Flavonoids of Citrus. IX. Some New C-Glycosylflavones and a Nuclear Magnetic Resonance Method for Differentiating 6- and 8-C-Glycosyl Isomers

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6-C-β-D-Glucopyranosyldiosmetin and 8-C-β-D-glucopyranosyldiosmetin have been isolated from citrus species and their structures determined from nmr and other spectral data. In addition, 2''-O-β-D-xylosylvitexin, a di-C-glycosylapigenin, and a di-C-glycosyldiosmetin have been obtained. The chemical shifts of acetyl methyl bands in the nmr spectra of various acetylated C-glycosyl compounds are discussed. It is shown that these shifts as well as other spectral and chromatographic data can be used to determine the position of substitution of the glucosyl residue.

C-Glycosylflavones have been found in about twenty different plant families.[2,3] Earlier publications[4-6] dealt briefly with the occurrence of these compounds in citrus fruits (Rutaceae), where they accompany a large array of other flavonoids, principally O-glycosides and O-permethylflavones. For the most part the C-glycosylflavones of *Citrus* occur in very low concentration and are difficult to isolate. We obtained the compounds described here by chromatographing crude peel extracts on columns of silicic acid developed with chloroform–methanol or ethyl acetate–methanol.

Compound I, mp 267–268°, was found both in lemons (*C. limon*) and oranges (*C. sinensis*). Its ultraviolet spectra in neutral ethanol and with various added diagnostic reagents (Table I) establish it as a flavone. Comparison of these spectra with those of some commonly occurring flavone aglycones (see Chart I and Table I) indicates that it is derived from diosmetin. Since I moves faster than diosmetin on paper chromatography in aqueous solvents or on paper electrophoresis in aqueous sodium borate (Table II), it can be assumed that a glycosyl residue is present in the molecule. Nevertheless, prolonged treatment of the compound in hot 3 *N* hydrochloric acid fails to release any sugar, but leads instead to a slow, partial conversion into a different glycosylflavone (II).

Compound II, mp 243–245°, is a natural constituent of lemons, but was not found in oranges. Its ultraviolet spectra are almost indistinguishable from those of diosmetin and I. It migrates faster than I on paper chromatography or electrophoresis and, when heated in acid, undergoes partial conversion into I. Both I and II yield isovanillic acid as a product of alkaline hydrolysis.

It is clear that we have in hand a pair of isomeric C-glycosylflavones, one of which is a 6-C-glycosyl derivative, the other an 8-C-glycosyl derivative. This conclusion is based (a) on the obvious presence of a glycosyl residue, (b) on its failure to be released by acid hydrolysis, and (c) on the interconversion of I and II in acid solution. Analogous Wessley–Moser interconversions of 6- and 8-C-glycosyl isomers have been observed before, for example, in vitexin and isovitexin[4] or orientin and isoorientin.[7]

The remaining structural questions have to do with the identification, configuration, and point of attachment of the glycosyl residue. Because of the very small amount of material on hand, it was impractical to ozonize or otherwise oxidize the flavone portion of the molecule to obtain the free sugar. However, in cases where the identity of the sugar residue of C-glycosyl compounds has been clearly established, it has always been found to be β-D-glucosyl. That this holds for compounds I and II is indicated by nmr data, which also show the configuration and point of attachment of the glucosyl residue.

Table III catalogs the band positions of the aliphatic acetyl methyl groups of various acetylated 8- and 6-C-glucosylflavones (groups A and B, respectively) and of a number of miscellaneous acetylated C-glucosyl compounds (group C). The acetyl bands of the 8-substituted flavones form a pattern quite distinct from that of the acetyl bands of the 6-substituted and miscellaneous group. Thus, in 8-C-glucosylflavones both the 2''-O-acetyl band (δ 1.70–1.73) and the 6''-O-acetyl band (δ 1.90–1.95) occur at consistently higher field than they do in the other groups of compounds (δ 1.77–1.83 for the 2''-O-acetyl in groups B and C and 1.98–2.04 for the 6''-O-acetyl in groups B and C). There is, in fact, essentially no difference in the spectra of the group B and C compounds in spite of wide variations in structure. The distinct character of the spectra of the group A compounds must be due to some special structural feature, most likely the proximity of the glucosyl residue to the B ring of the flavone.

The unusually high field position of the 2''-O-acetyl band in the various compounds listed in Table III is due primarily to shielding by the aromatic A ring to which the glycosyl residue is linked, since this acetyl is situated mainly in the diamagnetic region above or below the plane of the A ring[5,8,9] (see Figure 1). The slightly greater shielding of the 2''-O-acetyl in group A compounds compared with group B and C compounds is of some diagnostic value and can be explained on the assumption that the 2''-O-acetyl in group A is shielded not only by the A ring of the flavone but to some extent by the B ring. The greater shielding of the 6''-O-acetyl in group A can be accounted for on the assumption that it lies in the diamagnetic region

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(2) (a) J. Chopin in "Actualités de Phytochimie Fondamentale," 2nd Series, C. Mentzer, Ed., Masson et Cie., Editeurs, Paris, 1966, p 44; (b) H. Wagner in "Comparative Phytochemistry," T. Swain, Ed., Academic Press Inc., New York, N. Y., 1966, p 309.

(3) J. B. Harborne, "Comparative Biochemistry of the Flavonoids," Academic Press Inc., New York, N. Y., 1967.

(4) R. M. Horowitz and B. Gentili, *Chem. Ind.* (London), 498 (1964).

(5) R. M. Horowitz and B. Gentili, *ibid.*, 625 (1966).

(6) J. Chopin, B. Roux, and A. Durix, *Compt. Rend.*, **259**, 3111 (1964).

