In this mechanism the formation of a Michaelis complex would not be necessary *a priori*. Apparent Michaelis behavior might be observed if the enzyme would react directly with the substrate in a bimolecular reaction $EH + S \rightarrow HE\text{-}P_2 + P_1$, where at high substrate concentrations the hydrolysis of the enzyme-glucoside might become rate limiting. However, in this case K_S would not be finite (Fig. 2A).

The observation that the pK of the essential ionizable group in the free enzyme and in the Michaelis complex is 4.0 as compared to about 4.2 in the enzyme-glucoside does not contradict the supposition that only one such group is involved. The small difference in pK could be accounted for by the covalent bond between glucose and a neighboring group in the enzyme. Unlike the preliminary adsorption of S in the Michaelis complex the formation of this bond might result in a shift of electrons toward the ionizable group, *i. e.*, in a weakening of its acidity. The magnitude of the pK suggests that it is a carboxyl group.

Cunningham recently has proposed a theory for the mechanism of action of hydrolytic enzymes.[2] This theory is based on consecutive "acylation" and "deacylation" reactions similar to glucoside formation and subsequent hydrolysis in our scheme. However, it is thereby assumed that, unlike the present case, the rates of both these reactions increase with decreasing hydrogen ion concentration from which it would follow that with increasing pH the rate approaches a maximum instead of displaying an optimum. The theory may be applicable to the case of chymotrypsin,[14] but without modification it could not explain the pH optimum of β-glucosidase.

(14) G. H. Dixon and H. Neurath, *J. Biol. Chem.*, **225**, 1049 (1956).

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[CONTRIBUTION FROM WESTERN REGIONAL RESEARCH LABORATORY[1]]

Characterization of Coumestrol, a Naturally Occurring Plant Estrogen

BY E. M. BICKOFF, R. L. LYMAN, A. L. LIVINGSTON AND A. N. BOOTH

RECEIVED NOVEMBER 11, 1957

The estrogen coumestrol, previously isolated from ladino clover, has been characterized as 6',7-dihydroxybenzofuro[3',2',-3,4]coumarin. Confirmation of this structure was accomplished by a series of progressive degradations involving (a) alkaline methylation to 2-(2',4'-dimethoxyphenyl)-6-methoxybenzofuran-3-carboxylic acid; (b) decarboxylation by pyrolysis of the acid to 2-(2',4'-dimethoxyphenyl)-6-methoxybenzofuran; (c) cleavage of the double bond by ozonolysis to form 4-methoxy-2-(2',4'-dimethoxybenzoyl)-benzoic acid; and (d) final hydrolysis of the ester to 2,4-dimethoxy- and 2-hydroxy-4-methoxybenzoic acids.

Introduction

Following the isolation of an estrogenic compound from ladino clover,[2] preliminary investigations[3] resulted in the proposed structure (Fig. 1, IV), and the name "coumestrol" for the compound.

The presence of two free hydroxyl groups was confirmed by the formation of a diacetate and dimethyl ether derivative.[2] The empirical formula $C_{15}H_8O_5$ differed from that of a typical flavonoid only by the absence of two hydrogen atoms. Fusion studies yielded only resorcinol and β-resorcylic acid, giving an indication of the number and position of the hydroxyl groups on the two rings. The ultraviolet absorption spectrum of coumestrol differed markedly from the known estrogenic isoflavones, and was more characteristic of a flavone. However, comparison of coumestrol with a known flavone (7,2',4'-trihydroxyflavone) having the required hydroxyl pattern indicated by the fusion products was sufficiently different to rule out flavones as a possibility.

