3′-Methoxycoumestrol from Alfalfa: Isolation and Characterization

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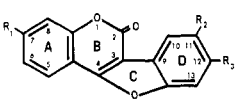
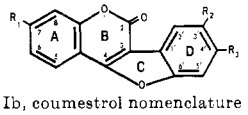
The isolation of a new coumestan, $C_{18}H_{10}O_6$, and its characterization by degradative and spectral studies are described. Synthesis confirmed the structure as 7,12-dihydroxy-11-methoxycoumestan for which the common name 3′-methoxycoumestrol is proposed.

Twelve phenolic compounds have been isolated from alfalfa (*4*) and their structures determined (*1–3, 5, 6, 18, 19, 24, 25*) (Table I). An additional compound, previously designated as Compound III (*4*), was obtained in admixture with coumestrol from which it could not be separated readily. This paper reports the procedures that were subsequently employed for its separation from coumestrol and for its characterization as the 3′-methoxy derivative of coumestrol (I, $R_1 = R_3 = OH$, $R_2 = OCH_3$).

Coumestrol and Compound III were initially separated in limited quantities by paper chromatography. Elemental analysis indicated that Compound III was a monomethoxy compound. Formation of a diacetyl and a dimethoxyl derivative demonstrated the presence of two hydroxyl groups. The UV spectrum of Compound III (3′-methoxycoumestrol) was very similar to that of coumestrol (*1*) (I, $R_1 = R_3 = OH$, $R_2 = H$), suggesting that these compounds were structurally related. The λ_{max} of 3′-methoxycoumestrol in alcohol (351 mμ, Peak III, Table II) underwent a bathochromic shift to 373 mμ in the presence of sodium acetate, indicating the presence of a hydroxyl group in the 7-position (*15*). The λ_{max} did not shift in the presence of boric acid–sodium acetate, suggesting that 3′-methoxycoumestrol did not contain an *ortho*-dihydroxyl grouping (*14*).

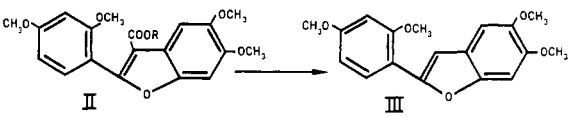


Ia, coumestan nomenclature



Ib, coumestrol nomenclature

To establish the ring substitution pattern of Compound III, it was desirable to degrade the compound to its benzofuran derivative. Since it was difficult to obtain sufficient material for this purpose by paper chromatography, the mixture of Compound III (3′-methoxycoumestrol) and coumestrol was taken to the *ortho*-methoxycinnamic acid derivatives via methylative ring opening, followed by hydrolysis. These derivatives were separated by countercurrent distribution (CCD) and the *ortho*-methoxycinnamic acid derivative (II, R = H) of 3′-methoxycoumestrol was decarboxylated to the benzofuran (III).



II III

The 60-Mc. proton magnetic resonance (PMR) spectra of the methyl ether and the benzofuran derivatives of 3′-methoxycoumestrol in tetrachloroethane and deuterochloroform, respectively, were used to establish the ring substitution pattern. The spectra of the benzofurans of the four related coumestans, coumestrol (*1*), trifoliol (*18*), medicagol (*19*), and sativol (*24*), were used to assist in the proton assignments.

Decarboxylation of 3′-methoxycoumestrol methyl ether left the position of an apparent *ortho-para* doublet ("X" portion of the ABX multiplet; splitting = 9 cps.) unchanged, at $\tau = 2.18$, while a broadened singlet at $\tau = 2.61$ was shifted upfield by 0.31 p.p.m. As was shown previously (*18*), these bands can be assigned only to the 5- and 10-proton positions. Since decarboxylation has an appreciable effect at only the 10-position, the *ortho-para* doublet must be assigned to H-5 and the broadened singlet to H-10. Therefore, the A ring must be substituted only at the 7-position, and the D ring at both the 11- and 12-positions. The functional group assignment was confirmed by comparison of the methyl ether of 3′-methoxycoumestrol with 7,11,-12-trimethoxycoumestan, with which it was identical (*19*).

