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CHOLESTEROL GLUCOSIDE IN TOBACCO

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Abstract—The sterol and sterolin fractions of flue-cured tobacco have been investigated. It has been confirmed that the major components in the sterol fraction are stigmasterol, β-sitosterol, campesterol and cholesterol and these compounds are present as their glucosides in the sterolin fraction. Cholesterol glucoside has not previously been reported in tobacco, nor in any other plant.

INTRODUCTION

THE PRESENCE of stigmasterol, β-sitosterol and campesterol in tobacco either in the free form or as esters or bound with D-glucose is now well established,[1] and recently free cholesterol[2, 3] and cholesteryl esters[3, 4] have been found. Our GLC investigation of tobacco sterol and sterolin fractions has confirmed that cholesterol is present in the former and shown that cholesterol glucoside is a small but significant component of the latter. Since its original discovery in plant material cholesterol has been shown to be widely distributed in the plant kingdom in the free and esterified form[5] but the existence of cholesterol as its glucoside appears hitherto to have remained undetected.

RESULTS AND DISCUSSION

The tobacco sterolin fraction was obtained from the methanol extract in a water, light petroleum, methanol sequential extraction of shredded flue-cured leaf. Purification was limited to a single recrystallization from ethanol, the aim being to diminish non-steroidal contaminants and at the same time to minimize loss of minor steroidal components. The sterol glycosides were subjected to acidic hydrolysis and the resulting free sterols effectively separated from remaining impurities at this stage by TLC. The free sterols, their acetates and trimethylsilyl (TMS) ethers were further investigated by GLC and identification (Table 1) made on the basis of retention time comparison with authentic compounds. In this way we have confirmed reports,[6, 7] based on mass spectral as well as GLC data, that the three major sterols involved in the tobacco sterolin fraction are stigmasterol, β-sitosterol and campesterol. By our procedure a fourth sterol was revealed and identified beyond reasonable doubt as

[1] R. L. STEDMAN, *Chem. Rev.* **68** (2), 153 (1968).
[2] B. RICHARDSON, J. R. BAUR, R. S. HALLIWELL and R. LANGSTON, *Steroids* **11** (3), 231 (1968).
[3] C. E. COOK, M. E. TWINE, C. R. TALLENT, I. HARPER, G. HEUNISCH, J. B. LEWIS and M. E. WALL, *Phytochem.* **8**, 1025 (1969).
[4] C. E. COOK, M. E. TWINE and M. E. WALL, *Experientia* **23**, 987 (1967).
[5] Reviewed in References 2, 3 and 4.
[6] A. G. KALLIANOS, F. A. SHELBURNE, R. E. MEANS, R. K. STEVENS, R. E. LAX and J. D. MOLD, *Biochem. J.* **87**, 596 (1963).
[7] H. E. WRIGHT, JR., W. W. BURTON and R. C. BERRY, JR., *J. Org. Chem.* **27** (3), 918 (1962).

cholesterol (see also Ref. 8). The possibility that free cholesterol was present as such in the sterol glycoside mixture was discounted when on refluxing the mixture with benzene, a good solvent for the free sterol, concentrating and gas chromatographing the supernatant, no peak attributable to cholesterol was apparent.

TABLE 1. GAS CHROMATOGRAPHY OF TOBACCO STEROLS, STEROLINS AND SUGARS, AUTHENTIC COMPOUNDS AND THEIR DERIVATIVES ON SE-30

Compound	Relative retention time*			Assignment	Conc.† per cent of total sterol fraction
	Free	Acetate	TMS ether		
1 Authentic compounds					
Octacosane	1·00	—	—		
Cholesterol	1·89	2·70	2·30		
Campesterol	2·45	3·56	3·00		
Stigmasterol	2·65	3·88	3·28		
β-Sitosterol	3·09	4·48	3·80		
Tobacco-free sterols					
Peak 1	1·90	2·73	2·28	Cholesterol	12
Peak 2	2·46	3·58	3·01	Campesterol	21
Peak 3	2·66	3·90	3·28	Stigmasterol	51
Peak 4	3·10	4·46	3·81	β-Sitosterol	16
Sterols ex glycosides					
Peak 1	1·89	2·71	2·31	Cholesterol	3
Peak 2	2·45	3·54	3·03	Campesterol	18
Peak 3	2·65	3·88	3·28	Stigmasterol	53
Peak 4	3·08	4·47	3·81	β-Sitosterol	26
2 Authentic cholesterol					
β-D-Glucoside	—	—	1·00		
Tobacco sterolins					
Peak 1	—	—	1·00	Cholesterol glucoside	
Peak 2	—	—	1·26	Campesterol + stigmasterol glucosides	
Peak 3	—	—	1·38	β-Sitosterol glucoside	
3 Equilibrated glucose					
α-D-Glucose	—	—	1·00		
β-D-Glucose	—	—	1·34		
Tobacco sterolin sugars					
Peak 1	—	—	1·00	α-D-Glucose	
Peak 2	—	—	1·34	β-D-Glucose	

* Relative to octacosane in Section 1. Relative to authentic cholesterol β-D-glucoside TMS ether in Section 2. Relative to α-D-glucose TMS ether in Section 3.

