Phytochemistry, 1966, Vol. 5, pp. 439 to 455. Pergamon Press Ltd. Printed in England

THE GLYCOFLAVONOID PIGMENTS OF *VITEX LUCENS* WOOD*

MARGARET K. SEIKEL, JULIANA H. S. CHOW and LINDA FELDMAN

Wellesley College, Wellesley, Mass., and Forest Products Laboratory,† Forest Service, U.S. Department of Agriculture, Madison, Wis. 53705‡

(*Received* 29 *September* 1965)

Abstract—Wood of *Vitex lucens* is a rich source of glycoflavonoid (*C*-glycosylflavonoid) compounds. In addition to the previously described apigenin derivatives vitexin (4′,5,7-trihydroxy-8-*C*-glucopyranosylflavone) and isovitexin (the 6-*C*-glucosyl isomer), the corresponding luteolin derivatives orientin and isoorientin have been discovered. Compounds of vitexin and orientin, which have xylose attached to the 8-glucosyl group, are also present. The most unusual constituents are eight compounds which appear to be 6,8-di-*C*-glycosyl derivatives of apigenin and luteolin. Several compounds in each series are interconvertible in hot acidic solution.

INTRODUCTION

THE original source of the flavone vitexin, which might be called the classical glycoflavonoid or *C*-glycosylflavonoid, was the New Zealand wood *V. lucens* T. Kirk (formerly *V. littoralis* A. Cunn).[1] Its isomer,[2] variously known as homovitexin,[1] saponaretin,[3] and isovitexin[2,4] (a single compound[5] with poorly defined properties), also occurs in the wood, as shown by paper chromatographic studies.[6,7] Such studies revealed that the wood also contained many other easily extractable flavonoid compounds[7,8] (Fig. 1). The predominant flavonoid compound was identified as a vitexin glycoside.[7] Although it was originally reported[7] as the 4′-rhamnoside,[9,10] new work has shown that it is a *C*-(*O*-xylosyl)-glucosylapigenin.

Continued work on the flavonoids centered first on the identification of the substances responsible for spots 13, 14, and 15 shown in Fig. 1. These were originally labeled the A–B group[7,11] and were of particular interest because of the orange–yellow color they assumed in u.v. light when the chromatograms were fumed with ammonia. This colour contrasted with the yellow–brown color of the other apigenin-based glycoflavonoids in the extracts and resembled that of lutonarin, the luteolin glycoflavonoid of barley.[12] Later the work was

* This work was supported in part by Research Grant G-9338 to Wellesley College from the National Science Foundation for which grateful acknowledgement is made.

† Maintained at Madison, Wis., in cooperation with the University of Wisconsin.

‡ Address to which inquiries should be addressed.

[1] A. G. PERKIN, *J. Chem. Soc.* **73**, 1019 (1898).
[2] R. M. HOROWITZ and B. GENTILI, *Chem. & Ind. (London)* 498 (1964).
[3] G. BARGER, *J. Chem. Soc.* **89**, 1210 (1906).
[4] L. H. BRIGGS and R. C. CAMBIE, *Tetrahedron* **3**, 269 (1958).
[5] R. C. CAMBIE, *Chem. & Ind. (London)* 87 (1959).
[6] R. C. CAMBIE, *New Zealand J. Sci.* **2**, 230 (1959).
[7] M. K. SEIKEL, D. J. HOLDER and R. BIRZGALIS, *Arch. Biochem. Biophys.* **85**, 272 (1959).
[8] R. C. CAMBIE, Personal communication.
[9] U. FIEDLER, *Arzneimittel-Forsch.* **5**, 609 (1955); *Chem. Abstr.* **50**, 2761 (1956).
[10] T. A. GEISSMAN and U. KRANEN-FIEDLER, *Naturwissenschaften* **43**, 226 (1956).
[11] M. K. SEIKEL, *Proceedings of a Symposium of the Plant Phenolics Group of North America*, p. 31. University of Toronto, Toronto, Canada (1963).
[12] M. K. SEIKEL and A. J. BUSHNELL, *J. Org. Chem.* **24**, 1995 (1959).

expanded to include preliminary characterization of many other minor glycoflavonoids in *V. lucens*, since material was available from larger scale isolation work.

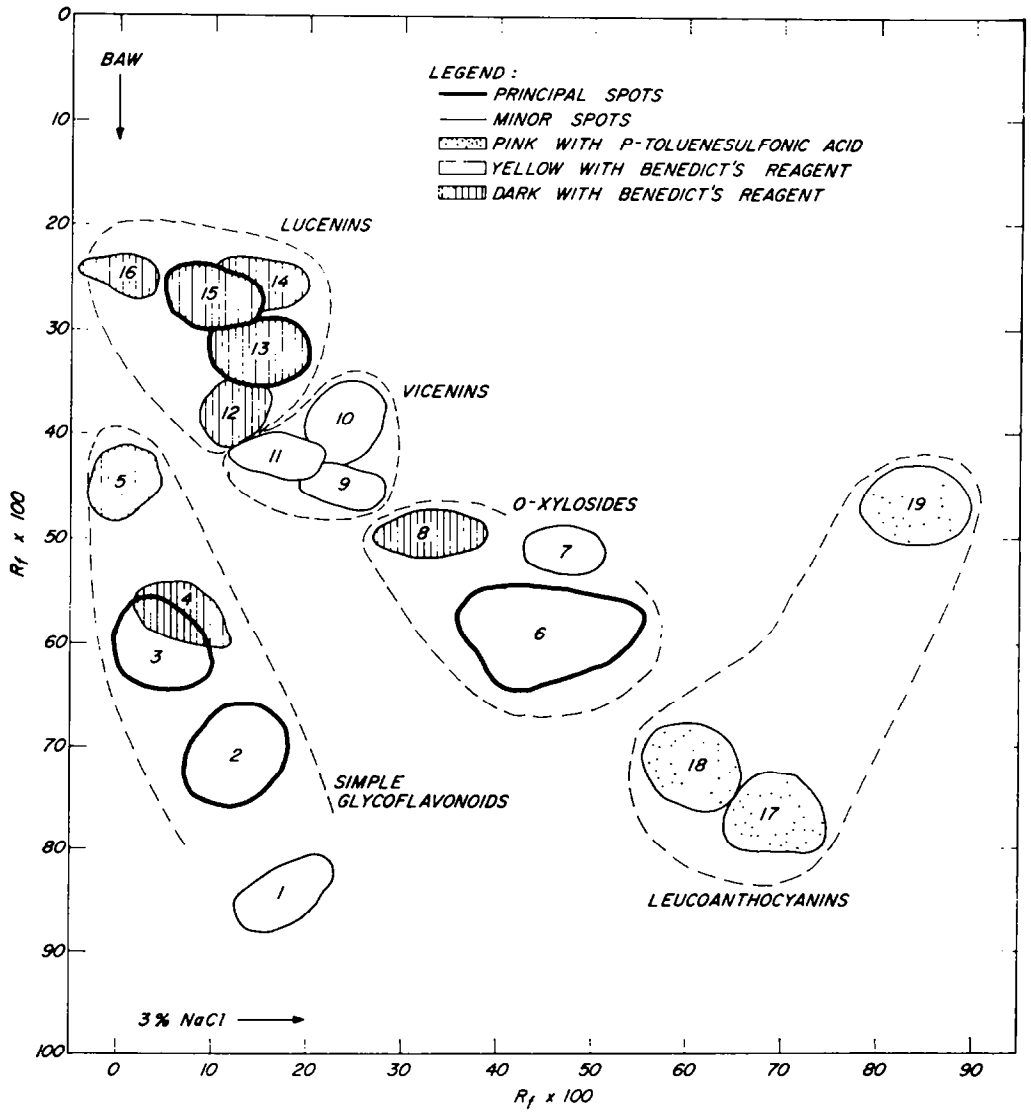


FIG. 1. CHROMATOGRAM OF FLAVONOID EXTRACTIVES OF *Vitex lucens* WOOD

RESULTS*

Chromatographic Mapping

As shown in Fig. 1, a total of nineteen flavonoid spots have now been distinguished on two-dimensional chromatograms of methanolic extracts of *V. lucens* wood in contrast with the nine previously noted.[7] The use of a richer sample of wood, of Benedict's spray, and of

