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TRIFOLIOL, A NEW COUMESTAN FROM LADINO CLOVER

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Abstract—A new coumestan, $C_{16}H_{10}O_6$, related to coumestrol, has been isolated from ladino clover. Degradation and synthesis of the compound established its structure as 3,7-dihydroxy-9-methoxy-6H-benzofuro[3,2,c][1]-benzopyran-6-one.

IN PREVIOUS communications, we established the occurrence of the estrogen-like compound coumestrol in alfalfa, ladino clover, and other legumes.[1,2] Further investigations of the phenolic constituents of ladino clover[3] led to a new compound, $C_{16}H_{10}O_6$ (XI), for which the trivial name *trifoliol* is proposed.

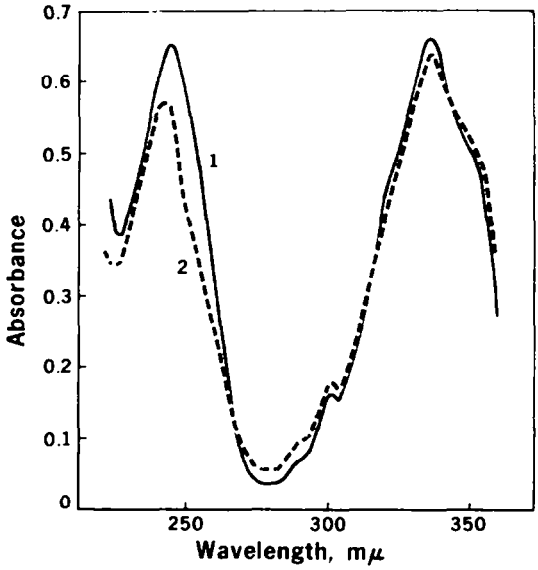


FIG. 1. UV spectra in ethanol of (1) trifoliol acetate, (2) 4′-O-methylcoumestrol acetate.

Analysis indicates that trifoliol contains one methoxy group and the formation of diacetyl and dimethyl derivatives indicates two phenolic hydroxyl groups. The UV spectrum of trifoliol diacetate is very similar (Fig. 1) to that of the acetate[4] of

[1] E. M. Bickoff, A. N. Booth, R. L. Lyman, A. L. Livingston, C. R. Thompson and F. DeEds, *Science* **126**, 969 (1957).

[2] R. L. Lyman, E. M. Bickoff, A. N. Booth and A. L. Livingston, *Arch. Biochem. Biophys.* **80**, 61 (1959).

[3] Manuscript submitted to *J. Org. Chem.* February 1964.

[4] L. Jurd, *J. Org. Chem.* **24**, 1786 (1959).

4′-O-methylcoumestrol (7-hydroxy-12-methoxycoumestan) (I, $R_1 = OH$, $R_2 = H$, $R_3 = OMe_3$). These spectra show that trifoliol and coumestrol are structurally related and, furthermore, that the methoxyl group of trifoliol is probably at the 12-position. The λ_{max} of trifoliol in alcohol (349 mμ) undergoes a bathochromic shift to 380 mμ in the presence of sodium acetate, indicating that one of the two free hydroxyl groups is located at the 7-position.[4] The λ_{max} of trifoliol does not shift with boric acid-sodium acetate, however, and, therefore, trifoliol does not contain an *o*-dihydroxyl grouping.[5]

7-Methoxy-12-hydroxycoumestan (I, $R_1 = OMe$, $R_2 = H$, $R_3 = OH$) and its benzofuran derivative (III, R = H) were model compounds for the analysis of the 60 mcs. NMR spectrum of trifoliol and its benzofuran derivative (III, R = OMe). Due to the low solubility of the parent phenols in most organic solvents, it was necessary to obtain their spectra from 5% solutions in D_7-dimethylformamide at 135°. The spectral data for these compounds is given in Table 1.

