Phytochemistry, 1965, Vol. 4, pp. 857 to 862. Pergamon Press Ltd. Printed in England

IDENTIFICATION OF THE STEROLS OF OAT SEED

B. A. KNIGHTS

Chemistry Department, University of Glasgow, Glasgow W.2, Scotland

(*Received* 16 *April* 1965)

Abstract—Gas chromatographic, chemical and mass-spectrographic evidence show that the three principal sterols in oat seeds are β-sitosterol, $\Delta^{5,24(28)}$- and $\Delta^{7,24,(28)}$-stigmastadienol respectively. Cholesterol, brassicasterol, campesterol, stigmasterol and Δ^{7}-stigmastenol have been identified as minor components of the mixture.

INTRODUCTION

THE fungus *Phytophthora cactorum* when grown on oat meal produced mature oospores, whereas when grown on a basal medium no oospores were formed.[1] The material in oat seed responsible for this phenomenon was found to be the sterol fraction[1,2] and therefore an investigation of the constituents of this mixture was undertaken. Previous workers[3] had shown β-sitosterol to be the principal component of this mixture. In addition two "avena sterols", present in the original mixture in 32 and 11 per cent respectively, were characterized and tentatively assigned the structures $\Delta^{5,11}$-stigmastadienol and $\Delta^{7,11}$-stigmastadienol respectively. Evidence for these structures was based on comparison with known physical properties of plant sterols, on data furnished from catalytic hydrogenation studies and the known response of Δ^{5}- and Δ^{7}-sterols to the Liebermann Burchard test.

RESULTS AND DISCUSSION

Extraction of crushed oat grains with petroleum ether afforded an orange brown oil (3–6 per cent) after removal of the phospholipids by precipitation with acetone. This oil was divided into three fractions by chromatography on alumina, these consisting chiefly of vegetable oils (triglyceride) and two yellow oils (diglyceride and monoglyceride respectively), Sterols were obtained from the vegetable oil and from the monoglyceride fractions by saponification followed by chromatography, crystallization and/or precipitation with digitonin of the nonsaponifiable material in each fraction. Little difference was noted in the two fractions or in fractions obtained from different varieties of oat seed when examined by gas chromatography (GLC). The principal compound detected by GLC had relative retention data (Table 1) on a number of columns corresponding to that for β-sitosterol confirming the findings of the earlier workers.[3] Minor peaks observed corresponded in retention data to cholesterol,* brassicasterol, campesterol† and stigmasterol. On polar stationary phases

* Cholesterol has been isolated from a plant source by Johnson *et al.*[4] and has been detected in plant sterol mixtures using GLC.[5,6]

† It has not been found possible[6] to resolve campesterol from its C-24 epimer Δ^{5}-ergostenol using GLC and therefore no information is available concerning the absolute stereochemistry at this position in the compounds studied in this work.

[1] C. G. ELLIOTT, M. E. HENDRIE, B. A. KNIGHTS and W. PARKER, *Nature* **203**, 427 (1964).
[2] J. ANTONIS LEAL, J. FRIEND and P. HOLLIDAY, *Nature* **203**, 545 (1964).
[3] D. R. IDLER, S. W. NICKSIC, D. R. JOHNSON, V. W. MELOCKE, H. A. SCHUETTE, and C. A. BAUMANN, *J. Am. Chem. Soc.* **75**, 1712 (1953).

TABLE 1. GLC RELATIVE RETENTION DATA*

Stationary phase	Principal oat seed sterol	β-Sitosterol	Δ5-Avenasterol	Fucosterol	Δ5-Avenasterol TMSi E†	Δ7-Avenasterol TMSi E†	Separation factor Δ5–Δ7	Separation factor Δ5–Δ7-Cholestenol TMSi
1% SE-30	3·3	3·25	3·3	3·3	4·27	4·88	1·14	
1% F-60	3·64	3·6	3·64	3·7	4·44	5·14	1·16	1·21
1% FS-1265	4·4	4·4	4·4	4·35	—	—	—	—
1% NGS	11·2	11·5	12·7	12·65	4·05	4·75	1·17	1·17
1% HiEFF-8B	10·8	10·4	12·5	11·8	3·93	4·72	1·2	1·2
1% HiEFF-8B+ 2% PVP‡	11·7	11·6	13·4	12·7	5·0	6·01	1·2	1·18

* Cholestane standard = 1·0, determined at 225°.
† TMSi E = Trimethylsilyl ether.
‡ PVP = Polyvinylpyrolidone.

such as cyclohexanedimethanolsuccinate (HiEFF-8B) two additional peaks were observed having peak area ratios to β-sitosterol which suggested that they corresponded to the two previously described avena sterols (Fig. 1).