* For abbreviations of developing solvents, see Experimental (p. 451).

the leucoanthocyanin spray accounts in part for this increase. Several are in low concentration. The chromatographic properties of all are listed in Table 1. Of these, at least fourteen appear to be glycoflavonoid in nature.

TABLE 1. PAPER CHROMATOGRAPHIC DATA ON FLAVONOID CONSTITUENTS OF *Vitex lucens*

Spot No.[a]	Identity	$R_f \times 100$ BAW/wNaCl[b]	Color in u.v. + NH_3[c]
1	—	87/27	Pale br
2	Isovitexin	72/19	Br
3	Vitexin	62/10	Y-br
4	Isoorientin (lutonaretin)	60/12	Light y-o
5	Orientin (lutexin)	46/5	Y
6	Vitexin-*O*-xyloside	63/46	Y-br
7	—	56/53	Pale y-br
8	Orientin-*O*-xyloside	55/40	O-y
9	Vicenin-3	50/29	Light br-y
10	Vicenin-2	45/30	Light br-y
11	Vicenin-1	46/23	Light br-y
12	?Lucenin-5	44/17	O-y
13	Lucenin-3	38/21	Deep o-y
14	Lucenin-2	34/22	O-y
15	Lucenin-1	34/16	Deep o-y
16[d]	Lucenin-4	31/6	O-y
17	(Leucoanthocyanin)	80/76	Colorless[e]
18	(Leucoanthocyanin)	77/61	Colorless[e]
19	(Leucoanthocyanin)	55/90	Colorless[e]

[a] See Fig. 1.
[b] See "Experimental" for abbreviations.
[c] br = brown; o, orange; y, yellow. For colors with Benedict's reagent, see Fig. 1.
[d] Not noted in sapwood extracts, perhaps because of low concentrations.
[e] Visualized as pink spots by spraying with *p*-toluenesulfonic acid and heating.

Methods of Separation and Isolation

For chromatographic, spectral, and hydrolytic studies of the close-lying lucenin group, chromatographically pure solutions of lucenins-1, -2, and -3 were obtained from crude methanolic extracts by the usual methods of preparative chromatography. When the unusual nature of these compounds had been established, methods for obtaining solid samples were investigated. Unsuccessful attempts included: (*a*) use of nylon (polyamide) columns[12] from which they eluted so slowly that almost complete decomposition had occurred; (*b*) use of Sephadex columns; (*c*) thin-layer chromatography on nylon layers which did not fractionate the individual compounds, only groups of compounds; and (*d*) countercurrent distribution[13] which gave a similar result to (*c*). Only methods employing paper were sufficiently selective to separate these closely related compounds. Cellulose columns, also run with crude extracts, separated the lucenins fairly well from other flavonoids and partially from each other, but final fractionation required preparative paper chromatography. Although a few crystals of lucenin-1 were obtained by this method, they were badly contaminated with dark-colored

[13] M. K. SEIKEL, A. J. BUSHNELL and R. BIRZGALIS, *Arch. Biochem. Biophys.* **99**, 451 (1962).

material. This approach was therefore abandoned because precautions for preventing oxidation on the paper sheets, which often led to complete loss of the compounds, were too cumbersome.

The most successful fractionation of the many closely related compounds was achieved by use of the rolled paper column (ChroMax) developed with the same reagents used for paper chromatography. This method was applied to a concentrated solution of the extractives, enriched in respect to the glycoluteolins by means of a lead acetate precipitation. With 1-butanol–acetic acid–water (BAW), it was possible to elute fairly well-separated fractions, particularly of the slower running lucenin group. The eluates were freeze dried to prevent the decomposition which resulted when the solvents were removed *in vacuo* at higher temperature. Further separation was achieved by using a second column developed and eluted with 5% acetic acid (A5); the eluates were similarly freeze dried. For further purification of certain compounds (for example, lucenin-3), it was necessary to repeat the fractionation with BAW. For final separation of the very close lying spots 9, 10, and 11 (the vicenins, which were far more stable than the lucenins), subsequent preparative paper chromatography had to be employed.

Compounds Isolated and their Characterization

Lucenins in solution. The chromatographically pure solutions of lucenins-1, -2, and -3 (spots 15, 14, and 13) mentioned above gave identical u.v. spectra and spectral shifts (see Table 3). These spectra were practically the same as those for orientin (lutexin), isoorientin (lutonaretin), and luteolin,[12] thus indicating a 3′,4′,5,7-tetrahydroxyflavone nucleus. The shifts with aluminum chloride, aluminum chloride–sodium acetate, boric acid–sodium acetate, and sodium acetate (Band II) indicated that the hydroxyl groups in positions 5-, 7- and 3′,4′-, were all free.[14, 15] The dark color with Benedict's spray also showed that the compounds contained an ortho dihydroxy grouping. By paper chromatography the three compounds were shown to be different from the known glycoluteolins orientin, isoorientin, and the glycoside lutonarin.[12] Their characteristic reactions to hot acid are discussed in a later section.

Lucenin-1 (*spot* 15). By the ChroMax column isolation method followed by crystallization from water and removal of a persistent iron contaminant, lucenin-1 was obtained as yellow crystals with an indefinite melting point. From 180 g of *V. lucens* heartwood, approximately 0·3 g of material pure enough to crystallize was isolated, but only 25 per cent of this was available after purification. It could not be recovered successfully from its trimethylsilyl ether used for the NMR studies. Molecular weight determination and elementary analysis showed that the molecule was larger and had a percentage of carbon lower than calculated for a monoglycosylluteolin, but they were in agreement with values calculated for a dihexosylluteolin. Alkaline degradation yielded 3,4-dihydroxyacetophenone and a trace of phloroglucinol, significantly less in proportion than was previously obtained from vitexin, lutonarin, and lutonarin 3′-methyl ether.[13] Attempted hydrolysis (reported in detail in a later section) produced no hydrolyzable sugar. NMR studies* of the trimethylsilyl ethers showed chemical shifts at: τ 2·27, poorly resolved quartet ($J_{meta}=2$ c/s, $J_{ortho}=8$ c/s); τ 2·78, doublet ($J=2$ c/s); and τ 3·10, doublet ($J=8$). These were interpreted to indicate

* Work of T. J. Mabry.

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

[15] L. JURD and T. A. GEISSMAN, *J. Org. Chem.* **21**, 1395 (1956).

protons at positions 6', 2', and 5', respectively, of the flavonoid B ring.[16] After gentle silylation, a signal at τ −3·5 was assigned to the 5-hydroxyl proton; this disappeared on complete silylation. Simultaneously, a singlet at τ 3·57, representing one proton, almost disappeared and a new singlet was found at τ 3·72, also integrating for one proton; this was assigned to the C-3 proton.[16-18] Thus lucenin-1 has no protons attached to the flavonoid A ring. Signals between τ 5 and 6·9 represent the sugar moiety and integrate for about fifteen protons.

