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THE IDENTIFICATION OF TWENTY-THREE 5-DEOXY- AND TEN 5-HYDROXY-FLAVONOIDS FROM *BAPTISIA LECONTEI* (LEGUMINOSAE)

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Abstract—The chemical investigation of all thirty-five compounds detected by u.v. light on a *Baptisia lecontei* paper chromatogram resulted in the identification of ten 5-hydroxyflavonoids, twenty-three 5-deoxyflavonoids and two coumarins. Thirteen new flavonoids were identified: the 7-*O*-glucosides and rhamnosylglucosides of 7,4′-dihydroxyflavone and 7,3′,4′-trihydroxyflavone, the 3-*O*-β-D-glucoside and 7-*O*-rhamnosylglucoside of fisetin, orobol 7-*O*-rhamnosylglucoside, daidzein 7-*O*-rhamnosylglucoside, 7,3′-dihydroxy-4′-methoxyisoflavone (calycosin) and its 7-*O*-β-D-glucoside and 7-*O*-rhamnosylglucoside, and the dihydroflavonol glycosides, lecontin and (+)-fustin 3-*O*-β-D-glucoside. The relative locations of the flavonoid spots on the 2D paper chromatogram are discussed with respect to the structures of the compounds.

INTRODUCTION

THIRTY-FIVE compounds were detected in u.v. light as dark-absorbing or fluorescent spots on a *Baptisia lecontei* two-dimensional paper chromatogram (Fig. 1) prepared from a methanol extract of *B. lecontei* leaves. Twenty-five of these compounds appeared as fluorescent spots and all but two were later shown to belong to the uncommon group of flavonoids lacking 5-oxygenation (5-deoxyflavonoids). This paper reports the isolation and identification of all thirty-five chromatographically detected compounds, including the previously unreported natural products: the 7-*O*-glucosides and 7-*O*-rhamnosylglucosides of 7,4′-dihydroxyflavone and 7,3′,4′-trihydroxyflavone, the 3-*O*-β-D-glucoside and 7-*O*-rhamnosylglucoside of fisetin, and the 7-*O*-rhamnosylglucosides of daidzein and orobol. The dihydroflavonol glycosides lecontin and (+)-fustin 3-*O*-β-D-glucoside, and the isoflavone calycosin and its 7-*O*-β-D-glucoside and 7-*O*-rhamnosylglucoside, were also isolated as new natural products from *B. lecontei*, and their structure determinations are described elsewhere.[1,17]

This work which represents one aspect of the broad biochemical systematic investigation of the genus *Baptisia*,[1-6] is the first study of the flavonoid components of *B. lecontei* and is to date the most complete chemical investigation carried out on any single *Baptisia* species.

RESULTS AND DISCUSSION

Identification of Flavones

The flavones luteolin (I) and apigenin (II) and their 7-*O*-β-D-glucosides (Ia, IIa) and 7-*O*-rhamnosylglucosides (Ib, IIb) are, with the exception of Ib, well-known natural products[7,8]

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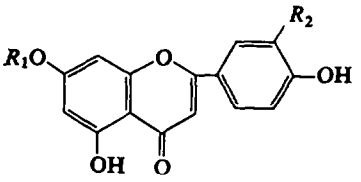
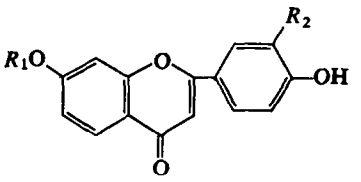
[1] K. R. MARKHAM and T. J. MABRY, *Tetrahedron*, in press.
[2] H. RÖSLER, T. J. MABRY and J. KAGAN, *Chem. Ber.* **98**, 2193 (1965).
[3] R. E. ALSTON, H. RÖSLER, K. NAIFEH and T. J. MABRY, *Proc. Natl Acad. Sci. U.S.* **54**, 1458 (1965).
[4] M. F. CRANMER and T. J. MABRY, *Phytochem.* **5**, 1113 (1966).
[5] P. LEBRETON, K. R. MARKHAM, W. T. SWIFT, III, OUNG-BORAN and T. J. MABRY, *Phytochem*, in press.
[6] T. J. MABRY, J. KAGAN and H. RÖSLER, *Phytochem.* **4**, 487 (1965).
[7] S. HATTORI, in *The Chemistry of Flavonoid Compounds* (edited by T. A. GEISSMAN), pp. 316–346. Pergamon Press, Oxford (1962).
[8] B. L. TURNER, *Pure Appl. Chem.* **14**, 189 (1967).

and were identified here by direct chromatographic, spectral and m.p. comparisons with authentic samples. Luteolin 7-*O*-rhamnosylglucoside is the least well documented and is previously described only in terms of a chromatographic spot[8] and a PMR spectrum.[9] The material isolated from *Baptisia lecontei* is well characterized and a PMR spectrum of the trimethylsilylated derivative indicated that it is a 7-*O*-rutinoside on the basis of the chemical shifts observed for the rhamnose H-1 and C—CH_3 signals (4·37 and 0·8–1·0 ppm respectively).

The occurrence of 7,3′,4′-trihydroxyflavone (III) and 7,4′-dihydroxyflavone (IV) in *B. lecontei* was established by direct comparison of isolated material with authentic samples. Each have previously been reported only once as natural products. Livingston and Bickoff in 1964–65 reported the isolation of IV from alfalfa (*Medicago sativa*)[10a] and IV together with III[10b] from ladino clover (*Trifolium repens*).



	R_1	R_2
(I)	H	OH
(Ia)	glu	OH
(Ib)	rh-glu	OH
(II)	H	H
(IIa)	glu	H
(IIb)	rh-glu	H



	R_1	R_2
(III)	H	OH
(IIIa)	glu	OH
(IIIb)	rh-glu	OH
(IV)	H	H
(IVa)	glu	H
(IVb)	rh-glu	H

The 7-*O*-β-D-glucosides (IIIa, IVa; spots 6,7) and 7-*O*-rhamnosylglucosides (IIIb, IVb; spots 10,11) of both 7,3′,4′-trihydroxyflavone and 7,4′-dihydroxyflavone are new compounds and, in each case, the aglycone obtained by acid hydrolysis was identified by chromatographic and spectral comparison with an authentic sample. With each of these *O*-glycosides the attachment of the sugar was shown to be at the 7-position by u.v. spectroscopy. In methanol with added NaOAc all four compounds failed to give the 10–20 nm bathochromic shift (relative to the methanol spectrum) of the short wavelength absorption peak observed for the respective aglycones (see Ref. 11). The 4′-hydroxyl group was shown to be free in all four compounds by the effect of NaOMe on the u.v. spectra[11] and the 3′,4′-dihydroxyl groups in IIIa and IIIb were detected by the spectral shifts caused by $AlCl_3$ and $AlCl_3$/HCl.[12] Both IIIa and IVa were hydrolyzed by β-glucosidase and the identity of the sugar as glucose was confirmed by paper chromatography. Compound IVa was shown to be a monoglucoside by quantitative estimation of the sugar liberated on hydrolysis, and compound IIIa (spot 6) must also be a monoglucoside since it occupies on a paper chromatogram, the same position relative to luteolin 7-*O*-glucoside (spot 8a) as does IVa (spot 7) to apigenin 7-*O*-glucoside (spot 9a). The sugars from the two other glycosides (spots 10,11) were identified in each case as rhamnose and glucose (in a 1 : 1 ratio) by gas chromatography,

[9] H. RÖSLER, T. J. MABRY, M. F. CRANMER and J. KAGAN, *J. Org. Chem.* **30**, 4346 (1965).