After acetylation it was found possible to separate the mono-unsaturated sterols (e.g. β-sitosterol) from di-unsaturated sterols by chromatography on a column of 25% silver nitrate on silica-gel; mono-unsaturated acetates were eluted with light petroleum containing a trace of ether, and di-unsaturated acetates with 30% ether in light petroleum. Examination using GLC of the first of these two fractions after deacetylation showed it to consist essentially of β-sitosterol with lesser amounts of cholesterol, campesterol and Δ^7-stigmastenol. These assignments were confirmed by determination of the ΔR_{Mr} values[7] obtained upon hydroboration of the double bond and upon oxidation with Jones reagent.[8] Two very minor components

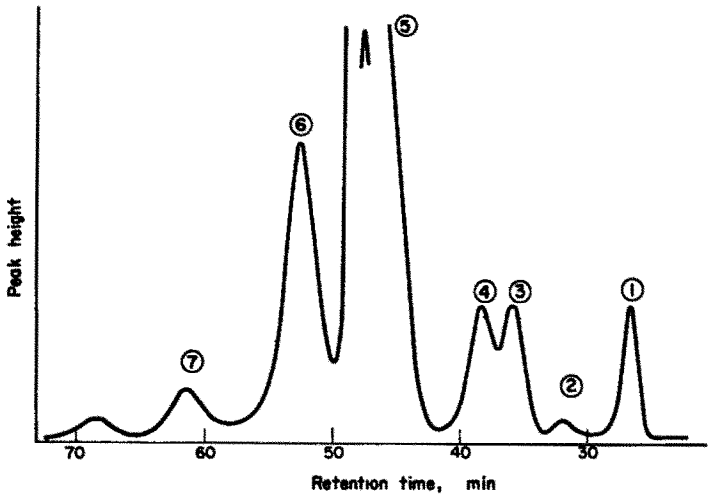


FIG. 1. GLC OF OAT SEED STEROL ACETATES ON 1% HiEFF-8B+2% POLYVINYLPYROLLINIDONE[17] ON ACID-WASHED GAS CHROM P. 225°.

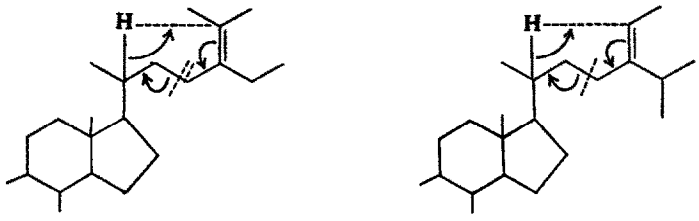
(1) Cholesterol; (2) Brassicasterol; (3) Campesterol; (4) Stigmasterol; (5) β-Sitosterol; (6) Δ^5-Avenasterol; (7) Δ^7-Avenasterol. On this system cholestane gives a retention time of 4·75 min.

were detected in this mixture and these may be Δ^7-cholestenol and a Δ^7-C_{28} sterol but such assignments are tentative.

Examination of the di-unsaturated fraction using GLC showed the presence of the two avena sterols together with small amounts of brassicasterol, stigmasterol and possible $\Delta^{7,22}$-stigmastadienol (α-spinasterol). Crystallization of this mixture afforded a nearly pure (90 per cent by GLC) sample of the Δ^5-avena sterol acetate.[3] The i.r. spectrum of this compound exhibited no peaks corresponding to a disubstituted double bond but exhibited peaks at 11·9 and 12·5 μ for the C_{5-6} double bond and at 12·40 for a second trisubstituted double bond. The parent ion (*m/e* 454) in the mass spectrum corresponded to a diunsaturated C_{29} sterol acetate. Additional information from the mass spectrum showed one nuclear double

