Medicagol, a New Coumestan From Alfalfa[1]

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A new coumestan, $C_{16}H_8O_6$, has been identified in alfalfa. Characterization was accomplished by degradative and proton magnetic resonance (p.m.r.) studies. Synthesis confirmed the structure as 7-hydroxy-11,12-methylenedioxycoumestan, closely related to the antifungal agent, pisatin.

The production of antibiotic substances by plants in response to injury or infection has recently received renewed attention. In some cases, the accumulation of these compounds and the concomitant shifts in metabolism are believed to be mechanisms for disease resistance in the plant. One such compound is the antifungal agent, pisatin, which was isolated from the pods of garden peas inoculated with fungal spore.[3]

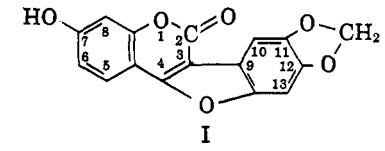
In studies on coumestrol levels in clover, we found that virus-infected plants were much higher in coumestrol than were comparable virus-free specimens.[4] More recently, two common leafspot diseases in alfalfa were found to cause abnormally high levels of coumestrol in the plant.[5] A number of other phenolic compounds that occur with coumestrol in the plant also build up to high levels during infection.

During a recent large-scale extraction of coumestrol (7,12-dihydroxycoumestan) from alfalfa meal,[6] a supply of these accompanying phenolics was obtained. The details involved in their isolation[7] and the characterization of one of them as 7,10-dihydroxy-12-methoxycoumestan were reported earlier (trifoliol).[8]

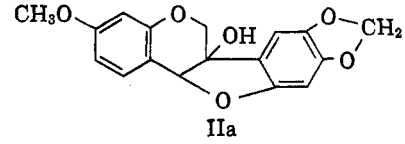
The present paper describes the characterization of an additional member of this series as 7-hydroxy-11,12-methylenedioxycoumestan (I) for which the trival name medicagol from the genus name for alfalfa, *Medicago*, is proposed.

The basic ring pattern of medicagol is very similar to that of the antifungal agent, pisatin[3] (IIa),[9] or its anhydro derivative (IIb).

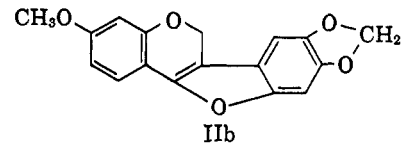
Medicagol (I) was obtained as a mixture with a second compound (II), from which it could not be separated by standard techniques such as column chromatography, countercurrent distribution (c.c.d.), or recrystallization. Thin layer chromatography (t.l.c.) on silica gel separated the acetates slightly. However, the loading per plate had to be limited to less than 30 γ, making it impossible to obtain a sufficient quantity of material for characterization.



I



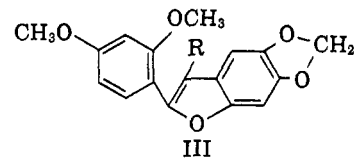
IIa



IIb

The similarity of the ultraviolet spectrum of this inseparable mixture to that of coumestrol, suggested that the compounds might also be coumestans. Since coumestans can systematically be degraded by methylative ring opening (III, R = $COOCH_3$), hydrolysis (III, R = COOH), and decarboxylation to their more soluble benzofuran derivatives (III, R = H), this offered a means of purification. The benzofuran could then be utilized in elucidating the structure of the parent phenolic from which it was derived. This procedure was therefore applied to the medicagol mixture. The methoxybenzofuran derivative of medicagol (III, R = H) was purified by fractional crystallization from ether.

