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APIGENIN 4′-*O*-β-D-GLUCOSIDE 7-*O*-β-D-GLUCURONIDE: THE COPIGMENT IN THE BLUE PIGMENT OF *CENTAUREA CYANUS*

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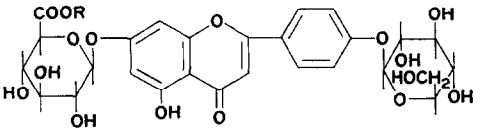
Key Word Index—*Centaurea cyanus*; Compositae; copigment; apigenin 4′*O*-β-D-glucoside 7-*O*-β-D-glucuronide.

Abstract—The copigment present in the crystalline blue pigment isolated from Blue Boy cornflowers (*Centaurea cyanus* L.) was identified as apigenin 4′-*O*-β-D-glucoside 7-*O*-β-D-glucuronide. The NMR spectra of aryl glucuronides are discussed.

INTRODUCTION

THE COPIGMENTATION of anthocyanins with other flavonoids and its effect on color were discussed earlier.[1] A much studied example of copigmentation is the complex blue pigment of the cornflower (*Centaurea cyanus* L.), which has been crystallized and also characterized.[2] In a previous study[3] carried out with a limited supply of material, the cornflower copigment was tentatively characterized as a bis-flavone glucoside which, on acid hydrolysis, appeared to yield 7-*O*-methylapigenin (genkwanin) and an unidentified second flavone. We have reexamined the copigment and have now identified it as apigenin 4′-*O*-β-D-glucoside 7-*O*-β-D-glucuronide (**5**). The compound was actually isolated as its methyl ester (**1**) but this turned out to be an artifact produced by the rapid esterification of (**5**) when dissolved in acidified methanol.



(**1**) R = Me
(**5**) R = H

RESULTS AND DISCUSSION

Compound (**1**), the putative copigment, appeared on chromatograms under UV light as a deep purple spot, changing to dull brown in ammonia vapor. Its UV spectrum and R_f values are given in Table 1. The spectral data were clearly those of a 7,4′-disubstituted

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[1] ASEN, S., STEWART, R. N. and NORRIS, K. H. (1972) *Phytochemistry* **11**, 1139.
[2] SAITO, N., MITSUI, S. and HAYASHI, K. (1961) *Proc. Japan Acad.* **37**, 485.
[3] ASEN, S. and JURD, L. (1967) *Phytochemistry* **6**, 577.

apigenin.[4] In addition, an IR band at 1740 cm^{-1} (Table 2) indicated that an ester group was likely to be present.

TABLE 1. R_f VALUES AND SPECTRAL PROPERTIES OF THE FLAVONOID COPIGMENT ISOLATED FROM THE CORNFLOWER BLUE PIGMENT AND ITS ACID-HYDROLYSIS PRODUCTS

Compound	R_f (×100) in†					λ_{max} (nm) in EtOH					
						Band I				Band II	
	15% HOAc	H_2O	BAW	Forestal	2-PFW	Alone	+NaOEt	+$AlCl_3$	+H_3BO_3 +NaOAc	Alone	+NaOAc
Isolated flavonoid:											
Apigenin 4′-*O*-β-D-glucoside 7-*O*-β-D-glucuronide methyl ester (**1**)	46	14	32	—	—	318	374‡	383	318	269	269
Acid hydrolysis products:											
Apigenin (**2**)	—	—	92	82	20	336	397§	383	337	269	277
Apigenin-4′-*O*-β-D-glucoside 7-*O*-β-D-glucuronide (**5**)	37	82	17	—	—	317	374‡	383	317	269	269
* Apigenin 4′-*O*-β-D-glucoside (**6**)	16	6	61	—	—	325	374‡	384	326	270	278
Apigenin 7-*O*-β-D-glucuronide (**4**)	29	38	50	—	—	333	391§	384	335	269	269
Apigenin 7-*O*-β-D-glucuronide methyl ester (**3**)	35	4	74	—	—	333	392§	383	335	269	269
Authentic:											
Apigenin	—	—	92	82	20	336	397§	383	337	269	277
Apigenin 7-*O*-β-D-glucuronide	29	38	50	—	—	333	391§	384	335	269	269
Apigenin 7-*O*-β-D-glucuronide methyl ester	35	4	74	—	—	333	392§	383	335	269	269