TABLE 1. SHIELDING VALUES[1] OF TRIFOLIOL AND SEVERAL MODEL COMPOUNDS (SPIN-SPIN COUPLING CONSTANTS[2] OF AROMATIC PROTONS)

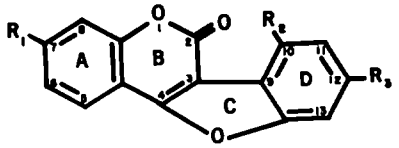
Compound[3]	H-3	H-5	H-6	H-8	H-10	H-11	H-13
Trifoliol ($R_1 = R_2 = OH$, $R_3 = OCH_3$)		2·17(9)	2·97(6·2)	3·03		3·58(1·5)	3·20(1·5)
Coumestrol ($R_1 = R_3 = OH$, $R_2 = H$)		2·10(9·5)	Complex 2·80–3·00		2·22(9)	Complex 2·80–3·00	
Trifoliol benzofuran (III, $R = OCH_3$)	2·83(1)	2·10(9·5)	3:42(6·5, 2)	3·49		3·70(2)	3·36(2, 1)
Coumestrol benzofuran (II, R = H)	2·90(1)	2·10(9·5)	3:40(8, 2·5)	3·48	2·60(8·5)	3·20(8, 2·5)	3·00

[1] in τ units.
[2] First order approximation in c/s.
[3] I, IA in DMF-D_7 at 135°. V, VA in $CDCl_3$ at 30°.

Decarboxylation of coumestrol leaves the position of the 2·10 τ doublet unchanged while the second *ortho* doublet now occurs at 2·60 τ. Since decarboxylation would not be expected to have an appreciable effect on the shielding of the 5-proton at that position the 2·10 doublet can be assigned to this proton. Conversely decarboxylation would be expected to have a major effect on the shielding of H-10. On this basis a single, low-field doublet centered at 2·18 τ in the trifoliol spectrum ($J \cong 9$ c/s) can be assigned to H-5. Decarboxylation effectively provides the A ring with a second electron donor (O-9) which will increase the shielding of the *ortho* and *para* positions of about 0·4 ppm.[6] Consequently H-6 and H-8 are assigned on the basis of the observed upfield shifts and their spin-spin splitting patterns. The only positions for the oxygen substituents in the D ring compatible with all spectral data are C_{10} and C_{12}. The 3·20 doublet is tentatively assigned to H-13 on the basis of decarboxylation shifts. The methoxyl resonance of trifoliol occurs at 6·18 τ.[7]

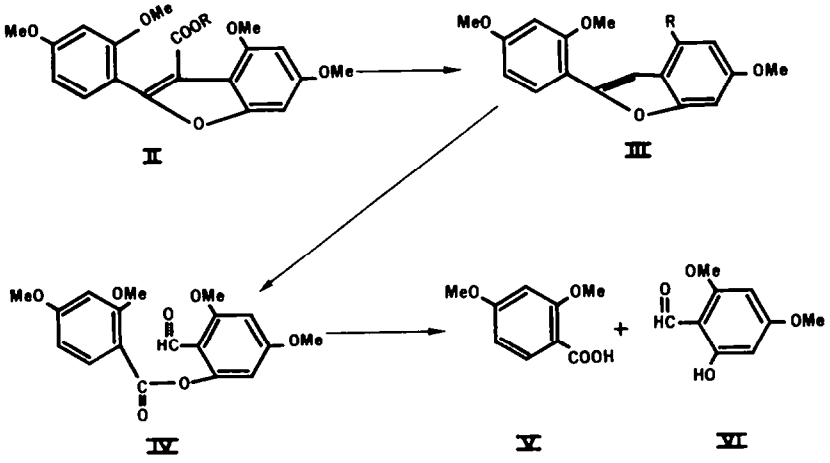
[5] L. Jurd, *Arch. Biochem. and Biophys.* **63**, 376 (1956).
[6] P. Diehl, *Helv. Chim. Acta* **44**, 829 (1961).
[7] A complete discussion of the NMR spectra of a group of substituted coumestans is now in preparation.

