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THE CONSTITUTION OF A CRYSTALLINE, BLUE CORNFLOWER PIGMENT

SAM ASEN

Crops Research Division, U.S. Department of Agriculture, Beltsville, Maryland, U.S.A.

and LEONARD JURD

Western Regional Research Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Albany, California, 94710

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Abstract—A new, crystalline, deep blue pigment has been isolated from cornflowers (*Centaurea cyanus* L.). The pigment is an iron complex of 4 molecules of cyanidin 3,5-diglucoside and 3 molecules of a "bisflavone" glucoside. The "bisflavone" glucoside is liberated on acid decomposition of the blue pigment, and chromatographic examination indicates that it is a *single* substance. On acid hydrolysis, however, it yields 7-*O*-methylapigenin and a second flavone which appears to be new and has not yet been positively identified. It is spectrally identical with 7-*O*-methylapigenin, and on the basis of its R_f values it may be the 7-*O*-methyl derivative of vitexin. The reported properties of commelinin, the blue pigment of *Commelina communis* L., are strikingly similar to those of the cornflower pigment and suggest that these two pigments have an essentially identical type of structure.

INTRODUCTION

THE structural modifications which red anthocyanins undergo in forming stable, blue plant pigments are obscure. It is generally believed, however, that blue colors result from the formation of ill-defined anthocyanin complexes with organic copigments *or* with metals, copigmentation and metal chelation frequently being considered to be unrelated processes.[1] The influence of a number of model organic copigments on the color and stability of cyanidin 3-glucoside was recently determined quantitatively.[1] In aqueous solutions at pH 3–6·5 these compounds did not measurably affect either the color or the stability of the anthocyanin. In the presence of metal (aluminum) salts, however, quercitrin, chlorogenic acid and catechin form stable copigment–metal–anthocyanin coordination complexes. These model studies therefore, indicated that copigmentation reactions occur only with metal chelates of anthocyanin anhydro bases.

In support of this conclusion a blue, crystalline pigment has now been isolated from cornflowers (*Centaurea cyanus* L.) and its constituent units have been identified.* This pigment has not previously been described; and in the following discussion it is referred to as cyanocentaurin.[2]

* Following the preparation of this manuscript, it was brought to the authors' attention that N. SAITO and K. HAYASHI, *Sci. Rept. Tokyo Kyoiku Daigaku* **12B**, 39 (1965) and N. SAITO, S. MITSUI and K. HAYASHI, *Proc. Japan Acad.* **37**, 485 (1961) have described the isolation of a blue pigment from cornflowers which yields some peptide, carbohydrate, cyanin, iron, potassium, magnesium and 16% flavone on hydrolysis. The observations reported in the present paper, therefore, confirm and extend the earlier Japanese results.

[1] For detailed discussion and literature references see L. JURD and S. ASEN, *Phytochem.* **5**, 1263 (1966).

[2] The trivial name centaurein has been given to a glucoside of 4′,6,8-trihydroxy-3,3′,7-trimethoxyflavone, isolated from *Centaurea jacea* L. (L. FARKAS, L. HÖRHAMMER and H. WAGNER, *Tetrahedron Letters* 727 (1963)).

RESULTS AND DISCUSSION

An amorphous blue compound, protocyanin, has been isolated from cornflowers by Bayer.[3,4] He reported that protocyanin is a macromolecular coordination complex of cyanin, polygalacturonic acid, iron and aluminum. Protocyanin, therefore, differs markedly from the cyanocentaurin described in this communication.