(7) B. H. Koeppen, C. J. B. Smit, and D. G. Roux, *Biochem. J.*, **83**, 507 (1962).

(8) W. E. Hillis and D. H. S. Horn, *Aust. J. Chem.*, **18**, 531 (1965).

(9) R. A. Eade, W. E. Hillis, D. H. S. Horn, and J. J. H. Simes, *ibid.*, **18**, 715 (1965).

TABLE I

ULTRAVIOLET ABSORPTION SPECTRA (λ_{max} IN mμ) OF FLAVONES, *C*-GLYCOSYLFLAVONES, AND THEIR ACETYL DERIVATIVES[a]

Compd	In EtOH[b]	In EtOH–NaOAc[c]	In EtOH–NaOH[d]	In EtOH–AlCl₃[e]
I	252, 272, ~290, 343	281, ~320, ~370	270, ~303, ~385	259, 280, ~296, 355, ~380
I heptaacetate (Ia)	~260, 319			
II	253, 272, 344	280, ~318, ~376	271, ~302, ~390	259, 281, ~295, 358, ~380
II heptaacetate (IIa)	~258, 320			
III	272, 333	281, 384	281, ~334, 400	279, 306, 349, ~382
IV	274, 335	283, 385	283, ~337, 404	282, 305, 350, ~380
IV peracetate (IVa)	258, 298, ~312			
V	~257, 274, 344	283, ~315, ~385	269, ~280, ~400	~265, 282, ~300, 357, ~380
V peracetate (Va)	~257, 314			
Apigenin (VI)	269, 335	277, ~300, 380	277, ~329, 399	278, 303, 345, ~382
Apigenin triacetate (VIa)	254, 299			
Luteolin (VII)	255, 268, ~295, 350	269, ~277, ~325, 392	267, ~335, 406	273, ~300, ~332, 426
Luteolin tetraacetate (VIIa)	~254, 259, 298			
Chrysoeriol (VIII)	252, 270, 346	278, ~321, 395	265, 335, 410	263, 278, ~296, 359, ~385
Chrysoeriol triacetate (VIIIa)	240, ~260, 308			
Diosmetin (IX)	252, 269, 344	278, ~321, ~362	271, ~302, ~382	261, 278, ~295, 358, ~383
Diosmetin triacetate (IXa)	~255, 318			
Vitexin (X)	270, 333	280, ~300, 384	281, ~333, 400	278, 305, 346, ~382
Vitexin heptaacetate (Xa)	254, 398, ~310			
Isovitexin (XI)	273, 336	280, ~300, 383	280, ~333, 404	281, 305, 349, ~380
2″-*O*-β-D-Xylosylvitexin (XII)	271, 332	280, ~305, 386	281, ~333, 400	278, 305, 346, ~380

[a] The symbol ~ indicates either a shoulder or band of relatively low intensity. [b] Absolute ethanol. [c] Fused sodium acetate. [d] One drop of 1% aqueous sodium hydroxide added to a 3-ml cuvette. [e] Excess crystalline aluminum chloride.

TABLE II

MIGRATION OF FLAVONES AND *C*-GLYCOSYLFLAVONES ON PAPER CHROMATOGRAPHY AND ELECTROPHORESIS

Compd	R_f value in 10% HOAc	R_f value in 30% HOAc	R_f value in *n*-BuOH–HOAc–H₂O (20:6:15)	R_f value in EtOAc–HCO₂H–H₂O (10:2:3)[a]	Electrophoretic migration[b]
I	0.13	0.42	0.58	0.39	0.48
II	0.30	0.61	0.68	0.47	0.75
III	0.65	0.74	0.62	0.39	1.86
IV	0.50	0.68	0.55	0.15	1.80
V	0.39	0.64	0.52	0.13	1.35
Apigenin (VI)	0.03	0.28	0.94	0.87	0.30
Diosmetin (IX)	0.02	0.23	0.90	0.86	0.08
Vitexin (X)	0.20	0.46	0.63	0.43	1.00
Isovitexin (XI)	0.39	0.63	0.72	0.51	1.31
2″-*O*-β-D-xylosylvitexin (XII)	0.65	0.74	0.62	0.39	1.88
Vicenin-2 (XIII)	0.47	0.65	0.55	0.18	1.80
Orientin (XIV)	0.12	0.38	0.42	0.28	1.15
Isoorientin (XV)	0.27	0.56	0.55	0.38	1.50

[a] Upper phase. [b] In 0.1 *M* aqueous sodium borate, *ca.* 900 V, 20–40 mA, *ca.* 3 hr, Whatman No. 1 paper. The migrations are given relative to vitexin = 1.00.

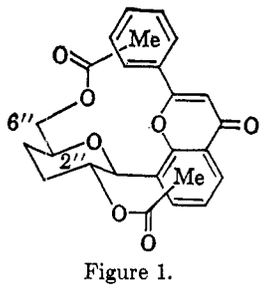


Figure 1.

of the B ring of the flavone nucleus. Models show this to be a likely conformation (see Figure 1). Since any similar shielding can obviously be ruled out in groups B and C, the 6″-*O*-acetyl band of these compounds occurs at the "normal" position (δ 1.98–2.04), *i.e.*, the same position as the 6-*O*-acetyl band of simple glucose derivatives such as D-glucose 2,3,4,6-tetraacetate (δ 2.03).[10]

(10) The assignments in Table III for the acetyl bands of D-glucose 2,3,4,6-tetraacetate are based on a comparison of the spectra of various partially acetylated glucoses (unpublished data).