That coumestrol might be a coumarin derivative was suggested[4] by the fact that its blue fluorescence was not appreciably affected by exposure to ammonia and that the bathochromic shift with alkali was much less than that expected from a flavone. A coumarin-type structure was demonstrated by methylation under alkaline conditions followed by saponification.[5] This reaction formed an *o*-methoxycarboxylic acid which gave an equivalent weight of 331 upon titration of the carboxyl group with methanolic potassium hydroxide solution. A naturally occurring 4-phenylcoumarin (dalbergin) had recently been isolated by Ahluwalia, *et al.*[6] How-

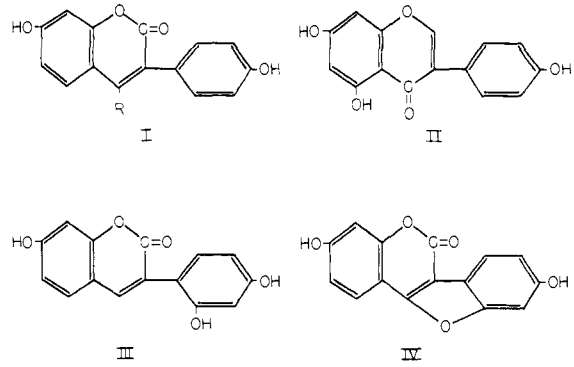

Fig. 1.—Structural formulas of Mentzer's estrogen (I), genistein (II), coumarin derivative related to coumestrol (III) and coumestrol (IV).

(1) Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture. Article not copyrighted.

(2) E. M. Bickoff, A. N. Booth, R. L. Lyman, A. L. Livingston, C. R. Thompson and G. O. Kohler, *J. Agr. Food Chem.*, in press.

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

(4) We are indebted to Prof. E. Jorgensen, University of California Medical School, for this observation.

(5) F. W. Canter and A. J. Robertson, *J. Chem. Soc.*, 1875 (1931).

(6) V. K. Ahluwalia and T. R. Seshadri, *ibid.*, 970 (1957).

ever, a sample of their compound failed to exhibit any of the characteristics of the estrogen.

Although a wide variety of organic compounds have been reported to have estrogenic properties[7] the only coumarins shown to be estrogenic to which we have found reference are certain 3-phenyl derivatives (Fig. 1, I) synthesized by Mentzer and his co-workers.[8] Of interest is the structural similarity of these compounds to the naturally occurring estrogenic isoflavones, like genistein (Fig. 1, II). A possible 3-phenylcoumarin structure which would degrade into β-resorcylic acid and resorcinol is shown in Fig. 1, III. Although this compound fails to fit our analytical data for the estrogen since it would form a triacetate and trimethoxyl derivative, the closely related furano compound (Fig. 1, IV) does fit completely. Nevertheless, direct evidence for such a structure was lacking.

In order to verify the "furano" type of structure, a series of stepwise degradations of the estrogen were undertaken, patterned after those described by Govindachari, *et al.*,[9] for wedelolactone. This compound with the exception of additional hydroxyl and methoxyl groups appeared to have the identical skeletal structure as that proposed for coumestrol. The *Chemical Abstracts* name for this compound would be 3,9-dihydroxy-6H-benzofuro-[3,2,c][1]benzopyran-6-one.[10]

The following series of reactions definitely established the structure of coumestrol as 6′,7-dihydroxybenzofuro[3′,2′,3,4]coumarin (Fig. 1, IV). Methylation with dimethyl sulfate and methanolic potassium hydroxide gave a trimethyl ether methyl ester which was saponified readily to the corresponding 2-(2′,4′-dimethoxyphenyl)-6-methoxybenzofuran-3-carboxylic acid (Fig. 2, I).

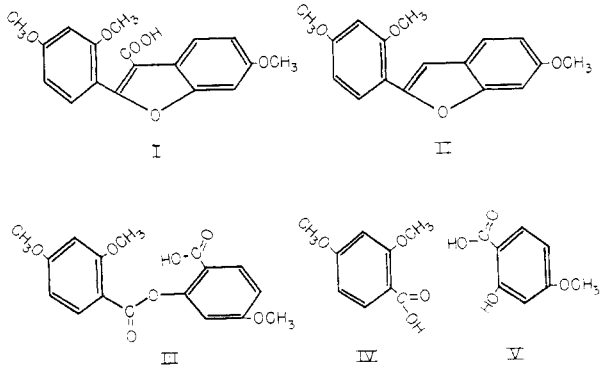

Fig. 2.—Structural formulas of stepwise degradation products of coumestrol.