Lucenin-2 (*spot* 14). This minor constituent was studied only in solution, as no chromatographically pure fractions of it could be isolated from the ChroMax column.

Lucenin-3 (*spot* 13). Although the concentration of this lucenin in the crude extracts appeared equivalent to that of lucenin-1, isolation of a significant amount of pure crystalline material has proved to be impossible. Failure is attributable both to (1) poor separation from preceding and succeeding fractions on the ChroMax column developed by BAW, and similar R_f values in acetic acid to components of both these fractions; and (2) difficulty of crystallization, including great solubility in water, gel formation, and months required for crystals to form. These properties are reminiscent of the behavior of isovitexin.

Lucenin-4 (*spot* 16). Small amounts of the minor constituent lucenin-4 were isolated from the ChroMax columns, but no attempts were made to crystallize the amorphous solid. It was characterized as a "lucenin" by its spectral characteristics (Table 2) and its reaction to Benedict's spray.

Vicenins-1, -2, *and* -3 (*spots* 9–11). A few crystals of each of the three vicenins were isolated by preparative paper chromatography. The ease with which they were obtained in a crystalline state and the lack of iron contamination contrasted markedly with difficulties which had delayed the completion of the lucenin work for years. The three gave identical u.v. spectra (see Table 2). These absorption characteristics were identical with those of vitexin and isovitexin[22] and showed that they too were probably apigenin derivatives with all the phenolic hydroxyl groups free. These are very minor components of the extracts, so too little material was available for analytical work.

Orientin (*spot* 5). A solution containing this substance (which gives a distinctively yellow spot in u.v. light when fumed with ammonia) was isolated from a ChroMax column fraction and was shown by its spectra (Table 2) to be a luteolin derivative. It was identified by cochromatography with samples of authentic orientin[19,20] and lutexin from barley[12] in three solvents (BAW, A15, and EAW).

Isoorientin (*spot* 4). Amorphous samples were obtained from the ChroMax column work, and it was identified by cochromatography in the above three solvents, as well as in AtW and aNaCl. The authentic sample used was obtained from barley.[12,19-21]

Orientin-xyloside (*spot* 8). A solution of this minor constituent was obtained from the macro-isolation work. Its spectra were identical with those of the other glycoluteolins (Table 2). After hydrolysis (see later) the products were identified as orientin (by spectra and by cochromatography in four solvents) and xylose (by TLC).

Vitexin-xyloside (*spot* 6). When the sugar was isolated from the hydrolysis mixtures obtained from the vitexin glycoside responsible for spot 6,[7] it was shown by cochromato-

[16] M. K. SEIKEL and T. J. MABRY, *Tetrahedron Letters* No. 16, 1105 (1965).
[17] T. J. MABRY, J. KAGAN and H. RÖSLER, *Phytochem.* **4**, 177 (1965).
[18] T. J. MABRY, J. KAGAN and H. RÖSLER, *Nuclear Magnetic Resonance Analysis of Flavonoids*. University of Texas Publ. No. 6418 (1964).
[19] L. HÖRHAMMER, H. WAGNER and F. GLOGGENGIESSER, *Arch. Pharm.* **291/63**, 126 (1958).
[20] L. HÖRHAMMER, H. WAGNER and H. S. DHINGRA, *Arch. Pharm.* **292/64**, 83 (1959).
[21] B. H. KOEPPEN, C. J. B. SMIT and D. G. ROUX, *Biochem. J.* **83**, 507 (1962).

graphy on paper to be xylose; this finding contradicts the earlier identification of the glycoside as a rhamnoside based on cochromatography with a known glycoside.[7] Crystalline samples of this xyloside, the major constituent, were later obtained from a Craig distribution separation, and its identification was confirmed by analysis and by a study of its hydrolysis products.

TABLE 2. ULTRAVIOLET ABSORPTION SPECTRAL DATA

Substance	Band I, λ_{max} in mμ					Band II	Material[a] used
	95% ethanol	+$AlCl_3$	+$AlCl_3$ +NaOAc	+H_3BO_3 +NaOAc	+NaOEt	+NaOAc	
Lucenin-1	352[b]	392[c]	410[c]	383[c]		271[c]	Crystals
	353	392	422	385	415	282	PC eluate
Lucenin-2	352	390	415	385		281	PC eluate
Lucenin-3	354	390	422	383	414[d]	282	PC eluate
Lucenin-4	351	386	414	380	415	281	ChroC residue
Lucenin-5	353	387 sh.	421				[d]
[Other bands for all lucenins[e]	272	360	278	265		*f*	]
	261 ± 1	300 sh.					
		280					
Orientin (lutexin)[g]	351	388	414	371	409 dec.	276	PC eluate
Isoorientin (lutonaretin)[g]	350	386	414	377	415 dec.	279	ChroC residue
Orientin xyloside[g]	348	386	413	374	408 dec.	279	ChroC residue
Vicenin-1	336	381	No shift		404	282	PC eluate
Vicenin-2	334	380	No shift		402	282	PC eluate
Vicenin-3	335	380	No shift		402	282	PC eluate
[Other bands for all vicenins[e]	273	342	No shift		333	*f*	]
		304			283		
		280					
Isovitexin[h]	337				401	279	Crystals

[a] Abbreviations: sh. = shoulder; PC = paper chromatography; ChroC = ChroMax column.
[b] Log ε = 4·34; Band II a and b, 272 (4·27) and 260 (4·25).
[c] In methanol–water (1:1).
[d] By PC from a hydrolysis solution.
[e] Values given by 4 compounds (lucenins), 2–3 compounds (vicenins); all values within 2 mμ of wavelength given.
[f] Band I is very sensitive to slight changes of pH.
[g] Other bands closely similar to bands of lucenins.
[h] Log ε = 4·30; Band II, 272 (4·30).

Isovitexin (saponaretin) and vitexin. Samples of these classical glycoflavonoids were obtained, as byproducts of the macro-isolation work, by means of a basic lead acetate precipitation. The usual difficulties were encountered in attempting to crystallize the very soluble isovitexin.[22, 5]

Effect of Hydrolytic Conditions

Application of mild hydrolytic conditions for extended periods of time (refluxing with 1 N hydrochloric acid in 50% methanol) to the newly discovered glycoflavonoids allowed a detailed study to be made of the courses of the reactions. The compounds fell into three classes.

[22] M. K. SEIKEL and T. A. GEISSMAN, *Arch. Biochem. Biophys.* 71, 17 (1957).

Orientin- and vitexin-xyloside. Both xylosides underwent complete hydrolysis to the corresponding aglycons and xylose in 1–2 hr. They are thus simple *O*-xylosides of the glycoflavones, in this case xylosides of the *C*-glycoside groups (see "Discussion").

