Phytochemistry, 1968, Vol. 7, pp. 651 to 654. Pergamon Press. Printed in England

LIPIDS OF RUST FUNGI—II.

STIGMAST-7-ENOL AND STIGMASTA-7,24(28)-DIENOL IN FLAX RUST UREDOSPORES

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(*Received* 19 *October* 1967)

Abstract—Gas chromatographic, chemical, infrared-, NMR- and mass-spectrographic evidence shows that the principal sterols of flax rust uredospores are stigmast-7-enol and stigmasta-7,24(28)-dienol. Stigmasta-5,7-dienol was indicated as a minor component.

INTRODUCTION

VERY little has been reported on the sterols of rust fungi. The principal sterol of wheat stem rust has been reported to be ergost-7-enol,[1] but the sterols of other rust fungi have not been previously reported. The sterols of flax rust uredospores are primarily located within the cell since the surface lipids of flax rust uredospores (to be reported elsewhere) do not contain sufficient amounts of sterols to account for the amount of sterols observed in the present paper. The sterols reported here are therefore not a result of contamination with some flax plant sterols when the uredospores were collected.

RESULTS AND DISCUSSION

From 100 mg of uredospores, about 22 mg of lipid were extracted; of this, 6 mg were nonsaponifiable lipid. The major nonsaponifiable lipids are sterols, but there are lesser amounts of carotenes, paraffin hydrocarbons and other unidentified classes of lipids. The sterols were separated from the other nonsaponifiable lipids by column chromatography.

Gas–liquid chromatography of the free sterols in a 1 per cent SE-30 column indicated that the major sterols had a retention time of 3·82 relative to cholestane. The relative retention time of ergost-7-enol was found to be 2·94; therefore, the sterols of flax rust uredospores are not ergost-7-enol. Neither a reported relative retention time on SE-30 nor a sample of stigmast-7-enol was available for comparison, but a relative retention time of 3·86 to 3·88 was calculated by the method of Recourt and Beerthuis.[2] Gas–liquid chromatography of the sterol fraction as trimethylsilyl ethers on neopental glycol succinate demonstrated the presence of three sterols in the ratio of 10, 68 and 22 per cent.

The sterol fraction was separated into three components by preparative thin-layer chromatography on silver nitrate–silica gel plates. The u.v. absorption spectrum of the

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[1] F. W. HOUGEN, B. M. CRAIG and G. A. LEDINGHAM, *Can. J. Microbiol.* **4**, 521 (1958).

[2] J. H. RECOURT and R. K. BEERTHUIS, *Determination of Sterols*, p. 30, Monograph No. 2. The Society of Analytical Chemistry, London (1964).

component making up 10 per cent of the sterol fraction had absorption maxima at 272, 282 and 294 nm with relative absorbance of 0·96, 1·00 and 0·59 respectively. The absorption spectrum has the characteristic triplet absorption bands of a conjugated 5,7 diene system of sterols. This evidence and the retention time of this component indicate that the sterol might be stigmast-5,7-dienol. Due to insufficient sample, further structural studies were not carried out on this component.

The two major sterols were labeled "upper sterol" and "lower sterol" in accord with their separation on silver nitrate–silica gel thin-layer plates. When the plates were sprayed with vanallin–sulfuric acid spray or acetic anhydride–sulfuric acid spray, both upper sterol and lower sterol were "fast reacting" and gave brown colors at the same time that cholesterol gave the characteristic violet–red color. Ergot-7-enol also gave a brown color under the same conditions, indicating that both flax rust sterols have a 7–8 double bond.

Upper Sterol—Stigmast-7-enol

After the upper sterol was recrystallized twice from methanol the resulting long needles had a melting point of 145° and the acetate melted at 156°. In an extensive study of sterols, Barton[3] found that the acetates of simple 3β-ol sterols have higher melting points than the free sterol only when a 7–8 double bond is present in the sterol molecule. This evidence indicates that the upper sterol has a 7–8 double bond. Idler *et al.*[4] reported the melting point of stigmast-7-enol as 146° and of the acetate at 159°.

