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FLAVONOIDS IN GENOTYPES OF *TRIFOLIUM SUBTERRANEUM*—I.

THE NORMAL FLAVONOID PATTERN OF THE GERALDTON VARIETY

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Abstract—Leaves of the Geraldton variety of subterranean clover (*Trifolium subterraneum* L.) have been found to contain kaempferol, quercetin, isorhamnetin, 4′,7-dihydroxyflavonol, fisetin, luteolin, 4′,7-dihydroxyflavone, 3′,4′,7-trihydroxyflavone, isoliquiritigenin and liquiritigenin. Three new compounds, 2′,4,4′-trihydroxy-3-methoxychalcone (homobutein), 4′,7-dihydroxy-3′-methoxyflavone (geraldone) and 4′,7-dihydroxy-3′-methoxyflavonol (geraldol), have also been found and have been synthesized. These flavonoids exist in clover predominantly as glycosides, nine of which have been isolated and characterized by chromatographic and spectrophotometric methods. Five of the glycosides have not been described previously.

INTRODUCTION

ALTHOUGH subterranean clover is known to contain large amounts of various isoflavones,[1] and coumestrol[2] and anthocyanins[3] in its leaves, little is known of its other flavonoids. Recent experiments using the Geraldton variety of subterranean clover have produced mutant lines deficient in isoflavones.[4] In order to understand more fully the genetic effects produced in these mutants, information on the other flavonoids present in subterranean clover is essential since these compounds are biogenetically closely related to the isoflavones. In this paper we report the isolation and identification of some of the hitherto unrecognized flavonoids of this plant.

RESULTS

Geraldton subterranean clover contains a variety of flavonoids, but most of these occur only as minor constituents compared with the isoflavones. In the mutant B763 however,[4] the flavonoid pattern is qualitatively similar to that of Geraldton but differs quantitatively in that the isoflavone levels are reduced and those of other flavonoids increased.[5] This mutant

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1 E. WONG, *J. Sci. Food Agr.* **14**, 376 (1963); A. B. BECK, *Australian J. Agri. Res.* **15**, 223 (1964); C. M. FRANCIS and A. J. MILLINGTON, *Australian J. Agri. Res.* **16**, 565 (1965).

2 R. L. LYMAN, E. M. BICKOFF, A. N. BOOTH and A. L. LIVINGSTON, *Arch. Biochem. Biophys.* **80**, 61 (1959).

3 J. A. CARPENTER, Ph.D. Thesis, University of Western Australia (1961).

4 C. M. FRANCIS and A. J. MILLINGTON, *Australian J. Agri. Res.* **16**, 565 (1965).

5 E. WONG and C. M. FRANCIS, unpublished results.

was therefore used as a more convenient plant source for the isolation and characterization of the flavonoids.

The known compounds kaempferol, quercetin, isorhamnetin, 4′,7-dihydroxyflavonol (IIIa), fisetin, luteolin, 4′,7-dihydroxyflavone (IIa), 3′,4′,7-trihydroxyflavone (IIb), isoliquiritigenin (Ia) and liquititigenin were identified by chromatographic (Table 1) and spectrophotometric methods. Two-dimensional paper chromatographic and spectrophotometric comparisons with authentic specimens confirmed the identification. The two 5-deoxyflavones (IIa) and (IIb) have only recently been described as natural products.[6] These compounds were isolated from clover by means of column and paper chromatography and

TABLE 1. CHROMATOGRAPHIC PROPERTIES OF FLAVONOIDS FROM SUBTERRANEAN CLOVER

Compounds identified	R_f in		Colour reaction*		
	BeAW	30% HOAc	u.v.	u.v. + NH_3	Others†
Quercetin	0·08	0·12	Y	Y	
Fisetin	0·08	0·15	bY	bY	a
3′,4′,7-Trihydroxyflavone	0·13	0·26	bV-B	bY-Gr	
Luteolin	0·17	0·18	dk	Gr-Y	
4′,7-Dihydroxyflavone	0·28	0·37	bW-V	bB-Gr	
4′,7-Dihydroxyflavonol	0·31	0·19	bY	bY	a
Kaempferol	0·33	0·15	Y	Y	
Isoliquiritigenin	0·44	0·22	dk	O	
Liquiritigenin	0·44	0·70	V	V	b
Geraldone (IIc)	0·43	0·29	blB	bGr-Y	
Geraldol (IIIc)	0·45	0·16	bY	bY	a
Isorhamnetin	0·45	0·12	Y	Y	
Homobutein (Ib)	0·54	0·18	dk	O-R	

* b = bright, dk = dark, l = light, B = blue, R = red, Y = yellow, Gr = green, V = violet, O = orange, W = white.

