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OXIDATION PRODUCTS OF ACYLATED ANTHOCYANINS UNDER ACIDIC AND NEUTRAL CONDITIONS

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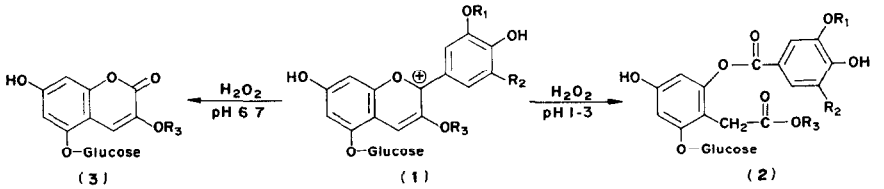
Abstract—Acylated anthocyanidin-3 5-diglucosides are oxidized with H_2O_2 under acidic conditions to acylated *ortho*-benzoyloxyphenylacetic acid esters When the same reaction is carried out under neutral conditions, the reaction product is the 3-*O*-acyl-glucosyl-5-*O*-glucosyl-7-hydroxy coumarin

INTRODUCTION

HYDROGEN peroxide oxidation of anthocyanins and other flavonoids has been used as an essential tool for the identification of the sugar substituents in the 3-position of the above compounds [1] Upon nucleophylic attack of the H_2O_2 at the 2-carbon of the molecule,[2,3] the heterocyclic ring is cleaved between C_2 and C_3 to form *ortho*-benzoyloxyphenylacetic acid esters (**2**) of the malvone type [4,5] These esters are easily hydrolyzed under alkaline conditions to the B-ring acid, the sugar substituent of the 3-position, and the elusive 2,4,6-trihydroxyphenylacetic acid, or its sugar derivatives, depending on the nature of the original compound



When heated in neutral aqueous solutions, anthocyanidin-3,5-diglucosides break down to the 3,5-di-(*O*-β-D-glucosyl)-7-hydroxycoumarin[6] (**3**, R_3 = glucose) This compound, having a strong UV-fluorescence and easily detectable on the chromatograms, has its original A and C-rings intact and can be used for the identification of anthocyanidin-3,5-diglucosides Thus, the combination of the method of Chandler and Harper and the degradation of the anthocyanin to the coumarin derivative can provide essential evidence for the structure of anthocyanins Anthocyanidin-3-glucosides do not form coumarin derivatives

[1] CHANDLER, B V and HARPER, K A (1961) *Australian J Chem.* **14,** 586
[2] SONDHEIMER, E and KERTESZ, Z I (1951) *Food Res* **17,** 288
[3] JURD, L (1966) *Tetrahedron* **22,** 2913
[4] KARRER, P and de MEURON, G (1932) *Helv Chim Acta* **15,** 507
[5] HRAZDINA, G (1970) *Phytochemistry* **9,** 1647
[6] HRAZDINA G (1971) *Phytochemistry* **10,** 1125

Based on the properties of the anthocyanidin-3,5-diglucosides to form the above compounds under acidic or neutral conditions, it was expected that acylated anthocyanidin-3,5-diglucosides, recently isolated in our laboratory,[7] would behave similarly under the above described conditions to form the acylated derivatives of the malvone and coumarin diglucoside types respectively