The location of the lone methoxyl group at either the 11- or 12-position could not be determined by PMR spectroscopy. However, the location of the methoxyl group at the 11-position was suggested by UV spectroscopy. The shifts of Peaks I, II, III (Table II) of Compound III were similar to the comparable shifts of coumestrol and 7,11,12-trihydroxycoumestan, both of which have a hydroxyl group at the 12-position. However, Peak I of 4′-methoxycoumestrol split into two peaks upon treatment with alkali. This suggested that the methoxyl group of Compound III is not located at the 12-position but is probably at the 11-position.

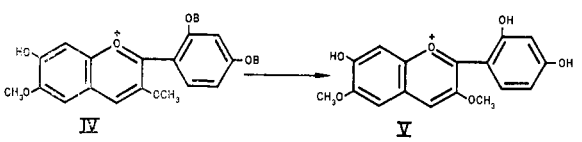
Definite assignment of the methoxyl group to the 11-position and confirmation of the structure of 3′-methoxycoumestrol were accomplished by unequivocal synthesis of the compound from 2,4 - dihydroxy - 5 - methoxybenzaldehyde and ω-2,4-dibenzyloxyacetophenone by means of Jurd's procedure (*16*) for synthesis of coumestans. The natural and synthetic compounds were identical.

Table I. Phenolic Compounds in Alfalfa

Common Name	Trivial Name	Compound No.	Ref.
Coumestrol	7,12-Dihydroxycoumestan		(*1, 4*)
Salicylic acid	2-Hydroxybenzoic acid		(*4*)
Tricin	5,7,4′-Trihydroxy-3′,5′-dimethoxyflavone		(*2, 4*)
Trifoliol	7,10-Dihydroxy-12-methoxycoumestan		(*18*)
Medicagol	7-Hydroxy-11,12-methylenedioxycoumestan	I	(*19*)
4′-Methoxycoumestrol	7-Hydroxy-12-methoxycoumestan	II	(*5*)
3′-Methoxycoumestrol	7,12-Dihydroxy-11-methoxycoumestan	III	
	7,4′-Dihydroxyflavone	IV	(*3*)
	3′,4′,7-Trihydroxyflavone	V	(*6*)
Lucernol	6,7,12-Trihydroxycoumestan	VI	(*24*)
Sativol	8,12-Dihydroxy-7-methoxycoumestan	VII	(*24*)
	11,12-Dimethoxy-7-hydroxycoumestan	VIII	(*25*)

Table II. Ultraviolet Spectra of Coumestans

Compound	Peak	λ_{max}, mμ EtOH	λ_{max}, mμ 0.1N NaOH
Coumestrol	I	244	282
	II	304	312
	III	344	381
4′-Methoxycoumestrol	I	242	242, 270
	II	302	312
	III	342	380
7,11,12-Trihydroxycoumestan	I	248	258
	II	308	322
	III	353	388
3′-Methoxycoumestrol	I	248	267
	II	308	315
	III	351	382



Experimental

Isolation. A solution of the mixture of coumestrol and Compound III in tetrahydrofuran was applied to Whatman 3MM paper (18 × 22½ cm.) as a thin band at a maximum level of 4 mg. of Compound III per sheet. The papers were developed in a solvent system consisting of isopropyl alcohol and concentrated ammonium hydroxide (2:1) by downflow development until the leading edge of the Compound III zone had come within 1 inch of the bottom of the paper (36 to 40 hours). The Compound III zone was recovered from the paper by repeated extractions with boiling methanol. The entire process was repeated twice to complete the separation of the two compounds. The crude Compound III was acetylated to give white needles (260 mg.), m.p. 282–283° C.

Analysis. Calculated for $C_{20}H_{14}O_8$: C, 62.8; H, 3.67; OCH_3, 8.11; CH_3CO, 22.5. Found: C, 62.8; H, 3.86; OCH_3, 7.99; CH_3CO, 22.2.

Deacetylation. An ice-cold solution of the acetate (225 mg.) (I, $R_1 = R_3 = OOCCH_3$, $R_2 = OCH_3$) in 25 ml. of 0.5% methanolic potassium hydroxide was stirred at zero degrees for 30 minutes. The ice bath was removed, and the stirring was continued until complete solution had occurred (1½ hours). The yellow reaction mixture was poured into ice water, acidified, and filtered. The gelatinous solid was recrystallized from acetone to give a straw-colored solid (135 mg.), m.p. 329.0–329.5° C. dec.

