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Identification of Stigmasteryl D-Glucoside in Aged Burley Tobacco

H. E. WRIGHT, JR., W. W. BURTON, AND R. C. BERRY, JR.

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Stigmasteryl D-glucoside was identified as the major constituent in a mixture of three sterolins isolated from aged Burley tobacco. The amount of steryl glucoside so isolated corresponded to 0.04% of dry leaf weight. Partial characterization of the other two sterolins in the mixture, corresponding in amount to 0.03% of dry leaf weight, indicated they were probably the glucosides of β-sitosterol and campesterol.

Reports concerning the characterization of sterols in tobacco, notably the extensive investigations of Stedman and co-workers, have been cited by Johnstone and Plimmer[1] in a recent review of the subject. Dymicky and Stedman[2] have since reported the occurrence of campesterol in flue-cured tobacco. Although the glucosides of β-sitosterol[3] and γ-sitosterol[4] are known to occur in tobacco, the isolation of a glycoside of stigmasterol has not heretofore been reported. Grossman and Stedman[5] and Kosak *et al.*,[6] in reporting the occurrence of free stigmasterol in flue-cured tobacco and tobacco smoke, respectively, commented on the possible occurrence of the sterol in glycosidic form.

During an ether extraction of aged Burley tobacco leaf, which had been preextracted exhaustively with pentane, a buff-colored precipitate accumulated in the extraction flask. Recrystallization of the precipitate from ethanol (decolorizing carbon) yielded a colorless crystalline product which gave positive Liebermann-Burchard and Molisch tests, indicating it to be a sterolin fraction. The contaminating substance associated with the sterolin fraction as isolated, and responsible for its color, was proteinaceous in nature. Hydrolysis of the recrystallized sterolin fraction with 0.36 *N* sulfuric acid in absolute ethanol yielded the sterol and carbohydrate moieties. The mass spectra of the sterol fraction indicated it to be a mixture of three sterols. Analysis of the mixture by mass spectrometry[7] provided evidence, with respect to each of the constituent sterols, on the quantitative composition of the mixture, the molecular weight (parent mass peak), and the nature of the C-24 alkyl substituent in the side chain of the sterol molecule.

TABLE I

MASS SPECTRAL DATA ON STEROLS FROM HYDROLYZATE OF STEROLIN MIXTURE

Sterol	Proportion of Mixture	Molecular Weight	C-24 Alkyl Substituent
I	60%	412	Ethyl
II	25%	414	Ethyl
III	15%	400	Methyl

It was determined by two-dimensional paper chromatography that glucose was the only carbohydrate of the sterolin hydrolyzate. The identification of glucose was confirmed by preparation of the *p*-nitrophenylhydrazone derivative. Quantitative determinations of the hydroxyl and glucose contents of the sterolin mixture were in agreement with the corresponding calculated values based on the mass spectra data. As the sterolin mixture was isolated as an insoluble precipitate in the ether extract of the Burley tobacco preextracted with pentane, it was considered that the method of isolation precluded the probable presence of any free sterol in the mixture. Thus, the method of isolation, the identification of glucose as the only sugar in the sterolin mixture, and the quantitative determinations of hydroxyl and glucose contents of the sterolin mixture led to the conclusion that each of the sterols comprising the mixture was present as a glucoside.

The ultraviolet spectrum of the sterol mixture isolated from the hydrolyzate of the sterolin fraction indicated the absence of conjugated unsaturation. The infrared spectrum exhibited absorption bands at 800 cm.$^{-1}$ and 840 cm.$^{-1}$ (indicative of the configuration $R_2C{=}CHR$), characteristic of 5,6-unsaturation in the sterol B ring, and at 972 cm.$^{-1}$ (indicative of a *trans* disubstituted ethylene configuration RCH=CHR), characteristic of 22,23-unsaturation in the sterol side chain.[8,9] The sterol mixture was acetylated and the resulting steryl acetate mixture was then