Degradation established the second hydroxyl at the 10-position to give structure (I, $R_1 = R_2 = OH$, $R_3 = OMe$):



I

In alkaline methyl sulphate, trifoliol formed a tetramethyl ether methyl ester (II, R = Me) which was hydrolyzed to the carboxylic acid (II, R = H). Decarboxylation of the acid occurred on heating to give the benzofuran (III, R = OMe). The benzofuran was ozonized at −65° in methylene chloride–chloroform solution and the product reduced to an aldehyde with triethyl phosphite. Crystalline 4,6-dimethoxy-2(2′,4′-dimethoxybenzoyl)benzaldehyde (IV) and its two hydrolysis products, 2,4-dimethoxybenzoic acid (V) and 2-hydroxy-4,6-dimethoxybenzaldehyde (VI) were thereby isolated.

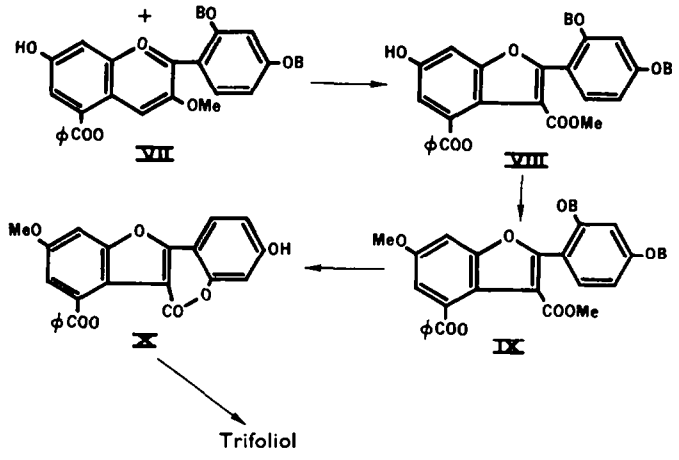


II III IV V VI

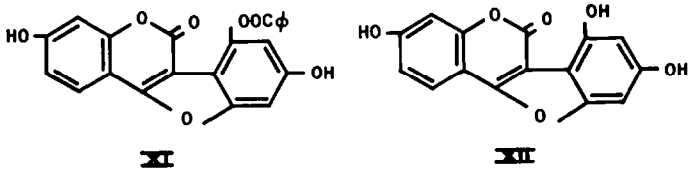
The structure of trifoliol as (I, $R_1 = R_2 = OH$, $R_3 = OMe$), indicated by the above spectral and degradative evidence, was confirmed unequivocally by its synthesis from 3-methoxy-5-benzoyloxy-7-hydroxy-2′,4′-dibenzyloxyflavylium chloride. Peroxide oxidation of the flavylium salt[8] (VII) gave the intermediate benzofuran (VIII) which was methylated (IX) and then debenzylated to give 10-benzoyloxy-7-hydroxy-12-methoxy coumestan (X). Alkaline hydrolysis of (X) yielded 7,10-dihydroxy-12-methoxy coumestan (I, $R_1 = R_2 = OH$, $R_3 = OMe$). The UV (Fig. 2) and IR spectra, R_f values, and m.p. of this product and of its diacetate were identical with those of trifoliol and its diacetate in every respect.

The parent trihydric phenol (XII) from which trifoliol is derived was also prepared from the benzofuran (VII). This was debenzylated to yield the monobenzoate (XI)

[8] L. Jurd, *Tetrahedron Letters* No. 18, 1151 (1963).



which on alkaline hydrolysis gave 7,10,12-trihydroxycoumestan (XII). In addition, alkaline hydrolysis of the dimethyl ether of the benzoate (XI) gave 10-*hydroxy*-7,12-*dimethoxycoumestan*, identical with the ether obtained by monomethylation of trifoliol.



We believe that this is the first reported isolation of a natural compound containing a substituted phloroglucinol-like structure in ring D (I, $R_1 = R_2 = OH$, $R_3 = OMe$).