Heating, decarboxylated this acid to yield the product 2-(2′,4′-dimethoxyphenyl)-6-benzofuran (Fig. 2, II). Ozonolysis of this product, and then treatment with hydrogen peroxide gave 4-methoxy-2-(2′,4′-dimethoxybenzoyl)-benzoic acid (Fig. 2, III). This compound did not crystallize, but was identified by chromatographic and spectral comparison with a synthetic sample. Hydrolysis gave the 2,4-dimethoxy- and 2-hydroxy-4-methoxybenzoic acids.

From these results, it is clear that the compound (Fig. 2, III) could occur only through the rupture of the double bond in a furan ring during ozonolysis. Since mild alkaline hydrolysis cleaved this compound into 2,4-dimethoxybenzoic acid and 2-hydroxy-4-methoxybenzoic acid, the presence and position of the furan ring, as well as the position of the hydroxyl groups on the two rings, were established.

The compound of structure IV (Fig. 1), has been obtained by unequivocal synthesis and proved identical in all respects with natural coumestrol. The details of this work will appear in a subsequent publication.

Experimental

We were greatly aided by the application of a silicic acid chromatostrip method.[11,12] Unless otherwise stated, the solvent system used to develop the chromatostrip was a mixture of ether and petroleum ether (63° to 70°), in the ratio of 7 to 3.

2-(2′,4′-Dimethoxyphenyl)-6-methoxybenzofuran-3-carboxylic Acid (Fig. 2, I).—To an acetone solution of 50 mg. of estrogen was added a small amount of sodium carbonate and the mixture was brought to boiling. One milliliter of dimethyl sulfate was added and 10% methanolic potassium hydroxide solution was pipetted in so as to keep the solution alkaline (solution turned yellow at *p*H 10–11). Periodically a few extra drops of dimethyl sulfate was added. After 1 hour, further addition of alkali did not turn the solution yellow. The mixture was acidified and the acetone removed. A chromatostrip migration showed, under ultraviolet light, a single bright blue fluorescing spot (R_f 0.67). The compound was extracted with ether, which was evaporated. Crystallization was from methanol and water. The white needles (40 mg.) melted at 98° and gave a negative Folin–Denis test. Since they were not soluble in alkali, it was assumed that the compound was the methyl ester of 2-(2′,4′-dimethoxyphenyl)-6-methoxybenzofuran-3-carboxylic acid.

Anal. Calcd. for $C_{15}H_6O_2(OCH_3)_4$: C, 66.6; H, 5.27; OMe, 36.3. Found: C, 66.7; H, 5.44; OMe, 36.1.

Hydrolysis of 50 mg. of the ester with 10% methanolic potassium hydroxide solution for 1 hour and subsequent acidification, showed, when migrated on silicic acid, a single bright blue fluorescent spot lower than before (R_f 0.44). Evaporation of the methanol, extraction with ether and subsequent removal left an oil that crystallized from methanol and water into slender needles (35 mg.), m.p. 178°. This material was soluble in sodium carbonate solution and CO_2 was evolved upon melting.

Anal. Calcd. for $C_{15}H_7O_3(OCH_3)_3$: C, 65.9; H, 4.87; OMe, 28.9; COOH, 13.7. Found: C, 66.1; H, 5.08; OMe, 28.0; COOH, 13.7.

2-(2′,4′-Dimethoxyphenyl)-6-methoxybenzofuran (Fig. 2, II).—Fifty milligrams of 2-(2′,4′-dimethoxyphenyl)-6-methoxybenzofuran-3-carboxylic acid (Fig. 2, I) was heated to 240–260° under a gentle stream of nitrogen. As the acid decomposed, the product distilled and condensed along the sides of the tube. After one hour, the decarboxylation was completed and the tube was rinsed out repeatedly with ether. The ether solution was extracted once with sodium carbonate solution, washed with water, dried and evaporated. The product obtained showed as a single dark absorption spot (R_f 0.86) on the chromatostrip when viewed under a short wave length (2540 Å.) ultraviolet lamp. A faint purple fluorescent spot was evident under the longer wave length (3660 Å.). Crystallization from methanol and water gave fine white needles (30 mg.), m.p. 82°.