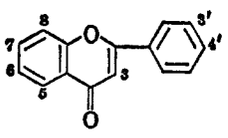
A consequence of the interaction of the 2″- and 6″-*O*-acetyl groups with the B ring and C-7 substituent in 8-substituted flavones is that these compounds exhibit hindered rotation about the C-1″–C-8 bond. Even at temperatures as high as 30–40° they appear to exist largely in two principal rotational conformations,[11] as discussed earlier by Eade and coworkers.[9] The existence of the conformers, which is markedly temperature dependent, is evidenced in the nmr spectrum at 35° by the splitting or broadening of the bands of certain aromatic protons and acetyl groups, particularly the 6″-*O*-acetyl group (Table III). Since the glycosyl portion of the group B and C compounds has a smaller rotational barrier, their spectra are generally sharp in all regions, with the possible exception of the slightly broadened 2″-*O*-acetyl band.

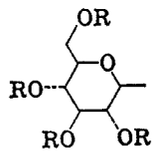
A further point of difference between the spectra of the acetylated 8-*C*-glucosylflavones and the other acetylated *C*-glucosyl compounds lies in the band posi-

(11) One of these is the conformation shown in Figure 1; in the other the glucosyl residue is rotated 180° about the C-1″–C-8 bond.

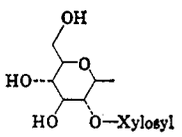
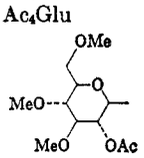
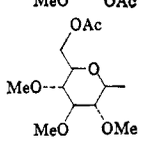
CHART I

FLAVONE SUBSTITUTION PATTERNS





Glu, R = H
Ac_4Glu, R = Ac

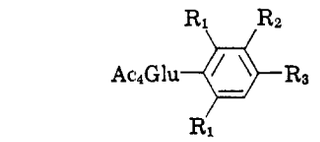
Compd	3	5	6	7	8	3′	4′
III, XII	...	OH	...	OH		...	OH
IV, XIII	...	OH	Glycosyl	OH	Glycosyl	...	OH
IVa	...	OAc	Peracetylglycosyl	OAc	Peracetylglycosyl	...	OAc
V	...	OH	Glycosyl	OH	Glycosyl	OH	OMe
Va	...	OAc	Peracetylglycosyl	OAc	Peracetylglycosyl	OAc	OMe
VI	...	OH	...	OH	...	...	OH
VIa	...	OAc	...	OAc	...	...	OAc
VII	...	OH	...	OH	...	OH	OH
VIIa	...	OAc	...	OAc	...	OAc	OAc
VIII	...	OH	...	OH	...	OMe	OH
VIIIa	...	OAc	...	OAc	...	OMe	OAc
IX	...	OH	...	OH	...	OH	OMe
IXa	...	OAc	...	OAc	...	OAc	OMe
X	...	OH	...	OH	Glu	...	OH
Xa	...	OAc	...	OAc	Ac_4Glu	...	OAc
XI	...	OH	Glu	OH	...	...	OH
XIa	...	OAc	Ac_4Glu	OAc	...	...	OAc
XIV	...	OH	...	OH	Glu	OH	OH
XIVa	...	OAc	...	OAc	Ac_4Glu	OAc	OAc
XV	...	OH	Glu	OH	...	OH	OH
XVa	...	OAc	Ac_4Glu	OAc	...	OAc	OAc
XVIa	...	OAc	...	OAc	Ac_4Glu	...	OMe
XVIIa	...	OMe	...	OMe	Ac_4Glu	...	OMe
XVIIIa	...	OMe	...	OMe		...	OMe
XIXa	...	OMe	...	OMe		...	OMe
XXa	...	...	...	OAc	Ac_4Glu	...	OAc
XXIa	...	...	...	OMe	Ac_4Glu	...	OMe
XXIIa	...	OMe	Ac_4Glu	OMe	...	...	OMe
XXIIIa	OAc	OAc	Ac_4Glu	OMe	...	...	OAc
XXXIIa	...	OAc	...	OAc	...	...	OMe

tions of the 3″- and 4″-*O*-acetyl groups. In the 8-substituted flavones these bands occur in two discrete ranges, δ 2.01–2.03 and 2.08–2.10, respectively, while in the 6-substituted and miscellaneous compounds they overlap or occur very close together in the range δ 2.03–2.08. Since the 4″-*O*-acetyl lies near the axis of rotation of the glucosyl ring, it should be relatively insensitive to the rotational conformation of the molecule. In the 8-substituted flavones the signal at δ 2.08–2.10 is a sharp singlet and is, therefore, assigned to the 4″-*O*-acetyl. The band at δ 2.01–2.03 is usually broadened or split and is assigned to the 3″-*O*-acetyl. Furthermore, models show that in one of the principal postulated conformations the 3″-*O*-acetyl is likely to be shielded to some extent by the B ring.[12,13]

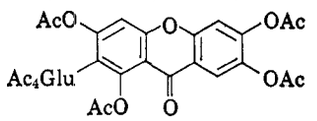
Using the band positions of the various sugar acetyl groups and sharpness of the spectra as criteria for determining the point of substitution, we conclude

(12) The same assignments for the 3″- and 4″-*O*-acetyls have been given by Eade, Hillis, Horn, and Simes[9] but the converse assignments are given by Hillis and Horn.[8]

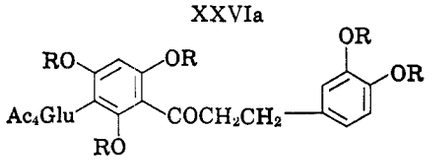
(13) The spectrum of the isoflavone, puerarin hexaacetate (XXXIa), appears to be of a "hybrid" nature, in which the 2″-*O*-acetyl signal conforms to the group A pattern while the 3″-, 4″-, and 6″-*O*-acetyl signals conform to the group B, C pattern. Models show that the 2″-*O*-acetyl group is roughly equidistant from the B ring in 8-substituted flavones or isoflavones, but the other acetyl groups are much further removed from the B ring in 8-substituted isoflavones than in 8-substituted flavones. It is also of interest that the 6- and 8-substituted flavanones, hemiphloin heptaacetate (XXXa) and isohemiphloin heptaacetate (XXIXa), are reported to give closely similar spectra.