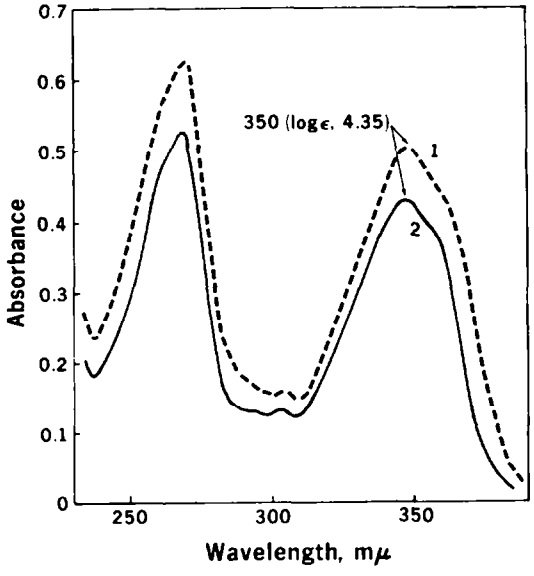


FIG. 2. UV spectra in ethanol of (1) trifoliol, (2) synthetic 4′,7-dihydroxy-6′-methoxybenzofuro[3′,2′,-3,4]coumarin.

This is of particular biosynthetic interest because it was thought that only the A ring of flavonoids arises from phloroglucinol.[9]

Although mouse bioassay indicated that trifoliol has no estrogenic activity, the parent phenol (XII) was as active as coumestrol (Table 2).

TABLE 2. ESTROGENIC ACTIVITY OF COUMESTROL AND RELATED COMPOUNDS

Diet	Dose level mg	Uterine weight[a] mg
Control	—	10·2
Coumestrol	0·4	30·9
	0·5	49·5
Trifoliol	5·0	10·0
	10·0	9·8
	15·0	11·0
Parent phenol of trifoliol	0·5	49·5

[a] Mean uterine wt of 5 mice.

The name coumarino-coumarone has been applied to the class of compounds of which coumestrol is a representative.[10] However, this name is objectionable according to the Ring Index published by the American Chemical Society. The trivial name coumestan has been proposed for the skeletal structure of the heterocyclic, four-ring system having the systematic name, 6H-benzofuro[3,2,c][1]benzopyran-6-one.[11] This name was employed in a recent review discussing the various classes of organic substances of plant origin[12] and is used in this paper. The list of naturally-occurring coumestans is growing and now includes wedelolactone,[13] norwedelolactone,[14] erosnin,[15] psoralidin,[16] coumestrol[17] and trifoliol.

EXPERIMENTAL

Isolation. A detailed account of the isolation of this compound was given in an earlier paper.[3] Briefly, it involved suspending the acetone solubles from ladino clover meal in chloroform, extracting with alkali and reacidifying the aqueous extract to precipitate a sediment. Countercurrent distribution of this sediment yielded a crude crystalline material from which trifoliol was obtained (XI).

Final purification was by recrystallization from N,N'-dimethyl formamide to give long rods, m.p. 332° (dec) λ_{max}^{EtOH} 349, 303, 270 mμ; λ_{max}^{NaOEt} 381, 293 mμ. The IR spectrum showed bands at 3350 (hydroxy), 1710 (lactone), 1670, 1630, 1605 (aromatic rings), and 1262 cm^{-1} (internal ether). (Found: C, 64·4; H, 3·48; OCH_3, 10·3. Calc. for $C_{16}H_{10}O_6$: C, 64·4; H, 3·36; OCH_3, 10·4%).

Alkaline fusion. Trifoliol (2 mg) was fused with ground KOH in a micro test tube. The solids were acidified and extracted with diethyl ether. Aliquots of the ether extract were spotted on sheets

[9] T. A. Geissman, *Modern Methods of Plant Analysis* (Edited by K. Paech and M. V. Tracey) Vol III; p. 450. Springer-Verlag (1955).

[10] T. R. Govindachari, K. Nagarajan and P. C. Parthasarathy, *Tetrahedron* **15**, 129 (1961).

[11] C. Deschamps-Vallet and C. Mentzer, *C.R. Acad. Sci., Paris* **251**, 736 (1960).

[12] C. Mentzer, *Memoires du Museum Naturelle* Serie D 1(1): 1–46. N. S., Paris, France (1960).

[13] T. R. Govindachari, K. Nagarajan, B. R. Pai and P. C. Parthasarathy, *J. Chem. Soc.* 545 (1957).

[14] N. R. Drishnaswamy and T. R. Seshadri, *Naturally-occurring phenyl-coumarins*, in the *Recent Progress in the Chemistry of Natural and Synthetic Colouring Matters and Related Fields* (Edited by T. Sore *et al.*) p. 235. Academic Press, New York and London (1962).