(7) U. V. Solmssen, *Chem. Revs.*, **37**, 481 (1945).

(8) C. Mentzer, P. Gley, D. Holho and D. Billet, *Bull. soc. chim. France, Ser. 5*, **13**, 271 (1946).

(9) T. R. Govindachari, K. Nagarajan and B. R. Pai, *J. Chem. Soc.*, 629 (1956).

(10) Leonard T. Capell, personal communication.

(11) J. G. Kirchner, J. M. Miller and J. G. Keller, *Anal. Chem.*, **23**, 420 (1951).

(12) R. L. Lyman, A. L. Livingston, E. M. Bickoff and A. N. Booth, *J. Org. Chem.*, in press.

Anal. Calcd. for $C_{14}H_7O(OCH_3)_3$: C, 71.8; H, 5.64; OMe, 32.8. Found: C, 71.5; H, 5.82; OMe, 32.4.

Ozonolysis of 2-(2′,4′-Dimethoxyphenyl)-6-methoxybenzofuran (Fig. 2, II).—A Welsbach ozonizer was used. Ozonization of 25 mg. dissolved in methanol was carried out by passing 0.0865 mmole of O_3 at 0° through the solution for 50 minutes. A few drops of hydrogen peroxide and water were added to the methanol and the solution left overnight to decompose the ozonide to the acid. The decomposition of the ozonide was not complete to the acid, however, and a number of other products were seen when the material was observed on the chromatostrips. The amounts of the products present were insufficient to be isolated, so identification was made by comparison with known synthetic compounds. Synthetic 2,4-dimethoxybenzoic acid and 2-hydroxy-4-methoxybenzoic acid were prepared as described in an earlier publication.[12]

Synthetic 4-Methoxy-2-(2′,4′-dimethoxybenzoyl)-benzoic Acid (Fig. 2, III).—To 2.6 g. of 2,4-dimethoxybenzoic acid was added 20 ml. of thionyl chloride and the mixture allowed to stand at room temperature for 24 hours. The excess thionyl chloride was removed from the acid chloride *in vacuo* and 1.5 g. of 2-hydroxy-4-methoxybenzoic acid, dissolved in 10 ml. of benzene containing 8 ml. of dimethylaniline, were added with agitation. After 3 hours, the benzene and aniline were removed under reduced pressure. The reddish colored oil failed to crystallize. The expected benzoyl ester appeared as a separate purple spot near the origin when migrated on chromatostrips in a solvent system of a mixture of acetone and petroleum ether (1:3).

In order to isolate the compound, the crude mixture was subjected to a 200-tube Craig countercurrent distribution. The solvent system consisting of acetone, ether, petroleum ether, and water (42:15:22:21) was employed. The ester appeared pure in tubes 90–102 and crystallized from methanol and water into white needles (200 mg.), m.p. 151°. Hydrolysis with 10% methanolic potassium hydroxide solution for 15 minutes on the steam-bath gave 2-hydroxy-4-methoxybenzoic acid and 2,4-dimethoxybenzoic acid exclusively.

Anal. Calcd. for $C_{14}H_7O_4(OCH_3)_3$: C, 61.5; H, 4.82; OMe, 28.1. Found: C, 61.6; H, 4.89; OMe, 28.1. The compound was relatively unstable, and decomposed into its two component acids when dissolved in methanol and allowed to stand at room temperature.