Two results indicated that medicagol has a hydroxyl group in the 7-position. (1) When medicagol acetate was treated with dimethyl sulfate in the presence of potassium carbonate a methoxy derivative formed.[10] (2) The λ_{max} of the ultraviolet spectrum of the parent phenols in ethanol (356 mμ) underwent a bathochromic shift to 380 mμ in the presence of sodium acetate.[11]



III

Elemental analysis of the methoxybenzofuran of medicagol gave an empirical formula of $C_{17}H_{14}O_5$. This compound could be derived from a coumestan having a methylenedioxy and a hydroxyl group. The presence of a methylenedioxy group was further indicated by a positive Hansen test[12] and by the p.m.r.

(1) Presented at the 147th National Meeting of the American Chemical Society, Philadelphia, Pa., April 1964.

(2) A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture. 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.

(3) D. R. Perrin and W. Bottomley, *Nature*, **191**, 76 (1961).

(4) E. M. Bickoff, A. L. Livingston, A. N. Booth, C. R. Thompson, E. A. Hollowell, and E. G. Beinhart, *J. Animal Sci.*, **19**, 4 (1960).

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(10) L. Jurd, *J. Am. Chem. Soc.*, **80**, 5531 (1958).

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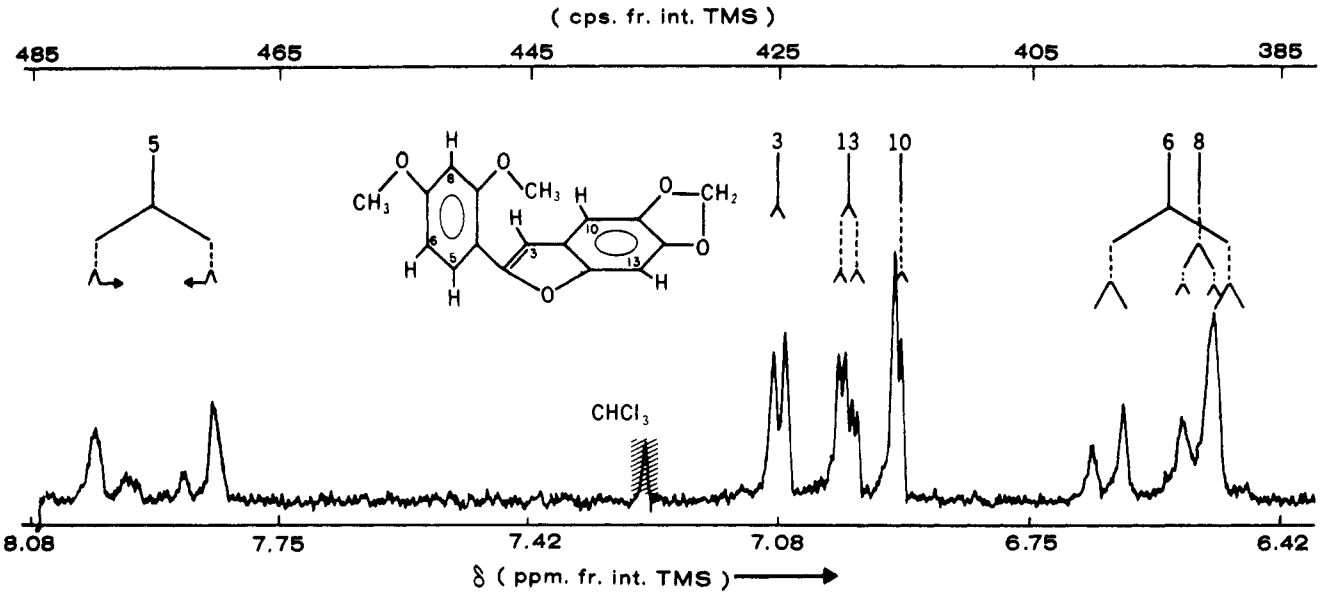
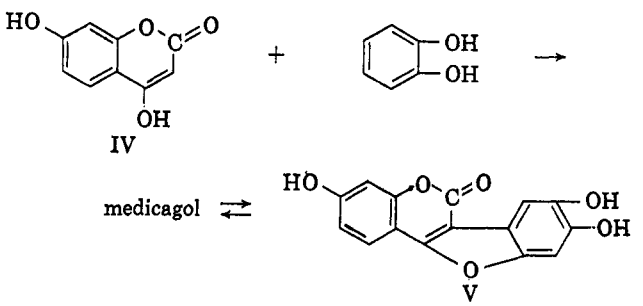