† a = Strong Y-Gr fluorescence, u.v. + visible light, b = $NaBH_4$/HCl → magenta.

TABLE 2. ULTRAVIOLET SPECTRAL PROPERTIES OF SOME FLAVONOID CONSTITUENTS OF CLOVER

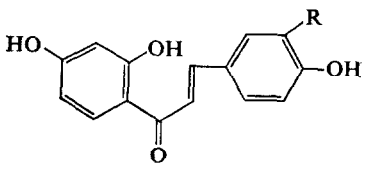
Compounds identified	λ_{max} (nm) in		
	EtOH (85%)	+ NaOH*	+ $AlCl_3$†
2′,4′,4-Trihydroxy-3-methoxychalcone (homobutein)	260, 380	278, 452	260, 418
4′,7-Dihydroxy-3′-methoxyflavonol (Geraldol)	246, 320, 362	unstable	260, 417
4′,7-Dihydroxy-3′-methoxyflavone (Geraldone)	236, 340	256, 337, 401	—
4′,7-Dihydroxyflavone	232, 329	253, 332, 392	—
3′,4′,7-Trihydroxyflavone	236, 342	256, 398	—

* Approx. 0·003 N in 85% EtOH.

† Approx. 0·2% in 85% EtOH.

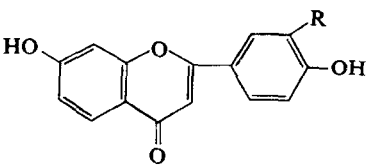
[6] A. L. LIVINGSTON and E. M. BICKOFF, *J. Pharm. Sci.* **53**, 1557 (1964); E. M. BICKOFF, A. L. LIVINGSTON and S. C. WITT, *Phytochem.* **4**, 523 (1965).

identified by direct comparison with authentic samples kindly provided by Dr. E. M. Bickoff. The spectral properties of these two compounds are included in Table 2 for comparative purposes.



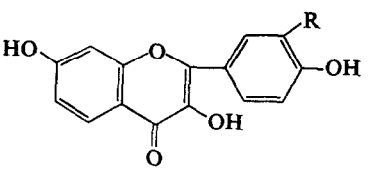
(I)

(a) R=H
(b) R=OCH_3



(II)

(a) R=H
(b) R=OH
(c) R=OCH_3



(III)

(a) R=H
(b) R=OH
(c) R=OCH_3

Column chromatography on polyamide followed by preparative paper chromatography resulted further in the isolation of three new natural products, to which the trivial names homobutein, geraldone and geraldol have been given. The colour reactions of these compounds (Table 1) resemble respectively those of the compounds (Ia), (IIa) and (IIb), and (IIIa) and (IIIb), suggesting their identity as 5-deoxyflavonoids. Their chromatographic (Table 1) and spectral properties (Table 2) suggest that they are the corresponding chalcone, flavone and flavonol having the 4′,7-dihydroxy-3′-methoxy substitution pattern (flavone numbering). 2′,4′,4-Trihydroxy-3-methoxychalcone (Ib), 4′,7-dihydroxy-3′-methoxyflavone (IIc), and 4′,7-dihydroxy-3′-methoxyflavonol (IIIc) were synthesized by standard methods (see Experimental) and were found to correspond in chromatographic and spectral properties with homobutein, geraldone and geraldol respectively. Further proof of identity was obtained from comparisons of the i.r. spectra of natural and synthetic materials. The flavonol however was not isolated in sufficient quantity from clover for confirmation by this means. 4′,7-Dihydroxy-3′-methoxyflavone has not been described previously.

Glycosides of most of the compounds identified above were obtained from the aqueous fraction of the clover extract (fraction B, see Experimental). Separation was again by means of column and paper chromatography. The chromatographic properties of these glycosides are given in Table 3.