[15] J. Eisenbeiss and H. Schmid, *Helv. Chim. Acta* **42**, 61 (1959).

[16] H. H. Khastgir, P. C. Duttagupta and P. Sengupta, *Tetrahedron* **14**, 275 (1961).

[17] E. M. Bickoff, R. L. Lyman, A. L. Livingston and A. N. Booth, *J. Amer. Chem. Soc.* **80**, 3969 (1958).

of Whatman No. 1 paper with and without known compounds. Following two-dimensional development in chloroform–acetic acid–water (2–1–1) (organic phase) and 20% KCl, the chromatograms were observed under UV lamps before and after treatment with ammonia, and then in visible light after spraying with diazotized sulfanilic acid. Spots were detected corresponding to resorcinol, 2,4-dihydroxybenzoic acid, and phloroglucinol.

Acetate (I, $R_1 = R_2 = OAc$, $R_3 = OMe$). Trifoliol (50 mg) was refluxed with fused sodium acetate and acetic anhydride for 3 min, then poured into ice water. The white solids were collected and recrystallized from acetone to give 52 mg of white needles, m.p. 243°, λ_{max}^{EtOH} 336, 301, 245 mμ. (Found: C, 62·9; H, 3·68, OCH_3, 8·08; CH_3CO, 22·5. Calc. for $C_{20}H_{14}O_8$: C, 62·8; H, 3·67; OCH_3, 8·11; CH_3CO, 22·5%).

Monomethyl ether ((I, $R_1 = R_2 = OMe$, $R_3 = OH$). Trifoliol, (0·10 g), Na_2CO_3 (0·25 g) and dimethyl sulfate (2 ml) were refluxed in dry acetone (75 ml) for 7 hr. The solution was concentrated to a few ml, poured into ice water and acidified. The white crystals which formed were collected and recrystallized from methanol to give 50 mg of crystals, m.p. 209–212°. The IR spectra showed a slight band at 3350 cm^{-1} (hydroxyl). (Found: C, 65·4; H, 3·97; OCH_3, 19·8. Calc. for $C_{17}H_{12}O_6$: C, 65·4; H, 3·85; OCH_3, 19·9%).

Dimethyl ether (I, $R_1 = R_2 = R_3 = OMe$). Trifoliol (0·10 g), K_2CO_3 (0·25 g), dimethyl sulfate (2 ml) and 75 ml dry acetone were refluxed 7 hr. The mixture was concentrated, diluted with ice water, and acidified. Crystalline solids formed which were collected and recrystallized from N,N'-dimethylformamide to give 80 mg of white crystals, m.p. 255–58°. (Found: C, 66·2; H, 4·34; OCH_3, 27·0. Calc. for $C_{18}H_{14}O_6$: C, 66·3; H, 4·30; OCH_3, 28·5%).

Tetramethylether–methyl ester (II, R = Me). Trifoliol diacetate (1·32 g) and K_2CO_3 (5 g) in dry acetone (350 ml) were brought to reflux and small quantities of dimethyl sulfate and 10% KOH in methanol were added alternately by a previously described procedure.[16] Upon dilution with water and acidification, crystals separated. Recrystallization from aqueous methanol gave 1·15 g colourless crystals, m.p. 126–28°. (Found: C, 64·6; H, 5·50; OCH_3, 41·4. Calc. for $C_{30}H_{30}O_7$: C, 64·4; H, 5·38; OCH_3, 41·7%).

o-*Methoxy cinnamic acid* (II, R = H). The above ester (1·1 g) was hydrolyzed in 300 ml of a 10% KOH in methanol solution for 1½ hr. Dilution with water and acidification gave colourless solids. The acid was recrystallized from diethyl ether to give 1·0 g crystals, m.p. 143–145°. (Found: C, 64·0; H, 5·34; OCH_3, 34·2. Calc. for $C_{19}H_{18}O_7$: C, 63·7; H, 5·03; OCH_3, 34·6%).