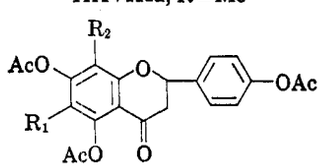
XXIVa, $R_1 = R_2 = R_3 = H$
XXVa, $R_1 = OMe$; $R_2 = Ac$; $R_3 = OAc$



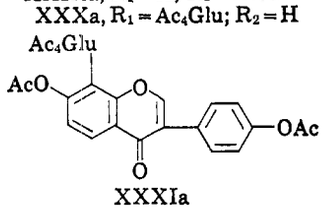
XXVIa



XXVIIa, R = Ac
XXVIIIa, R = Me



XXIXa, $R_1 = H$; $R_2 = Ac_4Glu$
XXXa, $R_1 = Ac_4Glu$; $R_2 = H$

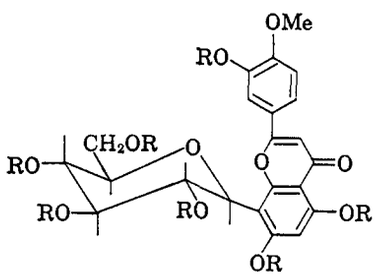


XXXIa

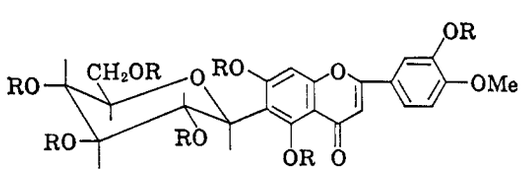
that compound I is an 8-substituted diosmetin and compound II is a 6-substituted diosmetin. The close correspondence in the acetyl band positions of Ia and IIa with those of the other compounds in their respective groups, all of which are *C*-β-D-glucosyl derivatives, leads us to infer that they too are *C*-β-D-glucosyl derivatives. Were this not the case one might expect to find perceptible differences in the spectra as a result of epimeric configurations in the sugar. In any case, the large coupling constant (10 Hz) of H-1″ in compound IIa (Table III) confirms the β configuration of the glucosyl radical and the equatorial configuration of the C-2″ acetoxyl group.

The nmr data in Table IV show the band positions of the aromatic and methoxyl protons in the acetyl derivatives of II, vitexin, isovitexin, and several simple flavones. The latter compounds—apigenin, acacetin, luteolin, chrysoeriol and diosmetin—give characteristic spectra that are useful in identifying *C*-glucosyl compounds derived from them. Comparison of the chemical shifts in Table IV shows, in agreement with the ultraviolet data of Table I, that compound II is derived from diosmetin, while the absence of an H-6 resonance confirms that it is 6 substituted.[14]

The problem of distinguishing 6- and 8-*C*-glucosyl isomers can be solved, as outlined above, by examining the chemical shifts of either the acetyl or aromatic protons. When only a small amount of compound is available the acetyl protons, because of greater signal strength, generally give more reliable information than do the aromatic protons, which sometimes cannot be discerned above the background. Confirmatory evidence can be obtained by comparing R_f values of the corresponding 6- and 8-*C*-glucosylflavones. In the solvents listed in Table II the 6-substituted compounds invariably migrate more rapidly than the corresponding 8-substituted compounds, and the same applies for paper electrophoresis in sodium borate solution.[15-17] According to these criteria compounds I and II can again be assigned as the 8 and 6 isomers, respectively. We conclude that I is 8-*C*-β-D-glucopyranosyldiosmetin and II is 6-*C*-β-D-glucopyranosyldiosmetin.



I, R = H
Ia, R = Ac



II, R = H
IIa, R = Ac

In addition to these monoglucosylflavones we have isolated small quantities of flavones that appear to contain more than one glycosyl residue. Compound III, obtained as a gum from orange peel, yields xylose and vitexin together with a small amount of isovitexin when hydrolyzed with acid or hemicellulase. Its ultraviolet spectra (Table I) are the same as those of 2″-*O*-β-D-xylosylvitexin,[5] as are its R_f values in several solvents and rate of migration on paper electrophoresis (Table II). 2″-*O*-β-D-Xylosylvitexin was isolated previously from *Vitex lucens* as a crystalline solid. The present compound, which was not obtained entirely pure, is believed to consist mainly of 2″-*O*-β-D-xylosylvitexin mixed with a small proportion of a glycosylisovitexin.

Compound IV was isolated from lemon peel as a crystalline solid, mp 233–236°. Its ultraviolet spectrum and spectral shifts are closely similar to those of apigenin, vitexin, and isovitexin. It migrates more rapidly than any of these compounds on paper chromatography in strongly aqueous solvents or on paper electrophoresis in aqueous sodium borate. When heated in hydrochloric acid it gives no sugar and does not isomerize appreciably. These results point to the presence of two *C*-glycosyl residues, which, because of the apparent lack of isomerization, are likely to be identical and located at the 6 and 8 positions of the

(14) There was an insufficient quantity of Ia available to determine reliable values for the aromatic protons. The methoxyl protons in Ia occur at δ 3.93.

(15) Other examples, not given in Table II, are cytisoside/isocytisoside[1,16] and swertisin/isoswertisin.[17]

(16) J. Chopin, M. L. Bouillant, and A. Durix, *Compt. Rend.*, **260**, 4850 (1965).