(1) R. A. W. Johnstone and J. R. Plimmer, *Chem. Revs.*, **59**, 885 (1959).
(2) M. Dymicky and R. L. Stedman, *Tobacco Sci.*, **3**, 179 (1959).
(3) M. Dymicky and R. L. Stedman, *Tobacco Sci.*, **2**, 99 (1958).
(4) V. R. Khanolkar, T. B. Panse, and V. D. Divekar, *Science*, **122**, 515 (1955).
(5) J. D. Grossman and R. L. Stedman, *Tobacco Sci.*, **2**, 115 (1958).
(6) A. I. Kosak, J. S. Swinehart, D. Taber, and B. L. Van Duuren, *Science*, **125**, 991 (1957).
(7) S. S. Friedland, G. H. Lane, Jr., R. T. Longman, K. E. Train, and M. J. O'Neal, Jr., *Anal. Chem.*, **31**, 169 (1959).
(8) G. Roberts, Beatrice S. Gallagher, and R. N. Jones, *Infrared Absorption Spectra of Steroids—An Atlas*, Vol. II, Interscience, New York, 1958.
(9) L. F. Fieser and Mary Fieser, *Steroids*, Reinhold, New York, 1959.

brominated in acetic acid, in accordance with the method of Windaus and Hauth.[10] The more insoluble steryl acetate tetrabromide fraction was separated from the soluble dibromide fraction by selective solvent extraction. Subsequent debromination and saponification of the tetrabromide fraction yielded stigmasterol, molecular weight 412.67 (Sterol I, Table I). The procedure of Windaus and Hauth has been the classical method for the separation of mono- and di-unsaturated sterols in mixtures. However, the method has been shown recently to be inefficient,[11,12] as was indeed substantiated by the low yield of stigmasterol obtained in the present work. The infrared spectrum of the isolated stigmasterol was identical with that of the authentic sterol. The identification of stigmasterol was confirmed by preparation of the acetate, benzoate, and 3,5-dinitrobenzoate derivatives. The melting points and specific optical rotations of these compounds were in agreement with the corresponding values reported in the literature.

From the foregoing evidence it was concluded that stigmasteryl D-glucoside was the major constituent of the sterolin mixture isolated from the leaf of aged Burley tobacco. Although the method of isolation of the sterolin mixture was not quantitative, the amount so isolated corresponded to 0.07% of dry leaf weight. Based on the mass spectral data the amount of stigmasteryl D-glucoside corresponded to 0.04% of dry leaf weight. Experimental evidence indicated that Sterols II and III (Table I) were probably β-sitosterol (molecular weight 414.69) and campesterol (molecular weight 400.66) respectively, each present in the sterolin mixture as the glucoside. However, the small amount of sample precluded the accumulation of sufficient evidence to constitute positive identification of Sterols II and III.

Bergmann,[13] in a comprehensive treatise on plant sterols, discussed the difficulties associated with sterol characterization, and emphasized that frequently they are isolated as complex mixtures of closely related compounds. The recognition and resolution of such mixtures is usually most difficult. With respect to this observation the present investigation attests to the value of mass spectrometry in the analysis of sterol fractions.

EXPERIMENTAL[14]