2(2,4-*Dimethoxyphenyl*)-6,8-*dimethoxybenzofuran* (III, R = OMe). The above acid (0·95 g) was heated under N_2 in a small tube at 225–30° for 1 hr. The solids were purified by chromatography on a column of silica gel. Recrystallization from ether gave 0·71 g crystalline rods, m.p. 126–127°. (Found: C, 68·8; H, 5·77; OCH_3, 38·8. Calc. for $C_{18}H_{17}O_5$: C, 69·0; H, 5·43; OCH_3, 39·6%).

Ozonolysis of the benzofuran. A solution of the benzofuran (0·40 g) in a 1:1 mixture of chloroform and methylene chloride (40 ml) was cooled to approximately −65° in a dry ice–methanol bath, and treated with a gentle stream of 8% ozone in oxygen for 3½ hr. At the end of this time thin layer chromatography indicated that all of the benzofuran had reacted with ozone. Triethyl phosphite (0·4 ml) was added to the flask. After the initial reaction subsided, the solution was slowly brought up to room temp, concentrated to an oil, and taken up in diethyl ether. The ethereal solution was extracted with dil. alkali, which was then acidified and re-extracted with diethyl ether. The ethereal solution of the acids was concentrated to dryness, and the solids recrystallized from hot water to give 38 mg crystals, m.p. 107°. An authentic sample of 2,4-*dimethoxybenzoic acid* (V) did not depress the m.p. The UV and IR spectra of the crystals and authentic 2,4-dimethoxybenzoic acid were also identical. (Found: C, 59·3; H, 5·46; OCH_3, 33·9. Calc. for $C_9H_{10}O_4$: C, 59·4; H, 5·50; OCH_3, 34·0%).

The above ethereal solution of the neutral fraction was concentrated to a volume of 10 ml and chromatographed on a column of silica gel. The column was developed with increasing concentrations of diethyl ether in Skellysolve B. A residue was obtained from the 20% ether fraction which, upon recrystallization from dil. ethanol, gave 30 mg colourless crystals, m.p. 71°, undepressed in admixture with authentic 2-*hydroxy*-4,6-*dimethoxybenzaldehyde* (VI). The UV and IR spectra were also identical. (Found: C, 59·7; H, 5·66; OCH_3, 32·9. Calc. for $C_9H_{10}O_4$: C, 59·4; H, 5·50; OCH_3, 34·0%).

Further developing the column with diethyl ether and evaporation of the solvent gave 44 mg of colourless crystals, m.p. 124–28°. Recrystallized from dil. ethanol to give 32 mg needle crystals, m.p. 133–135°. A synthetic sample of 4,6-*dimethoxy*-2-(2',4'-*dimethoxybenzoyl*)-*benzaldehyde* (IV)

did not depress the m.p. The UV and IR spectra were also identical. (Found: C, 61·8; H, 5·37; OCH_3, 34·6. Calc. for $C_{16}H_{16}O_7$: C, 62·4, H, 5·20; OCH_3, 35·8%).

Hydrolysis of the ester. The above ester (5 mg) was hydrolyzed with 0·01N KOH in methanol. Thin layer chromatography (TLC) was used to determine the rate of hydrolysis. At the end of 3 hr, two spots, one corresponding to 2,4-dimethoxybenzoic acid and a second corresponding to 2-hydroxy-4,6-dimethoxybenzaldehyde, were present. The solution was acidified and extracted with ether. The ether was washed ($NaHCO_3$ aq) and evaporated. A solution of the residue in ethanol gave a UV spectrum identical with 2-hydroxy-4,6-dimethoxybenzaldehyde. TLC of the isolated and synthetic compounds confirmed their identity.

The bicarbonate extract from the hydrolysis was acidified and extracted with ether. Evaporation of the ether resulted in a residue which gave a spectrum identical with that of synthetic 2,4-dimethoxybenzoic acid.

Synthesis of 2-hydroxy-4,6-dimethoxybenzaldehyde (VI). A solution of 2,4,6-trihydroxybenzaldehyde (5 g), K_2CO_3 (5 g) and dimethyl sulphate (30 ml) in 1 l. acetone was refluxed 3 hr. The solution was concentrated to an oil and crystallized from dil. ethanol to give plates (2·76 g) m.p. 71°, λ_{max}^{EtOH} 229, 277, 313 mμ. (Found: C, 59·4; H, 5·58; OCH_3, 33·4. Calc. for $C_9H_{10}O_4$: C, 59·4; H, 5·50; OCH_3, 34·0%).

