Phytochemistry, 1968, Vol. 7, pp. 803 to 808. Pergamon Press. Printed in England

NEW ISOFLAVONES FROM THE GENUS *BAPTISIA* (LEGUMINOSAE)

K. R. MARKHAM,* T. J. MABRY and T. W. SWIFT, III

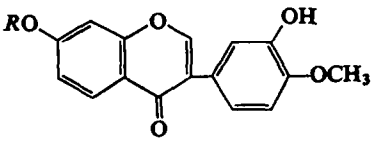
The Cell Research Institute and Department of Botany,
The University of Texas at Austin

(*Received* 8 *November* 1967)

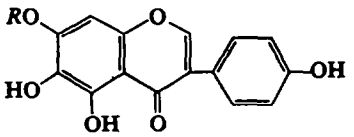
Abstract—6-Hydroxygenistein and its 7-*O*-rhamnosylglucoside have been isolated from *Baptisia hirsuta*, and 7,3′-dihydroxy-4′-methoxyisoflavone (calycosin), its 7-*O*-β-D-glucoside and 7-*O*-rhamnosylglucoside, have been isolated from *B. lecontei*. All are new natural products. 6-Hydroxygenistein and calycosin are the probable precursors of a number of known naturally occurring isoflavones.

INTRODUCTION

Two new biosynthetically significant isoflavone aglycones have been isolated and characterized as a result of our current chemotaxonomic investigation of the genus *Baptisia*. The first, 7,3′-dihydroxy-4′-methoxyisoflavone (I) for which we propose the name *calycosin*, was detected in *Baptisia calycosa* and subsequently isolated from *B. lecontei*, together with its 7-*O*-β-D-glucoside (Ia) and 7-*O*-rhamnosylglucoside (Ib). The second, 5,6,7,4′-tetrahydroxyisoflavone (6-hydroxygenistein, II), was isolated from *B. hirsuta* along with its 7-*O*-rhamnosylglucoside (IIa).



(I) R = H
(Ia) R = glu
(Ib) R = rh-glu



(II) R = H
(IIa) R = rh-glu

Calycosin and 6-hydroxygenistein are of interest biosynthetically since they are both probably the previously unencountered precursors of several well-known naturally occurring isoflavones. For example, four methylated derivatives of 6-hydroxygenistein are known as natural products, but the parent compound (II) has never been found.[1,2] If methylation in flavonoids is a late biosynthetic step, as has recently been suggested,[3,4] then II is the probable precursor of all its methylated derivatives. The occurrence of both II and its 6-*O*-methyl

* Present address: Chemistry Division, D.S.I.R., Petone, New Zealand.

[1] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 92. Academic Press, London (1967).
[2] W. D. OLLIS, in *The Chemistry of Flavonoid Compounds* (edited by T. A. GEISSMAN), p. 357. Pergamon Press, Oxford (1962).
[3] E. MOUSTAFA and E. WONG, *Phytochem.* 6, 625 (1967).
[4] J. B. HARBORNE, in *The Chemistry of Flavonoid Compounds* (edited by T. A. GEISSMAN), p. 593. Pergamon Press, Oxford (1962).

ether, tectorigenin (VII), in the genus *Baptisia*,[5] tends to support this hypothesis. Calycosin is the least highly methylated naturally occurring 7,3',4'-trioxygenated isoflavone known, and as such could well precede in the biosynthetic sequence such isoflavones as 7,3',4'-trimethoxyisoflavone (cabreuvin) and 7-hydroxy-3',4'-methylenedioxyisoflavone (psuedobaptigenin). Indeed, calycosin co-occurs with pseudobaptigenin in *B. lecontei*,[6] and *ortho*-methoxy phenols are proven precursors of their methylenedioxy equivalents in certain alkaloids.[7,8]

RESULTS

Calycosin (I) and its Glycosides (Ia, Ib)

Calycosin (I), its 7-*O*-β-D-glucoside (Ia) and its 7-*O*-rhamnosylglucoside (Ib) were each isolated by repeated two-dimensional paper chromatography of the aqueous methanol extract of *Baptisia lecontei* stem and leaf.