Analysis. Calculated for $C_{16}H_{10}O_6$: C, 64.4; H, 3.38; OCH_3, 10.4. Found: C, 64.2; H, 3.51; OCH_3, 10.9.

Dimethyl Ether (I, $R_1 = R_2 = R_3 = OCH_3$). 3′-Methoxycoumestrol (60 mg.), potassium carbonate (500 mg.), dimethyl sulfate (0.36 ml.), and dry acetone (100 ml.) (dried over anhydrous potassium carbonate) were refluxed for 2 hours. The reaction mixture was cooled, filtered, and taken to dryness in vacuo. Recrystallization from methanol gave white needles (60 mg.), m.p. 205–207° C.

Analysis. Calculated for $C_{18}H_{14}O_6$: C, 66.3; H, 4.29; OCH_3, 28.8. Found: C, 65.9; H, 4.35; OCH_3, 28.2.

Degradations. The following reactions were carried out under nitrogen, and their progress was followed by thin layer chromatography (TLC) on Silica Gel G employing ether and Skellysolve B (7:3) as developer.

Tetramethyl Ether-Methyl Esters. The mixture of Compound III and coumestrol (10.8 grams), anhydrous potassium carbonate (11.0 grams), dimethyl sulfate (48 ml.), and dry acetone (2400 ml.) was refluxed for 3 hours. The mixture was maintained basic during the course of the reaction by the addition of 10% potassium hydroxide in methanol. The solution was filtered, concentrated to an oil, and crystallized from aqueous methanol to give 13.1 grams of a white solid.

o-Methoxycinnamic Acid. A solution of the above mixed esters (12.0 grams) in 10% methanolic potassium hydroxide (1200 ml.) was refluxed with stirring for 3½ hours. The reaction mixture was diluted with ice water (2000 ml.) and acidified to give 11.5 grams of a white solid. A total of 36 grams of the mixed *o*-methoxycinnamic acids was prepared in this manner.

The mixed acids (36.0 grams) were recrystallized several times from ether to yield a mother liquor enriched in the acid of Compound III (14.0 grams). The mother liquor was taken to dryness, and the two acids were separated by CCD in a robot-operated 100-tube instrument (200-ml. capacity), employing ether–methanol–Skellysolve B–water (5:10:5:15) as the developing solvent. After 800 transfers, the contents of tubes 20 to 70 in the instrument were concentrated to dryness, and the solids were recrystallized from methanol to give 1.9 grams of the acid of Compound III, (II, R = H) m.p. 208–208.5° C.

Analysis. Calculated for $C_{19}H_{18}O_7$: C, 63.7; H, 5.18; OCH_3, 34.3. Found: C, 63.7; H, 5.05; OCH_3, 34.3.

2 - (2′,4′ - Dimethoxyphenyl) - 5,6-dimethoxybenzofuran (III). A mixture of this acid (1.0 gram) and powdered glass (1.0 gram) was heated at 230° C. for 1 hour, cooled, and extracted with ether. The ether solution was concentrated to dryness, and the solids were recrystallized from ether to give 625 mg. of colorless platelets, m.p. 157° C.

Analysis. Calculated for $C_{18}H_{17}O_5$: C, 68.8; H, 5.74; OCH_3, 39.5. Found: C, 69.0; H, 5.77; OCH_3, 39.8.

3,6 - Dimethoxy - 7 - hydroxy - 2′,4′-dibenzyloxyflavylium Chloride (IV). A solution of 2,4-dihydroxy-5-methoxybenzaldehyde (4.9 grams) (*23*) and ω-methoxy - 2,4 - dibenzyloxyacetophenone (12.75 grams) in ethyl acetate (50 ml.), and ether (150 ml.) was cooled in an ice bath and treated with HCl gas for 30 minutes. The flavylium salt rapidly separated as red needles. After standing for 72 hours at 0° C., the red crystals were collected, washed with ether, and air-dried (15.25 grams), m.p. 188–189.5° C. dec.

3,6 - Dimethoxy - 7 - hydroxy - 2′,4′-dihydroxyflavylium Chloride (V). A mixture of the flavylium salt (15.0 grams), acetic acid (60 ml.), and concentrated hydrochloric acid (60 ml.) was heated on a steam bath for 1 hour. The thick red paste was diluted with 10% aqueous hydrochloric acid (840 ml.) and layered with a 1 to 1 mixture of Skellysolve B and benzene (420 ml.). After being cooled overnight at 0°, the red solids were collected and washed with ethanol and 10% hydrochloric acid (1:1) giving 8.33 grams of a red solid which had no definite melting point.