(17) M. Komatsu, T. Tomimori, and M. Ito, *Chem. Pharm. Bull.* (Tokyo), **15**, 263 (1967); M. Komatsu and T. Tomimori, *Tetrahedron Lett.*, 1611 (1966).

TABLE III
CHEMICAL SHIFT OF ACETYL METHYL AND BENZYLIC (H-1'') PROTONS OF ACETYLATED *C*-GLUCOSYL COMPOUNDS IN DEUTERIOCHLOROFORM[a]

Compd	Chemical shift and coupling [δ, ppm (*J*, Hz)] 4''-OAc	3''-OAc	6''-OAc	2''-OAc	H-1''	Ref[b]
8-Substituted Flavones (Group A)						
Vitexin heptaacetate (Xa)	2.09	2.01	1.91[c]	1.73	4.98 (10)	5
Cytisoside hexaacetate (XVIa)	2.08	2.01	1.92	1.73	*d*	16
5,7,4'-Tri-*O*-methylvitexin tetraacetate (XVIIa)	2.09	1.98	1.93[c]	1.71	5.25 (10)	
5,7,4',3'',4'',6''-Hexa-*O*-methylvitexin acetate (XVIIIa)	...	...	...	1.72	5.06 (10)	5
5,7,4',2'',3'',4''-Hexa-*O*-methylvitexin acetate (XIXa)	...	...	1.93	...	5.00 (10)	5
Orientin octaacetate (XIVa)	2.09	2.02	1.95[e]	1.72	*b*	
Bayin hexaacetate (XXa)	2.10	2.02[g]	1.90[h]	1.72	*d*	8, 9
7,4'-Di-*O*-methylbayin tetraacetate (XXIa)	2.10	2.01[g]	1.91[h]	1.70	*d*	8, 9
8-*C*-β-D-Glucopyranosyldiosmetin heptaacetate (Ia)	2.08	2.02	1.94[e]	1.71	*f*	
Range for group A	(2.08–2.10)	(1.98–2.02)	(1.90–1.95)	(1.70–1.73)		
6-Substituted Flavones (Group B)						
Isovitexin heptaacetate (XIa)	2.08	2.08	2.04	1.83	4.91 (10)	
5,7,4'-Tri-*O*-methylisovitexin tetraacetate (XXIIa)	2.07	2.05	2.02	1.77	5.14 (10)	
Isoorientin octaacetate (XVa)	2.08	2.08	2.02	1.82	4.85 (9.5)	
Keyakinin heptaacetate (XXIIIa)	2.05	2.05	2.01	1.80	5.25	*i*
6-*C*-β-D-Glucopyranosyldiosmetin heptaacetate (IIa)	2.07	2.07	2.02	1.81	4.86 (10)	
Miscellaneous C-Glucosyl Compounds (Group C)						
C-β-D-Glucosylbenzene tetraacetate (XXIVa)	2.04	2.03	1.98	1.78	4.36	8
C-β-D-Glucosyldi-*O*-methylphloracetophenone pentaacetate (XXVa)	2.07	2.07	2.04	1.80	*d*	17
Mangiferin octaacetate (XXVIa)	2.07	2.05	2.01	1.79	4.92 (10)	
Aspalathin nonaacetate (XXVIIa)	2.06	2.03	2.00	1.78	4.72 (9–10)	*j*
3,4,2',4',6'-Penta-*O*-methylaspalathin tetraacetate (XXVIIIa)	2.09	2.06	2.03	1.78	5.34 (9–10)	*j*
Isohemiphloin heptaacetate (XXIXa)	2.03	2.03	2.00	1.80	~5.4	8
Hemiphloin heptaacetate (XXXa)	2.05	2.05	2.03	1.83	*d*	8
Combined ranges for groups B and C	(2.03–2.08)	(2.03–2.07)	(1.98–2.04)	(1.77–1.83)		
Other Compounds						
D-Glucose 2,3,4,6-tetraacetate	2.10	2.10	2.03	2.02	...	
Puerarin hexaacetate (XXXIa)	2.07	2.07	2.05	1.72	*d*	8

[a] Spectra from this laboratory were determined at 30–35°; spectra quoted from the literature are assumed to be at about the same temperature. Tetramethylsilane was used as internal standard. [b] Numbers are text references. [c] This band is split, the other branch occurring at δ 2.03. [d] Not reported. [e] This band is split, but the other branch was not clearly discernible at this temperature. [f] Insufficient sample was available to discern this proton clearly. [g] See ref 12. [h] A detailed temperature study of the splitting of this and other bands in the spectrum is given in ref 9. [i] W. E. Hillis, and D. H. S. Horn, *Aust. J. Chem.*, **19**, 705 (1966). [j] B. H. Koeppen and D. G. Roux, *Tetrahedron, Lett.*, 3497 (1965); *Biochem. J.*, **99**, 604 (1966).

TABLE IV
CHEMICAL SHIFT OF AROMATIC AND METHOXYL PROTONS OF ACETYLATED FLAVONES IN DEUTERIOCHLOROFORM[a]