Isolation of sterolin mixture from Burley tobacco. Multiple 360-g. samples of aged Burley tobacco, leaf web, U. S. type 31, 1953 crop, 7% moisture, ground to a 1-mm. particle size, were extracted with pentane in a Soxhlet apparatus (30 g. per thimble) for 20 hr. The dried tobacco residue was next extracted with ether in a similar manner for 20 hr. The crude buff-colored sterolin mixture accumulated as an isoluble precipitate during the course of the ether extraction. The ether extracts were combined and concentrated to *ca.* one-fifth the original volume. The crude mixture was then centrifuged, washed successively with ether, ethanol, and acetone until the supernatant solution was colorless, and dried *in vacuo*. The dried mixture, 350 mg. from 360 g. of tobacco, was suspended in 150 ml. of ethanol and placed on the steam bath for 30 min. Norit A activated carbon, *ca.* 0.5 g., was added and heating continued for an additional 15 minutes. The mixture was filtered on Whatman No. 42 filter paper, using a heated funnel. After chilling the filtrate in the refrigerator for 90 min., the precipitated sterolin mixture was centrifuged, washed with ethanol and acetone, and dried *in vacuo*. Recrystallization of the dried material several times from ethanol gave a colorless product, m.p. 289–290° dec., $[\alpha]^{25}D$ −51.7° in pyridine. Yields of 246 mg. of recrystallized sterolin mixture were thus obtained from 360 g. of tobacco, corresponding to 0.07% of dry leaf weight.

Anal. Calcd. on the basis of mass spectral data: C, 72.98; H, 10.28. Found: C, 73.28; H, 10.43.

Hydroxyl content of sterolin mixture. A 25-mg. sample of the steryl glucoside mixture in 1 ml. of pyridine containing 10% acetic anhydride was refluxed for 5 min. The cooled solution was diluted with carbon dioxide-free water and titrated with 0.1 *N* sodium hydroxide solution, using phenolphthalein indicator. A blank determination was made similarly.

Anal. Calcd. on the basis of mass spectral data: hydroxyl, 11.86. Found: hydroxyl, 11.89.

Glucose content of sterolin mixture. A 25-mg. sample of the steryl glucoside mixture in 10 ml. of acidified absolute ethanol (1 ml. of concentrated sulfuric acid per 100 ml. of absolute ethanol) was refluxed for 20 hr. The cooled solution was carefully neutralized with dilute sodium hydroxide solution, using phenolphthalein indicator, and then made slightly acid by the addition of 4 drops of 0.5 *N* sulfuric acid. Following complete removal of ethanol by rotary vacuum evaporator and air stream, the residue in 10 ml. of 1 *N* sulfuric acid was refluxed for 3 hr. to hydrolyze any ethyl glucoside. The cooled solution was neutralized with dilute sodium hydroxide solution and diluted with 35 ml. of water. Alkaline ferricyanide reagent, 10 ml. (2.5 g. of potassium ferricyanide and 1 g. of sodium carbonate in 100 ml. water), was added and the solution heated in a boiling water bath for 15 min. The cooled solution was acidified with 5 ml. of 12 *N* sulfuric acid and titrated with 0.025 *N* ceric sulfate reagent,[15] using erio-glaucine indicator (0.1% in water). A blank determination was made in a similar manner.

Anal. Calcd. on the basis of mass spectral data: Glucose, 31.41. Found: Glucose, 30.90.

Hydrolysis of sterolin mixture. The steryl glucoside mixture was hydrolyzed according to the procedure described by Swift.[16] A 250-mg. sample in 15 ml. of acidified absolute ethanol (1 ml. of concentrated sulfuric acid per 100 ml. of absolute ethanol) was refluxed for 20 hr., dissolution of the sample taking place in *ca.* 4 hr. The hydrolyzate was diluted

(10) A. Windaus and A. Hauth, *Ber.*, **39**, 4378 (1906).

(11) J. A. Campbell, D. A. Shepherd, B. A. Johnson, and A. C. Ott, *J. Am. Chem. Soc.*, **79**, 1127 (1957).

(12) R. A. Donia, A. C. Ott, and N. Drake, *Anal. Chem.*, **29**, 464 (1957).

(13) W. Bergmann, *Ann. Rev. Plant Physiol.*, **4**, 383 (1953).

(14) Infrared spectra were determined with a Perkin-Elmer Model 21 spectrophotometer equipped with a sodium chloride prism on KBr pellets of the compounds. Ultraviolet spectra were determined with a Beckman DK-2 ratio recording spectrophotometer. Melting points were determined on a Fisher-Johns melting point apparatus. Optical rotations were determined with a Schmidt and Haensch polarimeter at 25° in concentrations of *ca.* 20 mg. per ml. in a 1-dm. tube.