Synthetic 3′-Methoxycoumestrol Acetate. The above salt (7.84 grams) was suspended in methanol (120 ml.) and water (60 ml.), warmed on a steam bath, and oxidized with H_2O_2 (30%) (30 ml.). After 15 minutes, concentrated sulfuric acid (30 ml.) was added, and the heating was continued for an additional 15 minutes. The reaction mixture was diluted with an equal volume of water and cooled to give 0.98 gram of a dark gummy solid. This crude product was purified in the same manner as described for the natural compound. The crude synthetic Compound III was then acetylated to give 65 mg. of a white solid, m.p. 281–282° C. No depression of mixed melting point was obtained with the natural product. The infrared and PMR spectra of the natural and synthetic compounds were identical.

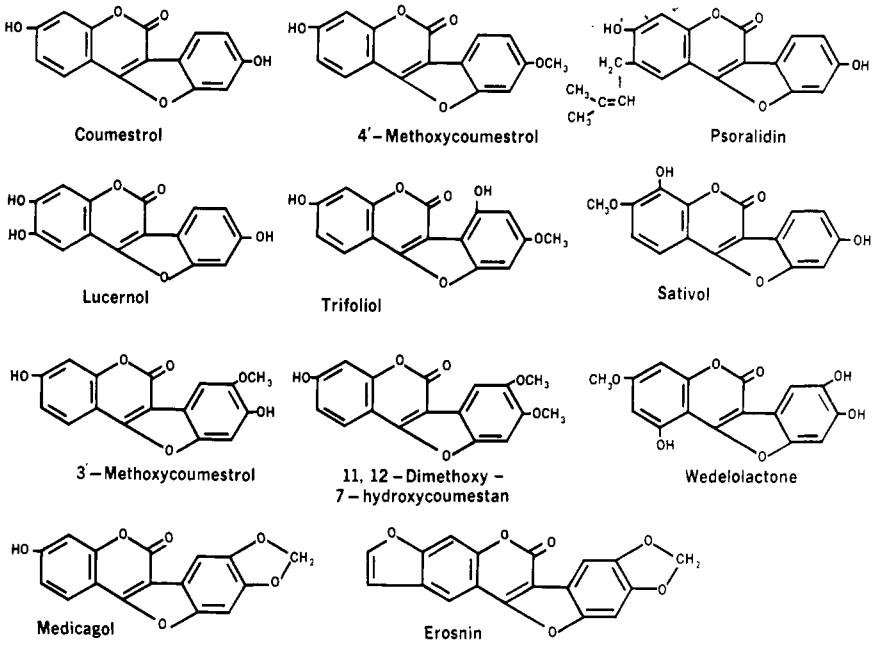


Figure 1. Naturally occurring coumestans

Results and Discussion

The compound, 3'-methoxycoumestrol, is the eighth coumestan isolated thus far from alfalfa. There is paper chromatographic evidence for the presence of additional members of the series (*4*). In addition, three other coumestans have been isolated from other plant sources (*10*, *12*, *17*). The structures of all these naturally occurring coumestans are presented in Figure 1. Coumestrol has the simplest structure of the series and, therefore, the others might be considered as further elaboration of the coumestrol molecule. Although the biosynthetic pathway of coumestrol has been worked out by Grisebach (*13*), no similar studies have thus far been carried out for any of the other coumestans.

The coumestrol content of alfalfa may rapidly increase more than 180-fold as a result of pathogenic attack of the plant (*20*). More recently, it has been found (*21*) that selection for increased disease resistance significantly lowered coumestrol content. The level of the accompanying coumestans is also related to the degree of infection (*7*).

According to Farkas and Király (*11*), various fungal, bacterial, and viral infections are known to result in the formation of aromatic compounds including coumarin derivatives. The authors' results with the coumestans would seem to confirm another example of induced synthesis of aromatic compounds in plants. Compounds biosynthesized in response to pathogenic attack of the plant and having the ability to act as plant protective agents are known as phytoalexins (*9*). A number of known phytoalexins, including pisatin (*22*) and trifolirhizin (*8*), have structures closely related to the coumestans.

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