R_3 = H$
Delphinidin 3-(6 *O p* coumarylglucoside) $R_1 = R_2$ = OH, $R_3 = H$

Malvidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside was found to be the major acylated pigment in the Ives grape and it is assumed to be identical with the *p*-coumaryl 3,5-diglucosides of malvidin, which have been reported to occur in the Vitaceae [9,14] It is of interest that the spectral data here reported of this pigment differs from those published for tibouchinin, a malvidin-3,5-diglucoside acylated with *p*-coumaric acid, which was identified in the petals of *Tibouchina semidecandra* [15]

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Delphinidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside and cyanidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside, the minor acylated anthocyanidin-3,5-glucosides of Ives, have been found previously in *Commelina communis* (awobanin)[25] and in *Perilla nankinensis*[26] respectively

The occurrence of anthocyanidin-3-*p*-coumarylglucosides in the genus *Vitis* is well documented[2,3,5,6,8,12,14] and the position of acylation has been in some cases determined [13] The three isolated anthocyanidin-3-*p*-coumarylglucosides of Ives, similarly to the malvidin-3-*p*-coumarylglucoside obtained from *Vitis vinifera*,[13] had the *p*-coumaryl group attached to the 6-OH of the glucose molecule The presence of anthocyanidin-3-monoglucosides acylated with acetic acid,[11] which have been reported to exist in *Vitis vinifera* could not be established in Ives grapes

EXPERIMENTAL

Materials Ives grapes were grown in the experimental vineyard of the N Y State Agricultural Experiment Station Geneva N Y The ripe grapes were harvested on 4 October 1971 hot pressed and the juice was kept frozen at −40 until used Immediately before chromatography the juice was centrifuged to remove the precipitated solids

TLC Carried out on Eastman cellulose sheets in the following solvent systems 1 BAW (4 1 5) 2 BAW (4 1 2), 3 BuOH-2N HCl (1 1), 4 1% aq HCl, 5 AcOH conc HCl-H_2O (15 3 82) and 6 2% aq AcOH The last was used for identification of the alkaline hydrolysis products

Isolation of the pigments All solvents used in the column chromatographic separation contained 1 ml 4 N HCl/l The grape juice (3 l l) was percolated through an MN-SC6 polyamide column (5 × 50 cm) which was prepared in H_2O The column was washed with H_2O until tasteless and eluted with a gradient aq EtOH soln (2000 ml H_2O in mixing flask 2000 ml EtOH in reservoir) Three fractions were obtained Fr 4 (0-900 ml) Fr *B* (1500-2700 ml) and Fr *C* (2800-3500 ml) Fraction *A* containing the residual 3 5 diglucosides of malvidin peonidin petunidin cyanidin and delphinidin (identified in the above 5 solvent systems with authentic pigments) were only weakly adsorbed to the polyamide Fractions *B* and *C* were evaporated to dryness dissolved in a small amount of MeOH (0 01% HCl) and precipitated with Et_2O Yield Fr *B* 3 75 g Fr *C* 0 87 g

Fraction *B* (2 0 g) was dissolved in 75 ml 30% aq EtOH and adsorbed to a PVP column (2 5 × 15 cm) which was connected to a second PVP column (5 × 35 cm) both equilibrated with 30% aq EtOH After three faster-migrating pigment bands B_1 B_2 and B_3 were eluted onto the second PVP column (5 × 35 cm) the columns were disconnected and eluted separately with the above solvent Elution of the 5 × 35 cm column provided 3 (B_1 B_3) pigment fractions The fractions were evaporated to dryness dissolved in a small amount of MeOH (0 01% HCl) and precipitated with Et_2O Thus, chromatographically pure (5 solvents) *malvidin*-3-(6-O-p-*coumarylglucoside*)-5-*glucoside* 79 3 mg and *petunidin*-3-(6-O-p-*coumarylglucoside*)-5-*glucoside* 139 5 mg were obtained from Fractions B_1 and B_3 respectively Fraction B_2 provided after rechromatography on PVP (2 5 × 40 cm) using 30% EtOH 65 4 mg *peonidin*-3-(6-O-p-*coumarylglucoside*)-5-*glucoside*