[4] D. F. JOHNSON, R. D. BENNETT and E. HEFTMANN, *Science* **140**, 198 (1963).
[5] R. K. BEERTHIUS and J. H. RECOURT, *Nature* **186**, 372 (1960).
[6] M. J. THOMPSON, W. E. ROBBINS and G. L. BAKER, *Steroids* **2**, 505 (1963).
[7] B. A. KNIGHTS, *J. Gas Chromatogr.* **2**, 160 (1964).
[8] K. BOWDEN, I. M. HEILBRON, E. R. H. JONES and B. C. L. WEEDON, *J. Chem. Soc.* **39** (1946).

bond (*m/e* 213) and one side chain double bond (*m/e* 255, 296 and 356 respectively involving loss of 139+60−side chain+acetate; 98+60 and 98). The loss of 98 from the parent ion may be explained by postulating a 1:6 hydride shift associated with a double bond at either C-24 or C-24-28 (Fig. 2).

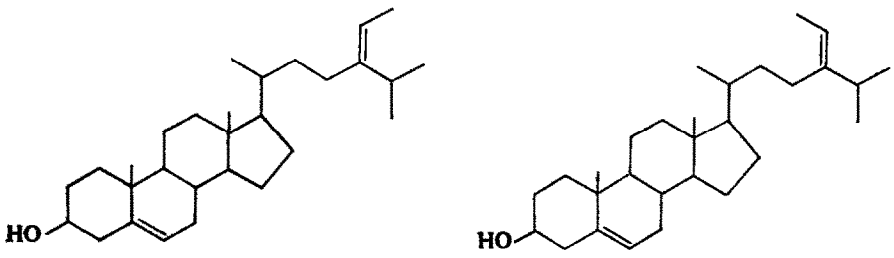


C-24 C24-28

FIG. 2.

The nuclear magnetic resonance spectrum of this compound showed two vinyl protons indicating the structure with the side chain double bond in position C-24-28 to be correct. This structure is the same as that for fucosterol. However, it proved possible to resolve these two compounds by GLC using HiEFF-8B as stationary phase (Table 1). Further information was obtained by subjecting the Δ^5-avena sterol to essentially the same degradation procedure as described by Bergmann and Klosty[9] for fucesterol. Osmium tetroxide was substituted for ozone and after cleavage of the diols with periodate, the mixture was oxidized with Jones reagent.[8] Thin layer chromatography of the final product afforded a substance found to have identical retention times with those expected for 24-ketocholesteryl acetate upon GLC. This assignment was confirmed by mass spectrometry.

Since the Δ^5-avena sterol and fucosterol[9] both give 24-keto cholesterol upon degradation of the side chain and since they have different retention times upon GLC, it is clear that these two compounds represent the two possible isomers (I and II) about the double bond (Fig. 3).



(I) (II)

FIG. 3.

Dusza[10] has synthesized 29-isofucosterol and from spectroscopic evidence has assigned the structure (I) to fucosterol and the structure (II) to 29-isofucosterol. The melting point gas chromatographic mobility and i.r. spectrum in the region 11·9–12·5 μ of 29-isofucosteryl acetate are identical with the corresponding data for the Δ^5-avena sterol obtained in this work. The occurrence together in plant sterol mixtures of both isomers at C-29 has not been

[9] W. BERGMANN and M. KLOSTY, *J. Am. Chem. Soc.* 73, 2935 (1951).
[10] J. P. DUSZA, *J. Org. Chem.* 25, 93 (1960).

reported but Schreiber and Osske[11] have isolated the isomer of citrostadienol[12] from *Solanum tuberosum* thus providing additional evidence for the separate occurrence of both isomers at C-29 in nature.

The second avena sterol is known to have a Δ^7-double bond and from its GLC mobilities on a number of columns (Table 1) together with the evidence of the earlier workers[3] it apparently has the same structure in the side chain as the Δ^5-compound.

EXPERIMENTAL

Varieties. Varieties of oat seed used were *Avena sativa*, Cultivar, Star, Victory, Blenda and *Avena fatua*. (From Hasler and Co. Ltd., Dunmow, Essex.)

Extraction of seeds. Crushed seeds were extracted in a Sohxlet extractor with petroleum ether (b.p. 40–60°) until no more colour was removed. The resultant solution was evaporated to dryness and the residue dissolved in acetone to precipitate the phospholipids. Filtration and evaporation afforded an orange-brown oil in 3–6 per cent yield depending on the variety of seed used.