Calycosin appeared as a u.v. fluorescent spot on paper which occurred in the isoflavone aglycone region of the chromatogram, indicating that it was a 5-deoxyisoflavone. This was confirmed by the u.v. spectrum which, with peaks at 212, 247, 258sh, and 288 nm, closely resembled that of 7,3',4'-trihydroxyisoflavone [λ_{max} (MeOH) 215, 247, 257sh, 290 nm]. The PMR spectrum confirmed the 7,3',4'-oxygenation pattern and revealed the presence of a single methoxyl group which must be at the 3'- or 4'-position since the 7-hydroxyl was shown to be free by the bathochromic effect (10 nm) of NaOAc on Band II of the u.v. spectrum.[9]

The position of the free hydroxyl group on the B-ring was determined as C-3' by comparing the PMR spectrum of the aglycone (I) in hexadeuteriodimethylsulfoxide, with that of the acetylated 7-*O*-β-D-glucoside (Ia) in the same solvent. In the PMR spectrum of the aglycone, the H-2', 5', 6', 6 and 8 signals occurred as a complex multiplet in the range 6·8–7·06 ppm, while in the spectrum of the acetylated glycoside, the H-2' and H-8 signals were clearly recognizable at 7·37 and 6·90 ppm, respectively.

Thus, on acetylation of the B-ring hydroxyl group, the H-2' signal had moved at least 0·31 ppm downfield. This shift is typical for flavonoid B-ring protons *ortho*, but not *meta*, to the acetylated hydroxyl group. For example, when 7,4'-dihydroxyisoflavone 7-*O*-glucoside (daidzin)* or 5,7,4'-trihydroxyisoflavone 7-*O*-rhamnosylglucoside (sphaerobioside)* were acetylated, signals of the *ortho* protons (H-2', 6') shifted 0·36 and 0·33 ppm downfield, compared with 0·18 and 0·16 ppm for the *meta* protons (H-3', 5'). Thus the 0·31 ppm (or larger) shift observed for the H-2' signal in calycosin, strongly suggests that H-2' is *ortho* to the free hydroxyl group and therefore that calycosin is 7,3'-dihydroxy-4'-methoxyisoflavone (I).

Compound Ia, calycosin 7-*O*-β-D-glucoside, was recognized as the *O*-β-D-glucoside by acid and β-glucosidase hydrolyses, both of which yielded calycosin and glucose. The sugar was located at the 7-position by the lack of change of the u.v. spectrum of Ia on the addition

* The PMR spectrum of a sample of synthetic diadzin in d_6-DMSO showed the H-2',6' and the H-3',5' signals at 7·45 and 6·86 ppm ($J = 8·5$ c/s), respectively; in the acetylated derivative they appeared at 7·63 and 7·22 ppm, respectively. The values for sphaerobioside (TMS ether in CCl_4) and sphaerobioside acetate (in $CDCl_3$) were reported in part by H. Rosler *et al.* (Ref. 2).

[5] K. R. MARKHAM, unpublished work associated with the current chemotaxonomic survey of the genus *Baptisia*.

[6] K. R. MARKHAM and T. J. MABRY, 7, 791 (1968).

[7] D. H. R. BARTON, G. W. KIRBY and J. B. TAYLOR, *Proc. Chem. Soc.* 340 (1962).

[8] S. KIRKWOOD and M. SRIBNEY, *Nature* **171**, 931 (1953).

[9] L. JURD, in *The Chemistry of Flavonoid Compounds* (edited by T. A. GEISSMAN), pp. 108–154. Pergamon Press, Oxford (1962).

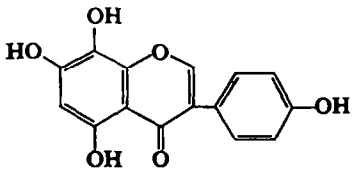
of NaOAc[9] (cf. the 10 nm bathochromic shift of the short wavelength peak observed for the aglycone). A monoglucoside structure for Ib was required by its R_f values on paper in TBA (0·60) and HOAc (0·58), which were similar to those of 7,4′-dihydroxyisoflavone 7-*O*-β-D-glucoside (daidzin), R_f 0·60 (TBA) and 0·64 (HOAc), with which it occurs in *B. lecontei*. These values differ significantly from those of the diglycoside 7,4′-dihydroxyisoflavone 7-*O*-rhamnosylglucoside, R_f 0·4 (TBA) and 0·8 (HOAc).