* Obtained from β-glucuronidase hydrolysis of **5**.
† Determined by TLC on cellulose plates in: 15% HOAc, 15% acetic acid; BAW, *n*-butanol–HOAc–H_2O (6 : 1 : 2): forestal, HCl–HOAc–H_2O (3 : 30 : 10): and 2-PFW, 2-PrOH–HCOOH–H_2O (2 : 5 : 5).
‡ Decrease in extinction.
§ Increase in extinction.

Acid hydrolysis of (**1**) yielded the series of compounds **2**–**5**. Compound **2** appeared after 1 hr of heating and increased in concentration with time. It was indistinguishable from an authentic sample of apigenin (Table 1).

TABLE 2. IR BANDS (cm^{-1}) OF GLUCURONIDES AND RELATED COMPOUNDS

Compound	Ester carbonyl		Carboxyl carbonyl		Ketone	
	KBr	DMSO*	KBr	DMSO*	KBr	DMSO*
p-Nitrophenyl *O*-β-D-glucuronide	—	—	1725	1728	—	—
Apigenin 7-*O*-β-D-glucuronide (**4**)	—	—	1728	1726	1658	1655
Apigenin 4′-*O*-β-D-glucoside 7-*O*-β-D-glucuronide (**5**)	—	—	1726	—	1650	—
Apigenin 7-*O*-β-D-glucuronide methyl ester (**3**)	1741	1743	—	—	1660	1654
Apigenin 4′-*O*-β-D-glucoside 7-*O*-β-D-glucuronide methyl ester (**1**)	1740	—	—	—	1662	—
D-glucuronic 3,6-lactone	1760†	1788†	—	—	—	—

* Dimethyl sulfoxide solution.
† γ-Lactone carbonyl.

The key substance in identifying the copigment was compound **3**. It appeared after 1 hr and then gradually diminished in concentration. UV data indicated it was a 7-substituted

[4] For comparable examples see HARBORNE, J. B. (1967) *Comparative Biochemistry of the Flavonoids*, pp. 46–49, Academic Press, New York.

apigenin, while IR data showed that an ester carbonyl band at 1741 or 1743 cm^{-1} was still present (Table 2). The NMR spectrum in deuteriopyridine contained, among other features, a 3-proton singlet at 3·63 ppm (Table 3) which could be attributed to either a methyl ester or methyl ether. Assuming it was an ester, it seemed likely that it would be a glucuronide methyl ester. In fact, both the NMR and IR spectra of **3** were found to be identical with those of an authentic sample of apigenin 7-*O*-β-D-glucuronide methyl ester. This sample had been isolated earlier from snapdragons as the free glucuronide[5] and had been esterified by passage through a polyvinylpyrrolidone column eluted with methanol containing 1 ml 2N HCl/l. **3** was unaffected by β-glucuronidase, but yielded apigenin 7-*O*-β-D-glucuronide on saponification with alkali.

