Phytochemistry, 1968, Vol. 7, pp. 1765 to 1772 Pergamon Press. Printed in England

BIOSYNTHESIS OF ISOFLAVONES—XVII.*

IDENTIFICATION AND BIOSYNTHESIS OF COUMESTANES IN *SOJA HISPIDA*

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(*Received* 7 *May* 1968)

Abstract—Two coumestanes were isolated for the first time from seedlings of *Soja hispida*. One compound was identified as coumestrol. The other compound, sojagol, is a new coumestane the structure of which was established as 7-hydroxy-5′,5′-dimethyl-6′-oxa-12,13-cyclohexenocoumestane (IV). When 7,4′-dihydroxyisoflavanone-[T] and 7,4′-dihydroxyflavanone-[2-^{14}C] with a T/^{14}C ratio of 1·43 was fed to the seedlings, the T/^{14}C ratio in the isolated coumestrol from stem and leaves was 1·80 and in daidzein 1·40, a result which indicates that the isoflavanone is a better precursor for coumestrol than the flavanone. The isolated sojagol was also radioactive but the total amount of radioactivity was too small to determine the exact rate of incorporation.

INTRODUCTION

DURING our investigation of the dehydrogenation of dihydrodaidzein to daidzein in *Soja* seedlings[1] we noticed two fluorescent spots on the chromatograms which had the characteristic colour of coumarins or coumestanes. Since the co-occurrence of isoflavones and coumestanes would be of biogenetic interest we have now investigated the nature of these compounds more closely.

RESULTS

Coumestanes in Soja hispida

The coumestanes were isolated from the roots of 4-week-old *Soja* seedlings. After enzymic hydrolysis of the glycosides by the glycosidases present in the roots, the ether extract of the aqueous phase was chromatographed on silica gel with benzene and increasing amounts of ethanol. The fractions with the strongest fluorescence in the u.v. were rechromatographed in the same manner. In this way, two strong fluorescent compounds (S_1 and S_2) were partially separated from each other.

The compound in the latter fraction (S_2) was further purified on preparative silica gel plates with benzene/dioxane/acetic acid (90:25:4), R_f 0·35. S_2 had the same R_f as coumestrol (I) in six different solvent systems on paper or thin-layer plates (Table 1) and had an identical u.v. spectrum (Table 2). After methylation with methyl iodide and K_2CO_3 in acetone the methyl ether was purified by TLC and sublimation. It had the same chromatographic and spectral properties as coumestrol dimethyl ether and possessed an identical i.r. spectrum. S_2 is therefore identical with coumestrol.[2] The concentration of coumestrol (u.v., A_{343}) was 0·05% relative to the dry weight of the roots.

* Part XVI: H. GRISEBACH and H. ZILG, *Z. Naturforsch.* **23b**, 494 (1968).

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[1] H. GRISEBACH and H. ZILG, *Z. Naturforsch.* **23b**, 494 (1968).

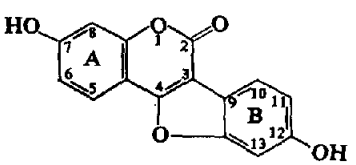
[2] E. M. BICKOFF, R. L. LYMAN, A. L. LIVINGSTON and A. N. BOOTH, *J. Am. Chem. Soc.* **80**, 3969 (1958).

TABLE 1. R_f VALUES OF COUMESTANES

Compound	R_f*					
	On paper			On silica gel plate		
	I	II	III	IV	V	VI
Coumestrol (S_2)	0·48	0·49	0·43	0·62	0·25	0·40
S_1	0·80	0·64	0·72	0·72	0·35	0·49
4′-Methylcoumestrol	0·60	0·49	—	0·72	0·41	—
Medicagol	0·58	0·49	—	0·76	—	—
7-Hydroxy-11,12-dimethoxycoumestan	0·37	—	—	0·59	0·25	—
Trifoliol	0·57	—	—	—	—	—
Psoralidine	—	0·58	—	—	0·35	—

* Key: I=acetic acid/H_2O/HCl (50:35:15); II=benzene/acetic acid/H_2O (125:72:3); III=50% acetic acid; IV=acetone/petrol. ether, 60–70° (2:1); V=ether/petrol. ether, 60–70° (7:3); VI=chloroform/isopropanol (9:1).

