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OCCURRENCE AND BIOSYNTHESIS OF 4',6-DIHYDROXY-AURONE IN SOYBEAN

EDMON WONG

Plant Chemistry Division, D.S.I.R., Palmerston North, New Zealand

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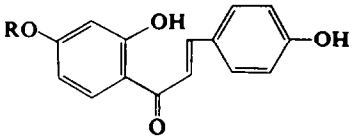
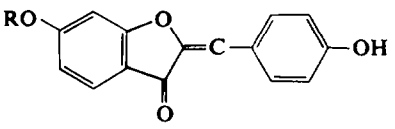
Abstract—Soybean seedlings (*Soja hispida*) have been shown to contain the chalcone isoliquiritigenin and a new aurone (hispidol) characterized by chromatographic and spectrophotometric means as 4', 6-dihydroxy-aurone. The biogenetic relationship of these two compounds was demonstrated by incorporation experiments using ^{14}C-isoliquiritigenin-4'-glucoside and cell-free extracts. Radioactivity was incorporated into the aurone and its 6-glucoside. The synthesis of hispidol and its 6-glucoside by ferricyanide oxidation of the corresponding chalcones is also reported.

INTRODUCTION

SOYBEAN (*Soja hispida*) has long been known to contain the isoflavones daidzein,[1] daidzin[1] and genistin[1, 2]. The presence of other flavonoid compounds has now been revealed by paper chromatography and the detection of isoliquiritigenin (I) and the corresponding 4',6-dihydroxyaurone (III) as minor constituents of soybean seedlings is reported in this paper. The aurone, now named hispidol, has not hitherto been recognized as a natural product.



(I) R=H
(II) R=Glucosyl



(III) R=H
(IV) R=Glucosyl

The biogenetic relationship of these of these two compounds was studied by incorporation experiments using ^{14}C-isoliquiritigenin-4'-glucoside (II) and cell-free extracts of soybean seedlings. Radioactive flavonoid products were studied by chromatographic and spectrophotometric methods and by radioautography and liquid scintillation counting.

RESULTS

Identification of Hispidol and Isoliquiritigenin

Two-dimensional paper chromatograms of soybean seedling extracts revealed trace amounts of hispidol and isoliquiritigenin. After isolation by preparative paper chromatography they were identified by chromatographic and spectrophotometric methods (Tables 1 and 2) by comparison with authentic specimens. 4',6-Dihydroxyaurone has previously

[1] E. WALZ, *Ann. Chem.* **489**, 118 (1931).
[2] E. D. WALTERS, *J. Am. Chem. Soc.* **63**, 3273 (1941).

been synthesized by condensation of 6-hydroxycoumaranone and *p*-hydroxybenzaldehyde.[3] Following unpublished work on chalcone–aurone conversion quoted by Dean[4] we have found that it could be very conveniently obtained from isoliquiritigenin by oxidation with alkaline ferricyanide.

TABLE 1. CHROMATOGRAPHIC PROPERTIES OF FLAVONOID COMPOUNDS FROM SOYBEAN

Compound identified as	R_f*							Colour reaction‡	
	BeAW	30% HOAc	2 N NH_3	BAW	30% iPrOH	BeE†	BePF†	u.v.	u.v. + NH_3
Isoliquiritigenin	0·44	0·27	0·32	0·88	0·06	0·22	0·50	dk	bY-O
4′,6-Dihydroxyaurone (hispidol)	0·41	0·24	0·30	0·87	0·06	0·15	0·47	bY-Gr	bO-Y
Hispidol-6-glucoside	0·10	0·47	0·14	0·26	0·43			bGr-Y	bY-O

* For composition of solvents, see Experimental.
† TLC on silica gel.
‡ dk = dark, b = bright, Y = Yellow, O = Orange, Gr = Green.

TABLE 2. ULTRAVIOLET ABSORPTION MAXIMA FOR FLAVONOIDS FROM SOYBEAN

Compound identified as	λ_{max} (mμ) EtOH (85%)	NaOH*
Isoliquiritigenin	242, 370	436
4′,6-Dihydroxyaurone (hispidol)	255, 386	248, 454
Hispidol-6-glucoside	255, 360,† 397	289, 351, 478

* 0·003 N in 85% EtOH.
† Inflection.

Incorporation Experiments

Buffer extracts of soybean seedlings were incubated with isoliquiritigenin-4′-glucoside (carbonyl-^{14}C) and products after extraction were examined by two-dimensional chromatography and radioautography. Isoliquiritigenin and liquiritigenin (4′,7-dihydroxyflavanone) were found to be the chief radioactive products, together with several spots not normally detected as soybean constituents. One of these, a yellow compound, possessed an aurone type u.v. absorption spectrum with maxima at longer wavelengths than those of hispidol (Table 2). This is consistent with the compound being hispidol glycosylated at the 6-hydroxyl group.[3] On hydrolysis it yielded hispidol, identified by chromatographic and spectrophotometric means, and glucose, identified chromatographically. The identity of the compound as hispidol-6-glucoside (IV) was fully established by its synthesis from isoliquiritigenin-4′-glucoside by ferricyanide oxidation. Synthetic hispidol-6-glucoside was identical with the product obtained from the cell-free extract in chromatographic behaviour (Table 1) and spectral properties (Table 2).