Lucenin-2 and vicenin-2. These two compounds are apparently quite stable to hydrolytic

TABLE 3. RESULTS OF ACID TREATMENT OF THE LUCENINS

Spot appearing	$R_f \times 100$		Time of appearance from			
	BAW	aNaCl	Lucenin-1	Lucenin-2	Lucenin-3	Lucenin-4
Major spots						
Lucenin-1	32	15	Original	—	3–6 hr	30 min
Lucenin-2[a]			—	Original	—	—
Lucenin-3	35	20	1 hr	—	Original	6 hr
Minor spots						
Lucenin-4	30	4	20–30 min	—	3–6 hr	Original
(Lucenin-5)[b]	43	16	12 hr	—	15 min	12 hr
(Lucenin-6)[c]		6	—	—	—	30 min
Degradation a[d]	22	1	12–30 hr		11–24 hr	12 hr
Degradation b	68	7	24 hr		29–46 hr	29–46 hr

[a] Acid treatment of lucenin-2 was continued for only 5 hr in preliminary work.
[b] Identity of spot with lucenin-5 in original extract assumed from position on two-dimensional chromatogram.
[c] Trace spot formed initially from lucenin-4, disappearing within 12 hr.
[d] Distinctively yellow with ammonia in u.v., resembling orientin.

TABLE 4. RESULTS OF ACID TREATMENT OF THE VICENINS

Spot appearing	$R_f \times 100$		Time of appearance from		
	BAW	aNaCl	Vicenin-1	Vicenin-2	Vicenin-3
Major spots					
Vicenin-1	44	25	Original	—	4 hr
Vicenin-2	42	32	—	Original	—
Vicenin-3	47	29	3 hr	—	Original
Minor spots					
(Vicenin-4)[a]	43	8	10 min	—	30 min
(Vicenin-5)[a]	53	23	3–6 hr	—	10 min
Degradation c[b]	38	3	6–22 hr	5 hr	4 hr

[a] Minor spots never seen with crude extracts (unlike lucenins-4 and -5).
[b] Distinctively yellow-green in u.v. after (a) $AlCl_3$ spray and (b) fuming with ammonia in addition.

conditions. Lucenin-2 showed no changes in 5 hr (Table 3). Vicenin-2, subjected to longer heating, showed slow accumulation of a compound which gave a characteristic color test on paper (see Table 4) and which is classed as a degradation product.

Lucenins-1, -3, and -4 and vicenins-1 and -3. These five compounds produced sooner or later a considerable proliferation of spots when the reactions were followed by paper chromatography (see Tables 3 and 4). It was demonstrated that within each group the compounds

were eventually converted into an equilibrium mixture which contained each of the compounds in the group plus some additional minor components. In each case new spots, specific for each individual compound, appeared on the chromatograms within a few minutes after refluxing commenced. It then generally required an hour or more before the major components of the equilibrium mixtures (vicenins-1 or -3 and lucenins-1 or -3) began to build up. After 2 days, the mixtures gave identical two-dimensional chromatograms. To substantiate the identity of spots in the equilibrium mixture, five products from the reaction of acid with lucenin-1 and three from vicenin-1 were isolated in solution. In each family, the spectra of the products were identical and their R_f values coincident with those of characterized lucenins and vicenins.

In both families, in addition to the spots which appeared to be connected with the equilibria, a second series of spots running slowly in the aNaCl began to build up in 6–24 hr. The concentration of these increased continually; they were therefore considered to be degradation products. It should be noted, however, that after 5 days of heating, the reaction mixture from lucenin-1 still contained large amounts of the original compound plus lucenin-3 and minor equilibrated lucenins in addition to the degradation products. One of these showed the color reactions of orientin with ammonia in u.v. light, but cochromatography proved them to be different. The most prominent degradation product from vicenins-1 and -3 gave the same color reaction and R_f values as the spot from vicenin-2.

Effect of Boric Acid on Paper Chromatography of the Glycoflavonoids

In an attempt to distinguish between the glycosyl side chains, all the glycoflavonoids available were chromatographed in the presence of free boric acid by a method previously used on sugars.[39] Results of the preliminary study are shown in Table 5; variations in the

TABLE 5. RETARDATION OF GLYCOFLAVONOIDS BY BORIC ACID ON PAPER

Compound	Ratio 1[a]	Ratio 2	Ratio 3	Compound	Ratio 1	Ratio 2	Ratio 3
Apigenin	100			Luteolin	78		
Vitexin	77	100		Orientin (lutexin)	41	58	100
Isovitexin (saponaretin)	79			Isoorientin (lutonaretin)	44		110
Violanthin[b]	75	99		Lucenin-1			65
Vicenin-1	67	98		Lucenin-2			62
Vicenin-2	85	105		Lucenin-3			62
Vicenin-3		101		Lucenin-4			68
Vitexin-4′-rhamoside		92		Lucenin-5 (?)			66
Vitexin-xyloside		97		Lutonarin (isoorientin-7-glucoside)		48	85
Saponarin (isovitexin-7-glucoside)		100	207	Orientin-xyloside			85
Scoparin (3-*O*-methyl-orientin)		100	214				
3′-*O*-methyllutonarin		100	204				

[a] Ratio 1 $= \frac{R_f \text{ in BBW}}{R_f \text{ in BW}} \times 100$; Ratio 2 $= \frac{R_{\text{vitexin}} \text{ in BBW}}{R_{\text{vitexin}} \text{ in BW}} \times 100$; Ratio 3 $= \frac{R_{\text{orientin}} \text{ in BBW}}{R_{\text{orientin}} \text{ in BW}} \times 100$

[b] From H. Wagner.

ratios within ±10 per cent are not considered significant. A reduction in R_f value of the spot resulted from the presence of (*a*) a 3′,4′-dihydroxyl grouping (compare Jurd's similar results with boric acid–sodium acetate[42]), (*b*) one *C*-glycosyl group, (*c*) a second *C*-glycosyl

group in the 3',4'-dihydroxy compounds, and (*d*) an *O*-glycoside group in the 3',4'-dihydroxy compounds although seemingly to a lesser extent. The lack of significant effect by a second glycosyl residue in the 4'-hydroxy compounds seemed to be real and consistent, occurring even with the compounds of known structure.

DISCUSSION

An array of glycoflavonoid (*C*-glycosylflavonoid) compounds, has been discovered in the extractives of *V. lucens* wood. In addition to the three previously reported, eleven new ones have now been characterized. Except for some trace constituents, all the flavonoids studied appear to possess the C–C glycosyl link. While a similar homogeneity also has been observed in barley,[12, 13, 22] glycoflavonoids have been more often accompanied by simple flavonoids (i.e., in wheat,[23] roobois tea,[21] and eucalypt kinos[24]). Simple flavonoids as well as glycoflavonoids occur in other *Vitex* species: orientin, isoorientin, and luteolin-7-glycoside in the leaves but not in the wood of *V. megapotamica*;[25] casticin in the leaves of *V. agnus-castus*, *V. negundo*, and *V. trifolia* (but not *V. lucens*).[25, 26] Vitexin alone was reported from V. *peduncularis*.[27, 28]

Only two fundamental flavonoid nuclei are present in the entire array, namely of the apigenin (4',5,7-trihydroxy) and luteolin (3',4',5,7-tetrahydroxy) hydroxylation patterns. This is strikingly seen by the complete similarity of the spectra, including all the spectral shifts, of all members of each group (Table 2). Even the *O*-glycosides show the same shifts, indicating that all the phenolic hydroxyls are free.