S_1 was purified from the first fractions by paper and thin-layer chromatography. The chromatographic properties of S_1 in comparison with other coumestanes are listed in Table 1. After sublimation, S_1 melted at 284–286°. The u.v. spectrum of S_1 is very similar to that of coumestrol (Table 2). The λ_{max} of S_1 in methanol (347 nm) undergoes a bathochromic shift to 375 nm in the presence of sodium acetate, indicating that the free hydroxyl group in S_1 is located at the 7-position[3] (see formula I). The further bathochromic shift in the presence of sodium methylate is only 5 nm, indicating that there is no free hydroxyl group at C-12; the cause of this very small bathochromic shift after addition of sodium methylate is not clear. The λ_{max} of S_1 does not shift upon addition of boric acid–sodium acetate and, therefore, S_1 does not contain an *o*-dihydroxyl grouping.[4]



Coumestrol (I)

In the i.r. spectrum of S_1 (Fig. 1) the lactone carbonyl band appears at 1700 cm^{-1} and an OH-stretching band at 3278 cm^{-1}. The bands at 2985 and 2941 cm^{-1} are attributable to CH_3-stretching, the band at 806 cm^{-1} to out of plane deformation vibration for two adjacent aromatic protons and the band at 1251 cm^{-1} to an aryl ether.

In the mass spectrum of S_1 (Fig. 2) the molecular ion is present at *m/e* 336 and important fragment ions at *m/e* 280 (base peak), 252, 224, 196 and 168. Metastable peaks at 233 and 227 prove that *m/e* 336 loses 56 mass units and *m/e* 280 loses 28 units. The second fragmentation corresponds to loss of CO followed by further loss of CO fragments, which has also been observed for other coumarins.[5]

[3] L. JURD, *J. Org. Chem.* **24**, 1786 (1959).
[4] L. JURD, *Arch. Biochem. Biophys.* **63**, 376 (1956).
[5] R. A. W. JOHNSTONE, B. J. MILLARD, F. M. DEAN and A. W. HILL, *J. Chem. Soc.* (C) 1712 (1966).

TABLE 2. SPECTRAL PROPERTIES OF COUMESTROL AND S_1

	λ_{max} nm (log ϵ) in MeOH*	In MeOH +NaOAc	In MeOH +H_3BO_3/NaOAc	In 0·002 m NaOMe
Coumestrol (synthetic)	244 (4·40); 305 (4·00); 343 (4·46)	313; 364	—	250; 280; 312; 380
Coumestrol (from *Soja*)	244; 304; 344	313; 367	347	250; 280; 310; 380
S_1	255 (4·40); 305 (3·90); 347 (4·40)	312; 375	347	275; 312; 380

* Figures underlined indicate major peaks.

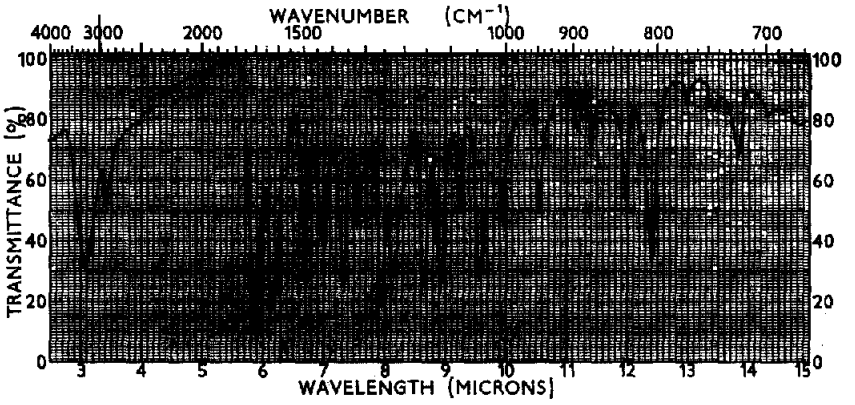


FIG. 1. I.r.-SPECTRUM OF S_1 (SOJAGOL) IN KBr. PERKIN-ELMER, INFRACORD.