RESULTS AND DISCUSSION

When malvidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside (**1**, R_1 = Me, R_2 = OMe, R_3 = 6-*O*-*p*-coumarylglucose) was oxidized under acidic conditions the colour of its solution faded considerably slower (4 hr) than that of the corresponding malvidin-3,5-diglucoside (15 min) The slower reaction rate is most likely caused by a decrease in activity of the C-2 position by steric hindrance, or by the combination of both The oxidation product(s) contrary to that observed with malvidin-3,5-diglucoside did not crystallize from the reaction mixture TLC of the reaction mixture showed the presence of *p*-coumaric acid, syringic acid, malvone, and 3 other compounds, presumably fragments of the original oxidation product The purification and isolation of the primary oxidation product *p*-coumarylmalvone (**2** R_1 = Me, R_2 = OMe R_3 = 6-*O*-*p*-coumarylglucose) was greatly hampered by the instability of this compound in neutral and acidic solvents, producing continuously syringic and *p*-coumaric acids, malvone and a Gibbs purple compound during the work-up process With the combination of column and cellulose thin layer chromatography a small amount of the original oxidation product *p*-coumarylmalvone could be obtained for spectral characterization The λ_{max} of *p*-coumarylmalvone was found to be 10 nm higher than that of malvone (Table 1) apparently caused by the overlapping of the absorption curves from malvone (λ_{max} 284 nm) and *p*-coumaric acid (λ_{max} 310 nm) A spectrum identical to *p*-coumarylmalvone was obtained from a solution of malvone and *p*-coumaric acid in equimolar amounts Alkaline hydrolysis of *p*-coumarylmalvone produced the same fragments as that of malvone in addition to *p*-coumaric acid identified by both GLC and TLC Similarly acidic hydrolysis of the compound followed by alkaline hydrolysis gave identical breakdown products to malvone, in addition to *p*-coumaric acid

TABLE 1 SPECTRAL AND CHROMATOGRAPHIC PROPERTIES OF THE ANTHOCYANIN OXIDATION PRODUCTS

Compound	λ_{max} (nm) MeOH	MeOH + MeONa	R_f Solvent* 1	2	3	4	5	6
p-Coumarylmalvone	294	356 sh 341 310 sh 240	0·72	0·76	0·72		0·75	0·62
Malvone	284	334 242	0·88	0·87	0·88	0·25	0·62	
3-(6-*O*-*p*-Coumarylglucosyl)-5-glucosyl-7-hydroxycoumarin	315 300 sh 260 250 sh	No shift	0·65	0·71	0·63	0·27	0·58	
3,5-di-*O*-β-D-Glucosyl-7-hydroxycoumarin	329 260 sh 248 sh	378 276 251	0·28	0·45	0·26	0·47	0·70	

* See Experimental

[7] HRAZDINA G and FRANZESI A J (1973) in print

Attempts were made to isolate and identify a Gibbs purple compound (R_f 0 20 and 0 83 in solvents 1 and 6 respectively), presumably the 2-glucosido-4,6-dihydroxyphenylacetic acid, present in both hydrolyzates (e g that of malvone and *p*-coumarylmalvone) Because of the instability of the compound, only negligible amounts (< 1 mg) could be obtained The MS of this compound showed the molecular ion at 346 *m/e* and fragments at 181 *m/e* (346–glucose, the fragment having the *p*-quinoid structure), 166 *m/e* ($C_8H_6O_4$, the lactone form of phenylacetic acid derivatives and decarboxylation, respectively decarbonylation products of this at *m/e* 139 and 138

$$(181 \xrightarrow[+2H]{-CO_2} 139,\ 166\text{–}CO \longrightarrow 138,\ m^*\ 114\ 72),$$

supporting the structure of the Gibbs purple compound as the 2-glucosido-4,6-dihydroxyphenylacetic acid

Upon oxidation of malvidin-3-(6-*O*-coumarylglucoside)-5-glucoside or of a pigment mixture containing the 3-(6-*O*-*p*-coumarylglucoside)-5-glucosides of cyanidin, peonidin, delphinidin, petunidin and malvidin in a 0 1 M AcONa solution, a blue fluorescent compound in UV light, the 3-(6-*O*-*p*-coumarylglucosyl)-5-glucosyl-7-hydroxy coumarin (**3**, R = 6-*O*-*p*-coumarylglucose) was produced The spectral characteristics of this coumarin derivative (Table 1) differed from that of the 3,5-di-*O*-β-D-glucosyl-7-hydroxy coumarin, (**3**, R = glucose) obtained from anthocyanidin-3,5-diglucosides [6] The difference in the λ_{max} is caused by the superimposition of the spectrum of *p*-coumaric acid on that of the coumarin diglucoside, as is the case with *p*-coumarylmalvone Approximately equimolar concentrations of the coumarin diglucoside and *p*-coumaric acid gave identical spectra with the reaction product Alkaline hydrolysis of the reaction product yielded *p*-coumaric acid and the 3,5-di-*O*-β-D-glucosyl-7-hydroxy coumarin, both identified with authentic reference compounds by TLC in 5 solvents and spectral comparison