4,6-*Dimethoxy-2-(2′,4′-dimethoxybenzoyl)-benzaldehyde* (IV). A solution of 2-hydroxy-4,6-dimethoxybenzaldehyde (2 g), thionyl chloride (1 ml), pyridine (1 ml) and methylene chloride (30 ml) were refluxed for a total of 9½ hr. Following chromatography on a column of silica gel, an oil was isolated which, upon crystallization from diethyl ether, gave 215 mg of white plates, m.p. 133–134°, λ_{max}^{EtOH} 222, 267, 286 (shoulder) mμ. (Found: C, 62·2; H, 5·32; OCH_3, 35·3. Calc. for $C_{18}H_{18}O_7$: C, 62·4; H, 5·20; OCH_3, 35·8%).

3-*Methoxy-5-benzyloxy-7-hydroxy-2′,4′-dibenzyloxyflavylium chloride* (VII). A solution of 2-O-benzoylphloroglucinaldehyde (2·58 g) and ω-methoxy-2,4-dibenzyloxyacetophenone (3·62 g) in ethyl acetate (40 ml) and diethyl ether (100 ml) was cooled in an ice-bath and treated with HCl gas for 20 min. The flavylium salt rapidly separated as orange-red needles. After standing 15 hr at 0°, the red crystals were collected, washed with ether, and air-dried (6·0 g), $\lambda_{max}^{EtOH\ 0.5\%\ HCl}$ 514, 277 mμ. The flavylium salt did not melt but began to decompose to a black solid at 145–150°.

7,10,12-*Trihydroxycoumestan* (XII). A solution of 3-methoxy-5-benzoyloxy-7-hydroxy-2′,4′-dibenzyloxyflavylium chloride (2·0 g) in warm methanol (75·0 ml) was diluted with water (15·0 ml) and 30% H_2O_2 (5·0 ml). The solution lost colour rapidly and an almost colourless, oily product began to separate. After 10 min, the mixture was diluted with water and extracted with ether. The ether solution was washed with water and dil. $NaHCO_3$ aq, dried (Na_2SO_4), and evaporated to a yellow gum. This was dissolved in hot glacial acetic acid (30 ml) and slowly diluted with conc. HCl aq (30 ml). The solution was heated on a steam-bath for 40 min during which an orange-red, crystalline solid separated. Water was added and the solids were collected and digested with aqueous methanol to remove most of the red impurity. The crude 10-benzoyloxy-7,12-dihydroxycoumestan thus obtained (0·32 g) crystallized from acetone–methanol as almost colourless needles, m.p. 303° (dec), λ_{max}^{EtOH} 344, 303, 239 mμ, λ_{max}^{NaOEt} 389, 282 mμ. (Found: C, 67·7; H, 3·36. Calc. for $C_{22}H_{12}O_7$: C, 68·0; H, 3·11%).

Acetylation of the monobenzoate in boiling acetic anhydride–sodium acetate for 1 min gave the 10-benzoyloxy-7,12-diacetoxycoumestan, colourless needles from acetone–methanol, m.p. 258°, λ_{max}^{EtOH} 343, 329, 297, 235 mμ. (Found: C, 66·1; H, 3·55. Calc. for $C_{26}H_{16}O_9$: C, 66·1; H, 3·41%).

Methylation of the monobenzoate with methyl iodide, potassium carbonate, and acetone gave the 10-benzoyloxy-7,12-dimethoxycoumestan. This crystallized from acetone–methanol as colourless, granular crystals which sinter at 237–238° and melt at 248°; λ_{max}^{EtOH} 341, 300, 238 mμ. (Found: C, 69·4; H, 3·95. Calc. for $C_{24}H_{16}O_7$: C, 69·2; H, 3·87%).

Alkaline hydrolysis of this benzoate yielded 10-hydroxy-7,12-dimethoxycoumestan m.p. 211° (trifoliol monomethyl ether, m.p. 209–212°).