Analysis of the spectral data[7] for the glycosides (Table 4) allowed the positions of glycosidic linkage to be positively assigned in all cases. Sugars and aglycones were identified chromatographically after acid hydrolysis and in every case the sugar component present was found to be glucose. With the exception of the kaempferol and quercetin glycosides the

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

TABLE 3. CHROMATOGRAPHIC PROPERTIES OF THE FLAVONOID GLYCOSIDES OF CLOVER

Compounds identified	R_f in				Colour reaction*	
	5% HOAc	BAW (6:1:2)	30% HOAc	BeAW	u.v.	u.v. + NH_3
Isoliquiritigenin 4-glucoside	—	—	0·52	0·10	dk	O
Homobutein 4-glucoside	—	—	0·43	0·13	dk	O—R
3′,4′,7-Trihydroxyflavone 7-glucoside	0·04	0·36	0·49	—	lB	Y
4′,7-Dihydroxyflavone 7-glucoside	0·12	0·55	—	—	lB-Gr	Gr
Geraldone 7-glucoside	0·07	0·42	—	—	W-B	bY
4′,7-Dihydroxyflavonol 4′-glucoside	0·04	0·50	0·53	—	bY	Y
Geraldol 4′-glucoside	0·04	0·40	0·48	—	bY	Y
Kaempferol 3-glucoside	0·26	0·74	0·67	0·07	dk	Y
Quercetin 3-glucoside	0·20	0·61	0·60	0·03	dk	Y

* For key, see Table 1.

amounts of material isolated were not sufficient for further study. Sugar/aglycone ratios were not established but the general chromatographic properties of the glycosides would suggest that they are monoglucosides. The following glucosides were isolated and thus identified:

Isoliquiritigenin 4-glucoside. The bathochromic shift with concomitant decrease of ϵ in EtOH–NaOH indicates that the 4′-OH is free. Synthetic isoliquiritigenin-4′-glucoside gives a shift with increased ϵ under the same conditions. Presence of a free 2′-OH is also indicated by the shift with $AlCl_3$. This glucoside has recently been reported as a constituent of *Glycyrrhiza glabra.*[8]

Homobutein 4-glucoside. Spectral data similarly indicate that the 2′- and 4′-hydroxyl groups are free.

4′,7-Dihydroxyflavone 7-glucoside. The absence of a hypsochromic shift of the long wavelength band compared with the aglycone (Table 2) indicates that it is not a 4′-glucoside. This was confirmed by the large bathochromic shift in EtOH–NaOH without a decrease in relative intensity, indicating a free 4′-OH.

Geraldone 7-glucoside and *3′,4′,7-trihydroxyflavone 7-glucoside.* Both assigned as the 7-glucoside for the same reasons as given above. Confirmation of the presence of a free 3′,4′-dihydroxy grouping in the latter compound was provided by the bathochromic shift with $NaOAc–H_3BO_3$.

4′,7-Dihydroxyflavonol 4′-glucoside. The spectrum of this compound in alkali is stable; therefore either the 3- or 4′-hydroxyl must be glycosylated. That the 3-OH is free is indicated by the large shift with $AlCl_3$. This 4′-glucoside has been provisionally identified in gorse flowers.[9].

Geraldol 4′-glucoside. Assigned as the 4′-glucoside for similar reasons as above.

Kaempferol 3-glucoside. The colour under u.v. suggests a 3-glycoside, confirmed by the spectral stability in alkali. The small bathochromic shift with $AlCl_3$ is ascribable to the 5-OH. This glucoside, recrystallized from aqueous ethanol, had m.p. 179–180° after thorough drying (astragalin,[10] m.p. 178°).

[8] V. I. LITVINENKO, *Chem. Abstr.* **60**, 6700 (1964).
[9] J. B. HARBORNE, *Phytochem.* **1**, 203 (1962).
[10] F. M. DEAN, *Naturally Occurring Oxygen Ring Compounds*, p. 324, Butterworth, London (1963).