Calycosin 7-*O*-rhamnosylglucoside (Ib) was incompletely characterized due to the small amount of material isolated. It gave calycosin on acid hydrolysis, together with a 1:1 ratio of rhamnose and glucose, and its R_f values, 0·4 (TBA) and 0·75 (HOAc), were those expected for an isoflavone rhamnosylglucoside. Structure Ib is therefore assigned to this compound on the basis of the above information and the fact that a number of other flavonoid 7-*O*-rhamnosylglucosides have also been isolated from *B. lecontei*.[6]

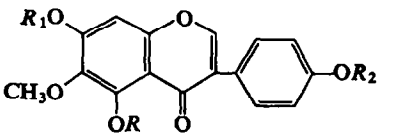
6-*Hydroxygenistein* (*II*) *and its Rhamnosylglucoside* (*IIa*)

6-Hydroxygenistein was isolated from a dark, u.v.-absorbing spot which ran in the isoflavone region of the *B. hirsuta* paper chromatogram. Its u.v. spectrum [λ_{max} 269, 340sh, nm] was typical of isoflavones containing three oxygen substituents in the A-ring such as tectorigenin [λ_{max} 268, 320sh, nm] and iridin [λ_{max} 268, 332sh, nm],[9] the B-ring substitution pattern having little effect on the position of Band II, the major absorption band. The presence of a tri-oxygenated A-ring in this compound was also suggested by its extreme instability towards alkali; for example, the spot on a paper chromatogram turned irreversibly blue when treated with ammonia vapor.

A u.v. spectral analysis of 6-hydroxygenistein revealed the presence of a free 5-hydroxyl group (11 nm $AlCl_3$/HCl shift) and an *ortho*-dihydroxyl system ($AlCl_3$ shift*). The *ortho*-dihydroxyl group was ascribed to the A-ring, since shifts are not usually observed for B-ring *ortho*-dihydroxyl groups in isoflavones, cf. orobol which gave no shift under the same conditions. Confirmation of the oxygenation pattern was provided by the PMR spectrum of its trimethylsilylated derivative, which showed only one A-ring proton signal (6·32 ppm), thereby establishing the A-ring oxidation pattern as either 5,6,7 or 5,7,8. The B-ring was established as 4′-mono-oxygenated by the presence in the PMR spectrum of doublets (J = 9 c/s) at 7·35 ppm and 6·80 ppm representing the H-2′,6′ and H-3′,5′ protons, respectively. Since methoxyl signals were absent in the PMR spectrum, only two structures, 5,6,7,4′-tetrahydroxyisoflavone (II) and 5,7,8,4′-tetrahydroxyisoflavone (III), correspond to the above data.



(III)



(IV) R = H, R_1 = glu, R_2 = H
(V) R = H, R_1 = H, R_2 = CH_3
(VI) R = CH_3, R_1 = H, R_2 = CH_3
(VII) R = H, R_1 = H, R_2 = H

* Details for the application of the spectral analysis of flavonoids in the presence of $AlCl_3$ and $AlCl_3$/HCl for the detection of *ortho*-dihydroxyl groups will be described in a forthcoming publication.

It was not possible to distinguish unequivocally between structures II and III for this substance by u.v. or PMR spectroscopy, but II was favored by the PMR spectrum which exhibited the A-ring proton signal at 6·32 ppm, a value close to that earlier reported for the C-8 proton in tectoridin (IV, 6·40 ppm).[10] Values for the C-6 protons in the 5,7,8-oxygenated isoflavones (not yet found naturally occurring[2]) were not available for comparison. Final proof of structure II was obtained by conversion of its 7-*O*-rhamnosylglucoside (IIa) to a mixture of 5,7-dihydroxy-6,4'-dimethoxyisoflavone (V, irisolidone) and 7-hydroxy-5,6,4'-trimethoxyisoflavone (VI), the structures of which were proven by direct comparison with authentic material synthesized from tectoridin (IV). The synthesis of V and VI from tectoridin was carried out in the same manner that was used to obtain them from the 7-*O*-rhamnosylglucoside (IIa), that is, methylation with diazomethane followed by acid hydrolysis. The two products from the 7-*O*-rhamnosylglucoside were shown to be identical with those from tectoridin by cochromatography on paper and on a polyamide thin-layer, and by u.v. and PMR spectroscopy. The PMR and u.v. spectra of V (synthesized from IIa) were also identical with those of the natural product, irisolidone (V), thereby providing final proof of structure II for the *Baptisia* isoflavone.