Contrary to an earlier assumption,[6] it seems now more likely, that the production of the coumarin derivatives is not caused by thermal degradation of the anthocyanidin-3,5-diglucosides and their acyl derivatives, but by a Bayer–Villiger type oxidation of their anhydrobases involving the alternate migration of the B-phenyl ring to the O^+ on the 2-position of the molecule [8] In a later stage of the reaction, the B-ring is subsequently lost and the coumarin derivative is formed

EXPERIMENTAL

The 3-(6-*O*-*p*-coumarylglucoside)-5-glucosides of malvidin, peonidin, petunidin, cyanidin and delphinidin were isolated from Ives grapes

TLC was carried out on Eastman cellulose sheets in the following solvents (1) BAW (4 1 5), (2) BAW (4 1 2), (3) BuOH–2 N HCl (organic phase), (4) 1% aq HCl, (5) AcOH–conc HCl–H_2O (15 3 82), (6) 2% aq AcOH

p-Coumarylmalvone 100 mg malvidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside was dissolved in 4 ml H_2O, 2 drops of conc HCl was added and the pigment oxidized with 1 ml 30% H_2O_2 for 4 hr At this time, the original dark red colour of the soln faded to very light pink The above soln was applied to a polyamide column (17 × 2 5 cm, prepared in H_2O), the column was washed with H_2O and eluted with 30% aq EtOH Four fractions were obtained Fr 1 (0–80 ml), Fr 2 (80–160 ml), Fr 3 (160–200 ml), and Fr 4 (220–640 ml) Fr 4, containing the acylated malvone was evaporated to dryness, dissolved in 5 ml abs MeOH, precipitated with Et_2O, filtered, and dried Yield 51 mg pink powder This preparation (51 mg), containing 3 impurities in lesser amounts, was dissolved in 4 ml 30% aq EtOH, applied to a freshly prepared polyamide column, washed with 500 ml 15% aq EtOH and eluted with 500 ml 50% aq EtOH Fractions containing the *p*-coumarylmalvone (260–400 ml) were evaporated to dryness the residue dissolved in 5 ml abs MeOH and

[8] JURD, L (1972) *Structural and Functional Aspects of Phytochemistry* (RUNECKLES, V C and TSO, T C, eds), Recent Advances in Phytochemistry Vol V pp 135–164 Academic Press, New York

precipitated with an excess of Et_2O Yield 23 mg TLC of the above compound in solvents 1 4 and 6 showed that it was contaminated with small amounts of syringic and *p*-coumaric acids

Spectroscopic characterization 10 mg of the above compound was dissolved in 1 ml MeOH streaked on cellulose TLC plates (1 per solvent) and further purified in solvents 1 3 and 5–6 respectively (R_fs 0 72 0 76 0 72, 0 75 and 0 62 dark band in UV light blue fluorescent after spraying with Na_2CO_3) After each purification step the band containing *p*-coumarylmalvone was scraped off eluted with MeOH and the eluate applied to a new plate Following the final purification in solvent 6 the UV-quenching band with R_f 0 62 was scraped off eluted with MeOH the eluate evaporated to dryness and redissolved in 4 ml abs MeOH The so obtained solution was chromatographically pure in 5 solvents and was used for the determination of the spectra Reference spectra of malvone[5] and malvone + *p*-coumaric acid both at 10^{-5} mol concentration were recorded in abs MeOH