[10a] E. M. BICKOFF, A. L. LIVINGSTON and S. C. WATT, *Phytochem.* **4**, 523 (1965).

[10b] A. L. LIVINGSTON and E. M. BICKOFF, *J. Pharm. Sci.* **53** (12), 1557 (1964).

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

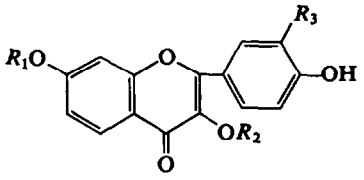
[12] Details for the application of the spectral analysis of flavonoids in the presence of $AlCl_3$ and $AlCl_3$/HCl for the detection of *ortho*-dihydroxyl groups will be described in a forthcoming publication.

and, although no quantitative sugar analyses were carried out on these compounds, the R_f values in TBA and HOAc indicated that they must be diglycosides and not tetraglycosides. On the basis of this evidence, together with the sugar location and aglycone information discussed above, these diglycosides are assigned structures IIIb and IVb.

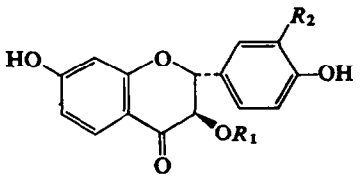
Identification of Flavonols and Dihydroflavonols

The identification of the flavonols from spots 3a and 4a as the known compounds 7-4',dihydroxyflavonol (VI) and fisetin (V) respectively has been described earlier,[1] but the glycosides, fisetin 3-*O*-β-D-glucoside (Va, spot 17) and the tentatively identified fisetin 7-*O*-rhamnosylglucoside (Vb, spot 8b) are reported here for the first time. The only fisetin glycoside that has previously been mentioned in the literature is fisetin 7-*O*-glucoside, which was isolated in 1966 from the heartwood of *Rhus succedanea*.[13]

The material from spot 17 hydrolyzed with both acid and β-glucosidase to give fisetin and 1 mole-equivalent of glucose. A u.v. spectral analysis[11, 12] of this glucoside showed that the 7,3'- and 4'-hydroxyl groups were free and that the 3-hydroxyl was blocked. On this basis the spot 17 compound must be fisetin 3-*O*-β-D-glucoside (Va).



	R_1	R_2	R_3
(V)	H	H	OH
(Va)	H	glu	OH
(Vb)	rh-glu	H	OH
(VI)	H	H	H



	R_1	R_2
(VII)	H	H
(VIIa)	glu	H
(VIII)	H	OH
(VIIIa)	glu	OH

The compound from the weak, yellow fluorescent spot 8b was not fully characterized but did give fisetin on acid hydrolysis. Its color characteristics corresponded to those reported for fisetin 7-*O*-glucoside[13] (cf. the 3-glucoside which is blue); however, the R_f value in 15 per cent HOAc (0·33) was considerably higher than those recorded for fisetin 7-*O*-glucoside (0·04 in 6 per cent HOAc and 0·25 in 30 per cent HOAc). This information suggests that the compound from spot 8b is a fisetin 7-diglycoside and the proximity of spot 8b to the 7-rhamnosylglucoside spots of 5,7,3',4'-tetrahydroxy- and 7,3',4'-trihydroxyflavones (spots 8 and 10) is consistent with it being fisetin 7-*O*-rhamnosylglucoside.

We reported earlier[1] on the dihydroflavonols, (+)-7,4'-dihydroxydihydroflavonol (VII, spot 15) and (+)-fustin (VIII, spot 15a) and their new 3-*O*-β-D-glucosides, lecontin (VIIa, spot 21a) and (+)-fustin 7-*O*-β-D-glucoside (VIIIa, spot 20).

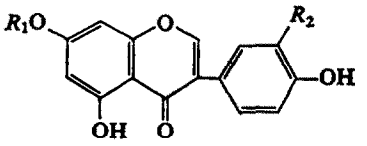
Identification of Isoflavones

The *B. lecontei* isoflavones occur as either dark-absorbing or fluorescent compounds when viewed on the paper chromatogram under u.v. light.

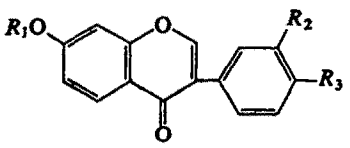
(*a*) *Dark* u.v.-*absorbing isoflavones.* Only four dark, u.v.-absorbing isoflavone spots, numbers 5c, 5e, 18 and 16, are visible on the *B. lecontei* 2D paper chromatogram (Fig. 1),

[13] W. E. Hillis and T. Inoue, *Phytochem.* **5**, 483 (1966).

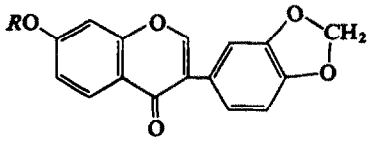
and these have been shown to be genistein (IX), orobol (X), genistein 7-*O*-rhamnosylglucoside (sphaerobioside,[2] IXa) and orobol 7-rhamnosylglucoside (Xa), respectively. The first three are known compounds and were identified by direct comparisons with authentic material. Orobol 7-rhamnosylglucoside, however, has not been reported before as a natural product. A PMR spectrum of the material from spot 16 was consistent with structure Xa and, in support of this, acid hydrolysis produced orobol together with rhamnose and glucose. The rhamnosylglucose unit must be attached to the 7-hydroxyl group of orobol since the spectrum of the glycoside was unaffected by the addition of NaOAc. In the spectrum of orobol itself the short wavelength band shifted bathochromically by 10 nm under the same conditions.