6-Hydroxygenistein 7-*O*-rhamnosylglucoside (IIa) was established as a rhamnosylglucoside both by acid hydrolysis, which yielded the aglycone together with a 1:1 mixture of rhamnose and glucose, and by its R_f values, 0·40 (TBA) and 0·64 (HOAc), which approximate to those of other similar rhamnosylglucosides [such as sphaerobioside, R_f 0·45 (TBA), R_f 0·68 (HOAc), also found in *B. hirsuta*]. The sugar location was determined by a u.v. spectroscopic study of the two compounds, (V) and (VI), produced by methylation and hydrolysis of the rhamnosylglucoside. Compound VI, which PMR evidence required to have three methoxyl groups, gave the same u.v. spectrum in the presence of NaOAc as it did with NaOMe and no change was observed on the addition of $AlCl_3$, thus indicating that the single free hydroxyl group in VI must be the 7-hydroxyl. In compound V the 5- and 7-hydroxyls were shown to be free, as evidenced by the 10 and 8 nm bathochromic shifts observed in the u.v. spectrum on the addition of $AlCl_3$/HCl and NaOAc, respectively. On the basis of the above data the 6-hydroxygenistein glycoside from *B. hirsuta* is assigned structure IIa. It is interesting to note that all seven flavonoid glycosides so far identified in *B. hirsuta*[5] are 7-*O*-rhamnosylglucosides; these include the independently characterized apigenin, luteolin and genistein rhamnosylglucosides.

EXPERIMENTAL

Baptisia lecontei plant material* was collected near Tampa, Florida, and *B. hirsuta* material† was collected near Harold, Florida. Melting points are uncorrected and u.v. spectra were determined using a Beckman DB-G spectrophotometer. PMR spectra, unless otherwise stated, were determined relative to tetramethylsilane as internal standard using a Varian A-60 spectrometer. Paper chromatograms were run on Whatman 3 MM paper (46 × 57 cm) at 25° using *t*-BuOH:HOAc:H_2O, 3:1:1 (TBA), 15% HOAc (HOAc) or the benzene layer of a mixture of benzene:HOAc:H_2O, 6:7:3 (Bz) as solvent. Paper chromatographic sugar analyses were carried out using the solvent mixture EtAc:Pyridine:H_2O, 12:5:4, and gas chromatographic sugar analyses were carried out according to the method of Kagan and Mabry.[11]

Extraction procedure. The ground plant material was extracted for 2 days with 20% aqueous methanol at

* Voucher specimen: TEX 257790, Univ. of Texas Herbarium, Austin, Texas.
† Voucher specimen: TEX 257112, Univ. of Texas Herbarium, Austin, Texas.

[10] T. J. MABRY, J. KAGAN and H. RÖSLER, *Nuclear Magnetic Resonance Analysis of Flavonoids*. University of Texas Publication No. 6418, Austin (1964).
[11] T. J. MABRY and J. KAGAN, *Anal. Chem.* 37, 288 (1965).

room temperature. The extract was evaporated under vacuum and applied to paper chromatograms, which were subsequently run in two dimensions (TBA and HOAc).