The array, however, is divided into three distinct groups with parallel members in the apigenin and luteolin families. First are the simple glycoflavonoids of already known structure. These include the classical glycoapigenins, vitexin and isovitexin (saponaretin),[1, 4, 5] and the glycoluteolins, orientin[19, 20] (lutexin)[12] and isoorientin[19, 20] (homoorientin, lutonaretin).[12] The two latter compounds have recently been found in *V. megapotamica* leaves.[25]

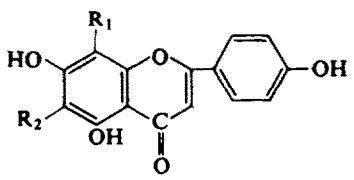
The second group comprises the *O*-xylosides of vitexin and orientin. Orientin xyloside must have the xylose residue attached to the glucosyl side chain, since spectral shifts showed that all the phenolic hydroxyl groups were free. Vitexin xyloside was previously reported to be a 4'-rhamnoside.[7] Reinterpretation of the large base shift (64 mμ)[7] suggests that the 4'-hydroxyl group as well as the 5- and 7-hydroxyl groups are free.[14] Comparison with the orientin derivative supports a side-chain *O*-glycosidic bond for the xylose. It has similar properties to the vitexin-*O*-xyloside which Horowitz isolated from citrus.[2, 29] Several other glycosides of glycoflavones of this type have been previously reported, such as adonivernith, the xyloside of isoorientin,[30] and the rhamnosides of glycoflavones in wheat and oats.[23]

The third and most interesting group is a new type of glycoflavonoid which appears to be di-*C*-glycosyl derivatives of apigenin and luteolin. They have been named vicenins* (I) and lucenins (II) The properties of the several members of each family are closely similar; they can only be separated by paper chromatography. In the extracts, the concentrations of

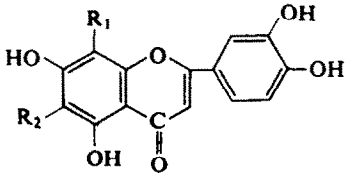
* In collaboration with J. W. McClure, University of Texas.

[23] J. B. HARBORNE and E. HALL, *Phytochem.* **3**, 421 (1964).
[24] W. E. HILLIS and A. CARLE, *Australian J. Chem.* **16**, 147 (1963).
[25] R. HÄNSEL, CH. LEUCKERT, H. RIMPLER and K. D. SCHAAF, *Phytochem.* **4**, 19 (1965).
[26] I. BELIČ, J. BERGANT-DOLAR and R. A. MORTON, *J. Chem. Soc.* 2523 (1961).
[27] V. N. SHARMA, *J. Sci. Ind. Res.* (*India*), **14B** 267 (1955).
[28] C. B. RAO and V. VENKATESWARLU, *J. Sci. Ind. Res.* (*India*) **21B**, 313 (1962).
[29] R. M. HOROWITZ, Private communication.
[30] L. HÖRHAMMER, H. WAGNER and W. LEEB, *Arch. Pharm.* **293/65**, 264 (1960).

the supposed di-*C*-glycosides of luteolin are greater than those of the apigenin derivatives, while with the mono-*C*-glycosyl derivatives the reverse is the case.



(I) Vicenins



(II) Lucenins

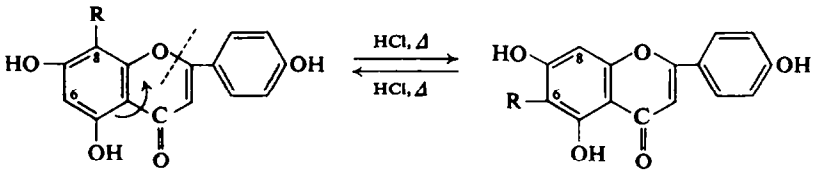
Evidence for the proposed di-*C*-glycosyl structure includes chromatographic, spectral, and hydrolytic data on seven of the compounds and a more detailed analytical study of lucenin-1 which has been briefly reported earlier.[16] The relative positions of the lucenins and vicenins on the two-dimensional chromatogram (Fig. 1) in comparison with the simple glycoflavonoids indicated a greater degree of hydroxylation. Spectral data showed that this was due neither to more phenolic hydroxyl groups nor to *O*-glycosides being present. Hydrolysis yielded no sugar released from lucenin-1, and the compounds were thus not *C*-glycosyl-*O*-glycosides. That more than one hexosyl side chain or a longer glycosyl one was present was substantiated by elution of the lucenins from Sephadex simultaneously with vitexin-xyloside. Their reluctance to crystallize was also suggestive of their having a large carbohydrate moiety. The greater retardation of the lucenins in the presence of boric acid on paper when compared with the mono-*C*-glycosylluteolins suggested the presence of another complexing group in the molecule.

Lucenin-1, a luteolin derivative, was the only compound isolated in crystalline form in sufficient amounts for analytical work. NMR work showed that this possessed a completely substituted flavonoid A-ring in contrast to vitexin and isovitexin whose spectra clearly show the presence of a 6- or an 8-proton respectively. Therefore, the compound must have two side chains and not one. Complete substitution of the A-ring would also account for the very low yield of phloroglucinol obtained on alkaline degradation in comparison with other *C*-glycosides. That the side chains are both glycosyl in nature is suggested by the analytical data and the large number of protons in the sugar portion of the NMR spectrum. Other than the fact that the analytical data checks best for hexopyranosyl or hexofuranosyl groups, the nature of the glycosyl groups R_1 and R_2 are unknown. Speculations from the hydrolysis results discussed below suggest that R_1 is not the same as R_2.

That the other lucenins and the vicenins are also di-*C*-glycosyl derivatives is suggested either by interconvertibility or by similarity in properties. The other major lucenin, lucenin-3, as well as the minor lucenin-4 and probably lucenin-5, are interconvertible in hot acid solution with lucenin-1. The vicenin group forms a smaller series of compounds, but it is parallel in all respects; vicenins-1 and -3 are interconvertible. Thus they are probably the analogous apigenin derivatives. In each family one compound does not interconvert, namely lucenin-2 and vicenin-2; outside of general chromatographic and spectral similarity, no direct evidence is available to support a di-*C*-glycosyl structure for these two. The fact that the vicenins have similar mobilities to vitexin and isovitexin in the presence of boric acid on paper cannot be taken as definite evidence against their di-*C*-glycosyl nature since violanthin, which has been shown to be of this type,[32] and saponarin, an *O*-glucoside, have similar mobilities.

The reactions of the vicenins and lucenins in hot hydrochloric acid is characteristic and revealing. It is well-substantiated now that simple glycoflavonoids exist in interconvertible

pairs in this medium: vitexin and isovitexin,[22, 31] orientin and isoorientin,[12, 31, 38] the 3'-methyl ethers of the latter pair,[13] and hemophloin and isohemophloin.[40] The latest NMR work suggests that this is due to 8-glycosyl–6-glycosyl isomerization taking place via pyran ring opening in the acid solution. (III to IV)[18, 29, 40] If both positions 6 and 8 bear glycosyl



(III) (IV)

substituents, one would expect either (1) no overall effect when treated with acid if $R_1 = R_2$ in formulae I and II or (2) two closely related compounds if $R_1 \neq R_2$. If the results obtained with the lucenins and vicenins are considered, the possibility of lucenin-2 and vicenin-2 being di-*C*-glycosyl compounds with $R_1 = R_2$ is immediately apparent. These will be called type A di-*C*-glycosylflavonoids. It is likely in the present case that R is the same as in vitexin and orientin (i.e. glucose). On the other hand if only the major spots on the chromatograms of the equilibrium mixtures are considered, lucenins-1 and -3 and vicenins-1 and -3 appear to have $R_1 \neq R_2$ and will be called type B. The multiplicity of minor spots which appear during hydrolysis and are still present at equilibrium remains to be explained. Since no such multiplicity occurs with the glucopyranosyl chain present in vitexin, it is suggested that the type B compounds of *V. lucens* possess one side chain of this or similar structure, but that the second one is a more reactive sugar residue (i.e. is itself an equilibrium of two or more forms in the hot acid solution). A variety of sugars (e.g. apiose derivatives, desoxy sugar residues, and residues with furan rings, etc.) are unstable in acid or react with methanol in acidic solution. Note that boric acid complexing failed to show any difference between type A and B compounds.