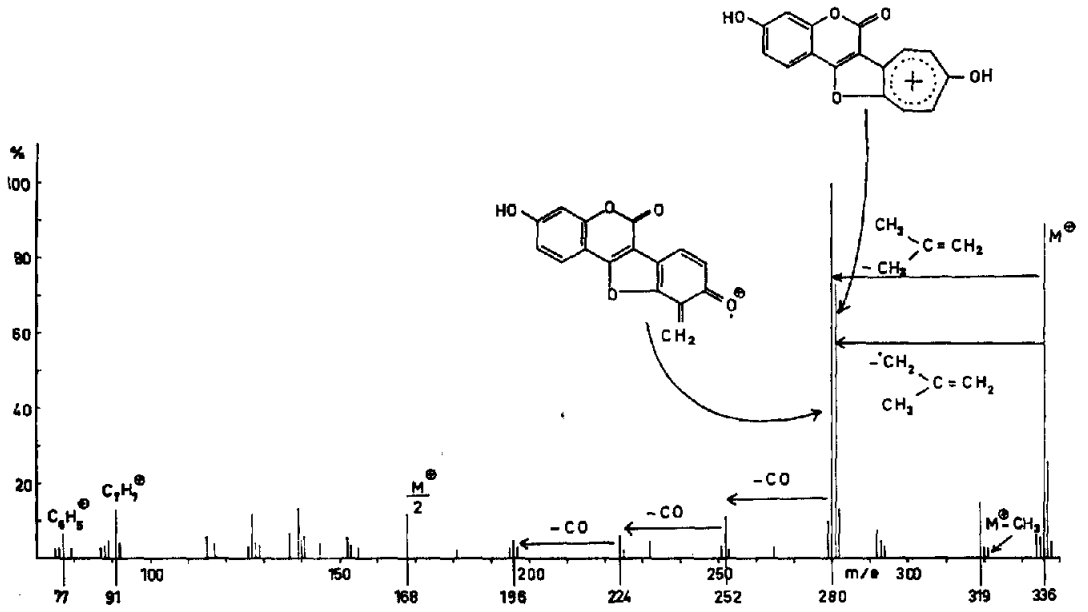
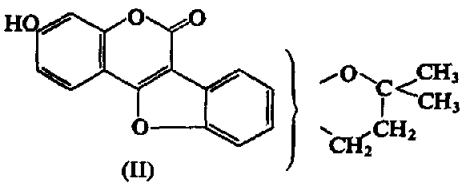


FIG. 2. MASS SPECTRUM OF S_1 (SOJAGOL). ATLAS CH4.

A high resolution mass spectrum gave the following elemental compositions of the molecular ion and of the two most intense peaks.

Found	Required	Formula
336·0992 ± 0·0015	336·0998	$C_{20}H_{16}O_5$
281·0433 ± 0·0028	281·0450	$C_{16}H_9O_5$
280·0351 ± 0·0028	280·0371	$C_{16}H_8O_5$

On the basis of the known substitution pattern in coumestanes, the molecular formula and the u.v. data the following partial structure (II) can be assigned to S_1.



(II)

Taking into account the NMR data (see below) the interpretation of the mass spectrum of S_1 is shown in Fig. 2.

The base peak at *m/e* 280 is formed by loss of isobutene (C_4H_8) from the molecular ion by a retro-Diels–Alder fragmentation. The ion *m/e* 281 could arise by loss of an isobutenyl radical with hydrogen transfer to the ether oxygen. In contrast to the mass spectrum of 2,2-dimethylchroman, an intense peak representing loss of a methyl group is not observed.[6]

