The Flavonoid Glycosides of *Dahlia variabilis*

IV. 3-Glucosido-5-arabinosidocyanidin from the Variety "Dandy"

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A method for the identification of anthocyanins on a micro-scale has been developed and applied to an anthocyanin separated from the petal extracts of the blue dahlia "Dandy". The anthocyanin was identified as 3-glucosido-5-arabinosidocyanidin.

In Part I of this series ¹ the presence of a cyanidin glycoside, yielding glucose and arabinose on hydrolysis, was reported in the petal extracts of the variety "Dandy". Further attempts to characterise this compound using methods similar to those developed for the identification of flavone glycosides were then unsuccessful due to the instability of cyanidin in solution. Investigation of the causes of this instability has resulted in the successful development of methods for the determination of the aglycone:sugar ratio and position of carbohydrate attachment in anthocyanins. Application of these methods to investigations of anthocyanins from natural sources gives more precise information regarding their structure than previously used micro-methods ^{2,3}.

As previously described (*cf.* Part I) large scale paper chromatography of the ethanolic hydrochloric acid extracts of the petals of the blue garden dahlia "Dandy" by butanol-acetic acid-water yields, besides a number of flavone glycosides, an anthocyanin which after further purification yielded an aglycone, tentatively identified as cyanidin, arabinose and glucose on hydrolysis. This glycoside was now further tested for homogeneity by running in a number of solvents. Using acetone-0.1 N hydrochloric acid (1:3) an impurity having a weak yellow fluorescence in ultra-violet light was removed and chromatograms of the ethyl acetate extract of the hydrolysed anthocyanin indicated the absence of further impurities. The aglycone which so far had been identified only by its R_F value in one solvent was now conclusively identified as cyanidin by its absorption spectrum in visible light and its R_F values in a number of other solvents ⁴ (Table 1).

All previous attempts to determine the molar aglycone:sugar ratio of this and other anthocyanins using the methods developed in Part I had so far been

unsuccessful due to the fading of the colour of the anthocyanidins in solution, making spectrophotometric determination of their concentration impossible. Such fading in acid solution in the light has previously been reported by Spaeth and Rosenblatt⁵. It was found that in 0.1 to 2 N hydrochloric acid solutions kept in darkness at room temperature no significant decrease in cyanidin concentration could be detected, whereas when similar solutions were exposed to light the cyanidin concentration decreased fairly rapidly. In six hours the concentration of a solution exposed to diffuse light had decreased by 10 % but in sunlight the decrease of concentration of the solution amounted to about 95 % of the cyanidin initially present. These experiments were repeated in nitrogen atmosphere and although the decrease in cyanidin concentration was slightly less, it was of the same order as in the previous cases. In no case could the cyanidin be restored by increasing the acid concentration. Model experiments on the hydrolysis of cyanin with 2 or 4 N aqueous hydrochloric acid at 100° yielded calculated amounts of cyanidin provided the hydrolysis was carried out in darkness. Thus it was possible to determine spectrophotometrically the cyanidin concentration in hydrolysed extracts of the "Dandy" anthocyanin.

In this connection the effect of the solvent on the main absorption peak of cyanidin and its glycosides should be mentioned. Thus the absorption maximum of cyanidin in 0.1 N 80–100 % ethanolic hydrochloric acid is at 545 m μ moving towards shorter wave-lengths on further decrease of ethanol concentration. In a solution containing 20 % ethanol the maximum lies at 530 m μ . Variations in the acid concentration has no influence provided this concentration is high enough to prevent colour-base formation. Similar solvent effects have been reported by Sondheimer and Kertesz⁶ in case of 3-glucosidopelarionidin.

The amount of arabinose in the hydrolysed extracts of the "Dandy" anthocyanin was determined after removal of the aglycone using the orcinol-ferric chloride method described by Fernell and King⁷ and the glucose was quantitatively determined directly by the revised anthrone method¹ applying corrections for arabinose and cyanidin. The results of these estimations gave a cyanidin:glucose:arabinose ratio of approximately 1:1:1.

The position of carbohydrate attachment was determined by identification of the compounds obtained on acid and alkaline hydrolysis of the product of methylation of the anthocyanin with dimethyl sulphate in aqueous alkaline solution. In such strongly alkaline solutions the heterocyclic ring of the anthocyanins is broken and chalcones formed but only when the 5-hydroxyl is blocked by a sugar molecule can the anthocyanidin methyl ether be reformed on acid hydrolysis⁸. Thus on methylation of cyanin (I), the chalcone (II) is obtained which yields (III) on hydrolysis.