TABLE 3. NMR SPECTRA OF FLAVONOID GLUCURONIDES AND RELATED COMPOUNDS [δ ppm(J_{Hz})]

Compound	Solvent/ temp.	Sugar Protons H-1	H-5	H-2,3,4	MeO	Aromatic Protons H-2',6' H-3',5'	H-6,8	H-3	Acetyls
Apigenin 7-β-glucuronide methyl ester (3)	d_5-Pyridine 100°	5·69(8)[a]	4·62(9)[b]	4·14–4·53[c]	3·63	7·79(9) 7·12(9)	6·75(2·5) 6·92(2·5)	6·70	
p-Nitrophenyl β-glucuronide methyl ester	d_5-Pyridine 100°	5·61(7·5)[a]	4·58(9)[b]	4·08–4·50[c]	3·68				
p-Nitrophenyl β-glucuronide methyl ester	d_5-Pyridine 22°	5·90(7·5)[d]	4·87(9)[b]	4·32–4·71[c]	3·72				
p-Nitrophenyl β-glucuronide (7)	d_5-Pyridine 22°	5·87(7·5)[e]	4·95(9)[b]	4·30–4·84[c]					
Apigenin 7-β-glucuronide methyl ester pentaacetate	$CDCl_3$ 22°	5·32[f]	4·30[g]	5·32[f]	3·73	7·88(9) 7·24(9)	6·98(2·5) 6·67(2·5)	6·56	2·44 2·34
Apigenin 7-β-glucuronide methyl ester pentaacetate	d_6-Acetone 22°	5·83(7·5)[b]	4·68(9·5)[b]	5·08–5·64[h]	3·67	8·08(9·5) 7·33(9·5)	7·32(2·5) 6·81(2·5)	6·65	2·07 [3]
p-Nitrophenyl β-glucuronide methyl ester triacetate	$CDCl_3$ 22°	5·34[f]	4·29[g]	5·34[f]	3·76	7·09(9) 8·21(9)			2·08 [3]
p-Nitrophenyl β-glucuronide methyl ester triacetate	d_6-Acetone 22°	5·80(7·5)[b]	4·69(9·5)[b]	5·00–5·63[i]	3·69	7·29(9) 8·24(9)			
Apigenin 4'-β-glucoside 7-β-glucuronide methyl ester octaacetate	$CDCl_3$ 22°	5·32[j]	4·24[k]	5·32[j]	3·72	7·82(9) 7·09(9)	6·98(2·5) 6·66(2·5)	6·53	2·44 2·07 [7]
Apigenin 4'-β-glucoside 7-β-glucuronide methyl ester octaacetate	d_6-Acetone 22°	5·85(7·5)[l] 5·63(7·0)[m]	4·70(9·5)[l]	5·05–5·56[l,m] 4·27	3·69	8·03(9) 7·26(9)	7·32(2·5) 6·83(2·5)	6·63	

[a]Quartet, spacing of strong outer lines 7·5–8 Hz. [b]Doublet. [c]3-Proton multiplet. [d]Poorly resolved doublet. [e]Triplet, changing to quartet at 100°. Decoupling experiments show this is coupled with a group of bands centered at 4·41 ppm. [f]Broad 4-proton singlet. [g]Triplet. [h]Nine-line, 3-proton multiplet. [i]Twelve-line, 3-proton multiplet. [j]8-Proton multiplet centered at 5·32 ppm., including H-1,2,3,4 of glucose and H-1,2,3,4 of glucuronic acid. [k]3-Proton multiplet including H-5 of glucuronic acid and H-6,6' of glucose. [l]Glucuronic acid proton(s). [m]Glucose proton(s). [n]H-6,6' of glucose.

Compound **4** appeared after 1 hr of acid hydrolysis and increased in concentration with time. It was a 7-substituted apigenin containing a carboxyl carbonyl absorbing at 1726 or 1728 cm^{-1}. The compound was hydrolyzed by β-glucuronidase but not by β-glucosidase. Spectral and chromatographic criteria proved its identity with the apigenin 7-*O*-β-D-glucuronide isolated from snapdragons.

Compound **5** appeared in the first hour of hydrolysis but then decreased in concentration until only a trace remained after 2 hr. It had the same UV spectral characteristics as the original flavonoid (**1**) but contained a carboxyl carbonyl (1726 cm^{-1}) rather than an ester carbonyl. Hydrolysis with β-glucosidase yielded **4**, while hydrolysis with β-glucuronidase yielded glucuronic acid and a new flavone (**6**). **6** was substituted only in

[5] ASEN, S., NORRIS, K. H. and STEWART, R. N. (1972) *Phytochemistry* **11**, 2739.