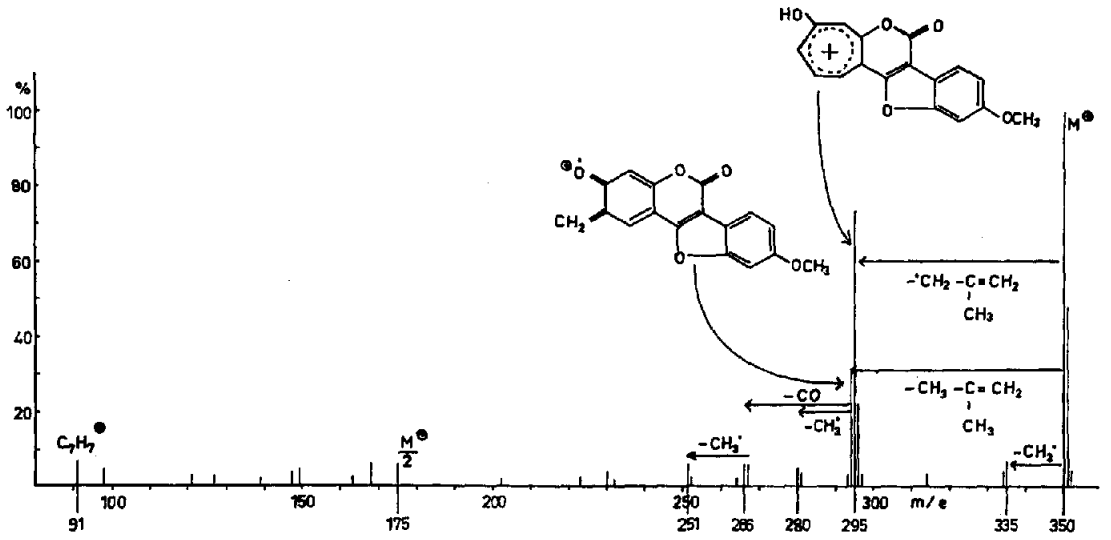
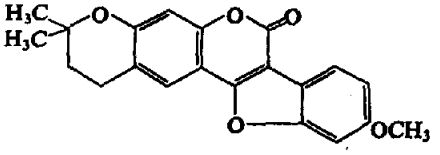


FIG. 3. MASS SPECTRUM OF ISOPSORALIDINE MONOMETHYL ETHER. ATLAS CH4.

However, the mass spectrum of isopsoralidine monomethyl ether (III),[7] Fig. 3, which was taken in comparison shows also only loss of a methyl fragment with weak intensity and, as main fragmentation, loss of C_4H_8 and C_4H_7 fragments.



Psoralidine monomethyl ether (III)

The mass spectrum of S_1 after treatment with CH_3OD proves that only one exchangeable proton is present (at C-7).

[6] B. WILLHALM, A. F. THOMAS and F. GAUTSCHI, *Tetrahedron* **20**, 1185 (1964).
[7] H. N. KHASTGIR, P. C. DUTTAGUPTA and P. SENGUPTA, *Tetrahedron* **14**, 275 (1961).

TABLE 3. τ-VALUES OF S_1 AND COUMESTROL (SPIN–SPIN COUPLING CONSTANTS*)

Compound	Methyl	Methylene	H-5	H-6 H-8	H-10	H-11	H-13
S_1	8·6	multiplets 8·1; 6·9	2·16 (9)	multiplet ~3·1 (9, 2·5)	2·43 (8)	~3·1 (8)	
Coumestrol			2·10 (9·5)	multiplet 2·80–3·00	2·22 (9)		multiplet 2·80–3·00

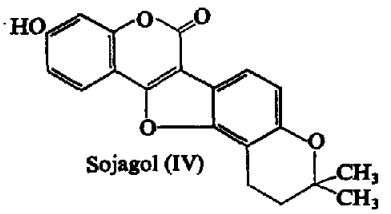
* First-order approximation in c/s.