Alkaline hydrolysis of p-*coumarylmalvone* 10 0 mg of the compound obtained via column chromatography was hydrolyzed in 1 ml 10% KOH for 30 min at room temp under N_2 in the dark The solution was acidified with 3 N HCl evaporated to dryness and extracted with 1 ml pyridine 0 2 ml of the extract was used for TLC identification with syringic and *p*-coumaric acids as comparison The remainder was silylated[9] and subjected to GLC analysis (15% DEXSIL on Gas Chrom Q 80–100 mesh, column dimensions 1 8 m × 3 mm, 181°, carrier gas N_2, 25 ml/min, on column injection) Malvone (10 mg) treated as above glucose syringic and *p*-coumaric acids were used for comparison

Attempted isolation of the phenylacetic acid glucoside 300 mg malvone was hydrolyzed in 3 ml 10% KOH at 100 for 10 min under N_2 in the dark The soln was cooled acidified to pH 2 3 with 3 N HCl and extracted with Et_2O (4 × 2 ml) and then with BuOH (3 × 2 ml) to remove syringic acid The aqueous layer (dark brown) was applied to a Sephadex G10 column and eluted with H_2O Two fractions were collected (Fr 1 0 125 ml, Fr 2 130–450 ml) Fraction 2, a mixture of 5 compounds on TLC (solvents 1 and 6), was evaporated to dryness, the residue dissolved in 5 ml MeOH and precipitated with Et_2O Yield 3 mg brown hygroscopic powder This compound was dissolved in 0 5 ml MeOH, further purified on TLC in solvent 6 scraped off the plate eluted with MeOH and precipitated with Et_2O to yield colorless flakes < 1 mg This preparation was chromatographically pure in 3 solvents (purple with Gibbs reagent) MS over 100 *m/e* M^+ 346 (6%) 181 (100%) 166 (24%) 139 (21%) 138 (18%)

3-(6-O-p-*coumarylglucosyl*-5-*glucosyl*-7-*hydroxycoumarin* 10 0 mg malvidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside was dissolved in 0 5 ml MeOH 4 ml 0 1 M aq Na-acetate added and the purple pigment solution oxidized with 0 2 ml 30% H_2O_2 for 4 hr The above solution was evaporated to dryness the residue extracted with 5 ml MeOH concentrated and the blue fluorescent compound (in UV) was purified on cellulose TLCs in solvents 1 and 4 (R_fs 0 65 and 0 27 respectively) Following purification in solvent 4 the blue fluorescent band was scraped off eluted with 10 ml MeOH evaporated to dryness and redissolved in 4 ml abs MeOH for spectral characterization Fraction B (10 mg) from the Ives pigments[7] containing the 3-(6-*O*-*p*-coumaryl-glucoside)-5-glucosides of malvidin peonidin petunidin cyanidin and delphinidin was treated as above

Alkaline hydrolysis of 3-(6-O-p-*coumarylglucosyl*)-5-*glucosyl*-7-*hydroxycoumarin* 10 0 Mg malvidin-3-(6-*O*-*p*-coumarylglucoside)-5-glucoside was treated as above the final methanolic eluate taken to dryness and hydrolyzed with 1 ml 10% aq KOH for 10 min at room temp under N_2 in the dark The hydrolyzate was acidified with 3 N HCl and extracted with Et_2O The Et_2O extract was used for TLC identification of the acyl moiety The aq soln was evaporated to dryness, extracted with 10 ml MeOH concentrated and chromatographed on a cellulose TLC in solvent 5 The blue fluorescent band (R_f 0 74) was scraped off, extracted with 15 ml H_2O the extract evaporated to dryness and the residue dissolved in 4 ml abs MeOH for spectral characterization 3 5-Di-*O*-*β*-D-glucosyl-7-hydroxycoumarin[6] prepared from 10 mg malvidin-3 5-diglucoside as above, was used for spectral comparison in the presence and absence of *p*-coumaric acid

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[9] MABRY T J and KAGAN T (1965) *Anal Chem* **37**, 288