Identification of 4-Methoxy-2-(2′,4′-dimethoxybenzoyl)-benzoic Acid (Fig. 2, III) as One of the Ozonolysis Products.—Following decomposition of the ozonide with hydrogen peroxide, the methanol was evaporated and the oil obtained dissolved in 5% sodium bicarbonate solution. Extractions with ether were made at *p*H 8 to 9, *p*H 7.0 and *p*H 4.0. The ether extract at *p*H 7.0 contained two prominent spots when migrated on a silicic acid strip in acetone and petroleum ether (1:3). The lower spot appeared identical to the synthetic benzoyl ester and was scraped off the glass strip, eluted with methanol and the solution concentrated. Migration in two other solvent systems assured purity as well as identity with the synthetic product. The two additional systems employed were ethyl acetate and petroleum ether (3:1) and ether and petroleum ether (7:3). Ultraviolet absorption spectra made from the synthetic compound isolated on silicic acid as well as the pure ozonolysis product isolated similarly were also identical. The absorption maxima were at 253 and 290 mμ and minima at 233 and 278 mμ.

Hydrolysis with 0.1 *N* methanolic potassium hydroxide solution of the synthetic and ozonolysis products resulted in formation of 2,4-dimethoxy- and 2-hydroxy-4-methoxybenzoic acid in both cases.

Identification of 2,4-Dimethoxybenzoic Acid (Fig. 2, IV) and 2-Hydroxy-4-methoxybenzoic Acid (Fig. 2, V) as Two of the Other Ozonolysis Products.—The ether extract at *p*H 4.0 contained mostly a material that corresponded to 2,4-dimethoxybenzoic acid. The R_f's of the ozonolysis product was identical with that of the synthetic 2,4-dimethoxybenzoic acid when migrated in the three solvent systems employed for identification of 4-methoxy-2-(2′,4′-dimethoxybenzoyl)-benzoic acid. The ultraviolet absorption spectra were also identical. The absorption maxima were at 253 and 287 mμ and the minima at 233 and 272 mμ.

The extract at *p*H 7 contained the 2-hydroxy-4-methoxybenzoic acid. The latter appeared as a blue fluorescent spot on a chromatostrip when viewed under a 2540 Å. ultraviolet light, while the 2,4-dimethoxybenzoic acid showed as a brown absorption area. The R_f's of the ozonolysis product was identical with that of synthetic 2-hydroxy-4-methoxybenzoic acid when migrated in the three solvent systems described above. The ultraviolet absorption spectra were also identical. The absorption maxima were at 250 and 293 mμ and the minima at 233 and 271 mμ.

Acknowledgment.—The authors gratefully acknowledge the assistance of L. M. White and G. Secor for all the elemental analyses; G. F. Bailey and E. Gong for ultraviolet absorption spectra; T. R. Govindachari and K. Nagarajan, Presidency College, Madras, India, for helpful suggestions and a sample of wedelolactone; T. R. Seshadri, University of Delhi, India, for a sample of dalbergin; and J. H. Simpson, Torry Research Station, Aberdeen, Scotland, for a sample of 7,2′,4′-trihydroxyflavone, all of which were used for comparison purposes.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, PURDUE UNIVERSITY]

Transmission of Electrical Effects Through Homoallylic Systems. I. The Synthesis and Physical Properties of a Series of 6-Arylcholesterols and of 6-Arylcholesteryl *p*-Toluenesulfonate Esters

BY RICHARD A. SNEEN

RECEIVED DECEMBER 16, 1957

The syntheses of 6-phenyl-, 6-*p*-anisyl-, 6-*p*-tolyl-, 6-*p*-chlorophenyl- and 6-*p*-nitrophenylcholesterol, and of the corresponding *p*-toluenesulfonate esters have been accomplished by the synthetic scheme outlined below.

As part of a study designed to assess the relative stabilizing effects of various substituents on the transition states of unimolecular solvolyses of homoallylic systems, the synthesis of a series of five 6-arylcholesterols was undertaken. This communication reports the successful synthesis of 6-phenyl-, 6-*p*-anisyl-, 6-*p*-tolyl-, 6-*p*-chlorophenyl- and 6-*p*-nitrophenylcholesterol. A kinetic study of the solvolyses of the *p*-toluenesulfonate esters of these substituted cholesterols is reported in a subsequent publication.[1]

The synthesis of all of the arylcholesterols began with the readily available cholestan-3β-ol-6-one acetate[2] (I). This keto acetate, when treated with

(1) R. A. Sneen, THIS JOURNAL, **80**, 3977 (1958).

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