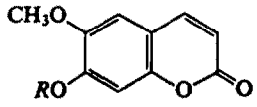
	R_1	R_2
(IX)	H	H
(IXa)	rh-glu	H
(X)	H	OH
(Xa)	rh-glu	OH



	R_1	R_2	R_3
(XII)	H	H	OH
(XIIa)	glu	H	OH
(XIIb)	rh-glu	H	OH
(XIII)	H	OH	OCH_3
(XIIIa)	glu	OH	OCH_3
(XIIIb)	rh-glu	OH	OCH_3



(XI) R = H
(XIa) R = rh-glu



(XIV) R = H
(XIVa) R = glu

(*b*) *Fluorescent isoflavones. B. lecontei* contains eight isoflavones which fluoresce on paper in u.v. light; one of these, pseudobaptisin (XIa, spot 21) is the major isoflavone in the species. Both pseudobaptisin and its aglycone pseudobaptigenin (XI, spot 5) have been reported only once before, the former from *B. tinctoria* in 1897[14] and the latter more recently from *Maackia amurensis*.[15] Both compounds (XI and XIa) were fully characterized in the present investigation and the physical data obtained were in good agreement with literature values. The PMR spectrum of pseudobaptisin, reported here for the first time, indicated that the disaccharide in pseudobaptisin is rutinose.

Daidzein (XII, spot 5b) is another u.v. fluorescent isoflavone which was found in the complex of spots in the lower left-hand corner of the *B. lecontei* 2D chromatogram. It was identified by comparison with authentic daidzein, a relatively common natural product.[16] Less common are the two glycosides of daidzein, the 7-*O*-β-D-glucoside (XIIa, spot 13) and the 7-*O*-rhamnosylglucoside (XIIb, spot 19a), which were also identified. The glycoside from spot 13 hydrolyzed with both acid and β-glucosidase to give daidzein and glucose, the

[14] K. GORTER, *Arch. Pharm.* **235**, 494 (1897); *Chem. Abs.* **1**, 469 (1907).
[15] H. SUGINOME and T. KIO, *Bull. Chem. Soc. Japan* **39**, 1541 (1966).
[16] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 92. Academic Press, London (1967).

glucose again being attached to the 7-hydroxyl group as indicated by the lack of change in the spectrum on the addition of NaOAc. Cochromatography of the glucoside with synthetic material confirmed the identification. Daidzein 7-*O*-rhamnosylglucoside (XIIb, spot 19a) is a new natural product; however, because it was isolated from *B. lecontei* in only small amounts, the identification rests largely upon the acid hydrolysis which produced daidzein together with a 1:1 ratio of rhamnose and glucose. This compound is considered to be the 7-*O*-rhamnosylglucoside of daidzein since (*a*), on the 2D paper chromatogram it occurs in the same general region as do all other *B. lecontei* isoflavone 7-*O*-rhamnosylglucosides (spots 16, 18, 19, 21) and (*b*), all flavone and isoflavone glycosides so far isolated from any *Baptisia* species have been 7-*O*-glycosides.

Another series of three u.v. fluorescent isoflavone spots (5a, 12 and 19), analogous to the daidzein series, are due to the presence of 7,3'-dihydroxy-4'-methoxyisoflavone (XIII), its 7-*O*-β-D-glucoside (XIIIa) and its 7-*O*-rhamnosylglucoside (XIIIb), respectively. These compounds are all new natural products, and we have proposed the name calycosin[17] for the aglycone; their identification is described elsehwere.[17]

Identification of Coumarins

The brilliant blue fluorescing spot 14 was identified in part by u.v. spectroscopy, as the coumarin scopoletin (XIV). The u.v. spectra recorded for this compound were typical for 6,7-dioxygenated coumarins[18] and the lack of an $AlCl_3$ shift indicated[12] that one of the hydroxyl groups was substituted. All diagnostic reagent shifts were identical with those observed for scopoletin (XIV), the 7-*O*-methyl isomer being excluded by the 50 nm bathochromic shift of the long wavelength absorption peak on the addition of NaOAc.[18] Cochromatography of this compound with scopoletin in TBA and HOAc solvents confirmed the identification. The 7-*O*-β-D-glucoside of scopoletin (XIVa, spot 22) was shown to be present in *B. lecontei*, when material from spot 22 hydrolyzed with β-glucosidase to give scopoletin.

General Discussion

Although the object of this work was to contribute to the biochemical systematic investigation of the genus *Baptisia*, a considerable amount of additional information was also obtained. For example, the vast array of new 5-deoxyflavonoids found in *B. lecontei*, representing every type of 5-hydroxyflavonoid in the plant, indicates that the 5-deoxy series of compounds may be much more common than was earlier thought, especially in the genus *Baptisia* where few examples have previously been recognized.

The geometrical relationship of one spot to another on the *B. lecontei* paper chromatogram (Fig. 1) is indicative of certain definite structural differences between the compounds concerned. For instance, the 5-deoxyflavonoids show as fluorescent spots in u.v. light and run consistently just ahead and slightly to the right of their 5-hydroxylated equivalents (cf. the pairs of spots: 8a,6; 9a,7; 8,10; 9,11; 1,2; 3,4; 5e,5a; 5c,5b; 18,19a). Another geometrical relationship noted involves B-ring oxidation patterns. 4'-Monohydroxy flavonoids are found consistently higher than, and to the left of, their 3',4'-dihydroxy equivalents. This is true for flavones (spots 1,3), 5-deoxyflavones (spots 2,4) and their glycosides (spots 9,8; 10,11), isoflavones (spots 5c,5e) and isoflavone glycosides (spots 16,18), 5-deoxyflavonols (spots 3a,4a), 5-deoxydihydroflavonols (spots 15,15a) and 5-deoxydihydroflavonol 3-glycosides (spots 20,21a). Spots of the mono- and di-glycosides of the same aglycone show yet

[17] K. R. MARKHAM, T. J. MABRY and T. W. SWIFT, III, *Phytochem.* 7, 803 (1968).
[18] M. HOROWITZ and B. GENTILI, *J. Org. Chem.* 25, 2183 (1960).