The i.r. spectrum of flax rust upper sterol closely resembles the i.r. spectrum of stigmast-7-enol[5] and ergost-7-enol. The mass spectrum, however, indicated that the sterol is of the stigmastenol type. The molecular ion peak is at mass 414 and, in addition, there are other strong peaks at masses 399, 273, 255 and 231. The loss of CH_3 group gives mass 399; loss of 10 carbon side-chain gives mass 273; loss of side-chain plus water gives mass 255 and loss of side-chain plus part of ring D and a hydrogen gives mass 231.

Lower Sterol—Stigmast-7,24(28)-dienol

The melting point of the twice crystallized lower sterol was 142°. Again the acetate melted at a higher temperature, 147°, indicating that this sterol also has a 7–8 double bond. When hydrogenated in a Brown Micro Hydro-Analyzer using Adams catalyst at a neutral pH, the lower sterol takes up 1 mole of hydrogen. Gas chromatography and i.r. analysis of the product indicated that the hydrogenated lower sterol is the same as the upper sterol. (stigmast-7-enol). The lower sterol, then, has one more double bond than the upper sterol.

The i.r. spectrum of the lower sterol is similar to the i.r. spectrum of stigmast-7-enol, but there is an additional absorption band at 820 cm^{-1}. A band at 800 to 840 is characteristic of a double bond with one position occupied by hydrogen and the remaining three occupied by carbon chains. The NMR spectrum of the lower sterol is very similar to the NMR spectrum of fucosterol.[6] The protons on the C-29 methyl group give a clearly defined doublet at 1·6 ppm from TMS with a coupling constant of about 6·7 c/s, as would be expected from an ethylidene group in the sterol side-chain. The mass spectrum confirms the presence of the ethylidene group in the lower sterol. The molecular ion peak is at mass 412 and additional strong peaks are present at masses 397, 314, 299, 271 and 231. The loss of a CH_3 group gives mass 397; loss

[3] D. H. R. BARTON, *J. Chem. Soc.*, 1116 (1946).
[4] D. R. IDLER, A. A. KANTUTSCH and C. A. BAUMANN, *J. Am. Chem. Soc.* **75**, 4325 (1953).
[5] D. R. JOHNSON, D. R. IDLER, V. W. MELOCHE and C. A. BAUMANN, *J. Am. Chem. Soc.* **75**, 52 (1953).
[6] W. R. NES, M. CASTLE, J. L. MCCLANAHAN and J. M. SETTINE, *Steroids* **8**, 655 (1966).

of seven carbons of the side-chain by allylic cleavage between carbons 22 and 23 gives mass 314; loss of a methyl group and the side-chain fragment gives mass 299; loss of the side-chain plus two hydrogens gives mass 231. Only small peaks for the loss of water and water plus other fragments were observed. A similar fragmentation pattern was observed for stigmasta-7,24(28)-dienol of oat seeds.[7]

To our knowledge, the only previous report of sterols of the stigmasterol group in fungi is a report of ergosterol and stigmasterol in *Debaromyces hansenii*.[8] Stigmast-7-enol and stigmasta-7,24[28]-dienol have, however, been reported in higher plants. Stigmast-7-enol was reported in wheat seed[4] and senita cactus,[9] and stigmasta-7,24(28)-dienol was reported in oat seed,[7, 10] but the two have not been previously reported together.

It is very likely that the sterols reported in this paper were synthesized in the host plant and that the flax rust uredospore is unable to synthesize its own sterol requirement (unpublished results). The possibility presents itself that the sterols obtained from the host are necessary for proper physiology of the flax rust uredospore and may therefore be a key to the obligate nature of the parasite. Our continuing research is designed to investigate these possibilities.

EXPERIMENTAL

Lipid Extraction and Saponification

The flax rust uredospores, physiological race one of *Melampsora lini* (Pers.) Lev. were grown on host plants (*Linum usitatissium* L.) of the Bison C.I. 389 variety. The uredospores were disrupted with a Wig-L-Bug, Model 6 electric mortar (Cresent Dental Mfg. Co., Chicago, Ill.). The grinding and lipid storage were carried out at 4° and under N_2. The ground uredospores were extracted for 18 hr with $CHCl_3$–MeOH (2:1 v/v), and the extract washed by the Folch technique.[11] The lower layer of the Folch wash was evaporated to near dryness under N_2 and the lipids were saponified using 0·5 N NaOH in 10 per cent aqueous MeOH at room temperature overnight. Nonsaponifiable lipids were extracted from the alkaline aqueous mixture with diethyl ether. The diethyl ether layers were combined, evaporated to dryness under N_2, and weighed.