10-Benzoyloxy-7,12-dihydroxycoumestan (0·2 g) was suspended in methanol (5 ml), diluted with 10% KOH aq (10 ml), and heated in a steam-bath for 10 min. The solid obtained on acidification with dil. HCl aq was recrystallized from methanol.

7,10,12-*Trihydroxycoumestan* (XII) separated as cream-coloured needles, m.p. 346–348°, λ_{max}^{EtOH} 351, 303, 270 mμ, $\lambda_{max}^{EtOH\text{-}NaOAc}$ 380, 272 mμ, $\lambda_{max}^{EtOH\text{-}NaOEt}$ 398, 292 mμ. (Found: C, 63·5; H, 2·81. Calc. for $C_{15}H_{16}O_6$: C, 63·4; H, 2·84%).

The *triacetate* of the above benzofurocoumarin crystallized from acetone–methanol as colourless, felted needles, m.p. 212°, λ_{max}^{EtOH} 342, 327, 296, 242 mμ. (Found: C, 61·6; H, 3·58; CH_3CO-, 31·2. Calc. for $C_{21}H_{14}O_9$: C, 61·5; H, 3·44; CH_3CO-, 31·5%).

7,10-*Dihydroxy*-12-*methoxycoumestan* (*trifoliol*). 3-Methoxy-5-benzoyloxy-7-hydroxy-2′,4′-dibenzyloxyflavylium chloride (2·0 g) was oxidized as described above. The ether extract of the oxidation products was dried (Na_2SO_4) and evaporated. The residual gum was methylated by heating under reflux with methyl iodide (10 ml), anhydrous K_2CO_3 (6 g), and acetone (50 ml) for 1 hr. The filtered acetone solution was evaporated; the residue was dissolved in hot acetic acid (40 ml) and slowly diluted with conc. HCl aq (40 ml) for 30 min. At the end of the addition, a crystalline solid began to separate. Water (20 ml) was added and the pink solid was collected and digested with aqueous methanol. The undissolved 10-benzoyloxy-12-methoxy-7-hydroxycoumestan, m.p. 288–289°, was collected (0·37 g). Recrystallized from acetone–methanol, it separated as colourless needles, m.p. 289–290°, λ_{max}^{EtOH} 343, 302, 240 mμ. (Found: C, 69·2; H, 3·65. Calc. for $C_{28}H_{14}O_7$: C, 68·7; H, 3·51%).

The above benzoate was heated on a steam-bath with methanol (5·0 ml) and 5% KOH aq (10 ml) for 10 min. On acidification with dil. HCl aq, colourless crystals separated. These were recrystallized from acetone–methanol. 7,10-*Dihydroxy*-12-*methoxycoumestan* was obtained as brittle, slightly yellow prisms, m.p. 332–333°, undepressed on admixture with trifoliol (0·2 g). The UV spectrum was identical with that of trifoliol. (Found: C, 64·6; H, 3·58; OCH_3, 10·7. Calc. for $C_{16}H_{10}O_6$: C, 64·4; H, 3·36; OCH_3, 10·4%).

The diacetate of the product, prepared by heating with acetic anhydride and sodium acetate for 1 min, crystallized from acetone–methanol as colourless, felted needles, m.p. alone and mixed with trifoliol diacetate, 243–244°. The UV and IR spectra of the diacetate and trifoliol diacetate were identical. (Found: C, 62·7; H, 3·59; CH_3CO-, 22·7. Calc. for $C_{20}H_{14}O_8$: C, 62·8; H, 3·67; CH_3CO-, 22·5%).

Bioassay. Following a previously described bioassay procedure,[18] trifoliol and its parent phenol were incorporated into the diets of weanling mice at levels of 5, 10, and 15 mg/mouse. For control purposes, coumestrol was also fed at levels of 0·4, 0·5, 0·6 mg/mouse.

No response was given by the mice fed trifoliol, whereas the mice given the parent phenol and the control mice that were fed coumestrol gave similar responses (Table 1).

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Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

[18] E. M. Bickoff, A. N. Booth, A. L. Livingston, A. P. Hendrickson and R. L. Lyman, *J. Animal Sci.* **18**, 1000 (1959).