That di-*C*-glycosylflavonoid of both types A and B are widely occurring is at once evident from a study of the literature. Hörhammer and Wagner and their coworkers have simultaneously worked out the structure of a di-*C*-glycosylapigenin violanthin, isolated from *Viola tricolor* roots.[32] No phloroglucinol was detected when this compound was fused with alkali, and it was unchanged by hot acid and hence belongs to type A, but it is chromatographically distinct from vicenin-2 and the other vicenins. Other evidence suggested that the side chains are different, one possibly being rhamnosyl. In their crude extract,* however, there are two other compounds chromatographically indistinguishable from the type A compounds vicenin-2 and lucenin-2. Chopin and coworkers have reported luteolin and apigenin-based glycoflavonoids from citrus which are unaffected by acid.[33] One closely resembles yet shows some chromatographic differences from lucenin-2,[34] but cochromatography of the apigenin glycoflavonoid with vicenin-2 suggested their identity. In *Lemna* and *Spirodela* species, McClure and Alston[35] have studied constituents which have paper chromatographic and spectral properties like the lucenins and vicenins. Since acidic treatment

* Courtesy of Professor Wagner and L. Rosprim.

[31] L. Hörhammer, H. Wagner, H. Nieschlag and G. Wildi, *Arch. Pharm.* **292/64**, 380 (1959).
[32] L. Hörhammer, H. Wagner, L. Rosprim, T. Mabry and H. Rösler, *Tetrahedron Letters* No. 22, 1707 (1965).
[33] J. Chopin, B. Roux, and A. Durix, *Compt. Rend.* **259**, 3111 (1964).
[34] J. Chopin, Personal communication.
[35] J. W. McClure and R. E. Alston, *Am. J. Botany* In press.

effected no change and since a direct comparison of the vicenin spot from *L. obscura* with vicenins-1, -2, and -3 yielded R_f values identical with vicenin-2, it would seem that these are type A di-*C*-glycosylflavonoids and probably the same as Hörhammer and Wagner's and as Chopin's compounds. In *Triticum* leaves, Harborne and Hall found, in addition to other glycoflavonoids, two new luteolin derivatives which on treatment with acid gave unidentified "aglycones".[23] This behaviour suggests type B. Unfortunately, no material was available for direct comparison, although chromatographic data suggest similarity to lucenins-1 and -4. However, a sample of *flavonoid B*, a glycoapigenin which King isolated from wheat germ[44] and Harborne identified in the leaves[23] and which was reported to yield only a small amount of *flavonoid C* on hydrolysis, was checked by chromatography and acid treatment. The material was found to be a mixture of two very closely related compounds which seem to be the acid-equilibrating vicenins-1 and -3. *Flavonoid C* seems to be vicenin-4, a minor component of the equilibrium mixture. Alliaroside, an apigenin derivative which Paris isolated from the leaves of *Alliaria officinalis*, gave a very low percentage of carbon on analysis and showed several spots on paper after acid treatment.[43] It was definitely not the same as vitexin; the proliferation of spots suggests the type B vicenins described here. Note, however, that two unidentified glycoflavonoids discovered by Hänsel and coworkers in *Vitex agnus-castus* wood[25] do not seem to have the properties of di-*C*-glycosyl derivatives. The R_f relationship of these to their acid-equilibrating isomers is far more similar to that of monoglycosyl compounds; also the apigenin derivative travels slower than vitexin in 50% acetic acid while the vicenins and violanthin travel faster.

Two final points should be mentioned—the instability of the lucenins and their ability to sequester iron. Both appeared more troublesome than with other luteolin derivatives which have been handled, but the effects were not investigated in detail.

EXPERIMENTAL

Material

Chipped sapwood of *Vitex lucens* (sample A),* black gum representing the methanolic extractives of sample A freed of butanol and ethyl acetate solubles (sample B),* and later heartwood (sample C)† were employed. Sample C was a deeper grayish-brown in color and was about seven times richer in flavonoid content than sample A; for example, a spectrographic estimation showed 1·0–1·2 g of flavonoid per 100 g of wood for sample A and 7·6 g per 100 g for C. The woods were ground in a Wiley mill to a fine powder.

Extraction

The woodmeal was extracted twice with cold methanol; 10 and then 5 ml/ g were used, and the mixtures were vigorously stirred for 1–2 hr under nitrogen. The first extract contained 80–85 per cent of the extractable flavonoids; another 10–15 per cent (consisting principally of the lucenin constituents) was obtained in the second. Aqueous extraction removed less material (60 per cent), and decomposition in aqueous solution was rapid; for example a loss of 29 per cent of the flavonoid material resulted in 4 days even under the storage conditions noted below. In some cases the methanolic extracts were evaporated *in vacuo*, in the presence of nitrogen, but this resulted in some loss.

* Samples A and B were gifts of Dr. R. C. Cambie, Department of Chemistry, University of Auckland, Auckland, New Zealand.

† Gift of Dr. R. M. Horowitz, Fruit and Vegetable Chemistry Laboratory, U.S. Department of Agriculture, Pasadena, Calif., U.S.A.

Storage

All extracts, solutions, eluates, and even solids were stored in the freezer under nitrogen.

Paper Chromatography

Whatman No. 1 and No. 3 MM paper were used for one- and two-dimensional and preparative paper chromatography. Developing solvents included the following: A5—5% acetic acid; A10—10% acetic acid; A15—15% acetic acid; AtW—acetone–water (1:1); BAW—butanol–27% acetic acid (1:1); BW—butanol saturated with water; BBW—butanol saturated with a saturated aqueous solution of boric acid; EAW—ethyl acetate–acetic acid–water (10:2:3); EPW—ethyl acetate–pyridine–water; IFW—isopropyl alcohol–formic acid–water (2:5:5); wNaCl—3% aq. sodium chloride; aNaCl—3% sodium chloride in 0·1 N hydrochloric acid. For use with BBW, paper was impregnated with boric acid by dipping it into the saturated aqueous solution; with the vicenins and lucenins it was necessary to overrun these sheets and the comparative ones (on unimpregnated paper developed with BW) 2 to 4 times longer than the original. Two-dimensional chromatograms were run on BAW-washed paper with BAW as the first developer and aNaCl or wNaCl as the second. Preparative runs were carried out first with BAW and then with A5, both overrun for 50–100 per cent of the original time to allow separation of the lucenins or the vicenins from each other.

Sheets were observed in u.v. light and in u.v. light in the presence of ammonia fumes. The following sprays were utilized: Spray I—1% aluminum chloride in ethanol; Spray II—Benedict's reagent (basic cupric citrate complex); Spray III—3% *p*-toluenesulfonic acid in ethanol followed by heating of the sheet. Spray I was used to brighten weak spots, as in the study of major and minor substances produced on hot acid treatment; Spray II was used to differentiate the 4′-hydroxyflavonoids from the 3′,4′-dihydroxy compounds;[36] and Spray III was for the detection of leucoanthocyanins.