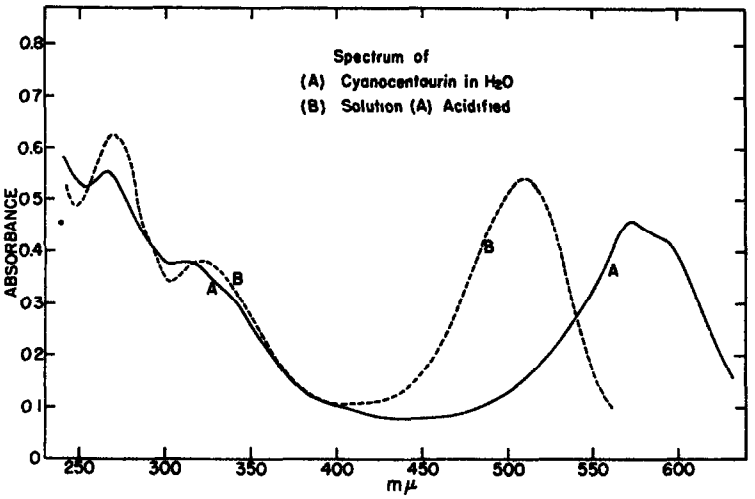


FIG. 1. SPECTRUM OF (A) CYANOCENTAURIN IN WATER (106·4 MG/L.) AND (B) 1 DROP HCl ADDED TO (A).

TABLE 1. R_f VALUES OF ANTHOCYANIN FROM CYANOCENTAURIN

	R_f in solvent*					
	A	B	C	D	E	F
Cyanocentaurin anthocyanin	0·52	0·48	0·10	0·23		
Cyanidin 3,5-diglucoside	0·51	0·46	0·13	0·23		
Cyanidin 3-glucoside	0·31	0·37	0·28	0·35		
Cyanocentaurin anthocyanidin					0·38	0·42
Cyanidin					0·39	0·42

* TLC on cellulose plates. (A) 10% aq. HOAc; (B) Acetone–2 N HCl, 1:3; (C) n-BuOH–2 N HCl, 1:1; (D) BAW, 6:1:2; (E) Paper chromatography, ascending, formic acid/HCl/water, 5:2:3; (F) TLC on silica gel, ethyl acetate/formic acid/water, 85:6:9.

In our investigation blue cornflowers were extracted by a procedure similar to that used by Hayashi, Abe and Mitsui[5] for the isolation of commelinin. From 40 g of fresh flowers 39 mg of cyanocentaurin were thereby obtained as a compactly crystalline, blue pigment. Cyanocentaurin forms stable, deep blue solutions in neutral aqueous media; and its spectrum (Fig. 1A) exhibits a broad absorption band at 570–600 nm (λ_{max} 573 nm). X-ray fluorescence measurements[6] established the presence of iron (roughly estimated at 1–2%) and a complete

[3] E. BAYER, *Chem. Ber.* **91**, 1115 (1958).
[4] E. BAYER, K. NETHER and H. EGETER, *Chem. Ber.* **93**, 2871 (1960).
[5] K. HAYASHI, Y. ABE and S. MITSUI, *Proc. Japan Acad.* **34**, 373 (1958).
[6] Kindly determined by Dr. H. C. LUKINS, Western Regional Research Laboratory.

absence of other metals. Suspended in cold dilute aqueous HCl cyanocentaurin immediately decomposes to yield a red anthocyanin (λ_{max} 510 nm) and a second compound (λ_{max} 319 nm) (Fig. 1B). The anthocyanin gives cyanidin on acid hydrolysis and it is spectrally (λ_{max} 525 nm in methanol–0·1% HCl) and chromatographically identical with cyanidin 3,5-diglucoside (Table 1).

The second compound (λ_{max} 319 nm, water) formed in this decomposition reaction migrates as a *single*, yellow fluorescent (under u.v. light + NH_3) spot on TLC cellulose plates and on paper chromatograms (Table 2). Extracted from the aqueous acid solution by means of ethyl acetate, and purified chromatographically, this compound has λ_{max} 269, 318 nm (ethanol), shifting to λ_{max} 290, 378 nm in alcoholic sodium ethylate (Fig. 2). With aluminum chloride the λ_{max} shifted to 279, 297, 330, 380 nm. These spectra indicate that the compound is a flavone glucoside. The relative intensities of the absorption peaks in the sodium ethylate