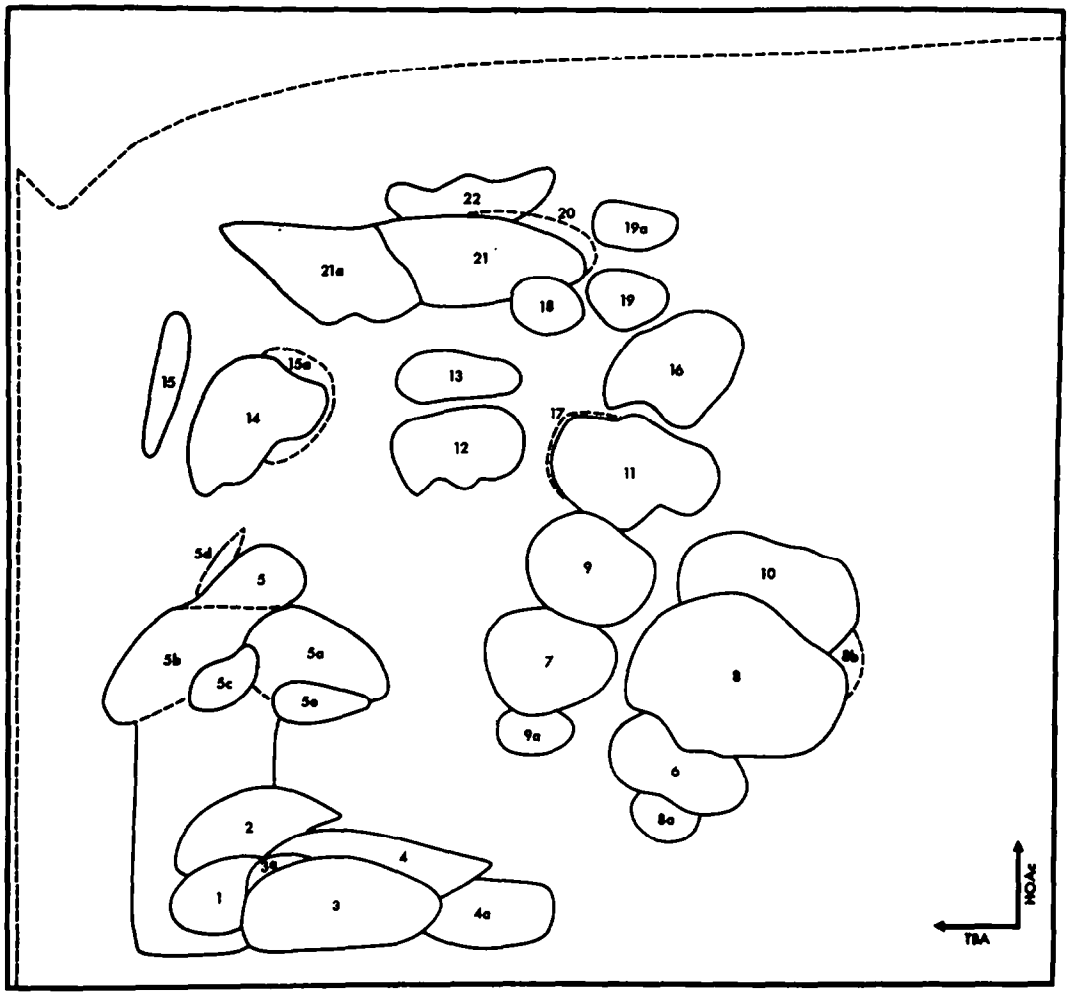


FIG. 1. PAPER CHROMATOGRAPHIC FLAVONOID PATTERN OF *B. lecontei*

Spot No.	Compound
1.	Apigenin (II)
2.	7,4′-Dihydroxyflavone (IV)
3.	Luteolin (I)
3a.	7,4′-Dihydroxylflavonol (VI)
4.	7,3′,4′-Trihydroxyflavone (III)
4a.	Fisetin (V)
5.	Pseudobaptigenin (XI)
5a.	Calycosin (XIII)
5b.	Daidzein (XII)
5c.	Genistein (IX)
5d.	Liquiritigenin?
5e.	Orobol (X)
6.	7,3′,4′-Trihydroxyflavone 7-*O*-glucoside (IIIa)
7.	7,4′-Dihydroxyflavone 7-*O*-glucoside (IVa)
8.	Luteolin 7-*O*-rutinoside (Ib)
8a.	Luteolin 7-*O*-glucoside (Ia)
8b.	Fisetin 7-*O*-rhamnosylglucoside (Vb)
9.	Apigenin 7-*O*-rhamnosylglucoside (IIb)
9a.	Apigenin 7-*O*-glucoside (IIa)
10.	7,3′,4′-Trihydroxyflavone 7-*O*-rhamnosylglucoside (IIIb)
11.	7,4′-Dihydroxyflavone 7-*O*-rhamnosylglucoside (IVb)
12.	Calycosin 7-*O*-glucoside (XIIIa)
13.	Daidzein 7-*O*-glucoside (XIIa)
14.	Scopoletin (XIV)
15.	(+)-7,4′-Dihydroxydihydroflavonol (VII)
15a.	(+)-Fustin (VIII)
16.	Orobol 7-*O*-rutinoside (Xa)
17.	7,3′,4′-Trihydroxyflavonol 3-*O*-glucoside (Va)
18.	Sphaerobioside (IXa)
19.	Calycosin 7-*O*-rhamnosylglucoside (XIIIb)
19a.	Daidzein 7-*O*-rhamnosylglucoside (XIIb)
20.	(+)-Fustin 3-*O*-glucoside (VIIIa)
21.	Pseudobaptisin (XIa)
21a.	Lecontin (VIIa)
22.	Scopoletin 7-*O*-glucoside (XIVa)

another geometrical relationship and this is exemplified by the apigenin (spots 9a and 9) and luteolin (spots 8a and 8) glucosides and rhamnosylglucosides. The addition of rhamnose to the glucose residue in a monoglucoside causes the compound to run higher and slightly to the right of the monoglucoside. This applied to isoflavones (spots 12, 19 and 13, 19a) and 5-deoxyflavones (spots 6, 10 and 7, 11) as well as to the luteolin and apigenin derivatives. All of these relationships are remarkably consistent for flavones, flavonols, dihydroflavonols and isoflavones, and for this reason have been found to be of value in the paper chromatographic screening of large numbers of *Baptisia* species for flavonoids.

Biogenetic Considerations

Flavonoids found in *B. lecontei* include dihydroflavonols, flavonols, flavones, isoflavones and possibly a flavanone, all with the same or closely related oxidation patterns. 5-Deoxy derivatives of all of these classes are found in abundance and, in the flavonol and dihydroflavonol groups, they are found to the exclusion of the 5-hydroxy derivatives. Every flavonoid group is represented by both 4′-hydroxy and 3′,4′-dihydroxy derivatives, the 3′,4′-dihydroxy representative of the 5-deoxyisoflavones being found as the further modified 3′-hydroxy-4′-methoxy derivative, calycosin (XIII). Calycosin is probably the precursor of the unusual 3′,4′-methylenedioxy isoflavone, pseudobaptigenin (XI), since *ortho*-methoxy phenols are known[19] to be precursors of methylenedioxy derivatives. If the precursor was the methylated chalcone or flavanone, then according to the currently accepted flavonoid biosynthetic scheme,[20,21] which places flavanones and chalcones in a central biosynthetic position from which all other flavonoid types diverge, one would also expect to find other methylated flavonoids; however this is not the case. Of the thirty-three flavonoids visible on the *B. lecontei* 2D paper chromatogram only three are methylated, calycosin (XIII) and its glucoside (XIIIa) and rhamnosylglucoside (XIIIb). These observations provide confirmatory evidence that methylation is a late step in flavonoid biosynthesis; a conclusion that had previously been suggested on the basis of genetic investigations[22] and chalcone-flavanone isomerase specificity studies.[21]