[3] T. A. GEISSMAN and J. B. HARBORNE, *J. Am. Chem. Soc.* **78**, 832 (1956).
[4] F. M. DEAN, *Naturally Occurring Oxygen Ring Compounds*, p. 614. Butterworth, London (1963).

^{14}C-Hispidol-glucoside was isolated by preparative chromatography and its activity determined by liquid scintillation counting (Table 3); the specific activity of hispidol obtained from hydrolysis of the glucoside was similarly determined. It can be seen from Table 3 that the specific activity of hispidol-glucoside, determined both before and after hydrolysis, agreed (within experimental limits) with that of the isoliquiritigenin-glucoside administered, indicating *de novo* synthesis of the aurone-glucoside in the cell-free system. Hispidol could not be detected on radioautographs from incorporation experiments because of its low concentration. It was isolated by preparative chromatography and found to be active on liquid scintillation counting (Table 3).

TABLE 3. RADIOACTIVITY OF FLAVONOID COMPOUNDS AFTER ISOLATION

Compound	No. of chromatographic purifications	Amount isolated* (μg)	Total activity (counts/min)†	Specific activity (counts/min per μmole $\times 10^{-3}$)
Isoliquiritigenin-4'-glucoside	4	890	38,670	18·3
Hispidol-6-glucoside	4	32·7	1,430	17·7
Hispidol from hydrolysis of glucoside	2	9·25	689	18·9
Hispidol	4	21·9	948	11·0

* Not quantitative.
† Counting efficiency 40 per cent.

In a previous incorporation study,[5] using ^{14}C-isoliquiritigenin and cell-free extracts of chana seedlings, three unknown radioactive products were reported. The one designated Y_3 has now been identified with hispidol with respect to chromatographic behaviour and spectral properties.

DISCUSSION

The number of naturally occurring aurones so far recognized is small and is limited to but a few plant families.[6] Frequently the aurone or aurone-glycoside is accompanied by the chalcone of corresponding structure suggesting a close biogenetic relationship between these two classes of compounds.[7] Dean[8] has discussed plausible mechanisms for the oxidative cyclization of chalcones to aurones. The *in vitro* conversion of chalcones to aurones has been demonstrated by Shimokoriyama and Hattori[9] using enzymic extracts of flowers of *Cosmos* or *Coreopsis* spp. Results from the present work provides further experimental evidence for biochemical conversion of chalcone to aurone and indicate that a chalcone is an intermediate in aurone biosynthesis. This lends further support to the hypothesis that a chalcone is the common C_{15}-intermediate for the biosynthesis of all classes of flavonoids.

[5] E. WONG, *Biochim. Biophys. Acta* **111**, 358 (1965).
[6] M. SHIMOKORIYAMA, In *The Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN), p. 286. Pergamon Press, Oxford (1962).
[7] M. SHIMOKORIYAMA and S. HATTORI, *J. Am. Chem. Soc.* **75**, 1900 (1953); T. A. GEISSMAN, J. B. HARBORNE and M. K. SEIKEL, *J. Am. Chem. Soc.* **78**, 825 (1956); J. B. HARBORNE and T. A. GEISSMAN, *J. Am. Chem. Soc.* **78**, 929 (1956); M. SHIMOKORIYAMA, *J. Am. Chem. Soc.* **79**, 214 (1957).
[8] F. M. DEAN, *Naturally Occurring Oxygen Ring Compounds*, p. 163. Butterworth, London (1963).
[9] M. SHIMOKORIYAMA and S. HATTORI, *J. Am. Chem. Soc.* **75**, 2277 (1953).

EXPERIMENTAL

Plant Material

Soya beans germinated in the dark for 8–10 days at 20° were used both for the chromatographic studies and incorporation experiments.

Extraction of Flavonoids from Seedlings

Extraction of seedlings was carried out as described for chana seedlings in a previous paper.[10] The seedlings were extracted with aqueous ethanol and the extract washed with petroleum ether to remove fatty material. Ethanol was removed by concentration *in vacuo* and the aqueous residue extracted with ether. The mixture of polyphenols obtained by evaporation of the ether extract was taken up in ethanolic solution for chromatography.

Chromatography

Solvent systems used for paper chromatography were (all compositions by volume): BeAW—benzene–acetic acid–water (125:72:3); BAW—*n*-butanol–acetic acid–water (120:30:50); 2 N ammonia, 30% acetic acid, 30% isopropyl alcohol.

Two dimensional chromatography was carried out in the systems BeAW and 30% HoAc, and BeAW and 2 N NH_3. For preparative chromatography Whatman 3 MM paper previously washed with dilute acetic acid and 85% ethanol was used. Spectra of compounds eluted from paper were measured against a blank eluted from an exact equivalence of paper.