TABLE 2. *R* VALUES OF FLAVONES FROM CYANOCENTAURIN

I. TLC on cellulose plates

Solvent	R_f A	R_f B
BAW (6:1:2)	0·27	
20% aq. HOAc	0·67	0·10, 0·30
iso PrOH/HCOOH/H_2O (2:5:5)		0·60, 0·75
H_2O/HOAc/HCl (10:30:3)		0·75, 0·60

II. Paper chromatography, ascending

Solvent	R_f A	B	Genkwanin	Apigenin	Vitexin
BAW (6:1:2)	0·12	0·64, 0·99	0·99	0·99	0·56
50% aq. HOAc		0·62, 0·76	0·73	0·67	
H_2O/HOAc/HCl (80:20:5)		0·15, 0·38	0·15	0·15	0·40
H_2O/HOAc/HCl (80:40:5)		0·30, 0·56	0·32	0·31	
HCOOH/3 N HCl (1:1)		0·30, 0·52	0·31	0·31	

(A) = the "bisflavone" glucoside ex cyanocentaurin.
(B) = the flavones formed by hydrolysis of (A) and cyanocentaurin.

spectrum, moreover, are typical of a flavone whose only free phenolic group is located at position 5.

Hydrolyzed in boiling N aqueous HCl for 1 hr the above glycoside gave glucose and *two* yellow fluorescent (u.v. light) compounds. As indicated in Table 2, the R_f values of these two yellow compounds differ considerably and, therefore, they were easily separated and eluted from cellulose plates. In alcohol, and in the presence of diagnostic reagents, the mixture of the two compounds and the individual, separated compounds have virtually indistinguishable spectra. Thus, in ethanol these aglycones have λ_{max} 269, 336 nm (Fig. 3A), indicating that they are derived from apigenin. With sodium ethylate the intensity of the long wavelength band increases, and it undergoes a pronounced bathochromic shift to 393 nm (Fig. 3B), suggesting that the 4′-hydroxyl group is free.[7] The aluminum chloride spectrum (Fig. 4A) shows the presence of a free 5-hydroxyl group. With sodium acetate the low wavelength band

[7] L. JURD, In *The Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN), p. 107. Pergamon Press, Oxford (1962).

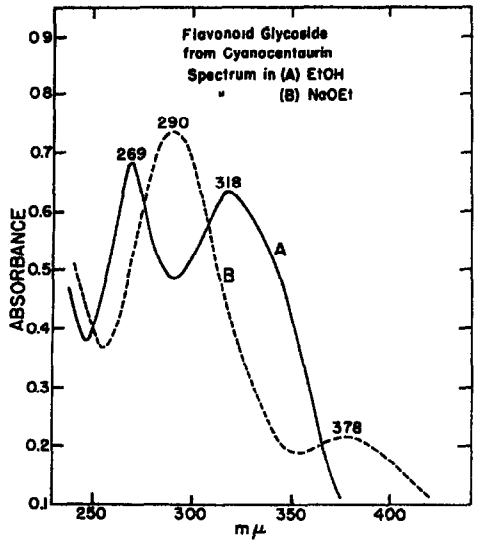


FIG. 2. SPECTRUM OF THE "BISFLAVONE" GLUCOSIDE FROM CYANOCENTAURIN IN (A) 95% ETHANOL (B) ETHANOLIC SODIUM ETHYLATE.