It is generally considered that loss of the 5-hydroxyl group in flavonoids occurs at an early stage in the biosynthetic pathway[23] and tracer work[20] indicates that this is before flavanone formation. If this is correct, then the remarkably consistent, parallel production of 5-deoxyflavonoids together with their 5-hydroxylated equivalents in *B. lecontei*, suggests that all enzymes involved in the transformation of chalcones through to flavones, isoflavones and flavonols and their glucosides and rhamnoglucosides, are non-specific with regard to 5-hydroxylation. This has recently been shown to be the case with at least one of these enzymes, chalcone-flavanone isomerase,[21] which does not distinguish between 2′,4,4′-trihydroxychalcone and 2′,4,4′,6′-tetrahydroxychalcone in the stereospecific conversion of chalcones to flavanones.

[19] D. H. R. Barton, G. W. Kirby and J. B. Taylor, *Proc. Chem. Soc.* 340 (1962); S. Kirkwood and M. Sribney, *Nature* **171**, 931 (1953).
[20] H. Grisebach, in *Recent Advances in Phytochemistry* (edited by T. J. Mabry), Appleton-Century-Crofts, New York (1967).
[21] E. Moustafa and E. Wong, *Phytochem.* **6**, 625 (1967).
[22] J. B. Harborne, in *The Chemistry of Flavonoid Compounds* (edited by T. A. Geissman), p. 608. Pergamon Press, Oxford (1962).
[23] R. W. Rickards, in *Recent Developments in the Chemistry of Natural Phenolic Compounds* (edited by W. D. Ollis), p. 4. Pergamon Press, Oxford (1961).

EXPERIMENTAL

Baptisia lecontei plant material (Voucher specimen No. 257790, University of Texas Herbarium, Austin) was collected near Tampa, Florida, by Dr. R. Long of the University of South Florida. Melting points are uncorrected. U.v. spectroscopy was carried out using a Beckman DBG spectrophotometer, and PMR spectra, unless otherwise stated, were determined using a Varian A-60 spectrometer with CCl_4 as solvent and tetramethylsilane as internal standard. Paper chromatograms were run on Whatman 3 MM paper (46 cm × 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. R_f values quoted for (Bz) are accurate only relative to one another. R_f values are quoted below in brackets, after the first mention of each constituent, in TBA and HOAc respectively. All colors of compounds on chromatograms were recorded in u.v. light.

Extraction Procedure

In a typical extraction, ground, dry, stem and leaf material (24 g) was extracted for 3 days with cold 25% aqueous MeOH (180 ml) to give a sticky green extract (5·3 g). This material was used for the *B. lecontei* type chromatogram (Fig. 1).

Isolation of Flavonoids by Column Chromatography

The extract (5 g), dissolved in aqueous methanol, was applied to a 25 cm × 5 cm (dia.) polyvinylpyrrolidone (Polyclar AT, General Aniline and Film Corp.) column, and elution was initiated with water. Elution was continued with water containing an increasing percentage of methanol up to 100%, and this was followed by elution with 0·3 N methanolic HCl, 1·1 N methanolic HCl and 4·5 N methanolic HCl, successively. Individual flavonoids were purified by repeated column chromatography of the appropriate fraction or fractions.

Quantitative Estimation of Individual Flavonoids

From a total extract of 5·3 g from 24 g of *B. lecontei* leaf and stem material (see Extraction Procedure) 143 mg were applied to ten paper chromatograms. These chromatograms were run in two dimensions (TBA and HOAc), dried, and spots that were reasonably clear of contamination were cut out and eluted with methanol. The solution of each compound so obtained was made up to a known volume (usually 20–30 ml) and the absorption spectrum was measured. Using known ϵ values for these or similar compounds, the approximate amount of each natural flavonoid in the original plant material was calculated. The results expressed as percentage weight of the dry plant material are as follows: compound Ib (2·0%), Xa (1·1%), IXa (0·3%), IIIb (0·15%), IIb (0·1%), IVb (0·08%), XIII (0·8%), XI/XII (0·08%), XIV (0·04%), IVa (0·01%), XIIIa (0·01%), XIIa (0·01%), IIIa (0·008%), Ia (0·004%). The yields of compounds VIIIa (0·0003%), XIa (1·0%) and VIIa (0·01%) were determined by weighing isolated material and for this reason are probably low relative to those listed above.

Methods of Sugar Analysis

(*a*) *Gas chromatography*. Using the method of Kagan and Mabry,[24] flavonoid glycosides were acid hydrolyzed and the sugars were isolated, trimethylsilylated in pyridine with hexamethyldisilazane and trimethylchlorosilane, and gas chromatographed* on a 6 ft × 0·25 in. stainless-steel column packed with chromosorb W which was coated with D.C. 560 (a Dow Corning chlorophenylmethyl silicone). Standards of trimethylsilylated rhamnose and glucose (α and β) forms were run prior to each identification.

(*b*) *Paper chromatography*. Using essentially the method reported by Pridham,[25] the glycoside was acid hydrolyzed and the resultant mixture was separated by paper chromatography in EtAc:Py:H_2O, 12:5:4. The sugars were detected using a *p*-anisidine HCl spray reagent,[25] the intensity of the spots so produced being measured spectrophotometrically following elution.

(*c*) *β-Glucosidase*. To establish the linkages in the flavonoid glucosides, each compound was dissolved in distilled water and treated with β-glucosidase (Sigma Chemical Co., St. Louis, Mo.) at 25° for 24 hr. The product was analyzed by paper chromatography.

Flavones

Luteolin (*I, spot* 3). The material from spot 3 (R_fs 0·77/0·07), which was isolated by 2D paper chromatography (TBA, HOAc), was spectrally and chromatographically identical with authentic luteolin.

Luteolin 7-O-*rutinoside* (*Ib, spot* 8). The compound from spot 8 (0·30/0·30) was isolated by column chromatography as a yellow crystalline solid (100 mg), m.p. 186° [unchanged on admixture with luteolin 7-*O*-

* A Research Specialities Model 600 dual-injection instrument with an argon ionization detector was used for this work.

[24] T. J. MABRY and J. KAGAN, *Anal. Chem.* 37, 288 (1965).