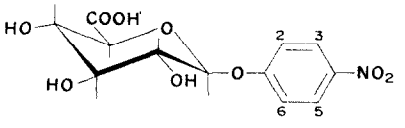
the 4′-position and was hydrolyzed by β-glucosidase to glucose and apigenin. Thus, **6** must be apigenin 4′-*O*-β-D-glucoside and **5** must be apigenin 4′-*O*-β-D-glucoside 7-*O*-β-D-glucuronide. **5** could also be obtained from **1** by alkaline saponification.

The results of acid, base and enzymic hydrolysis, together with UV and IR data, show that the isolated flavonoid (**1**) must be apigenin 4′-*O*-β-D-glucoside 7-*O*-β-D-glucuronide methyl ester. The NMR spectrum of the acetyl derivative of (**1**) confirmed that it contained an *O*-methyl group and was consistent in other respects with the assigned structure. However, comparison of the isolated flavonoid (**1**) with the copigment in its natural state (i.e. as constituted in the complex blue pigment) revealed that they were chromatographically distinct. The natural copigment was, in fact, identical with compound (**5**) (apigenin 4′-*O*-β-D-glucoside 7-*O*-β-D-glucuronide). In the isolation procedure the blue pigment had been dissolved in MeOH containing 2% HCl and had been allowed to stand 24 hr. This caused about 75% of the copigment (**5**) to be converted to its methyl ester (**1**). In non-esterifying solutions (2% HCl or 2% HCl in dioxane) the copigment remained as the free acid.

It is clear that glucuronides esterify rapidly, even at room temperature, and this must be taken into account when using esterifying media. β-Glucuronidase did not hydrolyze the glucuronide methyl esters reported here, although this might not be true for all glucuronide methyl ester substrates nor for all preparations of β-glucuronidase. Thus, failure of a glycoside to undergo hydrolysis with β-glucuronidase cannot be taken as assurance that the compound is not a glucuronide, but might indicate instead that the carboxyl group is esterified. Experiments are now under way to determine how the apigenin 4′-*O*-β-D-glucoside 7-*O*-β-D-glucuronide (**5**) functions as a copigment.

NMR spectra of aryl glucuronides

Glucuronide methyl esters and their acetyl derivatives have NMR spectral characteristics that make recognition of the basic structures relatively easy (Table 3). *p*-Nitrophenyl *O*-β-D-glucuronide (**7**), its methyl ester and methyl ester triacetate were chosen as model compounds.



(**7**)

In general, the sugar protons of *p*-nitrophenyl *O*-β-D-glucuronide and its derivatives gave spectral bands that were closely similar to those of the corresponding protons in derivatives of apigenin 7-*O*-β-D-glucuronide. In deuteriopyridine the H-1 band of the glucuronide residue was a triplet or poorly resolved doublet at 22°, changing to a well-defined quartet at 100° (*J* 7·5–8). The multiplicity of this band is attributed to virtual coupling since H-2 and H-3, which occur in the region of 4·46 ppm, are strongly coupled. The spectra of the acetylated compounds in $CDCl_3$ gave relatively little information. H-1,2,3 and 4 occur so close together (5·3 ppm) that they give the appearance of a 4-proton singlet. Similar results have been reported for the NMR

spectra of aromatic steroid glucuronides in $CDCl_3$.[6] In this solvent H-5 (4·30 ppm) gave rise to a triplet which is attributed to virtual coupling.