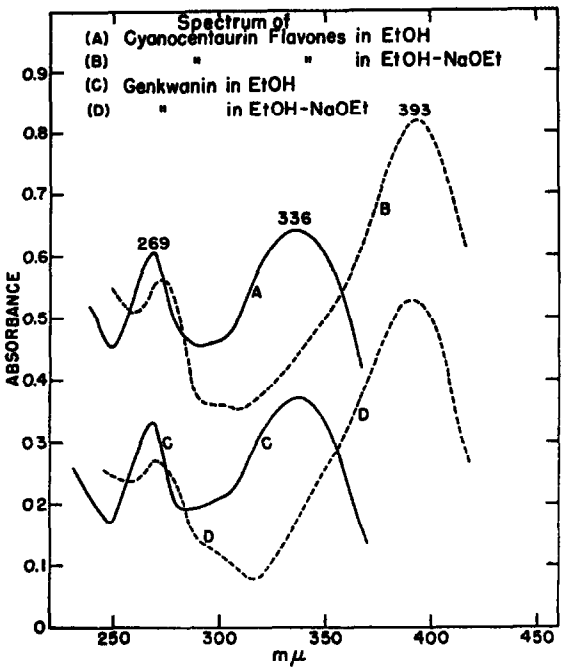


FIG. 3. SPECTRA OF (A) CYANOCENTAURIN FLAVONES IN ETHANOL, (B) CYANOCENTAURIN FLAVONES IN ETHANOLIC SODIUM ETHYLATE, (C) GENKWANIN IN ETHANOL, (D) GENKWANIN IN ETHANOLIC SODIUM ETHYLATE.

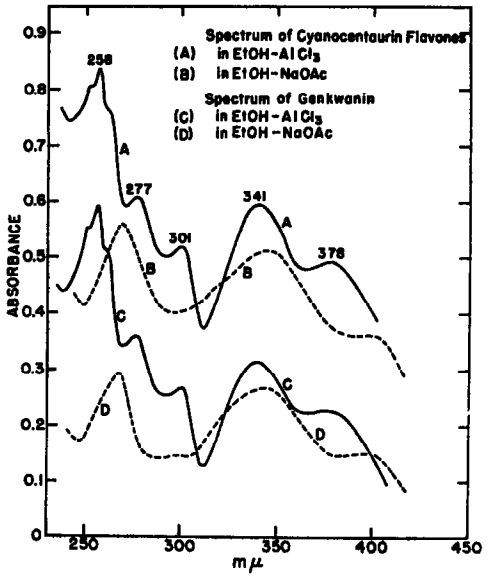


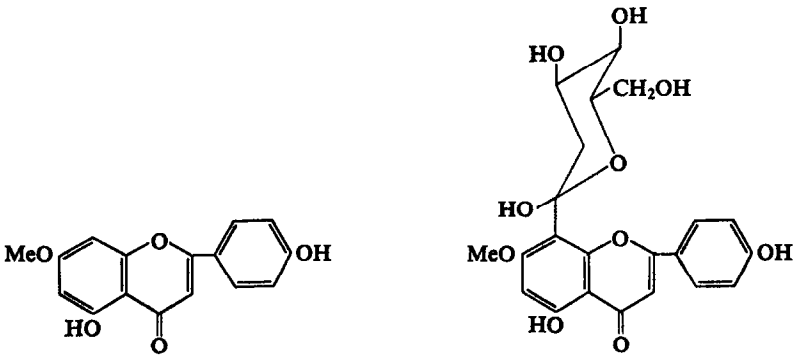
FIG. 4. SPECTRA OF (A) CYANOCENTAURIN FLAVONES IN ETHANOLIC ALUMINUM CHLORIDE, (B) CYANOCENTAURIN FLAVONES IN ETHANOLIC SODIUM ACETATE, (C) GENKWANIN IN ETHANOLIC ALUMINUM CHLORIDE, (D) GENKWANIN IN ETHANOLIC SODIUM ACETATE.

does not shift (Fig. 4B); and with boric acid–sodium acetate the position of the long wavelength band is unaffected, indicating the absence of a free 7-hydroxyl[8] and of an ortho-dihydroxyl group,[9] respectively. On the basis of these spectral changes these flavones are clearly related to 7-*O*-methyl apigenin (genkwanin) (I). The spectra of authentic genkwanin (Fig. 3C, D and 4C, D) are identical in all details with those of the cyanocentaurin aglycones. Chromatographic comparison of these aglycones with genkwanin (Table 2) in a variety of solvent systems confirms that one of these aglycones is genkwanin.[10] The second aglycone appears to be a new flavone whose structure can only be established when larger quantities are available. It is noteworthy, however, that (*a*) it is spectrally identical with 7-*O*-methyl apigenin; (*b*) its R_f values in aqueous solvents are considerably higher than those of 7-*O*-methyl apigenin. In BAW, on the other hand, the order is reversed, the compound having a lower R_f than 7-*O*-methyl apigenin. These R_f differences coincide with the relative R_f values of apigenin and vitexin in similar solvents, e.g.