[25] J. B. PRIDHAM, *Anal. Chem.* 28, 1967 (1956).

rhamnosylglucoside], λ_{max} (MeOH) 254, 265sh, 347·5 nm; λ_{max} (NaOMe) 264, 392 nm; λ_{max} ($AlCl_3$) 272, 295sh, 327, 425 nm; λ_{max} ($AlCl_3$/HCl) 268, 293, 362sh, 390 nm; λ_{max} (NaOAc) 258, 380 nm; λ_{max} (NaOAc/H_3BO_3) 257, 372 nm. It cochromatographed with authentic luteolin 7-*O*-rhamnosylglucoside in TBA and HOAc and gave a PMR spectrum identical with that published[6] for luteolin 7-*O*-rhamnosylglucoside. Acid hydrolysis of this glycoside produced luteolin, rhamnose and glucose.

Luteolin 7-O-β-D-*glucoside* (*Ia, spot* 8*a*). The spot 8a compound (0·38/0·16) was isolated in small amounts (0·5 mg) by 2D paper chromatography. The absorption properties of this compound were identical to those listed above for luteolin 7-*O*-rutinoside. It cochromatographed with authentic luteolin 7-*O*-glucoside in TBA and HOAc and hydrolyzed with β-glucosidase to produce luteolin.

Apigenin (*II, spot* 1). The material from spot 1 (0·87/0·11) was isolated by 2D paper chromatography and was spectrally and chromatographically identical with apigenin.

Apigenin 7-O-*rhamnosylglucoside* (*IIb, spot* 9). The spot 9 material (0·43/0·42) was isolated in small amounts (3 mg) by column chromatography. Acid hydrolysis of this compound gave apigenin, rhamnose and glucose. This glycoside was spectrally and chromatographically identical with authentic apigenin 7-*O*-rhamnosylglucoside [λ_{max} (MeOH) 266, 325 nm; λ_{max} (NaOMe) 247, 264, 387 nm; λ_{max} ($AlCl_3$) and ($AlCl_3$/HCl) 227sh, 274, 298, 348, 382 nm; λ_{max} (NaOAc) 265, 340, 390sh, nm; λ_{max} (NaOAc/H_3BO_3) as for MeOH].

Apigenin 7-O-β-D-*glucoside* (*IIa, spot* 9*a*). The compound from spot 9a (0·51/0·24) was not isolated due to the small amount present but its R_f values were identical with those for apigenin 7-*O*-glucoside.

7,3′,4′-*Trihydroxyflavone* (*III, spot* 4). The spot 4 compound (0·70/0·06) appeared light blue on paper and turned bright yellow-green with NH_3 vapor. It was isolated by 2D paper chromatography and by acid hydrolysis of material from spot 10. The spectra [λ_{max} (MeOH) 234, 250sh, 310sh, 340 nm; λ_{max} (NaOMe) 255, 310sh, 396 nm; λ_{max} ($AlCl_3$) 235, 302, 370, 450 nm; λ_{max} ($AlCl_3$/HCl) 235sh, 250sh, 310, 340, 407sh, nm; λ_{max} (NaOAc) 254, 310sh, 374 nm; λ_{max} (NaOAc/H_3BO_3) 253sh, 305, 363 nm] are identical with those of authentic 7,3′,4′-trihydroxyflavone as also are the R_f values and the color in u.v. light.

7,3′,4′-*Trihydroxyflavone*-7-O-*rhamnosylglucoside* (*IIIb, spot* 10). The compound from spot 10 (2–3 mg) (0·26/0·38) was isolated by 2D paper chromatography of the 30% methanol fractions from polyamide column chromatography of the crude plant extract. It appeared on paper as a light blue spot, which turned yellow with NH_3 vapor, and it had the following spectral properties: λ_{max} (MeOH) 245–255sh, 304, 340 nm; λ_{max} (NaOMe) 294, 303sh, 410 nm; λ_{max} ($AlCl_3$) 250sh, 300, 380 nm; λ_{max} ($AlCl_3$/HCl) as for MeOH; λ_{max} (NaOAc) 250, 300, 347, 398 nm; λ_{max} (NaOAc/H_3BO_3) 255sh, 300, 364 nm. Acid hydrolysis produced 7,3′,4′-trihydroxyflavone, identified by comparison with authentic material, and rhamnose and glucose (in a 1:1 ratio).

7,3′,4′-*Trihydroxyflavone* 7-O-β-D-*glucoside* (*IIIa, spot* 6). The spot 6 compound (0·35/0·19) was isolated in small amounts (0·5 mg) by 2D paper chromatography of suitable fractions from the original polyamide column. It had the same appearance on a paper chromatogram in u.v. light and in NH_3 as did spot 10, but the R_f values were significantly different. The u.v. spectra [λ_{max} (MeOH) 247sh, 300sh, 340 nm; λ_{max} (NaOAc) 250sh, 300sh, 347, 400sh, nm; λ_{max} (NaOAc/H_3BO_3) 255, 300sh, 367 nm] were similar to those of 7,3′,4′-trihydroxyflavone 7-*O*-rhamnosylglucoside (spot 10). Both β-glucosidase and acid hydrolysis gave 7,3′,4′-trihydroxyflavone and glucose.

7,4′-*Dihydroxyflavone* (*IV, spot* 2). The aglycone (0·85/0·11) present as spot 2 was isolated by 2D paper chromatography of both the crude plant extract and as the acid hydrolysis product of spot 11. It appeared light blue, turning a rich blue-green in NH_3 vapor. The u.v. spectra of this compound [λ_{max} (MeOH) 228, 253sh, 312sh, 327 nm; λ_{max} (NaOMe) 251, 262sh, 328, 386 nm; λ_{max} (NaOAc) 264, 307, 320sh, 364 nm; λ_{max} (NaOAc/H_3BO_3), ($AlCl_3$) and ($AlCl_3$/HCl) as for MeOH] and its appearance, and R_f values were identical with those of authentic 7,4′-dihydroxyflavone.

7,4′-*Dihydroxyflavone* 7-O-*rhamnosylglucoside* (*IVb, spot* 11). Repeated column chromatography and subsequent 2D paper chromatography of semi-pure column fractions, gave workable quantities (2–3 mg) of the glycoside from spot 11 (0·40/0·58). It appeared on paper as a light blue spot which turned bright blue-green in NH_3 vapor, and it had the following spectral properties: λ_{max} (MeOH) 225sh, 252, 309, 325 nm; λ_{max} (NaOMe) 253sh, 294, 303, 384 nm; λ_{max} ($AlCl_3$) as for MeOH; λ_{max} (NaOAc) 252, 304, 329, 380 nm; λ_{max} (NaOAc/H_3BO_3) as for MeOH. Acid hydrolysis of this glycoside gave 7,4′-dihydroxyflavone and rhamnose and glucose in a 1:1 ratio.