Compd	Chemical shift and coupling [δ, ppm (*J*, Hz)] H-3	H-6	H-8	H-2'	H-6'	H-3'	H-5'	CH_3O
Apigenin triacetate (VIa)	6.61	6.86 (2.5)	7.35 (2.5)	7.87 (8.5)	7.87 (8.5)	7.26 (8.5)	7.26 (8.5)	...
Acacetin diacetate (XXXIIa)	6.55	6.84 (2.5)	7.31 (2.5)	7.80 (9)	7.80 (9)	7.00 (9)	7.00 (9)	3.88
Luteolin tetraacetate (VIIa)	6.60	6.86 (2.5)	7.36 (2.5)	7.73 (~2.5)	~7.78 (~2.5, 9)	...	7.36 (9)	...
Chrysoeriol triacetate (VIIIa)	6.58	6.83 (2.5)	7.33 (2.5)	~7.38 (~2.5)	~7.45 (~2.5, 9)	...	7.13 (9)	3.90
Diosmetin triacetate (IXa)	6.55	6.83 (2.5)	7.33 (2.5)	7.56 (2.5)	7.71 (~2.5, 8)	...	7.05 (8)	3.90
6-*C*-β-D-Glucosyldiosmetin heptaacetate (IIa)	6.54	...	7.33	7.53 (~2)	7.71 (~2, 8.5)	...	7.06 (8.5)	3.90
Vitexin heptaacetate (Xa)	6.70[b]	6.84[b]	...	8.11; 7.95 (~9)[c]	8.11; 7.95 (~9)[c]	7.40 (9)	7.40 (9)	...
Isovitexin heptaacetate (XIa)	6.65	...	7.38	7.90 (9)	7.90 (9)	7.30 (9)	7.30 (9)	...

[a] Tetramethylsilane used as internal standard, temperature 30–35°. [b] Broad, poorly defined band. [c] H-2' and H-6' are nonequivalent at this temperature.

apigenin nucleus. We tentatively conclude that IV is 6,8-di-*C*-glycosylapigenin.

A number of flavones that are considered to be 6,8-di-*C*-glycosyl derivatives are known.[18,19] One of these, vicenin-2,[20] obtained from *Vitex lucens*, is thought to be a 6,8-di-*C*-glycosylapigenin in which the glycosyl residues are identical. A chromatographic comparison of IV with vicenin-2 indicates probable identity.[21] A noncrystalline *C*-glycosylapigenin from

(18) M. K. Seikel and T. J. Mabry, *Tetrahedron Lett.*, 1105 (1965).
(19) L. Horhammer, H. Wagner, L. Rosprim, T. Mabry, and H. Rösler, *ibid.*, 1707 (1965).
(20) M. K. Seikel, J. H. S. Chow, and L. Feldman, *Phytochemistry*, **5**, 439 (1966).
(21) Vicenin-2 was isolated from an extract of *Vitex lucens* wood by elution from a thin layer plate. It was not obtained crystalline but its properties correspond with those reported by Seikel and coworkers[20] for vicenin-2.

lemon peel, isolated recently by Chopin,[6] is also considered[1] to be identical with vicenin-2.

Compound V was obtained from lemon peel as an amorphous solid. Ultraviolet spectral data suggest that it is derived from diosmetin, whereas chromatographic and electrophoretic data indicate that it has two glycosyl residues. We regard 6,8-di-*C*-glycosyldiosmetin as a tentative structure for V. It is probably identical with the *C*-glycosyldiosmetin reported by Chopin.[6]

The results described here illustrate again the wide distribution of *C*-glycosylflavones. The presence in the lemon of apigenin *O*-glycoside[22] and 6,8-di-*C*-glycosylapigenin, as well as diosmin,[23] 6-*C*-glucosyldiosmetin, 8-*C*-glucosyldiosmetin, and 6,8-di-*C*-glycosyldiosmetin are interesting examples of the co-occurrence of *O*- and *C*-glycosyl derivatives of the same flavone. As a result of this and earlier isolation studies[22-26] it is known that lemons contain at least twenty different flavonoids or related compounds. It is not clear whether this demonstrates an unusually versatile synthetic capacity of the lemon or merely reflects the fact that this plant has been scrutinized more closely than most.

Experimental Section

Isolation of 8-C-Glucosyldiosmetin (I), 6-C-Glucosyldiosmetin (II), and 6,8-Gi-C-glycosylapigenin (IV) from Lemons.—The isolation procedure has been described in detail in an earlier publication.[24] "Calcium Flavonate Glycoside, Lemon" (a mixture of the calcium salts of crude lemon flavonoids)[27] in aqueous solution at pH 3 was extracted with 1-butanol. Evaporation of the 1-butanol extract gave a mixture of glycosides. The mixture (6 g) was separated into its constituents by chromatographing it on a column of 100 mesh silicic acid (1090 g) developed with methanol–chloroform in a stepwise gradient elution. The progress of the elution was monitored by paper chromatography (10% acetic acid) and uv spectra.

Compound I was eluted at a concentration of 10–11% methanol in chloroform. It followed limocitrin 3-β-D-glucoside.[25] Compound II followed I at a concentration of 11% methanol. Compound IV was eluted at 23–26% methanol, following eriocitrin. All three compounds appeared as dark spots on paper chromatograms under uv light. After assembling fractions and taking them to dryness the products were crystallized (see Table V).

TABLE V

Compd	Solvent	Mp, °C	Yield, mg
I	Methanol	267–268	7
II	Methanol	243–245	15
IV	Water	233–236	2

Acetyl derivatives Ia, IIa, and IVa were prepared by allowing the compounds to stand in acetic acid–pyridine at room temperature, followed by evaporation of the reagents under vacuum. None of the acetyl derivatives could be obtained in crystalline form.

Acid Treatment of Compounds I, II, and IV.—The samples were dissolved in ethanol made 3 *N* in hydrochloric acid by adding the concentrated reagent. The solutions were heated on the steam bath and cooled, the precipitates were filtered, and the filtrate was extracted with ethyl acetate. The ethyl acetate extract and the precipitate were combined and used for paper chromatography in these solvents: 10 and 30% acetic acid, butanol–acetic acid–water, and ethyl acetate–formic acid–water (see Table II). The chromatograms showed that compound I was partially converted into II after 5 hr of heating; compound II was partially converted into I after 1 hr; and compound IV remained essentially unchanged after 1 hr.