Column Chromatography

All columns were jacketed and cooled with tap water to prevent excess evaporation of the eluting solvents. Fractionation of the nonsaponifiable lipids on acid-treated Florisil as described by Carroll[12] was carried out using the stepwise elution apparatus of Hirsch and Ahrens.[13] The column was packed with 16 g of acid-treated Florisil, slurried in light petroleum. The column was loaded with approximately 100 mg of nonsaponifiable matter for chromatography. The elution sequence was light petroleum, 25 ml; diethyl ether in light petroleum (1:5 v/v), 20 ml; diethyl ether in light petroleum (1:3 v/v), 20 ml; $CHCl_3$, 40 ml; $CHCl_3$–MeOH (2:1 v/v), 20 ml. 5-ml fractions were collected and evaporated under N_2. Each concentrated fraction was assayed by TLC to determine the location of the individual lipid classes. Those fractions which contained a single class of lipid were combined for further examination.

Thin-Layer Chromatography

Sterols varying in unsaturation were separated on plates made by slurrying 50 g of Adsorbosil-1 with 20 g of $AgNO_3$ in 75 ml of water. The 8 × 8 in. thin-layers were 0·25 mm thick for analytical separation and 0·75 mm thick for preparative separations. After air drying, the coated plates were activated at 110° to 120° for 1 hr. These plates were kept in a dark dessicator to prevent blackening prior to use. The chromatoplates were developed in a solvent system of 0·5 per cent glacial acetic acid in $CHCl_3$.

To visualize the sterol spots, the TLC plates were sprayed with either the Liebermann-Burchard spray

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[9] W. B. HEED and H. W. KIRCHER, *Science* **149**, 758 (1965).
[10] B. A. KNIGHTS, *Phytochem.* **4**, 857 (1965).
[11] J. FOLCH, M. LEES and G. H. S. STANLEY, *J. Biol. Chem.* **226**, 497 (1957).
[12] K. K. CARROLL, *J. Am. Oil Chemists' Soc.* **40**, 413 (1963).
[13] J. HIRSCH and E. H. AHRENS, *J. Biol. Chem.* **233**, 311 (1958).

according to Waldi[14] or the vanallin–sulfuric acid spray according to Mathews.[15] Both sprays gave about the same colors on heating, but the vanallin–sulfuric acid spray appeared to give more distinct colors.

Gas–Liquid Chromatography

The sterols were chromatographed in silanized glass columns mounted in an Aerograph Gas Chromatograph, Model 665, utilizing a hydrogen flame detector. The free sterols were chromatographed on a 1·5 m × 4 mm i.d. column of 1 per cent SE-30 on Gas Chrom Z operated at 225° with a flow rate of 25 ml/min. The sterol trimethyl silyl ether derivatives[16] were chromatographed on a 1·5 m × 4 mm i.d. column of 2 per cent neopentyl glycol succinate on 2 per cent polyvinyl pyrrolidone treated Gas Chrom P. The column temperature was maintained at 230° with a flow rate of 55 ml/min of N_2.

Spectroscopy

The i.r. spectra were determined with KBr micropellets on both a Perkin Elmer 337 and a Beckman IR-4 instrument using beam condensers.

The NMR spectra were determined on a Varian A-60 instrument in deuterated chloroform at a concentration of 100 mg/ml.

The mass spectra were run on a RMU-6E Single Focusing Hitachi Mass Spectrometer using a Direct Introduction Inlet.

Acknowledgements—Published with the approval of the Director of the North Dakota Agricultural Experiment Station as Journal Article No. *124*. Supported in part by National Science Foundation Grant No. 23635. Thanks are due to Mr. A. H. Struck of Perkin-Elmer Corporation for the mass spectra determinations, and to Dr. L. J. Goad for mass spectra of fucosterol, and 24-methylene and 24-ethylene lophenol acetates.

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