7,4′-*Dihydroxyflavone* 7-O-β-D-*glucoside* (*IVa, spot* 7). The spot 7 compound (0·47/0·34) was isolated in small amounts (0·5 mg) by 2D paper chromatography of the crude plant extract, followed by a one-dimensional run in HOAc. The isolated glycoside had the following spectral properties: λ_{max} (MeOH) 222, 255sh, 305sh, 325 nm; λ_{max} (NaOMe) 254sh, 305sh, 385 nm; λ_{max} (NaOAc) 255sh, 303, 325, 380sh, nm; λ_{max} (NaOAc/H_3BO_3) as for MeOH; λ_{max} ($AlCl_3$) as for MeOH. Both β-glucosidase and acid hydrolyses gave 7,4′-dihydroxyflavone and glucose. The glycoside (42 μg) gave 0·9 mole-equivalents of glucose (15 μg) when analyzed by the Pridham method.

Flavonols

3′,4′,7-*Trihydroxyflavonol* (*fisetin, V; spot* 4*a*). The material from spot 4a could not be eluted satisfactorily from paper and was therefore isolated by column chromatography. It appeared on paper as a

brilliant yellow fluorescing spot (0·54/0·03). It cochromatographed with authentic fisetin and had spectra indistinguishable from those of fisetin.

3′,4′,7-Trihydroxyflavonol 3-O-β-D-glucoside (Va, spot 17). Spot 17 (0·40/0·57) appeared pale blue turning yellow in NH_3 vapor and was not visible on 2D paper chromatograms of the crude extract, since it was hidden by the stronger spot 11. The spot 17 compound was separated from the material of spot 11 by polyamide column chromatography and was purified by 2D paper chromatography. The compound so isolated (1 mg) exhibited the following spectal properties: λ_{max} (MeOH) 250sh, 309, 342 nm; λ_{max} (NaOMe) 256, 322, 405 nm; λ_{max} ($AlCl_3$) 274sh, 307, 380 nm; λ_{max} ($AlCl_3$/HCl) as for MeOH; λ_{max} (NaOAc) 256sh, 315, 360sh, nm; λ_{max} (NaOAc/H_3BO_3) 255sh, 305, 367 nm. Hydrolysis of this compound with either 5% HCl or β-glucosidase gave fisetin and glucose [22 μg (0·9 mole-equiv.) glucose were isolated from 66 μg of the glycoside by the Pridham method].

Fisetin 7-O-rhamnosylglucoside (Vb, spot 8b). Spot 8b appeared as a very weak, yellow fluorescing spot (0·20/0·35) and was unaffected by NH_3 vapor. The compound was not isolated, but a small amount of extract from spot 8b gave fisetin on acid hydrolysis.

7,4′-Dihydroxyflavonol (VI, spot 3a). The spot 3a compound (0·74/0·05) could not be eluted satisfactorily from paper or from polyamide. The best source of this substance was acid hydrolysis of lecontin (spot 21a), which gave it as a yellow powder, m.p. 275° (dec); λ_{max} (MeOH) 217sh, 257, 316, 355 nm; λ_{max} (NaOMe) 235sh, 273, 317sh, 325, 406 nm; λ_{max} ($AlCl_3$) 223, 254sh, 269, 320, 416 nm; λ_{max} ($AlCl_3$/HCl) as for $AlCl_3$; λ_{max} (NaOAc) 266, 316sh, 326, 376 nm; λ_{max} (NaOAc/H_3BO_3) as for MeOH. The material from both lecontin and spot 3a was identical in all respects with authentic 4′,7-dihydroxyflavonol.

Dihydroflavonols

The dihydroflavonol glycosides lecontin and (+)-fustin 3-*O*-glucoside were the subject of an earlier communication,[1] but some additional physical data are reproduced here.

Lecontin (VIIa, spot 21a). Lecontin appeared as a pale blue spot (0·77/0·80) and turned bright blue–green in NH_3 vapor. U.v. spectra: λ_{max} (MeOH) 230, 277, 310 nm; (log ε, 4·21, 4·11, 3·85); λ_{max} (NaOMe) 250, 339 nm, and λ_{max} (NaOAc) 256, 338 nm. The aglycone, *7,4′-dihydroxy-dihydroflavonol (VIII, spot 15)*, gave a very pale spot on paper (0·83/0·65) and had the following u.v. spectra: λ_{max} (MeOH) 229, 275, 311 nm (log ε, 4·28, 4·19, 3·93); λ_{max} ($AlCl_3$) 227sh, 308, 345sh, nm; λ_{max} ($AlCl_3$/HCl) as for MeOH; λ_{max} (NaOMe) 247, 296sh, 333 nm; λ_{max} (NaOAc) 255sh, 278, 333 nm; λ_{max} (NaOAc) as for MeOH.

(+)-Fustin *3-O-β-D-glucoside (VIIIa, spot 20).* Fustin glucoside appeared as a pale blue spot (0·57/0·78) which turned a bright yellow–green in NH_3 vapor. It had the following spectral properties: λ_{max} (MeOH) 230, 278, 310 nm (log ε, 4·20, 4·18, 3·80); λ_{max} (NaOAc) 255, 284, 337 nm; λ_{max} (NaOAc/H_3BO_3) 284, 318 nm; λ_{max} (NaOMe) 252, 337; λ_{max} ($AlCl_3$) as for MeOH. The aglycone, (+)-fustin (*VIII, spot 15a*), a pale spot on paper (0·75/0·64), was not visible on the original paper chromatogram due to the overlapping coumarin (spot 14), it was, however, detected in fractions from the polyamide column. Aerial oxidation converted it to fisetin.

Isoflavones

Sphaerobioside (IXa, spot 18). Sphaerobioside appeared as a dark purple spot (0·45/0·68) which was unaffected by NH_3 vapor. It was isolated as a white solid (7 mg), m.p. 202°, by column chromatography, and its u.v. spectra [λ_{max} (MeOH) 260, 325 nm; λ_{max} (NaOMe) 268, 305sh, 350sh, nm; λ_{max} ($AlCl_3$) and ($AlCl_3$/HCl) 270, 305sh, 377 nm; λ_{max} (NaOAc) and (NaOAc/H_3BO_3) as for MeOH], PMR spectrum in d_6-DMSO [8·83 singlet (H-2), 7·42 doublet J=9 c/s (H-2′,6′), 6·83 doublet J=9 c/s (H-3′,5′), 6·75 doublet J=2 c/s (H-8), 6·47 doublet J=2 c/s (H-6), 5·04 broad (glucose H-1), 4·57 broad (rhamnose H-1), 3·0–4·0 (sugar protons), 1·10 doublet J=6 c/s (rhamnose C—CH_3)] and m.p. were identical with those of authentic[2] sphaerobioside. Acid hydrolysis of IXa gave *genistein (IX, spot 5c)* (0·85/0·30), λ_{max} (MeOH) 259, 324sh, nm; λ_{max} (NaOMe) 273, 324sh, nm; λ_{max} ($AlCl_3$) and ($AlCl_3$/HCl) 225sh, 270, 307, 374 nm; λ_{max} (NaOAc) 269, 325 nm; λ_{max} (NaOAc/H_3BO_3) as for MeOH.