Calycosin (*I*). On the paper chromatogram calycosin appeared as a pale blue spot [R_f 0·79 (TBA), R_f 0·35 (HOAc), R_f 0·35 (Bz)] in u.v. light, changing to bright greenish blue in NH_3. Calycosin was isolated by elution from twenty 2D paper chromatograms and was obtained as a white solid (1·4 mg), m.p. 228–230°, by sublimation at 180° and 0·045 mm, λ_{max} (MeOH) 212, 247, 258sh, 288 nm; λ_{max} (NaOMe) 257, 330 nm; λ_{max} ($AlCl_3$) 212, 247, 260, 290sh, nm; λ_{max} (NaOAc) 257, 330 nm; λ_{max} (NaOAc/H_3BO_3) 258sh, 288 nm. A 100 Mc PMR spectrum was determined for the trimethylsilylated derivative in CCl_4 on a Varian HA-100 spectrometer. Signals appeared at: 8·09 doublet, J = 9 c/s (H-5), 7·80 singlet (H-2), 7·07 broad doublet, J = 10 c/s (H-6'), 6·66–6·88 multiplet (H-6,8,2',5') and 3·78 singlet (—OCH_3) ppm. An additional PMR of calycosin in d_6-DMSO (60 Mc) showed signals at 8·30 singlet (H-2), 8·0 doublet, J = 9 c/s (H-5), 6·8–7·06 multiplet (H-2',5', 6',6), 3·83 singlet (—OCH_3) ppm.

Calycosin 7-O-*β*-D-*glucoside* (*Ia*). Calycosin glucoside appeared as a pale blue spot [R_f 0·60 (TBA), R_f 0·58 (HOAc)] on paper in u.v./NH_3, which was invisible when viewed in u.v. light alone. The glycoside was isolated in a small amount (0·5 mg) from twenty 2D paper chromatograms and had the following spectra: λ_{max} (MeOH) 248, 254, 300sh, nm; λ_{max} (NaOMe) 255, 327 nm; λ_{max} (NaOAc) 249–253, 300sh, nm; λ_{max} (NaOAc/H_3BO_3) as for MeOH. Both acid and *β*-glucosidase hydrolyses gave the aglycone, which was chromatographically identical with 7,3'-dihydroxy-4'-methoxyisoflavone (calycosin), together with a single sugar which was identified gas chromatographically as glucose.

An acetate was prepared by treating the glucoside (0·5 mg) with pyridine/acetic anhydride at 25° overnight, and the PMR spectrum of the product (in the range 6·2–8·7 ppm) was determined in d_6-DMSO by using a time-averaging computer in conjunction with the 100 Mc spectrometer. Approximately 150 scans revealed signals at 8·32 singlet (H-2), 7·95 doublet, J = 9 c/s (H-5), 7·37 singlet (H-2'), 6·90 singlet (H-8) ppm together with lower intensity broad signals at: 7·26, 7·23, 7·10 and 7·02 (H-5',6',6) ppm.

Calycosin 7-O-*rhamnosylglucoside* (*Ib*). This material was isolated in a small amount (*ca.* 0·1 mg) from a pale blue spot, R_f 0·4 (TBA), R_f 0·75 (HOAc), on the *B. lecontei* chromatogram. It acid hydrolyzed to give 3',7-hydroxy-4'-methoxyisoflavone (calycosin) together with two sugars. The sugars were isolated in a 1:1 ratio and were paper chromatographically identical with rhamnose and glucose.

6-*Hydroxygenistein* (*II*). Crude material (5 mg) was isolated by methanol elution of a deep purple u.v.-absorbing spot [R_f 0·79 (TBA), R_f 0·36 (HOAc), R_f 0·05 (Bz)] on the *B. hirsuta* 2D paper chromatogram, and also by acid hydrolysis of the 7-*O*-rhamnosylglucoside. It was purified by sublimation at 180° and 0·025 mm, and subsequent recrystallization from ethyl acetate yielded 6-hydroxygenistein as fawn-colored crystals, m.p. 243–250° (dec), λ_{max} (MeOH) 212sh, 245sh, 269, 340sh, nm; λ_{max} (NaOMe) 257, 305, 329sh, nm (dec); λ_{max} (NaOAc) 248sh, 301, 325sh, 416 nm (dec); λ_{max} (NaOAc/H_3BO_3) 300sh, 337sh, 415sh, nm; λ_{max} ($AlCl_3$) 238, 274, 297sh, 352 nm; λ_{max} ($AlCl_3$/HCl) 234sh, 280, 329sh, nm. The PMR spectrum of the trimethylsilylated derivative in CCl_4 showed signals at: 7·76 singlet (H-2), 7·35 doublet, J = 9 c/s (H-2', 6'), 6·80 doublet, J = 9 c/s (H-3',5') and 6·32 singlet (H-8) ppm.