Preparative bands and spots from two-dimensional chromatograms were eluted with 50% methanol, or for spectral work with 95% ethanol, preferably in tanks filled with nitrogen.

Column Chromatography on Cellulose Powder

Large colums (57 × 4·4 cm) were filled with 150 g of Whatman standard grade cellulose powder suspended in BW. Sample B, 7·5 g, was dissolved in 35 ml of water and applied to the column. After a development time of 18–24 hr, 2 days were required to elute all flavonoids from the column with BW. The fractions were analyzed by paper chromatography, and combined fractions were concentrated for preparative paper chromatography by evaporation *in vacuo*.

Macro-Isolation by Means of ChroMax Column

Precipitation of lead salts. The unconcentrated methanolic extract, 1·5–2·0 l. from 100 g of wood, was vigorously stirred and treated (1) dropwise with 20 ml of diluted neutral lead acetate (4 ml of a saturated aqueous solution diluted immediately before use with four volumes of methanol), (2) dropwise, but quickly, with 95 ml of the same solution, and (3) by rapid addition of 100 ml of basic lead acetate (425 g/l. of lead subacetate); between each addition the lead salts were filtered on to a Celite mat. The first dirty yellow hygroscopic precipitate was discarded. The second ("dihydroxy") precipitate was vivid yellow, the third ("monohydroxy"), lemon yellow. The lead was immediately removed from the two

[36] H. Reznik and K. Egger, *Z. Anal. Chem.* **183**, 196 (1961).

lots of moist salts by suspending each in 300 ml of methanol and treating them respectively with 10 and 30 g of Dowex-50-8X ion-exchange resin in the acid form which had been washed free of colored impurities by methanol. The mixtures were stirred in a nitrogen atmosphere for 15 and 20 hr respectively before filtration. The "monohydroxy" precipitate was re-treated with a fresh batch of resin for about 5 hr. The clear yellow filtrates were analyzed chromatographically and spectrographically. They were concentrated *in vacuo* at room temperature, respectively, to a thick gum (called sample D) and to a syrupy solution (called sample E).

Fractionation by ChroMax columns. The Swedish pressurized ChroMax column,* with a cylinder of rolled paper 58 × 750 mm, was prepared for use by washing it with BAW for 2–4 days until 100 ml of eluate, which had originally been brown in color, left less than a milligram of white residue. During this time, the correct pressure was determined by applying 5-ml samples of the dye Sudan III in BAW; it was about 0·5 kg/cm^2. Columns for use with A5 were washed with this solvent, and methyl orange was used as a test dye. Washing and adjusting of pressure were carried out expeditiously as the column deteriorated with use. Solvent flow was continued between separate runs on the same insert, on which about three fractionations could be carried out successfully.

Columns developed with BAW were generally used for fractionating crude "dihydroxy" mixtures (sample D). The gum from the neutral lead treatment, in amount representing about 0·5–0·6 g of flavonoid material and equivalent to 200 g of sample A or 20–25 g of sample C, was dissolved in a minimum amount of BAW (about 20 ml), the solution filtered, and the filtrate applied to the column. The development with BAW required a day, the elution about 2 days. Approximately 100 fractions (about 15 ml each) were collected, stored as usual, and analyzed by paper chromatography. In some runs the concentration of flavonoids was determined spectrographically.

Columns developed with A5 were used for final separation of lucenins-1, -2, and -4, preliminary separation of the vicenins and orientin xyloside, and purification of lucenin-3 (which was contaminated badly with orientin). In one case, fractionation of the crude mixture was done with this solvent. Although it moved much faster than BAW, the small R_f values of several of the glycoflavonoids in this solvent required extended periods of elution and increase in acetic acid concentration to clear the column.

Freeze drying. Combined eluates of similar composition were freeze dried. The BAW ices stayed barely frozen at room temperature, and solid yellow or tan residues were obtained at the rate of 200 ml of eluate evaporated in 5–6 hr. The acetic acid ices were much more solid and evaporated more slowly.

Crude products. Amorphous yellow to brown residues were obtained from the freeze drying. After fractionation by two columns, several of the residues were chromatographically pure. Of these, *lucenin*-4 and *isoorientin*, both dark yellow, were used as such for further studies. Residues of *orientin* and its *xyloside* were dark and sometimes gummy. Extraction of these with cold 95% ethanol gave clear yellow solutions, which were used for further studies, and dark residues giving strong tests for iron.

Crystallization of Lucenins

Lucenin-1. Addition of a few drops of water per 100 mg to the fluffy, amorphous lucenin-1 obtained from freeze drying caused immediate solution and the start of crystallization at

* LKB Produkter AB, Stockholm 12, Sweden; LKB Instruments, Inc., Washington, D.C. 20014, U.S.A. Use of this product does not imply endorsement by the U.S. Department of Agriculture.

once or within an hour provided that the material was chromatographically pure. Traces of other lucenins inhibited crystallization markedly, so that only very low recoveries were obtained after many days; sometimes months were required for crystals to separate. Pure materials yielded 60–80 per cent recoveries after 2–3 days in the refrigerator. To remove a persistent iron impurity (detected by ferrocyanide and iron-free hydrochloric acid), the crystals (or crude amorphous materials) were extracted repeatedly with cold or slightly warm 95% ethanol (1–5 ml total ethanol per 10 mg) until only a small, dark residue remained. This represented 5–15 per cent of the original weight, was extremely soluble in water, and gave a strong iron test. The alcoholic extracts were evaporated to dryness *in vacuo* at room temperature, and water (less than 0·5 ml/10 mg) added. Four repetitions of the above procedure, with 1–10 days allowed for each crystallization, yielded pale yellow, transparent crystals (65–95 per cent recovery per repetition). These lost water of crystallization at once in air, leaving a dense, granular, yellow product, m.p. slow decomposition about 230° (Found: C, 53·03; H, 5·44; mol.wt. 573 ±10%. Calc. for $C_{27}H_{30}O_{16}$: C, 53·13; H, 4·95%; mol.wt. 610). The mol.wt. was determined on a vapor pressure osmometer in ethanolic solution.

Lucenin-3. Attempts to crystallize and purify amorphous samples of lucenin-3, obtained from the ChroMax column and slightly contaminated with orientin, by the methods used for lucenin-1 were far less successful. Crystals formed only when volumes of water were reduced to 1–2 drops; gels separated first, which sometimes over a period of months developed nuclei of crystals (definitely revealed by a polarizing microscope). From 113 mg after passage through a Sephadex column and many filtrations to remove traces of insoluble tars, six lots of crystals ranging in weight from 1·9 to 7·0 mg were obtained (19 mg total). This material was still contaminated with iron; and only a gel resulted after a second alcohol treatment and attempted crystallization (17 per cent recovery).

Separation of Vicenins

Refractionation of vicenin-rich residues on one or two ChroMax columns developed with A5 was used to separate the vicenins from orientin xyloside, orientin, and lucenins-3 and -5, as well as partially from each other. By paper chromatography in BAW and then in A5, with severe pruning of the paper bands and careful monitoring of the separations with overrun paper chromatograms, the three pure constituents were isolated from the residues of some eluates from the columns. Small residues obtained from the methanolic eluates of the bands after evaporation *in vacuo* at 40°, were crystallized by treating them with a few drops of water and allowing them to stand and partially evaporate for 1–2 weeks. Insufficient material was isolated for analytical work. Generally no iron tests were obtained with these materials.