Methylation and subsequent acid hydrolysis of the "Dandy" anthocyanin yielded an anthocyanidin methyl ether indicating that carbohydrate is attached to the 5-hydroxyl. In order to investigate the possibility of further glycosidation in the aromatic nuclei the cyanidin methyl ether was hydrolysed with 10 % aqueous sodium hydroxide solution, a treatment known to degrade 3-hydroxyflavylium compounds and anthocyanins to a phloroglucinol component and an acid component⁹, but no compound could be isolated. Experiments with a

3',4',5,7-tetra-*O*-methyl cyanidin showed that relatively large amounts of material had to be used in order to obtain enough degradation products to be detected even by paper chromatography. However, it was found that alkali treatment of methylated cyanin without previous acid hydrolysis gave much better yields of degradation products. When this technique was applied to the "Dandy" anthocyanin, phloroglucinol dimethyl ether and veratric acid were identified (Table 2). Since the cyanidin methyl ether obtained on acid hydrolysis of the methylated "Dandy" anthocyanin, when run alongside 3',4',5,7-tetra-*O*-methyl and 3,3',4',5,7-penta-*O*-methyl cyanidin on paper chromatograms, showed R_F identity with the former compound it must be concluded that the sugars are attached to the 3- and 5-hydroxy-groups.

Partial hydrolysis of the anthocyanin with 1 % aqueous hydrochloric acid yielded a monoside which after separation on streak chromatograms by butanol-acetic acid-water and further purification by running in acetone-0.1 N hydrochloric acid (1:3) on hydrolysis showed the presence of cyanidin and glucose together with traces of arabinose (Table 1). The behaviour of the monoside in a number of specific tests (*i.e.* stability towards ferric chloride, colour in sodium carbonate solution and regeneration on acidification of an alkaline solution) was identical with that of mecocyanin and 3-glucosidocyanidin but different from that reported for 5-glucosidocyanidin¹⁰ and shown by cyanidin and its 3',4',5,7-tetramethyl ether. Since León and Robinson¹¹ claim that they obtained 5-glucosidocyanidin on partial hydrolysis of cyanin, this latter compound was partially hydrolysed and the resulting monoside purified as described above. The monoside obtained showed all the above mentioned characteristics of a 3-glucoside. Both monosides, on methylation and hydrolysis with alkali, yielded phloroglucinol trimethyl ether and so conclusively proved to be 3-glucosido-5-arabinosidocyanidin. Consequently the "Dandy" anthocyanin must be 3-glucosido-5-arabinosidocyanidin. This compound has not previously been found to occur in Nature and is a new type of anthocyanin since all previously isolated pentose containing anthocyanins are of the type 3-pentose glucoside.

EXPERIMENTAL

Separation and purification of the "Dandy" anthocyanin. Petals (122 g) of the variety "Dandy" were crushed under 0.1 N ethanolic hydrochloric acid (600 ml), kept at 0° for one week and filtered (total solids 8.2 mg/ml, cyanidin 0.08 mg/ml). The extract was then stored at 0°.

Portions (25 ml) of the extract were applied to Whatman No. 3 MM paper 22 × 22" and the chromatograms developed with butanol-acetic acid-water (6:1:2). The separated anthocyanin band was cut out without previous drying and the glycoside eluted with 0.05 N aqueous hydrochloric acid as described in Part I. The eluate was applied as a streak to No. 3 MM paper and further purified by isopropanol-2 N hydrochloric acid. On elution as above the anthocyanin was tested for homogeneity on Whatman No. 1 paper in other solvents. Separation from a yellow fluorescing compound took place in acetone-0.1 N hydrochloric acid (1:3) and the whole eluate was rerun in this solvent. The anthocyanin solution obtained on eluting the final chromatograms was used for further investigation. R_F values and wave-lengths of maximum absorption of the "Dandy" anthocyanin and a few reference compounds are given in Table 1.

Identification of the anthocyanin. (a) *Qualitative hydrolysis.* A portion of the glycoside solution was made 2 N by the addition of concentrated hydrochloric acid and heated in the dark at 100° for 2 h., then cooled and the aglycone extracted with butanol. The extract was washed with water and after removal of the solvent the aglycone was dissolved in 0.1 N ethanolic hydrochloric acid. The aglycone was then identified by comparing its R_F values and main absorption maximum with those of synthetic standards ⁴ (Table 1).

The identification of the sugar components has been described in Part I of this series ¹.

Table 1. R_F values and wave-lengths of the main absorption peaks of the "Dandy" anthocyanin, the products obtained on partial and complete hydrolysis and of reference compounds.

Compound	R_F in					$\lambda_{\max}$ m μ (a)
	BuOH- HOAc- H ₂ O 6:1:2:	BuOH- 2N HCl	COMe ₂ - 0.1N HCl 1:3	H ₂ O- HOAc- conc. HCl 10:30:3	m-Cresol- 5.5 N- HCl- HOAc 1:1:1	
"Dandy" anthocyanin	0.08	0.03	0.32			533
Aglycone		0.68		0.50	0.69	545
Part. hydrolysed anthocyanin	0.18	0.11	0.19			536
Aglycone		0.68		0.50	0.69	545
Sugar	0.15					
Cyanin	0.07	0.02	0.32			538
Mecocyanin	0.13	0.10	0.55			537
Part. hydrolysed cyanin	0.18	0.11	0.19			536
3-Glucosidocyanidin	0.18	0.11	0.19			536
Cyanidin	0.48(b)	0.68		0.50	0.69	545
"	0.73(c)					
Glucose	0.15					
Arabinose	0.19					

(a) in 0.1 N ethanolic HCl. (b) chloride. (c) colour-base.