	H_2O/HOAc/HCl (80:20:5)	BAW
7-*O*-Methyl apigenin	0·15	0·99
Cyanocentaurin aglycones	0·15, 0·38	0·99, 0·64
Apigenin	0·15	0·99
Vitexin	0·40	0·56

[8] L. JURD and R. M. HOROWITZ, *J. Org. Chem.* **22**, 1618 (1957).
[9] L. JURD, *Arch. Biochem. Biophys.* **63**, 376 (1956).
[10] Apigenin and genkwanin have similar R_f values in the solvent systems studied. These compounds, however, are easily distinguished by differences in their alkaline spectra.

On the basis of this limited evidence it appears that the second aglycone may be a C-glycosyl-flavone, viz. the 7-*O*-methyl derivative II of vitexin.[11]



Thus, cyanocentaurin is an iron-cyanidin 3,5-diglucoside-"bisflavone" glucoside complex. The ratio of cyanidin to flavone nuclei in this complex is 2:3. This was determined by hydrolyzing the pigment in N HCl for 2 hr, and by measuring the spectrum (Fig. 5A) of the

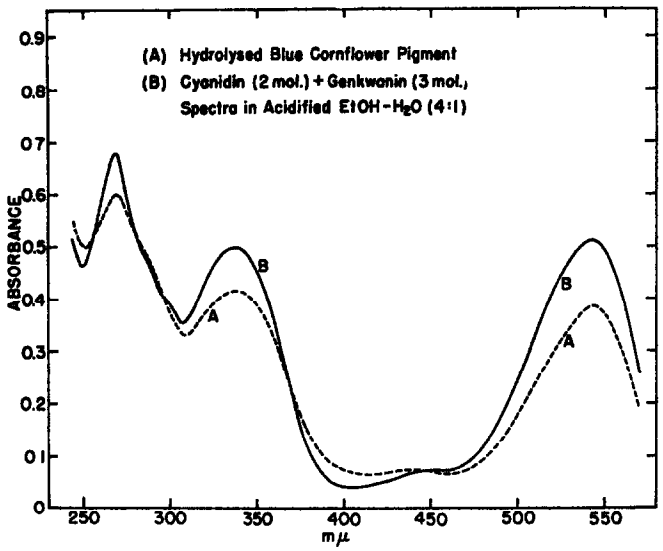


FIG. 5. (A) SPECTRUM OF HYDROLYZED CYANOCENTAURIN IN ACIDIFIED ETHANOL/WATER (80:20), (B) SPECTRUM OF CYANIDIN (2 MOL. EQUIVALENTS) + GENKWANIN (3 MOL. EQUIVALENTS) IN ACIDIFIED ETHANOL/WATER (80:20).

cyanidin–flavone mixture formed. The spectrum of a model, synthetic mixture of cyanidin chloride (2 mol. equivalents) and genkwanin (3 mol. equivalents), heated in acid solution under similar conditions, is shown in Fig. 5B. The positions and relative intensities of the

[11] 7-*O*-Methyl vitexin has not been described. 7-*O*-Methyl isovitexin (Swertisin), however, was recently isolated from *Swertia japonica* (Roem & Schult.) MAKINO, M. KOMATSU and T. TOMIMARI, *Tetrahedron Letters* **15**, 1611 (1966).

three absorption peaks in the cyanocentaurin hydrolysis spectrum agree well with those in the synthetic 2:3 mixture. The slight differences are attributed to variable cyanidin decomposition. Model experiments showed that in the boiling acid solutions in 2 hr, the amount of cyanidin decomposition varies from about 10–20%.