(15) W. Z. Hassid, *Ind. Eng. Chem., Anal. Ed.*, **9**, 228 (1937).

(16) L. J. Swift, *J. Am. Chem. Soc.*, **74**, 1099 (1952).

with 30 ml. of water and the precipitated sterols extracted with ether. The ether extract was washed with water and evaporated to dryness. Recrystallization of the dried material from 85% ethanol yielded 126 mg. of mixed sterols, m.p. 146–147.5°, $[\alpha]^{25}D$ −45.8° in chloroform.

Washings from the ether solution were combined with the extracted hydrolyzate and, after adjustment of *p*H to 5–6 with dilute sodium hydroxide, the solution was evaporated to dryness *in vacuo* at 60°. A mixture of the residue and 10 ml. of 0.5 *N* sulfuric acid was refluxed for 4 hr. to hydrolyze any ethyl glucoside. The hydrolyzate, after adjustment of *p*H to 5–6, was evaporated to dryness as above. The residue was taken up with a small volume of hot ethanol and centrifuged. The supernatant solution, concentrated to a final volume of *ca.* 1 ml., was reserved for carbohydrate identification.

Chromatography of glucose. Chromatograms, Whatman No. 1 chromatography paper, were run by the ascending technique. The chromogenic spray was *p*-anisidine phosphate.[17] The sample glucose cochromatographed with authentic glucose in all solvent systems. The following solvent systems, with corresponding glucose R_f values, were used: *n*-butyl alcohol–acetic acid–water (12:3:5) R_f 0.25, followed in the second direction by isopropyl alcohol–*n*-butyl alcohol–water (7:1:2) R_f 0.44; isopropyl alcohol–water (4:1) R_f 0.55, followed in the second direction by ethyl acetate–pyridine–water (12:5:4) R_f 0.57; *n*-butyl alcohol–pyridine–water (2:2:1) R_f 0.50, followed in the second direction by methyl ethyl ketone–propionic acid–water (15:5:6) R_f 0.28.

Glucose p-nitrophenylhydrazone. A mixture of the ethanolic glucose solution (1 ml.) from the hydrolyzate of the sterolin mixture and *p*-nitrophenylhydrazine (75 mg.) was heated in a water bath at 75° for 3 hours. After refrigeration for 72 hr. the *p*-nitrophenylhydrazone precipitate was centrifuged, washed several times with ethanol, and recrystallized from ethanol. Glucose *p*-nitrophenylhydrazone (22 mg.) separated as yellow platelets, m.p. 188–189° (lit.[18] for glucose *p*-nitrophenylhydrazone, 189°).

Anal. Calcd. for $C_{12}H_{17}O_7N_3$: C, 45.71; H, 5.43. Found: C, 45.51; H, 5.63.

Bromination of mixed steryl acetates and separation of stigmasteryl acetate tetrabromide. A mixture of 75 mg. of the sterols isolated from the hydrolyzate of the sterolin mixture, 0.5 ml. of dry pyridine, and 0.3 ml. of acetic anhydride was refluxed for 3–5 min. Water was added, the precipitate of mixed steryl acetates extracted with ether, and the ether extract washed with 0.5 *N* hydrochloric acid and water. After evaporation of the ether the steryl acetate mixture was recrystallized from ethanol. The yield was 64 mg., m.p. 137–138°, $[\alpha]^{25}D$ −51.9° in chloroform.

A mixture of 950 mg. of accumulated mixed steryl acetates, 9.5 ml. of ether, and 19 ml. of glacial acetic acid containing 5% bromine was placed in the refrigerator for 1 hr. The precipitate was centrifuged, washed several times with cold 50% ethanol, and dried *in vacuo* in a heated desiccator at 55°. The dried material was extracted with benzene–methanol (1:3) and with benzene–methanol (1:1). The insoluble residue of stigmasteryl acetate tetrabromide (358 mg.) was dried *in vacuo*, m.p. 189–192°. The melting point of stigmasteryl acetate tetrabromide prepared similarly from authentic stigmasterol was 188–189.5°.