TABLE 4. INCORPORATION OF LABELLED PRECURSORS INTO DAIDZEIN AND COUMESTROL IN *Soja* SEEDLINGS

	Roots			Stem and leaves		
	T	^{14}C	T/^{14}C	T	^{14}C	T/^{14}C
Precursor 7,4-Dihydroxyflavanone-[2-^{14}C] 3·42 μc Dihydrodaidzein-[T] 4·90 μc			1·43			1·43
Spec. activity (dpm/mmole)						
Daidzein	$2·4 \times 10^7$	$3·0 \times 10^7$	0·8	$2·18 \times 10^7$	$1·55 \times 10^7$	1·40
Coumestrol	$1·1 \times 10^7$	$1·4 \times 10^7$	0·79	$1·25 \times 10^7$	7×10^6	1·80
Dilution						
Daidzein	215	196		239	381	
Coumestrol	460	410		410	840	
Incorporation rate (%)						
Daidzein	0·05	0·08		0·04	0·04	
Coumestrol	0·02	0·03		0·002	0·002	
Change in T/^{14}C ratio						
Daidzein	100 → 56			100 → 98		
Coumestrol	100 → 55			100 → 126		

The position of the dimethylchroman ring can be deduced from the 60 mcs NMR spectrum. The spectral data for S_1 and for coumestrol[8] is given in Table 3. The position of the methyl singlet (in $CDCl_3$ as solvent) proves that the methyl groups cannot be located at a double bond (dimethylallyl substituent) and is consistent with the presence of a dimethylpyran ring. Since two neighbouring protons at C-5 and C-6 and a free hydroxyl group are present in ring A the pyran ring cannot be attached to this benzene ring.

Taking into account the known substitution pattern of coumestanes the following structure is proposed for S_1 which is consistent with all spectral data.



Sojagol (IV)

The low field multiplet for the methylene protons can also be explained by this structure because these protons would come under the deshielding influence of the furan ring. For this new coumestane we propose the trivial name sojagol.

Incorporation of 7,4'-Dihydroxyflavanone-[2-^{14}C] and 7,4'-Dihydroxyisoflavanone-[T] into coumestrol in Soja *seedlings*

Incubation of *Soja* seedlings with the above mixture of labelled flavanone and isoflavanone had been carried out earlier to investigate the ability of the seedlings to dehydrogenate the isoflavanone to the corresponding isoflavone (daidzein).[1] After coumestrol had been identified in the seedlings, this compound was isolated from the chromatograms and purified to radiopurity after dilution with synthetic coumestrol. The results are shown in Table 4. Sojagol was also radioactive in this experiment. However, the total amount of radioactive material isolated was too small to determine the exact incorporation rate.

DISCUSSION

Sojagol is the twelfth coumestane to be isolated from plants, all of which belong to the family Leguminosae.[9] The co-occurrence of the isoflavone daidzein and of coumestrol and sojagol in *Soja hispida* is of biogenetic and taxonomic interest. The co-occurrence of isoflavones and coumestanes has been found before in several clover varieties[10,11] and in lucerne.[11]

Biogenetically coumestanes belong to the class of isoflavones. An earlier investigation with lucerne (*Medicago sativa*) had shown that the labelling pattern in coumestrol with cinnamic-acid-[3-^{14}C], acetate-[1-^{14}C] and 4,2',4'-trihydroxychalcone-4'-glucoside-[β-^{14}C] as precursors is the same as in isoflavones.[12] Furthermore it was shown that daidzein is a

[8] A. L. LIVINGSTON, E. M. BICKOFF, R. E. LUNDIN and L. JURD, *Tetrahedron* **20**, 1963 (1964).
[9] A. C. JAIN, V. K. ROHATGI and T. R. SESHADRI, *Tetrahedron* **23**, 2499 (1967).
[10] E. M. BICKOFF, A. N. BOOTH, R. L. LYMAN, A. L. LIVINGSTON, C. R. THOMSON and F. DE EDS, *Science* **126**, 969 (1957); C. R. THOMSON, A. L. CURL and E. M. BICKOFF, *Analyt. Chem.* **31**, 838 (1959); C. M. FRANCIS, A. J. MILLINGTON and E. T. BAILEY, *Australian J. Agric. Res.* **18**, 47 (1967).
[11] R. L. LYMAN, E. M. BICKOFF, A. N. BOOTH and A. L. LIVINGSTONE, *Arch. Biochem. Biophys.* **80**, 61 (1959).
[12] H. GRISEBACH and W. BARZ, *Z. Naturforsch.* **18b**, 466 (1963); *Z. Naturforsch.* **19b**, 569 (1964).