Figure 1.—Aromatic region of the p.m.r. spectrum of medicagol benzofuran in deuteriochloroform at 60 Mc.

and infrared[13] spectra. The 60-Mc. p.m.r. spectrum of the benzofuran derivative of medicagol in deuteriochloroform indicates the presence of one aromatic methylenedioxy group (δ 5.93,[14] singlet, two hydrogens) and two aromatic methoxy groups (δ 3.92 and 3.82, each a singlet representing three hydrogens). Figure 1 shows an expanded sweep of the aromatic region from approximately 385 to 485 c.p.s. below TMS (δ 6.3 to 8.0). Integration showed the presence of six aromatic protons. The assignment given is the only one fully consistent with the areas of the various peaks, the known shielding effects of ether substituents on aromatic protons, a unique long-range coupling in benzofurans, and the usual *ortho*, *meta*, and *para* coupling constants. The spectra of the benzofurans of the two closely related coumestans, coumestrol and trifoliol, further confirm this assignment.[8] The low-field quartet centered at δ 7.92 has the typical appearance of the "X" portion of an ABX multiplet. The separation of the two weak inner lines confirms the assignment of the high field at δ 6.58 to the "AB" portion.[15] In the spectra of benzofuran derivatives only H-5 appears lower in field than δ 7.66 (electron-withdrawing effect of the *ortho* furanyl system) provided it is neither *ortho* nor *para* to an ether group. In the parent compounds H-10 also appears in this range apparently as a result of spatial deshielding by the 2-carbonyl. The splitting of the H-3 peak proves that H-13 is present since in benzofuran a 0.9-c.p.s. long-range coupling exists between these protons.[16]

Strong acid hydrolysis of the medicagol acetate mixture gave 7,11,12-trihydroxycoumestan (V) further indicating that the structure of medicagol must be 7-hydroxy-11,12-methylenedioxycoumestan.

Confirmation of this structure and of 7,11,12-trihydroxycoumestan was obtained by their syntheses from 4,7-dihydroxycoumarin (IV). By the procedure of Wanzlick[17] 4,7-dihydroxycoumarin was oxidatively coupled with catechol to give 7,11,12-trihydroxycoumestan. The ultraviolet and infrared spectra, R_f values, and melting point of the acetate of this compound and the acetate of the trihydroxycoumestan obtained by acid hydrolysis of medicagol were identical in every respect.



Methylenation of 7,11,12-trihydroxycoumestan with diiodomethane in acetone gave 7-hydroxy-11,12-methylenedioxycoumestan (medicagol). The natural medicagol mixture and its acetate were found to be chromatographically identical with the synthetic material.

Experimental

Preparation of the Benzofuran Derivatives.—The following reactions were carried out under nitrogen and were followed by t.l.c. on silica gel G with anhydrous diethyl ether–Skellysolve B (7:3) as the developing agent.

Methyl Methoxycinnamate (III, R = $COOCH_3$).—The medicagol acetate mixture[7] (2.4 g.), anhydrous potassium carbonate (2.4 g.), dimethyl sulfate (12.0 ml.), and dry acetone (600 ml.) were brought to reflux. Potassium hydroxide (10%) in methanol was added dropwise until the alkali no longer turned the solution yellow. The mixture was refluxed an additional 30 min., then filtered, concentrated to a small volume (50 ml.), and used directly in the next reaction. Small-scale preparation of the methyl methoxycinnamate from the parent phenol mixture gave the same product.