TABLE 4. ULTRAVIOLET SPECTRAL PROPERTIES OF THE FLAVONOID GLYCOSIDES OF CLOVER

Compounds identified	λ_{max} (nm) in			
	EtOH (85%)	+NaOH	+NaOAc+H_3BO_3‡	+$AlCl_3$
Isoliquiritigenin 4-glucoside	370	434+	—	415
Homobutein 4-glucoside	262, 380	275, 440†	—	420
3′,4′,7-Trihydroxyflavone 7-glucoside	243,* 307*, 343	295, 403	305, 369	—
4′,7-Dihydroxyflavone 7-glucoside	230, 254, 329	295, 305, 390	—	—
Geraldone 7-glucoside	234, 310*, 341	297, 410	—	—
4′,7-Dihydroxyflavonol 4′-glucoside	253, 317*, 355	275, 430	—	400
Geraldol 4′-glucoside	248, 320*, 358	278, 430	—	265, 416
Kaempferol 3-glucoside	266, 350	275, 329, 405	—	275, 302, 345, 392
Quercetin 3-glucoside	258, 362	272, 330, 412	264, 384	269, 300, 400

* Shoulder.
† ϵ decreased relative to parent peak in neutral spectrum.
‡ Excess solids in 85% EtOH.

Quercetin 3-glucoside. Assigned as the 3-glucoside for similar reasons as above. Further confirmed by the detection of the 3′,4′-dihydroxyl grouping with $NaOAc-H_3BO_3$. The compound was recrystallized from ethanol, m.p. 216–220° (isoquercitrin, m.p. 223–224°).[11]

Excepting for the glucosides for which references are given, the other glucosides have not apparently been previously described.

DISCUSSION

The flavonoids found in this work, together with the previously known isoflavones of subterranean clover,[1] all have one of the four possible substitution patterns derivable from a 5- or 5,7- substituted ring A combined with a 4′- or 3′,4′-substituted ring B. These are illustrated in the flavonol series, for example, by 4′,7-dihydroxyflavonol, fisetin, kaempferol and quercetin. The biogenetic relationship of these series of compounds and the effects of genetic factors on their relative distribution will be discussed in subsequent papers. The co-occurrence of homobutein, geraldone and geraldol, together with isorhamnetin, indicates that methylation of the 3′-OH is a common feature in clover. If this methylation is mediated by a single enzyme or enzyme system then it would appear to be a non-specific process with respect to the class of flavonoid. On the other hand, a 4′-O-methylating system specific to the isoflavones would seem also to be operative in this plant. This is clearly illustrated by the co-occurrence of genistein and biochanin A, daidzein and formononetin, and the 4′-O-methyl ether (pratensein) of 3′,4′,5,7-tetrahydroxyisoflavone.

EXPERIMENTAL

Extraction

Fresh subterranean clover plants (*ca.* 10 kg of Geraldton mutant B 763) were extracted with aqueous ethanol at 70° and the extract washed several times with petrol. ether. The ethanol was removed *in vacuo* at 50° and the aqueous residue extracted with ether to give the ether-soluble material (fraction A) and the aqueous fraction (fraction B).

Two-Dimensional Paper Chromatographic Analysis

Aliquots of A, equivalent to 3–6 g of plant material were analysed by two-dimensional paper chromatography in the systems BeAW and 30 per cent HOAc.[12] R_f values of compounds found are given in Table 1. The glucosides of quercetin, kaempferol, isoliquiritigenin and homobutein were all partly extracted into this fraction and could also be detected on the paper chromatogram (Table 2). A sample of B was hydrolysed with acid, extracted into ether, and similarly analysed by two-dimensional paper chromatography. A qualitatively similar pattern of flavonoids was obtained as for A, indicating that glycosides of these compounds existed in B.

Separation and Isolation of Components of Fraction A

(*a*) *Column chromatography.* A was taken up in ethanol and chromatographed on a column of polyamide (36 cm × 2·8 cm). Elution with ethanol resulted in fifty 25-ml fractions which were examined by paper chromatography. Fractions containing a similar range of components were bulked and used as starting materials for isolation by means of preparative paper chromatography.

(*b*) *Paper chromatography.* Column fractions were chromatographed successively in BeAW and 30 per cent HOAc on acid-washed Whatman 3MM paper. When necessary, individual components were further rechromatographed in these solvent systems until chromatographically and spectrophotometrically pure. In this way homobutein, geraldone, geraldol, 4′,7-dihydroxyflavone, 3′,4′,7-trihydroxyflavone, and the glucosides of kaempferol, isoliquiritigenin and homobutein were isolated. Geraldol adsorbs strongly to paper and elutes incompletely; only a very small amount of material was recoverable after successive chromatography.

[11] B. K. NORTJE and B. H. KOEPPEN, *Biochem. J.* **97**, 209 (1965).
[12] E. WONG, P. I. MORTIMER and T. A. GEISSMAN, *Phytochem.* **4**, 89 (1965).