6-*Hydroxygenistein* 7-O-*rhamnosylglucoside* (*IIa*). Compound IIa (4 mg) was isolated by methanol extraction of a deep purple u.v.-absorbing spot, R_f 0·40 (TBA), R_f 0·64 (HOAc), from twenty *B. hirsuta* paper chromatograms. This material was purified by 2D paper chromatography and had the following u.v. spectra: λ_{max} (MeOH) 269, 340sh, nm; λ_{max} (NaOMe) 259, 290sh, nm (dec); λ_{max} ($AlCl_3$) 239, 282, 325sh, nm; λ_{max} ($AlCl_3$/HCl) 242, 282, 325sh, nm; λ_{max} (NaOAc) 272, 340sh, nm; λ_{max} (NaOAc/H_3BO_3) 272, 340sh, nm. Acid hydrolysis of IIa gave 6-hydroxygenistein together with a 1:1 mixture of rhamnose and glucose, all of which were identified by paper chromatographic comparison with authentic material.

Conversion of IIa to 5,7-*dihydroxy*-6,4'-*dimethoxyisoflavone* (*V*) *and* 7-*hydroxy*-5,6,4'-*trimethoxyisoflavone* (*VI*). The 7-*O*-rhamnosylglucoside (IIa) (2–3 mg) in methanol (2 ml) was treated repeatedly over a period of 2 days with excess ethereal diazomethane until u.v. spectral analysis of the product (using $AlCl_3$ and NaOMe as diagnostic reagents) indicated that complete methylation of the phenolic hydroxyl groups had occurred. The product was then hydrolyzed with 5% HCl at 100° for 1 hr. Aglycone material was subsequently isolated by ether extraction of the hydrolysis mixture. One-dimensional paper chromatography of the extract in 15% acetic acid revealed two aglycone bands visible in u.v. light, one fluorescent, the other deep purple.

Methanol extraction of the fluorescent band yielded compound VI which had the following properties: R_f 0·88 (TBA), R_f 0·41 (HOAc), R_f 0·89 (Bz); λ_{max} (MeOH) 256, 310sh, nm; λ_{max} (NaOMe) and (NaOAc) 260, 331 nm; λ_{max} ($AlCl_3$) 256, 310sh, nm; the PMR spectrum in $CDCl_3$ showed signals at 7·82 singlet (H-2), 7·46 doublet, J = 9 c/s (H-2',6'), 6·97 doublet, J = 9 c/s (H-3', 5'), 6·88 singlet (H-8) and 4·03, 3·97, 3·84 singlets (—OCH_3) ppm.

Methanol extraction of the deep purple band yielded compound V, which had the following properties: R_f 0·86 (TBA), R_f 0·32 (HOAc), R_f 0·54 (Bz); λ_{max} (MeOH) 264, 334sh, nm; λ_{max} (NaOMe) and (NaOAc), 272, 337 nm; λ_{max} ($AlCl_3$) 274, 315sh, nm; the PMR spectrum in $CDCl_3$ showed signals at 7·89 singlet (H-2) 7·46 doublet, J = 9 c/s (H-2',6'), 7·0 doublet, J = 9 c/s (H-3', 5'), 6·53 singlet (H-8) and 4·03, 3·84 singlets (—OCH_3) ppm. This PMR spectrum was identical with that of authentic irisolidone (V).

Compounds V and VI were also synthesized from tectoridin (IV) by the procedure outlined above, and were spectrally and chromatographically identical with V and VI from the rhamnosylglucoside (IIa). The

chromatographic comparison included cochromatography on a polyamide thin layer in a solvent mixture of MeOH:H_2O:HOAc, 90:5:5.

Acknowledgements—We are indebted to Dr. B. Shoulders of the Chemistry Department, The University of Texas at Austin, for the determination of the 100 Mc PMR spectra. Support from the National Science Foundation (Grant GB-5548X), the National Institutes of Health (Grant GM-11111-04A1), and the Robert A. Welch Foundation (Grant F-130), is acknowledged.