Methoxycinnamic Acid (III, R = COOH).—The above solution of the ester was hydrolyzed by refluxing with 10% potassium hydroxide in methanol (350 ml.) for 1 hr. The mixture was added to ice–water (700 ml.) and stirred for 30 min. The white

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(14) Parts per million downfield from internal tetramethylsilane.

(15) J. A. Pople, W. G. Schneider, and H. J. Bernstein, "High Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., Inc., New York, N. Y., 1959, p. 132.

(16) J. A. Elridge and R. C. Foster, *J. Chem. Soc.*, 590 (1963).

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crystalline product was collected, washed with water, dried (sodium sulfate), and recrystallized from ether, giving 2.3 g. of product.

Methoxybenzofuran (III, R = H).—The above acid mixture (2.3 g.) was decarboxylated by heating at 180 to 185° for 3.5 hr. The dark residue was dissolved in ether (300 ml.), extracted with three 50-ml. portions of 10% sodium carbonate, washed with water (50 ml.), and dried (sodium sulfate). The ether solution was then evaporated to dryness (1.52 g.). The solids were dissolved in ether (200 ml.), and the solution was permitted to evaporate slowly. Successive crops of crystals were collected and their content was evaluated by t.l.c. Crop 1 contained 310 mg. of medicagol methoxybenzofuran, m.p. 162–63°. Recrystallization from methanol gave the analytical sample (220 mg.), m.p. 166–167°.

The infrared spectrum showed bands at 2841, 1260, and 1020 cm.$^{-1}$, characteristic of a methylenedioxy group.[13] This derivative also gave a positive Hansen test.[12] Absorption and integral p.m.r. spectra were obtained from a Varian A-60 spectrometer (60 Mc.) with deuteriochloroform at 32° as the solvent and tetramethylsilane (TMS) as an internal reference.

Anal. Calcd. for $C_{17}H_{14}O_5$: C, 68.5; H, 4.73; OCH_3, 20.8. Found: C, 68.9; H, 4.91; OCH_3, 21.4.

Hydrolysis of Medicagol Mixture.—The medicagol mixture (300 mg.) was hydrolyzed with 86% sulfuric acid (10 ml.) at room temperature for 15 min., then poured into ice–water (100 ml.) and adjusted to pH 6 with dilute potassium hydroxide. The aqueous solution was extracted with three 50-ml. portions of diethyl ether. The ether phase was dried (sodium sulfate) and concentrated to dryness, and the solids were purified by countercurrent distribution in a solvent system composed of acetone–ethanol–diethyl ether–Skellysolve B–water (10:5:10:2:1). Progress of the distribution was followed by t.l.c. Fractions were obtained after 100 transfers. Fraction 1, tubes 10–20, contained a material which gave a rapidly fading bluish fluorescence on chromatoplates (R_f 0.05). The solvent was removed and the solids were acetylated in the usual manner with fused sodium acetate and acetic anhydride. Crystallization of the acetate from acetone gave colorless crystals (80 mg.), m.p. 255–257°, undepressed with the synthetic sample of 7,11,12-triacetylcoumestan prepared as described below. The ultraviolet and infrared spectra were also identical.

Synthesis of 4,7-Dihydroxycoumarin (IV).—The procedure of Sonn[18] was used to prepare the coumarin. Recrystallization from water gave colorless needles, m.p. 266° dec., lit.[18] m.p. 265°.

Anal. Calcd. for $C_9H_6O_4$: C, 60.6; H, 3.37. Found: C, 60.6; H, 3.45.

7,11,12-Trihydroxycoumestan (V).—To a solution of 4,7-dihydroxycoumarin (4.0 g.), sodium acetate (12.0 g.), and catechol (2.0 g.) in 20 ml. of acetone–water (1:1), potassium ferrocyanide (15 g.) and sodium acetate (15 g.) in 60 ml. of water was added dropwise. Several crops of light tan crystals of the 7,11,12-trihydroxycoumestan were collected during the addition period. The trihydroxycoumestan (3.7 g.) was difficult to purify for analysis because it was only slightly soluble in the more common organic solvents and decomposed in solvents such as dimethylformamide. Its analysis was therefore confirmed by the preparation and analysis of its trimethoxy and triacetyl derivatives.