Isolation of sterols. The crude oil was chromatographed on Peter Spence type H alumina (4 g/g of oil). Successive elution with benzene, chloroform and ethyl acetate afforded three fractions. Fractions (1) and (3) were saponified with sodium hydroxide (an equal weight) in 1:1 aqueous ethanol and the nonsaponifiable material was obtained by continuous extraction with ether of the diluted (water) reaction mixture. The material from fraction (1) was chromatographed on alumina to remove hydrocarbons and aliphatic alcohols. From fraction (3) the sterols were isolated by crystallization, and from the mother liquors by digitonin precipitation.[13,14]

Chromatography of sterols. Thin layer chromatography was carried out on silica-gel G using 10% ethyl acetate in petroleum ether as developing solvent. Spots were detected by spraying the plate with ceric sulphate and heating until the colours developed (pink for Δ^5- and blue for Δ^7-sterols). When used for preparative separation prior to GLC, zones on TLC plates were detected using iodine vapour. After allowing the iodine to evaporate the indicated zones were cut out and the sterols eluted with ethyl acetate.

GLC was carried out on a Pye argon chromatograph equipped with a flash heater and arranged for septum injection into columns of 4 ft length. Column packings consisted of acid washed siliconized Gas Chrom P coated with various stationary phases (1% w/w) using well established methods.[15] The phases used were SE-30, F-60, FS-1265, HiEFF-8B, PEG-A and NGS (all obtainable from Applied Science Laboratories). Cholestane as internal standard was included in each chromatographic run. To separate mono- and di-unsaturated steroids, chromatography on 25% silver nitrate on silica-gel (140–200 mesh from Applied Science Laboratories) was used (10:1 ratio of adsorbent to sterol acetate). The mono-unsaturated sterol acetates were eluted with light petroleum containing 2% of ether and the di-unsaturated sterol acetates with 30% ether in light petroleum (b.p. 40–60°).

[11] K. Schreiber and G. Osske, *Tetrahedron* **20**, 2575 (1964).
[12] A. Weizmann and Y. Mazur, *J. Org. Chem.* **23**, 832 (1958).
[13] R. Schoenheimer and H. Dam, *Z. Physiol.* **215**, 59 (1933).
[14] W. Bergmann, *J. Biol. Chem.* **132**, 471 (1940).
[15] E. C. Horning, W. J. A. Vanden Heuvel and B. G. Creech, *Meth. Biochem. Anal.* **11** (1963).

Degradation of Di-unsaturated Sterols to 24-Ketocholesteryl Acetate

After removal of the 3β-acetoxyl group the free sterols were converted to *p*-toluenesulphonate esters by reaction in pyridine with *p*-toluenesulphonyl chloride, the product being isolated by addition of water and extraction with ether. These esters were solvolysed to the i-steroid-6β-acetates in acetic anhydride[16] and the product treated in ethereal pyridine with excess osmium tetroxide. Following the usual work-up procedure and oxidation of the free hydroxyls with sodium metaperiodate in aqueous acetone, the crude mixture was immediately treated with Jones reagent[8] until a permanent orange colour persisted. Partition between water and ether, followed by extraction of acid material from the ether layer with aqueous sodium carbonate solution, afforded a crude neutral mixture. When subjected to reverse i-steroid rearrangement in acetic acid and subsequent purification using thin layer chromatography this mixture afforded 24-ketocholesteryl acetate.

Spectroscopy. Routine i.r. spectra were run on a Unicam SP. 200 and the high resolution spectra on a Unicam SP. 100. The nuclear magnetic resonance spectrum of $\Delta^{5,24(28)}$-stigmastadienyl acetate was run on a Perkin Elmer 60 Mc. and the mass spectra were determined on an A.E.I. M.S. 9 using the hot box attachment.

Acknowledgement—Thanks are due to Dr. W. J. A. Vanden Heuvel for gifts of some of the packings for GLC columns and to Dr. W. R. Supina for a generous gift of 25% silver nitrate on silica-gel. The help and encouragement of Professor R. A. Raphael and of Drs. C. J. W. Brooks, C. G. Elliott and W. Parker are gratefully acknowledged. This work was carried out during the tenure of a D.S.I.R. research assistantship.

[16] E. S. WALLIS, E. FERNHOLZ and F. T. GEPHART, *J. Am. Chem. Soc.* **59**, 137 (1937).
[17] B. A. KNIGHTS, *J. Gas Chromatogr.* **2**, 338 (1964).