Isolation of Glycosides from Fraction B

One half of the total aqueous fraction (250 ml) was passed through a large polyamide column (48 cm × 4·5 cm) which was then washed exhaustively with water to remove sugary material. The column was then developed with ethanol, the first 500 ml of elutate was discarded, then twenty-five 100-ml fractions were collected. These were chromatographed on 3MM paper successively in 5 per cent HOAc, BAW (6:1:2),[12] and 30 per cent HOAc. Further chromatography in one or more of these solvent systems was usually necessary before chromatographically and spectrophotometrically pure materials were obtained. R_f values for the glycosides isolated are given in Table 3.

Hydrolysis of Glycosides

Pure samples of the glycosides were each hydrolysed with 1 ml N H_2SO_4 in 50 per cent ethanol under reflux for 45 min. The mixture, after removal of ethanol, was extracted with ether and the aglycone thus separated was identified on chromatographic and spectral evidence. The aqueous fraction was treated with solid $BaCO_3$, filtered, and examined by chromatography in EtOAc–pyridine–H_2O (120:50:40), EtOAc–HCO_2H (9:1·5:0·5:2), and BuOH–pyridine–benzene–H_2O (5:3:1:3). In all cases glucose was detected as the sugar present.

Synthetic 2′,4,4′-Trihydroxy-3-methoxychalcone (Ib, *Homobutein*)

This chalcone was prepared by condensation of resacetophenone (1·10 g) and vanillin (1·52 g) in alcoholic KOH (20 ml 50 per cent KOH, 5 ml EtOH) for 48 hr at 70°. The resulting product was purified by polyamide column chromatography, then recrystallized from ethanol, yielding orange–yellow crystals (0·5 g), m.p. 209°. The acetate, prepared by heating with acetic anhydride–pyridine for 1 hr at 100°, had m.p. 133° after recrystallization from ethanol (lit.[13] chalcone m.p. 210–211°, acetate 133°).

Synthesis of 4′,7-Dihydroxy-3′-methoxyflavone (IIc, *Geraldone*)

This flavone has not previously been described. It was synthesized from the above chalcone via the acetate and dibromide by procedures used for the synthesis of the isomeric 3′,7-dihydroxy-4′-methoxyflavone.[14] (1) *2′,4,4′-Triacetoxy-3-methoxychalcone dibromide.* To a stirred solution of homobutein acetate (214 mg) in CS_2 (5 ml) was added 0·8 ml of 10 per cent w/v solution of Br_2 in CS_2. The mixture was exposed to u.v. light for a short period and stirred for 4 hr at room temperature. The solvent was then removed and the solid residue repeatedly recrystallized from ethanol yielding colourless crystals, m.p. 164–165°. (2) *Debromination to geraldone.* The above dibromide (*ca.* 200 mg) was dissolved in 10 ml of 5 per cent NaOH in aqueous ethanol (1:1) and left to stand for 3 days. The solid product obtained on acidification was dissolved in ethanol (6 ml) and chromatographed as bands on twelve sheets of 3MM paper in 30 per cent HOAc. The eluted product was recrystallized twice from ethanol, yielding minute off-white crystals, m.p. 293–294° (decomp).

Synthetic 4′,7-Dihydroxy-3′-methoxyflavonol (IIIc, *Geraldol*)

To an ice-cold solution of homobutein (80 mg) in 3 ml 5 per cent NaOH was added 0·2 ml 30 per cent H_2O_2. The solution was allowed to stand for 4 days, then acidified. The mixture was extracted with ether and the ether-soluble material was chromatographed on four papers in BeAW. The flavonol band, detected by its bright yellow fluorescence under u.v. light, was eluted and the product recrystallized from aqueous alcohol, m.p. 307° (lit.[13] m.p. 307–308°).

Acknowledgements—The authors are indebted to Dr. E. M. Bickoff, U.S.D.A., Albany, for samples of 5-deoxyflavones, and to Dr. T. J. Mabry, University of Texas, for a gift of isorhamnetin.

[13] S. YAMAGUCHI, *Nippon Kagaku Zasshi* **84**, 148 (1963); *Chem. Abstr.* **60**, 5443 (1964).
[14] S. YAMAGUCH, *Nippon Kagaku Zasshi* **84**, 152 (1963); *Chem. Abstr.* **60**, 5444 (1964).