7,11,12-Trimethoxycoumestan.—The above trihydroxycoumestan (0.5 g.), potassium carbonate (2 g.), dimethyl sulfate (5 ml.), and dry acetone (400 ml.) were refluxed for 4 hr. The mixture was concentrated, diluted with Skellysolve B, and cooled. The crystals were collected and recrystallized from methanol to give colorless needles: m.p. 254°; λ_{max}^{EtOH} 347 mμ (log ε 3.42), 306 (3.84), 247 (3.02).

Anal. Calcd. for $C_{18}H_{14}O_6$: C, 66.3; H, 4.30; OCH_3, 28.5. Found: C, 66.4; H, 4.38; OCH_3, 28.4.

7,11-12-Triacetylcoumestan.—The triacetate was prepared in the usual manner with acetic anhydride and sodium acetate to give colorless needles: m.p. 255–57°; $\lambda_{max}^{CHCl_3}$ 344 mμ (log ε 3.32), 328 (3.42), 298 (3.12), 236 (3.34).

Anal. Calcd. for $C_{19}H_{12}O_8$: C, 62.0; H, 3.29; CH_3CO, 31.9. Found: C, 61.8; H, 3.83; CH_3CO, 31.7.

7-Hydroxy-11,12-methylenedioxycoumestan.—A mixture of 7,11,12-trihydroxycoumestan (0.5 g.), diiodomethane (5 ml.), and anhydrous potassium carbonate (5 g.) in 1200 ml. of anhydrous acetone was refluxed for 3 hr. The mixture was filtered and the filtrate was concentrated under vacuum to an oil. Purification of the oil was by countercurrent distribution employing the following solvent system: Skellysolve B–diethyl ether–water–methanol–dimethylformamide (2:5:1:3:0.04). Progress of the distribution was followed by t.l.c. A fraction containing a bluish white fluorescent spot, R_f 0.6 on chromatoplates (diethyl ether–Skellysolve B) (7:3), was collected and the solvent was removed under vacuum. The solids were sublimed to give colorless crystals (45 mg.): m.p. 324–325°; λ_{max}^{EtOH} 347 mμ (log ε 4.43), 309 (4.01), 245 (4.22).

Anal. Calcd. for $C_{16}H_8O_6$: C, 64.8; H, 2.70. Found: C, 64.9; H, 3.01.

The acetate was prepared in the usual manner with acetic anhydride and sodium acetate to give colorless needles: m.p. 262–263°; λ_{max}^{EtOH} 349 mμ (log ε 4.45), 333 (4.52), 298 (4.13), 239 (4.35).

Anal. Calcd. for $C_{18}H_{10}O_7$: C, 63.9; H, 2.96; CH_3CO, 12.1. Found: C, 63.6; H, 3.07; CH_3CO, 12.8.

The synthetic 7-hydroxy-11,12-methylenedioxycoumestan and its acetate were compared by t.l.c. with the parent and acetylated mixture of medicagol and compound II. The R_f values of natural medicagol in anhydrous ether–Skellysolve B (4:1) was 0.56, of the acetate 0.78; in chloroform 0.05, of the acetate 0.39; in ethyl acetate–Skellysolve B (1:1) 0.50, of the acetate 0.81; in ethyl acetate–Skellysolve B (1:3) 0.35, of the acetate 0.57; in anhydrous ether–Skellysolve B (7:3) 0.52; of the acetate 0.72. The R_f values of synthetic medicagol and its acetate were identical with those above.

Medicagol prepared by the Wanzlick reaction was found identical with a sample synthesized by Jurd from a flavylium salt,[19] by his new technique for the synthesis of coumestans.

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