Stigmasteryl acetate. Debromination of the stigmasteryl acetate tetrabromide was accomplished by refluxing for 2 hr. a mixture of the tetrabromide (358 mg.), zinc dust (358 mg.), and 50% glacial acetic acid in ethanol (18 ml.). The hot solution was poured into water and the stigmasteryl acetate extracted with ether. The ether extract was washed successively with a 5% solution of sodium carbonate, 0.5 *N* hydrochloric acid, and water. Evaporation of the ether and recrystallization of the residue several times from ethanol gave 170 mg. of stigmasteryl acetate, m.p. 139–141°; $[\alpha]^{25}D$ −51.3° in chloroform (lit.[6] for stigmasteryl acetate, m.p. 140–141°; $[\alpha]^{20}D$ −51.9°).

Anal. Calcd. for $C_{31}H_{50}O_2$: C, 81.88; H, 11.08. Found: C, 81.96; H, 11.02.

Stigmasterol. Saponification of stigmasteryl acetate was accomplished by refluxing for 2 hr. a mixture of 155 mg. of the acetate and 15 ml. of 1 *N* absolute ethanol solution of potassium hydroxide. The hot solution was poured into water and the stigmasterol extracted with ether. The ether extract was washed with 0.5 *N* hydrochloric acid and water. Evaporation of the ether and recrystallization of the residue several times from 85% ethanol gave 120 mg. of stigmasterol, m.p. 167.5–168.5°; $[\alpha]^{25}D$ −48.2° in chloroform (lit.[19] for stigmasterol, m.p. 168–169°; $[\alpha]^{29}D$ −47.3°).

Anal. Calcd. for $C_{29}H_{48}O$: C, 84.40; H, 11.72. Found: C, 84.27; H, 11.59.

Stigmasteryl benzoate. A mixture of 40 mg. of stigmasterol, 0.5 ml. of dry pyridine, and 0.1 ml. of benzoyl chloride was refluxed for 3–5 min. The hot solution was poured into water and the stigmasteryl benzoate extracted with ether. The ether extract was washed successively with a 2% solution of sodium bicarbonate, 0.5 *N* hydrochloric acid, and water. Evaporation of the ether and recrystallization of the residue several times from ethanol gave 42 mg. of stigmasteryl benzoate, m.p. 164–165°; $[\alpha]^{25}D$ −24.3° in chloroform (lit.[6] for stigmasteryl benzoate, m.p. 161–161.5°; $[\alpha]^{20}D$ −24.3°).

Anal. Calcd. for $C_{36}H_{54}O_2$: C, 83.67; H, 10.14. Found: C, 83.40; H, 10.14.

Stigmasteryl 3,5-dinitrobenzoate. A mixture of 50 mg. of stigmasterol, 1 ml. of dry pyridine, and 100 mg. of 3,5-dinitrobenzoyl chloride was refluxed for 3–5 min. The 3,5-dinitrobenzoate was isolated in a manner similar to that of the benzoate above. Recrystallization of the product several times from chloroform–ethanol (3:5) gave 44 mg. of stigmasteryl 3,5-dinitrobenzoate, m.p. 234.5–235.5°; $[\alpha]^{25}D$ −22.2° in chloroform (lit.[11] for stigmasteryl 3,5-dinitrobenzoate, m.p. 234–235°; $[\alpha]^{26}D$ −21.0°).

Anal. Calcd. for $C_{36}H_{52}O_6N_2$: C, 71.26; H, 8.31. Found: C, 70.88; H, 8.43.

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(19) A. C. Ott and C. D. Ball, *J. Am. Chem. Soc.*, 66, 489 (1944).