Isolation of Vitexin and Saponaretin

Large amounts of vitexin contaminated with saponaretin (at least 20 per cent) were obtained as byproducts in the macro isolation work on the lucenins by the following simple method. During storage in the refrigerator, the concentrated solutions of "monohydroxy" mixtures (sample E) from the basic lead acetate precipitations, slowly deposited bright yellow, crystalline precipitates, m.p. dec. 244–245°. A similar material was obtained from the appropriate early eluates of a cellulose powder column by evaporating them *in vacuo*, adding methanol to the original brown glass, and refrigerating. After 4 days, 33 mg of vitexin had separated, derived from 100 g of sapwood (sample A).

Vitexin. Extracting crude crystals (1·82 g) three times with 50 ml of boiling 95% alcohol

left 1 g of practically pure vitexin, m.p. dec. 262–263° (recorded dec. 264–265°)[37]. This was purified for NMR by two recrystallizations from dimethylcellosolve–water (4:1), 5 ml/100 mg, 55–65 per cent recovery after evaporation; this method removed an insoluble, fast-running (BAW) impurity but did not change the melting point.

Isovitexin (saponaretin). This was obtained from the first alcoholic extract of the crude vitexin. After removal of several precipitates (which were mainly vitexin), and concentration, the final filtrates set to gels of hair-fine needles which were about 80 per cent pure. Recrystallization from boiling absolute alcohol removed some but not all of the vitexin and required drastic evaporation of the filtrates to recover the isovitexin, m.p. dec. 216–217°, still contaminated with about 10 per cent vitexin. The crystals lost solvent of crystallization and their form on drying in the oven. A few crystals isolated from a dilute aqueous eluate of a Sephadex G-25 column decomposed at 230° and were chromatographically pure.

Isolation of Crystalline Vitexin Xyloside

One sample of E, derived by basic lead precipitation from 200 g of sample A, was partially freed of vitexin by filtration after 2 months of refrigeration and the residue of the filtrate subjected to Craig distribution between water and butanol–ethyl acetate (9:1) in a 60-tube apparatus. Following unseparated isovitexin and vitexin ($K=11$ and 9 respectively) in tubes 48–58, vitexin xyloside ($K=2{\cdot}9$) appeared in tubes 35–48. From the contents of tubes 40–47, 90 mg of pale yellow crystals growing in rosettes was obtained by (1) evaporation *in vacuo* at 60°, (2) solution of the resultant brownish resin in methanol and cooling, (3) removal of the first powdery precipitates appearing during 10 days, (4) concentration and addition of acetone, (5) cooling for 2 weeks, and (6) obtaining successive precipitates by adding more acetone and by concentrating. They were recrystallized three times by dissolving 40 mg in 2–3 drops of methanol, adding 5–10 ml of acetone, and allowing slow crystallization during at least a week. The stocky, bright yellow needles decomposed with an imperceptibly gradual evolution of a gas around 200° (Found: C, 55·24; H, 5·22. Calc. for $C_{26}H_{28}O_{14}$: C, 55·32; H, 4·99%).

Acidic Hydrolysis Methods and Identification of Products

Standard conditions and time study. Solid samples weighing 0·3 to 3·0 mg were dissolved in methanol and treated with an equal volume of 2 N hydrochloric acid (preferably iron-free) usually in the ratio of 1 ml of each solvent per milligram of solid. Concentrated eluates from paper were treated with the reagents so that the final solutions were 1 N in acid and 50 per cent in methanol. The mixtures were refluxed, and the course of reactions followed by paper chromatography of test samples, preferably with both BAW and aNaCl. Generally, *O*-glycosides were hydrolyzed completely in about 2 hr. Treatment of the di-*C*-glycosyl compounds was continued for at least 2 days, in some cases longer.

Extraction. Methanol was evaporated from the solutions and the glycoflavonoids were isolated from the 0·75–3·0 ml of aqueous residue by exhaustive extraction with 1-ml portions of butanol.

Separation and identification of glycoflavonoids. The residues obtained on evaporating

[37] W. H. EVANS, A. MCGOOKIN, A. ROBERTSON, L. JURD and W. R. N. WILLIAMSON, *J. Chem. Soc.* 3510 (1957).
[38] B. H. KOEPPEN, *Z. Naturforsch.* **19b**, 173 (1964).
[39] G. R. BARKER and D. C. C. SMITH, *Chem. & Ind. (London)* 19 (1954); D. J. BELL, F. A. ISHERWOOD and N. E. HARDWICK, *J. Chem. Soc.* 3702 (1954).
[40] W. E. HILLIS and D. H. S. HORN, *Australian J. Chem.* **18**, 531 (1965).

the butanol extracts were (1) identified by u.v. spectra and by cochromatography with authentic samples if they represented single compounds, (2) separated and purified first by paper chromatographic methods if they were multicomponent and then each component identified as in (1), or (3) identified with other multicomponent equilibrium mixtures by running two-dimensional cochromatograms.

Identification of sugars. The residual aqueous layers were freed of hydrochloric acid by treating them with the calculated amount of *N,N*-di-octylmethylamine in chloroform (0·5 g in 5 ml).[41] The residues of the washed aqueous layers were checked for sugars by paper chromatography [solvents, BAW (4:1:5), EAW (3:1:3), EPW (2:1:2), phenol saturated with water; spray, aniline phthalate] or by thin layer chromatography [plates, kieselguhr made up with 0·015 M sodium acetate; solvent, EPW (144:40:24); spray, ammoniacal silver, heated after spraying].

Alkaline Degradation

A 4-mg sample of amorphous lucenin-1 was degraded with concentrated aqueous potassium hydroxide by the method described for lutonarin[13] and the products identified by paper chromatography in BAW, A15, aNaCl, and 60% isopropyl alcohol.

Ultraviolet Spectra

Ultraviolet spectra, except for those of crude extracts, were determined in 95% ethanol. Diagnostic shifts[14] were determined by adding to solutions in the spectrophotometer cells (about 3 ml): (1) 3 drops of 5% aluminum chloride in ethanol; (2) the preceding plus excess powdered anhydrous sodium acetate (with time allowed for development); (3) 3 drops of 1 N sodium ethylate; (4) excess sodium acetate; and (5) the preceding plus 0·75 ml of a saturated solution of boric acid in ethanol.

Nuclear Magnetic Resonance Spectra

Trimethylsilylethers of lucenin-1 were prepared by the recently described procedure,[17] with reaction times of both 10 min and 4 hr. The spectra were measured in carbon tetrachloride on a Varian A-60 spectrometer with tetramethylsilane as an internal reference.

Acknowledgements—We wish to acknowledge the cooperation of Dr. T. J. Mabry whose NMR study gave definite evidence for the di-*C*-glycosyl theory, the gifts of *V. lucens* wood from Drs. R. C. Cambie and R. M. Horowitz, and the kindness of Drs. Hörhammer, Wagner, and Chopin in furnishing samples of their compounds for comparison.

[41] J. B. Harborne and H. S. A. Sherratt, *Biochem. J.* **65**, 23P (1957).
[42] L. Jurd, *J. Chromatog.* **4**, 369 (1960).
[43] R. R. Paris and P. Delaveau, *Compt. Rend.* **254**, 928 (1962).
[44] H. G. C. King, *J. Food Sci.* **27**, 446 (1962).