Alkaline Hydrolysis of Compounds I and II.—The acetyl derivatives Ia and IIa (about 5 mg each) were boiled in 40% aqueous potassium hydroxide (1 ml) for 45 min. The products were worked up in the usual way and chromatographed on paper with benzene–acetic acid–water (2:2:1 upper phase). Isovanillic acid was identified in both runs by its R_f value (0.54).

Isolation of 6,8-Di-C-glycosyldiosmetin (V) from Lemons.—"Lemon Bioflavonoid Complex"[27] (100 g) was extracted with boiling methanol. Evaporation of the methanol gave 63 g of crude glycosides. This was dissolved in water (500 ml) and the solution adjusted to pH 4.65. Crude fungal hemicellulase[28] (15 g) was added and the mixture kept at room temperature for 3 days. It was then extracted with four 50-ml portions of ether and ten 50-ml portions of ethyl acetate to remove flavonoid aglycones formed by hydrolysis of *O*-glycosides.

The remaining aqueous layer was concentrated under vacuum to a volume of 100 ml and this was diluted with 500 ml of methanol. The voluminous precipitate was centrifuged down and discarded. The supernatant was taken to dryness and the residue (47 g) was dissolved in water (120 ml) and extracted for 2 days with ethyl acetate in a liquid–liquid extractor. The aqueous layer was extracted with fifteen 30-ml portions of 1-butanol, the combined butanol extract was taken to dryness, and the residue was redissolved in water (40 ml) and reextracted with ten 25-ml portions of 1-butanol. This yielded a residue of 7 g, which was dissolved in 50% methanol (50 ml) and treated with 30% aqueous basic lead acetate (20 ml). The filtered precipitate was treated with hydrogen sulfide in the usual way to give 2.2 g of partially purified flavonoids. This material was further purified on a polyamide column (3.7 × 27 cm) eluted with 10–15% aqueous methanol. Evaporation of the eluate gave a residue of 0.7 g which was adsorbed in the usual way (see experiment below on isolation of I and II from oranges) onto 4 g of 100 mesh silicic acid. The dry powder containing the adsorbed flavonoids was slurried with ethyl acetate (25 ml) and this was added to the top of a 3.7 × 30 cm column of silicic acid prepared in ethyl acetate. The compounds were eluted with methanol–ethyl acetate using a stepwise gradient elution. 6,8-Di-*C*-glycosylapigenin (IV) was eluted when the concentration of methanol reached 12%; it crystallized from water (5 mg, mp 236°). 6,8-Di-*C*-glycosyldiosmetin (V) was eluted at a concentration of 13% methanol. The assembled fractions containing V were taken to dryness and the residue was dissolved in ethanol from which it precipitated as a gel (7 mg). Attempts to crystallize V and its acetyl derivative Va (prepared in hot acetic anhydride–pyridine) were unsuccessful.

Isolation of 8-C-Glucosyldiosmetin (I) and 2″-*O*-β-D-Xylosylvitexin (III) from Oranges.—"Orange Bioflavonoid Complex, Navel"[27] (25 g) was extracted with two 250-ml portions of chloroform under reflux and then with hot ethanol (100 ml). Evaporation of the ethanol afforded 16 g of crude glycosides. The glycosides (10 g) in methanol (100 ml) were adsorbed onto 100 mesh silicic acid (55 g) by adding the silicic acid in portions with shaking and finally evaporating under vacuum at room temperature to a dry powder. The dry powder was shaken with 100-ml portions of chloroform until the extracts were colorless. It was then slurried in chloroform and introduced to the top of an 8 × 71 cm column of silicic acid (1750 g) which had been prepared as a slurry in chloroform (7000 ml). The compounds were separated by methanol–chloroform in a stepwise gradient elution.

Compound I was eluted together with a blue fluorescing impurity at 18–20% methanol. It crystallized in very low yield from methanol and was identical in every respect (melting point, R_f, and ir and uv spectrum) with compound I obtained from lemons.

Compound III, accompanied by a moderate amount of naringenin 7-β-rutinoside,[29] was eluted with 24–25% methanol. It appeared as a dark spot on paper chromatograms under uv light. Assembled fractions containing III were extracted with several portions of ethyl acetate which removed most of the

(22) R. M. Horowitz and B. Gentili, *J. Org. Chem.*, **25**, 2183 (1960).
(23) R. M. Horowitz, *ibid.*, **21**, 1184 (1956).
(24) R. M. Horowitz and B. Gentili, *J. Amer. Chem. Soc.*, **82**, 2803 (1960).
(25) R. M. Horowitz and B. Gentili, *J. Org. Chem.*, **26**, 2899 (1961).
(26) B. Gentili and R. M. Horowitz, *Tetrahedron*, **20**, 2313 (1964).
(27) Manufactured by Sunkist Growers, Ontario, Calif. References to specific products or brands does not constitute endorsement by the U. S. Department of Agriculture.
(28) Crude preparations of the enzyme give better results in the hydrolysis than purified preparations.
(29) B. Gentili and R. M. Horowitz, *Bull. Nat. Inst. Sci. India*, No. 31, 78 (1965).

accompanying naringenin rutinoside. Compound III failed to crystallize, even when seeded with crystalline 2''-*O*-β-D-xylosylvitexin obtained from *Vitex lucens*. It was freely soluble in water. Compound III was indistinguishable from 2''-*O*-β-D-xylosylvitexin on paper chromatography in a variety of solvents or on paper electrophoresis (Table II). The two compounds were also indistinguishable (R_f = 0.18) on polyamide tlc using nitromethane–methanol (2:1).