Orobol 7-O-rhamnosylglucoside (Xa, spot 16). This compound appeared as a dark purple spot (0·33/0·65) which was unaffected by NH_3 vapor. It was isolated by column chromatography, along with sphaerobioside (genistein 7-*O*-rhamnosylglucoside) and was purified by paper chromatography in TBA. U.v. spectra: λ_{max} (MeOH) 219, 259, 286sh, 330sh, nm; λ_{max} (NaOMe) 260–267, 287sh, 360sh, nm; λ_{max} ($AlCl_3$) 269, 295sh, 370 nm; λ_{max} ($AlCl_3$/HCl) 270, 376 nm; λ_{max} (NaOAc) 259, 285sh, 330sh, nm; λ_{max} (NaOAc/H_3BO_3) 257–265, 290sh, 320sh, nm; PMR spectrum in d_6-DMSO: 8·37 singlet (H-2), 7·05 and 6·83 broad singlets (H-2′,5′,6′), 6·72 doublet J=2 c/s (H-8), 6·48 doublet J=2 c/s (H-6), 5·0 broad (glucose H-1), 4·58 broad (rhamnose H-1), 3·0–4·0 (sugar protons), 1·15 broad doublet (rhamnose C—CH_3) ppm. Acid hydrolysis of this compound gave *orobol (X, spot 5e)* (0·71/0·25) which was identified by cochromatography with authentic orobol. The hydrolysate also contained rhamnose and glucose (1:1).

Pseudobaptisin (IXa, spot 21). The spot 21 compound crystallized from all column fractions containing it, and recrystallized readily from MeOH/H_2O as fine white needles (0·9 g, 1·0% by wt. of dry plant material), m.p. 150–152°, and 248–250° after resolidification (lit.[14] 148–150° and 249–251°). Found: C, 56·85; H, 5·55;

O, 37·91. Calc. for $C_{28}H_{30}O_{14}$: C, 56·92; H, 5·14; O, 37·95%; λ_{max} (MeOH) 217, 247sh, 259, 289 nm, unchanged by any of the usual diagnostic reagents. PMR spectrum (trimethylsilyl ether in CCl_4): 8·17 doublet J = 9 c/s (H-5), 7·83 (H-2), 7·07, 6·93 and 6·83 (H-6,8,2′,5′,6′), 5·96 (O—CH_2—O), 5·0 broad (glucose H-1), 4·39 (rhamnose H-1), 3·35–3·85 (sugar protons), 0·9–1·1 (rhamnose C—CH_3); PMR spectrum (d_6-DMSO): 8·40 (H-2), 8·08 doublet J = 9 c/s (H-5), 7·24, 7·19, 7·05 (H-6,8,2′,5′,6′), 6·04 (O—CH_2—O), 5·07 (glucose H-1), 4·55 (rhamnose H-1), 3·1–3·9 (sugar protons), 1·1 doublet J = 6 c/s (rhamnose C—CH_3). On paper chromatography this compound appeared as a pale blue spot (0·55/0·75) which was unaffected by NH_3 vapor. Acid hydrolysis of XIa produced pseudobaptigenin together with rhamnose and glucose.

Pseudobaptigenin (*XI, spot* 5). The spot 5 compound (0·85/0·40 and 0·60 in Bz) was most conveniently isolated by acid hydrolysis of pseudobaptisin. It crystallized from methanol as white needles, m.p. 290–292° (lit.[26] 296–298°), λ_{max} (MeOH) 215sh, 248, 260sh, 294 nm; λ_{max} (NaOMe) 257, 295sh, 333 nm; λ_{max} (NaOAc) 256, 295sh, 333 nm; λ_{max} ($AlCl_3$), ($AlCl_3$/HCl) and (NaOAc/H_3BO_3) as for MeOH. This compound appeared as a pale blue spot which turned bright blue on contact with NH_3 vapor.

Daidzein (*XII, spot* 5*b*). The compound from spot 5b (0·87/0·35 and 0·15 in Bz) was best isolated as the acid hydrolysis product of the glucoside (spot 13). It cochromatographed with and the spectra were identical with those of authentic daidzein. Spot 5b was visible as a blue spot only in the presence of NH_3 vapor.

Daidzein 7-O-β-D-*glucoside* (*XIIa, spot* 13). The compound from spot 13 (0·60/0·64) was isolated by 2D paper chromatography of selected polyamide column fractions. It was obtained in small amounts (0·5 mg), was only visible in the presence of NH_3 vapor and had the following spectra: λ_{max} (MeOH) 248sh, 257, 303 nm; λ_{max} (NaOMe) 243sh, 278, 300sh, nm; λ_{max} ($AlCl_3$), ($AlCl_3$/HCl), (NaOAc) and (NaOAc/H_3BO_3) as for MeOH. Acid or β-glucosidase hydrolysis gave daidzein and glucose. The original glycoside cochromatographed with authentic daidzein 7-*O*-β-D-glucoside in both TBA and HOAc.

Daidzein 7-O-*rhamnosylglucoside* (*XIIb, spot* 19*a*). The compound from spot 19a (0·4/0·8) was isolated by 2D paper chromatography of the crude plant extract. Elution of this spot gave a small amount (< 0·5 mg) of a compound which on acid hydrolysis yielded daidzein and a 1 : 1 mixture of rhamnose and glucose.

Coumarins

Scopoletin (*XIV, spot* 14). Spot 14 (0·76/0·65) appeared bright blue and turned greenish in NH_3 vapor. The compound was isolated by paper chromatography, but was usually contaminated with a little dihydrofisetin (spot 15a). It did not hydrolyze with acid and although it cochromatographed (TBA, HOAc) with both scopoletin and esculetin its spectral properties were identical with those of scopoletin but not with those of esculetin.

Scopoletin 7-O-β-D-*glucoside* (*XIVa, spot* 22). The material from spot 22 appeared as a bright blue spot (0·60/0·85). It was isolated by paper chromatography and when hydrolyzed with β-glucosidase produced scopoletin together with glucose.

Flavanone

Spot 5d (*liquiritigenin?*). The material from spot 5d was not isolated, but appeared as an extremely weak light-colored spot running in the same position on a 2D chromatogram as did authentic liquiritigenin.

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[26] E. SPATH and E. LEDERER, *Chem. Ber.* 63, 743 (1930).