† Approximate values calculated by normalization from chromatograms of the acetates.

The sugar moiety of the tobacco sterolins has been investigated in some detail by other workers[6, 7] and found to be entirely D-glucose. However, in view of the low concentration

[8] C. L. KELLER, L. P. BUSH and C. GRUNWALD, *Agric. & Food Chem.* **17**, 331 (1969).

involved, the nature of the cholesterol glucoside sugar remained to be confirmed. The sugars obtained by hydrolysis were converted to their TMS ethers and gas chromatographed. Only two peaks were apparent and these corresponded to the TMS ethers of the α- and β-anomers in authentic equilibrated D-glucose.[9] To obtain further confirmation the tobacco sterol glycosides were converted to their TMS ethers and gas chromatographed. Although the major components were less well resolved than in the free sterol mixture, a minor component which co-chromatographed with authentic cholesterol β-D-glucoside TMS ether was well defined.

The free sterol fraction of tobacco was obtained from a light petroleum extract by alumina column chromatography. Column fractions were monitored for sterols by TLC, all sterol-containing fractions were combined and the sterols isolated by TLC prior to GLC investigation. The proportions of stigmasterol, β-sitosterol and campesterol were found to follow the reported trend[2] and it was confirmed that cholesterol is well represented. Minor contaminants with retention times equal to or shorter than that of cholesterol were evident in the sterol mixture gas chromatograms but these failed to form derivatives when the sterols were converted to their acetates and TMS ethers and, by virtue of the sterol derivatives' increased retention times, the contaminants were cleanly separated.

The level of cholesterol glucoside (*ca.* 3 per cent) in the sterolin fraction is considerably lower than that of unbound cholesterol (*ca.* 12 per cent) in the sterol fraction and enzyme systems responsible for glycoside synthesis can hardly be expected to behave in an entirely natural way at flue-curing temperatures (>60°). None the less, in a consideration of phytosterol metabolism, the presence of cholesterol glucoside in tobacco may not be without significance.

EXPERIMENTAL

Isolation and Hydrolysis of Tobacco Sterol Glycosides

Tobacco (810 g; shredded Virginia-type blend) was Soxhlet extracted sequentially with water, light petroleum and methanol. The methanol extract (19 g) was treated with ether and the crude sterol glycosides (970 mg; 0·12% of moist tobacco) separated by filtration, washed with ether and recrystallized once from ethanol. A portion of the glycoside mixture (281 mg) was hydrolysed by refluxing with conc. H_2SO_4 (0·2 ml) in ethanol (40 ml) for 20 hr and the free sterols obtained as a crystalline mixture (195 mg) by ether extraction from the evaporated and diluted reaction mixture. The sugars were prepared for GLC by adjusting the aqueous layer to pH 7 and removing water *in vacuo* at low temperature. Residual moisture was removed by azeotropic distillation with benzene.

Isolation of Tobacco-Free Sterols

Tobacco (2270 g) was extracted by percolation with light petroleum and the extract (69 g) chromatographed in two batches on alumina (700 g; Woelm; Activity Grade III) columns. Elution was commenced with light petroleum and continued with ether/petroleum mixtures. The sterols were eluted when the proportion of ether in petroleum was 40% and crystallized on standing. Total sterols after purification by TLC weighed 1·46 g (0·06% of moist tobacco).

Thin-Layer Chromatography

Fractions from the alumina column were monitored by TLC on silica gel (Kieselgel G) with benzene–EtOAc (4:1). The sterols were visualized as magenta spots by spraying with conc. H_2SO_4 and heating at 100° for 1 min. All sterol-containing fractions were combined and small samples (*ca.* 5 mg) prepared for GLC by preparative TLC using the above conditions. Large sterol samples were cleaned up by preparative TLC on ChromAR sheet (Mallinckrodt) with benzene.

[9] C. C. SWEELEY, R. BENTLEY, M. MAKITA and W. W. WELLS, *J. Am. Chem. Soc.* **85**, 2497 (1963).

Gas Chromatography

The free sterols and their acetates and TMS ethers were gas chromatographed on a Perkin–Elmer 800 dual column instrument equipped with a flame ionization detector. The 2-m stainless-steel columns were packed with 3·8% SE-30 on Diatoport S and maintained at 225°. The nitrogen carrier flow rate was 40 ml/min and octacosane was used as an internal standard. Sterol glycosides and sugars were gas chromatographed as their TMS ethers using the above conditions except that 1 m columns were used and maintained at 300° and 150° respectively.

Authentic Compounds and Derivatives

Cholesterol β-D-glucoside was prepared, following its discovery in tobacco, by the method of Meystre and Miescher.[10] Other compounds were obtained from commercial sources and derivatives were prepared by standard procedures.

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[10] CH. MEYSTRE and K. MIESCHER, *Helv. Chim. Acta* **27**, 231 (1944).