Thin-layer chromatography was carried out with plates of silica gel activated by heating at 110°. Solvent systems were: BeE—benzene–ethanol (10:1) and BePF—benzene–pyridine–formic acid (72:18:10).

Chromatographic data for compounds identified are given in Table 1. Ultraviolet examination of papers was made with a Hanovia 16 lamp, max. 365 mμ.

^{14}C-*Isoliquiritigenin-4′-glucoside*

Isoliquiritigenin-4′-β-glucoside (carbonyl-^{14}C), specific activity 20·6 μc/mmole, was prepared as previously described.[5]

Synthesis of Hispidol

To a stirred solution of isoliquiritigenin (72 mg) in 5% NaOH (1 ml) was added dropwise a solution of $K_3Fe(CN)_6$ (170 mg) in water (1 ml). After 3 hr stirring, the solution was acidified with dilute HCl and extracted repeatedly with ethyl acetate. The extract, after washing with water, was concentrated to dryness and the product recrystallized from aqueous ethanol, yielding yellow crystals, m.p. 287–290° (decomp.), after first sintering to minute needles (lit.[3] m.p. 288° decomp.). The synthetic compound was identical with a sample of 4′,6-dihydroxyaurone prepared by condensation of 6-hydroxycoumaran-3-one[11] and *p*-hydroxybenzaldehyde according to published procedures.[12]

Synthesis of Hispidol-6-glucoside

A solution of isoliquiritigenin-4′-glucoside (15 mg) in 1·3% NaOH (0·5 ml) was treated with a solution of $K_3Fe(CN)_6$ (26 mg) in water (0·5) in a similar manner to that described

[10] E. WONG, P. I. MORTIMER and T. A. GEISSMAN, *Phytochem.* **4**, 89 (1965).
[11] T. A. GEISSMAN and J. B. HARBORNE, *J. Am. Chem. Soc.* **77**, 4624 (1955).
[12] R. L. SHRINER and M. WITTE, *J. Am. Chem. Soc.* **61**, 2328 (1933).

above. The reaction mixture, after neutralization with dilute HCl was evaporated *in vacuo* to dryness. The crude product was taken up in absolute ethanol and purified by preparative chromatography successively in the solvent systems 30% HOAc and 2 N NH_3. The aurone-glucoside isolated was recrystallized from water yielding minute yellow cubes m.p. 191–192°.

^{14}C-Incorporation Experiment

Cell-free extracts were prepared in a similar manner as described previously for chana seedlings.[5] Batches of 100–120 g of seedlings (from 80 g dry beans) were macerated at 2° with 100 ml of 0·05 M tris–HCl buffer (pH 7·5) in a pestle and mortar. After straining through muslin the mixture was centrifuged for 30 min at 27,000 *g* and the supernatant was incubated at 25° with stirring with the ^{14}C-isoliquiritigenin-glucoside (6–8 mg). After 3 hr the mixture was boiled with an equal volume of ethanol and filtered. The alcoholic solution was concentrated *in vacuo* to a small volume and the aqueous residue extracted repeatedly with ether. The ether soluble material (*fraction A*) and the aqueous residue after extraction (*fraction B*) were both analysed by two-dimensional chromatography (solvents for *fr B*: BAW and 30% HOAc). Chromatograms were radioautographed for 3–4 weeks using Kodak royal blue X-ray films. The phenolic compounds were found to be located in *fraction A* but *fraction B* was found to contain the major portion of the hispidol-glucoside. Blank experiments were carried out using heat inactivated extracts (boiling 5–10 min). No hispidol-glucoside was found in these experiments.

Hispidol, hispidol-glucoside and isoliquiritigenin-glucoside were isolated by preparative chromatography (see above) for spectral determinations and liquid scintillation counting. Amounts isolated were calculated from the absorptivity measured at the λ_{max}. $E^{1\%}_{1cm}$ values (determined with synthetic materials in 85% ethanol) are as follows: isoliquiritigenin-4′-glucoside, $7{\cdot}23 \times 10^3$ (375 mμ); hispidol-6-glucoside $5{\cdot}87 \times 10^2$ (397 mμ); hispidol, $10{\cdot}8 \times 10^2$ (386 mμ).

A portion of the hispidol-glucoside isolated was hydrolysed by refluxing with N HCl in 50% ethanol for 1 hr. The products were partitioned in ether and water and the fractions analysed separately by paper chromatography. Glucose was identified by chromatographic comparisons with the authentic compound. Hispidol was eluted for spectral determinations and liquid scintillation counting.

Liquid Scintillation Counting

The compounds after isolation by means of paper chromatography were taken up in dioxane–naphthalene (5%) containing 0·7% PPO and 0·05% POPOP and counted in a liquid scintillation counter (Nuclear Enterprises, NE 8301). The counting efficiency was 40 per cent as measured by addition of internal standards.