Elution of the short PVP-column (2 5 × 15 cm) gave a mixture (106 mg) of cyanidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside and delphinidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside The fractions containing this pigment mixture were evaporated to dryness dissolved in 10 ml 30% aq EtOH and rechromatographed on a freshly prepared PVP-column (1 × 18 cm) using the above solvent The first fraction of this column (B_4) containing only a small amount of the pigment was evaporated to a small volume (*ca* 0 5 ml) and further purified on preparative cellulose TLC in solvent 1 After elution from the adsorbent with MeOH (25 ml 0 01% HCl), chromatographically pure (5 solvents) *cyanidin*-3-(6-O-p-*coumarylglucoside*)-5-*glucoside* was obtained Because of the small amounts found (< 1 mg) this pigment was characterized using its methanolic solution Evaporation of the second pigment fraction (B_5) and precipitation with Et_2O yielded 23 5 mg chromatographically pure (5 solvents) *delphinidin*-3-(6-O-p-*coumarylglucoside*)-5-*glucoside*

Fraction *C* of the polyamide separation (120 mg) was dissolved in 10 ml 30% EtOH applied to a freshly prepared PVP-column (2 × 20 cm) and eluted with 50% EtOH After a small contamination of the Fr *B* pigments were removed 3 pigment bands (C_1 C_3) were separated and eluted from the column Fraction *C* was evaporated to a small volume applied to a preparative cellulose TLC and further purified in solvent 1 This pigment was found to be identical in the above 5 solvents with an authentic sample of *malvidin*-3-(6-O-p-*coumarylglucoside*) and by spectral comparison Evaporation of the two other pigment fractions (C_2 and C_3) and precipitation of the pigments with Et_2O yielded chromatographically pure (5 solvents) *petunidin*-3-(6-O-p-*coumarylglucoside*) 3 5 mg and *delphinidin*-3-(6-O-p-*coumarylglucoside*) 3 7 mg No attempts were made to crystallize the pigments

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Alkaline hydrolysis of the pigments and identification of the hydrolysis products Alkaline hydrolysis was carried out according to Albach *et al*[3] with 5 mg samples of pigment fractions and pure pigments The concentrated Et_2O and isoPrOH extracts were chromatographed on cellulose TLCs in all solvents with reference compounds In the case of cyanidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside and malvidin-3-(6-*O*-*p*-coumarylglucoside) a 10 ml portion of the pigment solution was used It was found that a 2 hr hydrolysis destroyed the acylated delphinidin glucosides, these were hydrolyzed for 10 min, sufficient for a complete saponification

Determination of the p-coumaric acid content of the pigments Pigments (5×10^{-5} mol) were subjected to alkaline hydrolysis as above, the *p*-coumaric acid extracted with Et_2O, evaporated to dryness, dissolved in MeOH, and the concentration determined spectrophotometrically using ϵ 25 000 (determined from the monohydrate crystallized from cold solution[27]) *p*-Coumaric acid (5×10^{-5} mol), subjected to the same treatment, was used as control

Hydrolysis of the pigments and identification of the acyl sugar The acidic hydrolysis of the anthocyanins (10 mg) on Dowex 50W-X8 (5 g, wet wt) was carried out according to Birkofer *et al*[21] After extraction of the free *p*-coumaric acid, the aqueous layers were evaporated to dryness and the acyl glucoside further purified on steamed cellulose/silica gel TLC plates[28] with solvent 1 Following the elution of the compound (R_f 0 77) from the adsorbent with MeOH, the solution was evaporated to dryness and the residue silylated in 1 ml pyridine for GLC analysis (15% Dexsil on Gas Chrom Q, 80–100 mesh, column dimensions 18 × 0 32 cm stainless steel, carrier gas N_2, 25 ml/min, oven temp 263°, FID) Authentic 6-*O*-*p*-coumaryl and 4-*O*-*p*-coumaryl glucose (see Acknowledgements) was used as reference The acyl sugar was identified in all cases also on cellulose TLC in solvents 1, 2 and 4

H_2O_2 *oxidation—identification of the acyl sugars* 10 Mg pigment was dissolved in MeOH (2 ml) and oxidized with 30% H_2O_2 (0 4 ml) for 4 hr as reported by Harper and Chandler[19] After destruction of the excess H_2O_2 with Pd–C the soln was treated with NH_4OH (0 5 ml) on a steam bath for 5 min in N_2 atmosphere and evaporated to dryness The residue was then taken up in a small amount of H_2O for TLC or dissolved in 0 5 ml pyridine, silylated, and subjected to GLC analysis using 6-*O*-*p*-coumaryl and 4-*O*-*p*-coumaryl glucose as reference

Acknowledgements—The authors are grateful to Dr R E Kepner for samples of malvidin-3-(6-*O*-*p*-coumaryl-glucose), malvidin-3-glucoside acylated with acetic acid, 6-*O*-*p*-coumaryl and 4-*O*-*p*-coumaryl glucose Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No 2036

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