The most useful spectra were those of the acetylated derivatives in deuterioacetone. The doublets arising from H-1 and H-5 were well separated from a 3-proton multiplet (H-2,3,4). The chemical shifts and coupling constants of H-1 and H-5, as well as the pattern of the 3-proton multiplet, seem to be characteristic of this type of acetylated glucuronide methyl ester and should facilitate recognition of related compounds. Addition of a 4′-glucosyl residue, as in the acetyl derivative of (**1**), obviously complicates the spectrum, but the characteristic glucuronide features are still apparent.

EXPERIMENTAL

Before any hydrolysis or spectra determination isolated compounds were passed through a column of Sephadex LH20 (15 × 300 mm) with MeOH to eliminate any contamination from TLC. Except for preparative work, all chromatography was by TLC on plates containing a 250 μm layer of microcrystalline cellulose (Avicel). Solvents for sugar determinations were EtOAc–pyridine–H_2O (2 : 1 : 2) and *n*-BuOH–HOAc–H_2O (6 : 1 : 2). UV spectra were determined in EtOH using diagnostic reagents previously described.[7]

Isolation of blue pigment. Fresh flowers were dehydrated with acetone, filtered, air-dried, and then ground to pass a 40-mesh screen. The ground tissue was extracted with cold H_2O and the blue pigment crystallized by the method of Hayashi *et al.*[8]

Isolation of flavonoid (**1**). The crystalline blue pigment was dissolved in 2% HCl–MeOH and allowed to stand at room temp. for *ca* 24 hr. The precipitate that formed was filtered and the flavonoid was isolated by TLC on 20 × 20 cm plates containing a 2 mm layer of Avicel. The solvents used were C_6H_6–HOAc–H_2O (6 : 7 : 0·9) and *n*-BuOH–HOAc–H_2O (6 : 1 : 2). Apigenin 4′-*O*-β-D-glucoside 7-*O*-β-D-glucuronide methyl ester was removed from the cellulose with hot MeOH. On heating at 100° in Ac_2O pyridine it gave an *octaacetate*, crystallizing in clusters of fine needles from methanol, m.p. 229–231°.

Acid hydrolysis. Flavonoid (**1**) was acid hydrolyzed by refluxing in 2 N HCl–MeOH (1 : 1) for 4 hr. Samples were removed hourly and the intermediate compounds that were formed were isolated by TLC using n-BuOH–HOAc–H_2O (6 : 1 : 2).

Base hydrolysis. The compounds (8 mg in 4 ml 0·5 N NaOH) were warmed on a steam bath for 15 min. The soln was cooled, acidified and taken to dryness under reduced pressure at 40°. The free acid was dissolved in MeOH, the insoluble salt was filtered off and the soln was then passed through a column of Sephadex LH20 with MeOH.

Enzymic hydrolysis. The compounds (8 mg in 10 ml water) were kept for 17 hr at 37° with an equal weight of β-glucosidase or β-glucuronidase. The hydrolysate was thoroughly extracted with EtOAc and the sugar moiety in the aqueous residue was examined by methods previously described.[9]

Authentic compounds. Apigenin 7-*O*-β-D-glucuronide was isolated from Yellow Rocket snapdragons.[10] The methyl ester was formed by passing the free acid through a column (25 × 400 mm) of purified insoluble polyvinylpyrrolidone with MeOH containing 1 ml of 2 N HCl/l. *Apigenin 7-O-β-D-glucuronide methyl ester pentaacetate* was obtained by heating the compound in acetic anhydride–pyridine at 100°. *p*-Nitrophenyl *O*-β-D-glucuronide was esterified by allowing it to stand overnight in MeOH containing a trace of anhyd. HCl. The product was isolated by evaporating the solvent at room temp. under vacuum and was then acetylated in the usual way.

NMR spectra of aryl glucuronides. All measurements were made at a field frequency of 100 MHz. Spectra of non-acetylated compounds were determined in deuteriopyridine at 22° and 100°; those of the acetylated compounds were determined in deuterio-$CHCl_3$ and deuterioacetone at 22°.

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