Table 2. R_F values of the methylated "Dandy" anthocyanidin, of the products of alkali degradation and of reference compounds.

Compound	R_F in		
	H ₂ O-HOAc- conc. HCl 10:30:3	Toluene- HOAc-H ₂ O 4:1:5	BuOH-2 N Ammonia
"Dandy" anthocyanin	0.82		
Phenolic component		0.83	
Acid component			0.25
Part. hydrolysed anthocyanin, phenolic component		0.96	
Part. hydrolysed cyanin, phenolic component		0.96	
3,3',4',5,7-Penta-O-Me cyanidin	0.97		
3',4',5,7-Tetra-O-Me cyanidin	0.82		
Phloroglucinol		0.00	
» Me ether		0.18	
» di-Me ether		0.83	
» tri-Me ether		0.96	
Protocatechuic acid			0.02
Vanillic acid			0.06
isoVanillic acid			0.09
Veratric acid			0.25

(b) *Quantitative hydrolysis.* (i) Determination of aglycone concentration. A portion of the glycoside solution (1 ml) and concentrated hydrochloric acid (0.5 ml) was heated at 100° in total darkness for 30 min. On cooling, the solution was diluted with ethanol to 10 ml. The concentration of the aglycone was determined spectrophotometrically, standards being determined in the same medium. (Cyanidin: $\lambda_{\max}$ 545 m μ , ϵ 29 270).

(ii) Determination of sugar concentration. Arabinose. A portion of the glycoside solution was made 2 N by the addition of concentrated hydrochloric acid and heated at 100° for 2 h. On cooling the aglycone was extracted with butanol, the extract washed with water and the combined mother liquor and washings made up to 25 ml. The arabinose concentration of this solution was determined by the orcinol-ferric chloride method⁷ using a Fisher photoelectric colourimeter. No correction for glucose was necessary.

Glucose. Portions (5 ml) of the glycoside solution were taken to dryness in 3/4" test tubes. The glucose concentration was then measured directly using the revised anthrone method¹. Glucose and arabinose standards were measured simultaneously and a blank containing the corresponding amount of cyanidin was similarly measured. Corrections for arabinose and cyanidin were then applied. Results: Cyanidin:glucose:arabinose = 1:0.98:0.95; 1:0.96:0.87.

(c) *Determination of the position of sugar substituents.* A portion of the glycoside solution was carefully taken to dryness under reduced pressure at room temperature, and the glycoside was dissolved in 2–3 drops of 1 N aqueous sodium hydroxide solution. With extremely vigorous stirring freshly distilled dimethyl sulphate (0.5 ml) and 30 % aqueous sodium hydroxide (2 ml) were added dropwise during 1.5–2 h. in nitrogen atmosphere and the stirring was continued for another hour. A part of the mixture was neutralised with 2 N hydrochloric acid and made about 4 N with concentrated hydrochloric acid.

On heating at 100° for 30 min. the cyanidin methyl ether was extracted with chloroform, the extract washed with water and taken to dryness. The residue was dissolved in a small amount of acid ethanol and run on paper chromatograms alongside synthetic standards. Results are given in Table 2. To the remainder of the methylation mixture one half of its volume of 30 % aqueous sodium hydroxide was added and while nitrogen was passed through the solution, it was boiled for 4 h., acidified and boiled for 30 min. longer to hydrolyse any remaining glycosides. On cooling, the products of degradation were extracted with ether. The extract was washed free of acid and separated into a phenolic and an acid fraction. Both fractions were run on paper chromatograms alongside synthetic standards, the phenolic fraction in toluene-acetic acid-water (4:1:5)¹³, the spots being made visible with Millons reagent or diazotised *p*-nitroaniline, the acid fraction in butanol saturated with 2 N ammonia, the spots in this case being rendered visible by spraying with dilute aqueous silver nitrate followed by phenol saturated with aqueous sodium hydroxide¹³. Results are given in Table 2.

Identification of the monoside. A final portion of the glycoside solution was carefully taken to dryness and heated with 1 % aqueous hydrochloric acid at 100° for 30 min. The mixture was cooled, applied as a streak to No. 3 MM paper and separated with butanol-acetic acid-water. The monoside was eluted with 0.05 N 70 % ethanolic hydrochloric acid and separated from free sugars on paper chromatograms run in acetone-0.1 N hydrochloric acid. On final elution the monoside was identified in the usual way. Results of this identification are given in the tables.

Cyanin was similarly partially hydrolysed and the 3-monoside identified as described above.

Mecocyanin was partially hydrolysed and the 3-monoside separated in the same way.

The reference compounds needed for this investigation were prepared by recorded methods.

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