Hydrolysis of 2''-*O*-β-D-Xylosylvitexin (III).—A sample of compound III in aqueous 2 *N* hydrochloric acid was heated on the steam bath for 30 min. The solution was extracted with ethyl acetate. Evaporation of the extract afforded a mixture of vitexin and isovitexin, as shown by paper chromatography and electrophoresis. When the hydrolysis was carried out enzymatically at pH 4.6 using crude hemicellulase,[28] the ethyl acetate extract contained mainly vitexin together with a very small proportion of isovitexin. The presence of xylose in the aqueous layers remaining from these hydrolyses was demonstrated by paper chromatography.

Registry No.—I, 15822-81-8; Ia, 15895-78-0; II, 15822-82-9; IIa, 15895-77-9; III, 11044-10-3; VI, 520-36-5; VIa, 3316-46-9; VII, 491-70-3; VIIa, 1061-93-4; VIII, 491-71-4; VIIIa, 3162-04-7; IX, 520-34-3; IXa, 3162-05-8; X, 1397-60-0; Xa, 11040-83-8; XI, 11044-04-5; XIa, 11044-05-6; XIVa, 11044-08-9; XVa, 11044-03-4; XVIIa, 11044-09-0; XXIIa, 11044-06-7; XXVa, 6980-38-7; XXVIa, 11044-07-8; XXXIIa, 5892-39-7; D-glucose 2,3,4,6-tetraacetate, 10343-06-3.

Dihydroisocoumarins from a *Sporormia* Fungus

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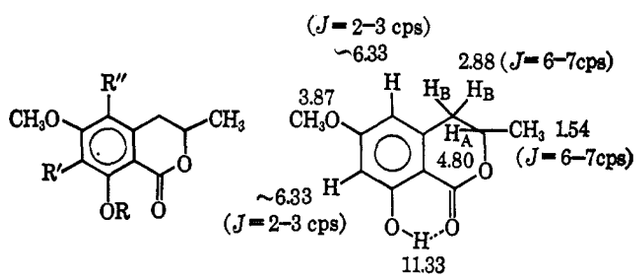
Three dihydroisocoumarins, 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin, 5,7-dichloro-3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin, and 7-chloro-3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin, have been isolated from a *Sporormia* fungus. The structures of the two new chlorinated dihydroisocoumarins have been established by spectral studies and by chemical conversion.

Sondheimer[1] isolated 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (I) from carrots which had developed a bitter taste during storage. Condon, *et al.*,[2-4] later associated the production of this fungitoxic substance in carrots with alterations in the normal metabolism of the carrot root tissue which they felt were possibly induced by the presence of fungi. Recently, Aue, *et al.*,[5] have reported the isolation of this same compound, which is sometimes referred to as 6-methoxymellein, from a submerged culture of the fungus *Sporormia bipartis* Cain. In our work on the metabolic products of *Sporormia affinis* Sacc., Bomm and Rouss, we have isolated not only 6-methoxymellein (I) but also the closely related halogenated compounds 5,7-dichloro-3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (IV) and 7-chloro-3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (V).

These findings and those of Aue, *et al.*,[5] suggest to us that the occurrence of I in fungal infected carrots might be due to the fungus itself. The presence of I in two *Sporormia* species may also be noteworthy in a chemotaxonomic sense.

For our purposes, the fungus *Sporormia affinis* was grown in submerged culture under standard conditions and the metabolic products were isolated after 120 hr by carbon adsorption followed by chromatography. A major product was identical in its physical and chemical properties with 6-methoxymellein; its identity was confirmed by comparison with an authentic specimen.[1]

In determining the structure of the two minor chlorinated metabolites, the nmr and mass spectra of I were quite revealing and it is appropriate to discuss them at this stage. The various peaks in the nmr spectrum are assigned pictorially in formula VIII.



VIII

I, R = R′ = R″ = H
II, R = CH_3; R′ = R″ = H
III, R = CH_3; R′ = R″ = Cl
IV, R = H; R′ = R″ = Cl
V, R = R″ = H; R′ = Cl
VI, R = CH_3; R′ = Cl; R″ = H
VII, R = CH_3; R′ = H; R″ = Cl

The three-proton doublet at 1.54 ppm (J = 6–7 cps) is attributed to the methyl group on the carbon bearing a single proton and attached to the electronegative oxygen. A doublet at 2.88 ppm is assigned to the virtually equivalent methylene protons H_B split by the single proton H_A of the asymmetric center. A sharp three-proton singlet at 3.87 ppm is due to the methoxy group and the one-proton multiplet at 4.80 ppm arises from the coupling of the methyl and methylene group with the single proton H_A. The aromatic region contains split signals (J = 2–3 cps typical for *meta*-coupled protons) for two barely separated protons at about 6.33 ppm and the exchangeable proton of the intramolecularly bonded hydroxyl group is observed at 11.33 ppm. The mass spectrum confirms the molecular weight by a peak at m/e 208 and contains a number of other significant peaks. The most abundant peak at m/e 164 (400%) arises because of loss of acetaldehyde. A metastable peak at 129.3 mass units confirms this loss from the molecular ion. The loss of $CH_3CO\cdot$ accounts for the peak at m/e 165 (100%). Elimination of CO from the molecular ion of I occurs as is evidenced by m/e 180 (9%) but is clearly not a

(1) E. Sondheimer, *J. Amer. Chem. Soc.*, **79**, 5036 (1957).
(2) P. Condon and J. Kuc, *Phytopathology*, **50**, 267 (1960).
(3) P. Condon and J. Kuc, *ibid.*, **52**, 182 (1962).
(4) P. Condon, J. Kuc, and M. H. Draudt, *ibid.*, **53**, 1244 (1963).
(5) R. Aue, R. Mauli, and R. P. Sigg, *Experientia*, **22**, 575 (1966).