Since acid decomposition of cyanocentaurin yields a *single* flavonoid glycoside which, in turn, is hydrolyzed to *two* flavone nuclei (genkwanin and (?) 7-*O*-methyl vitexin), the 2:3 cyanidin–flavone ratio obtained on hydrolysis of the blue pigment proves that cyanocentaurin must be an iron complex of 4 molecules of cyanidin 3,5-diglucoside and 3 molecules of a "bisflavone" glucoside. The "bisflavone" glucoside present in cyanocentaurin is clearly unusual; and at the present time the limited, available evidence does not indicate the nature of the acid labile linkage between genkwanin, the second flavone and glucose.

In 1958 Hayashi and co-workers[5,12] described the isolation and partial identification of commelinin, a crystalline blue pigment of *Commelina communis*. Comparison of the properties of cyanocentaurin with those reported for commelinin reveals striking similarities and suggests that these pigments have essentially analogous structures. Thus, in aqueous solutions, commelinin and cyanocentaurin have principal absorption maxima at 273, 316, 591 nm and 268, 314, 573–592, respectively. Treated with 2% aqueous HCl commelinin decomposes to yield a metal (identified as magnesium), an anthocyanin (λ_{max} 528 nm, an acylated delphinidin glycoside) and "an unknown substance" (λ_{max} 317 nm). By gravimetric and colorimetric analysis Hayashi determined that commelinin is a magnesium complex of 4 molecules of the anthocyanin and (?) 4 molecules of the unidentified, flavonoid-like[13] substance. Except for expected differences in the positions of the anthocyanin maxima, the spectrum of the commelinin acid-decomposition products is remarkably similar to that (Fig. 1B) from cyanocentaurin. Furthermore, as in the case of the cyanocentaurin "bisflavone" glucoside (λ_{max} 318 nm), the commelinin "unknown substance" (λ_{max} 317 nm) migrates as a *single*, yellow fluorescent compound, which on acid hydrolysis yields *two* yellow-fluorescent aglycones (R_f 0·69 and 0·89 in acetic acid/HCl/water, 5:1:5). The spectra of these aglycones were not described. It is of interest, however, that we have determined that in Hayashi's solvent system, apigenin and vitexin have R_f 0·66 and 0·83 respectively. Under u.v. light apigenin is yellow and vitexin is yellow–green. Hayashi's aglycone R_f 0·69 is yellow and R_f 0·89 is yellow–green. It is possible, therefore, that, as with cyanocentaurin, the unknown substance in commelinin is also a "bisflavone" glucoside, containing apigenin and vitexin (or 7-*O*-methyl apigenin and 7-*O*-methyl vitexin).

EXPERIMENTAL

Isolation of Cyanocentaurin

Macerated, fresh blue flowers (40 g) of *Centaurea cyanus* cv. Blue Boy, were extracted in a blender with water (2 × 300 ml). The filtered aqueous extract was concentrated to 15·0 ml under reduced pressure at 40°, diluted with ethanol (90·0 ml) and cooled. The precipitated blue pigment was collected, washed with absolute alcohol, redissolved in water (15·0 ml), filtered, treated with alcohol (30 ml) and cooled. Precipitated impurities were filtered and the filtrate was treated with more alcohol (45 ml). The crude blue pigment which separated was collected, redissolved in water (14·0 ml), and treated with alcohol (14·0 ml), then cooled

[12] S. MITSUI, K. HAYASHI and S. HATTORI, *Proc. Japan Acad.* **35**, 169 (1959).

[13] K. HAYASHI, In *The Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN), p. 248. Pergamon Press, Oxford (1962).

overnight. Precipitated impurities were filtered and the filtrate was treated with more alcohol (35·0 ml). On standing the blue pigment crystallized. It was recrystallized twice from water (3·0 ml) and alcohol (6·0 ml), dissolved in a minimum volume of water and passed through a 1×15 cm cellulose column. The column was eluted with 50% aqueous ethanol. Sufficient alcohol was added to the eluate to induce crystallization. After cooling the deep blue compact crystals of cyanocentaurin were collected (39 mg; yield, 0·1% of fresh flowers).

Acid decomposition and hydrolysis products of cyanocentaurin were examined chromatographically and spectrally (Cary 15 recording spectrophotometer, 1 cm silica cells) as described in the text.