better precursor than phenylalanine for coumestrol.[13] This result is compatible with the assumption that coumestrol is derived from daidzein.[14] On the other hand, the introduction of the oxygen function at C-2 could also occur at the dihydroisoflavone (isoflavanone) stage. In the present investigation we have therefore compared the incorporation of dihydrodaidzein-[T] and 7,4'-dihydroxyflavanone-[2-^{14}C] into coumestrol. Both compounds are incorporated into coumestrol to about an equal extent. The fact that the T/^{14}C ratio in the upper part of the plants is higher in coumestrol than in the supplied mixture indicates that dihydrodaidzein is a better precursor for coumestrol than the flavanone. However, since *Soja* seedlings are able to dehydrogenate dihydrodaidzein to daidzein[1] it is not possible to decide from this result whether the incorporation of dihydrodaidzein into coumestrol occurs via daidzein or by a direct route.

EXPERIMENTAL

Soja hispida plants were grown from a commercial variety of seeds (Fa. Henselwerk, Magstadt, Germany). For the isolation of coumestanes, 4-week-old field-grown plants were used. The tracer experiments were carried out with 2-week-old plants grown hydroponically in vermiculite.

Isolation of Coumestanes

Roots from about 4000 plants were chopped in water in a Waring blendor. The aqueous slurry was allowed to stand for 8 hr at 25° to hydrolyse the glycosides with the glycosidases present in the roots. The mixture was filtered through glass wool and the filtrate extracted with ether. The ether extract and the methanolic extract of the residue from filtration were combined and the solvent removed. The residue was taken up in ether, the etheral phase was extracted with 10% Na_2CO_3 and the aqueous phase was acidified and again extracted with ether. The ether extract was chromatographed on a silica gel column (Kieselgel S, Riedel de Haen) successively with benzene, benzene/ethanol (95:5) and benzene/ethanol (8:2). The middle fractions which showed the strongest fluorescence in u.v. light were combined and rechromatographed with the same solvents. The first fractions contained sojagol and the later fractions coumestrol. However, a complete separation of the two compounds was not obtained. The enriched fractions were therefore further purified by thin-layer and paper chromatography.

Coumestrol was purified by preparative TLC with benzene/dioxane/acetic acid (90:25:4), R_f 0·35, yield 35 mg. Five mg of coumestrol were methylated with CH_3I and K_2CO_3 in boiling acetone for 2 hr. Coumestrol dimethyl ether was purified by TLC with the solvent system $CHCl_3$/isopropanol (9:1) and had the same R_f as synthetic material[15] (R_f 0·75). Coumestrol dimethyl ether was further purified by sublimation at 180°/0·05 mm.

Sojagol was purified from the first fractions by paper chromatography in 50% acetic acid (R_f 0·72) and TLC with $CHCl_3$/isopropanol (9:1) (R_f 0·55). Sublimation at 240°/0·01 mm yielded colourless needles, m.p. 284–286°. Yield: 3 mg.

NMR Spectrum

The NMR spectrum was taken with a Varian A 60 with CAT attachment.

Determination of Radioactivity

The determination of ^{14}C and T was carried out in the gas phase according to the method of Simon *et al.*[16] with a proportional counter with an anticoincidence circuit (UNI ZS, Fa. Berthold, Wildbad, Germany).

Acknowledgements—We are indebted to Dr. G. Snatzke, Bonn, for the determination of the NMR spectrum and to Dr. H. Achenbach, Freiburg, and Dr. M. Hesse, Zürich, for the determination of the mass spectra. Reference substances were kindly supplied by Dr. E. N. Bickoff, Albany, and by Dr. P. Sengupta, Kalyani. The work was supported by Fonds der Chemischen Industrie.

[13] W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **21b**, 1113 (1966).
[14] E. C. BATE-SMITH, in *The Pharmacology of Plant Phenolics* (edited by J. W. FAIRBAIRN), p. 69, Academic Press, London (1959).
[15] E. M. BICKOFF and A. N. BOOTH, American Patent 2,987,398; *Chem. Abstr.* **56**, 5936 (1962).
[16] H. SIMON, H. DANIEL and J. F. KLEBE, *Angew. Chem.* **71**, 303 (1959).