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STEROLS OF THE LICHEN *PSEUDEVERNIA FURFURACEA*

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Abstract—A mixture of C_{27}, C_{28} and C_{29} sterols was isolated from the lichen *Pseudevernia furfuracea* and characterized by means of GLC and MS. Mono-, di- and tri-unsaturated sterols were identified as well as a small amount of fully saturated sterols (stanols). Only a part of the total sterols present in the lichen tissue was easily extractable with organic solvents. Another portion was only obtained after saponification of the lichen residue remaining after extraction with organic solvents. The composition of these two fractions differed considerably, the former contained predominantly 5α,8α-epidioxy-5α-ergosta-6,22-dien-3β-ol (ergosterol peroxide) and 24-ethylcholesta-5,22-dien-3β-ol while in the latter 24-ethylcholesta-5,22-dien-3β-ol and C_{28} triene sterols were the main components.

INTRODUCTION

Pseudevernia furfuracea (L.) (Zopf. *Parmelia furfuracea*) has been shown to contain atranorin, chloroatranorin, physodic acid, arabitol, mannitol, lichenin and tetrahydroxy fatty acids,[1,2] Sterols present in this species were identified as 'fungisterol' (ergost-7-en-3β-ol) and ergosterol.[3]

As previous work carried out in this laboratory with other lichen species revealed that the sterol composition of these organisms is complex and that a part of the total sterols could only be extracted from the tissue by saponification[4a,b] we decided to undertake a more detailed re-examination of the sterol components in *Pseudevernia furfuracea.*

RESULTS AND DISCUSSION

For the purpose of isolation of all the sterols present in *P. furfuracea* dry tissue was extracted successively first with acetone and then with $CHCl_3$–MeOH. The extracts were combined, taken to dryness and the lipids redissolved in diethyl ether leaving an insoluble residue which was shown to contain atranorin. The diethyl ether solution was washed with aqueous $NaHCO_3$ and NaOH to remove acidic components and the remaining neutral lipid was subjected to column chromatography on neutral alumina. Successive elution with light petrol.–diethyl ether mixtures gave crude steryl esters (21·9 mg) and crude free sterols (28·4 mg). A more polar material (76·1 mg) was then eluted with diethyl ether. The lichen residue remaining after the solvent extraction was saponified and the non-saponifiable lipids released were extracted into diethyl ether. The sterols of this fraction were also obtained by chromatography on neutral alumina. The mixtures of free sterols from both fractions were further purified by TLC on silica gel (solvent system III) then acetylated and separated by means of TLC on $AgNO_3$ impregnated silica gel (solvent systems I and II). The various

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TABLE 1. TLC, GLC AND MS DATA FOR THE ACETATES

	$R_f \times 100$*	Relative retention time†	M^+
Cholestan-3β-ol	54	1·40	430(28)
24-Methylcholestan-3β-ol		1·76	444(93)
24-Ethylcholestan-3β-ol		2·15	458(88)
24-Methylcholest-7-en-3β-ol	49	2·02	442(100)
24-Ethylcholest-7-en-3β-ol		2·46	456(100)
Cholest-5-en-3β-ol	44	1·36	—
24-Methylcholest-5-en-3β-ol		1·72	—
24-Ethylcholest-5-en-3β-ol		2·09	—
24-Ethylcholesta-5,22-dien-3β-ol	22	1·82	—
24-Methylcholesta-5,22-dien-3β-ol	14	1·53	—
24-Methylcholesta-5,8,22-trien-3β-ol	6 (40)	1·60	438(10)
24-Methylcholesta-7,24(28)-dien-3β-ol		2·07	440(42)
24-Methylcholesta-5,7,22-trien-3β-ol	3 (22)	1·68	—

* On silica gel G impregnated with 15% of $AgNO_3$, solvent system I or II (values in parenthesis).
† 3% SE-30, 270°, retention time of 5α-cholestan-3β-ol = 1·00.

steryl acetates were characterised by GLC and MS.[5,6] Some fractions which contained more than one component were analysed by combined GLC–MS. TLC and GLC properties of the steryl acetates isolated as well as their MS are presented in Table 1. The amounts of individual sterol components calculated on the basis of peak area on GLC are given in Table 2. A considerable difference can be seen between the composition of the sterols of the direct solvent extract and the sterols obtained after saponification of the residue. The former contained a small amount of stanols and Δ^7 sterols but did not contain any of the C_{28} trienes which were present in a relatively large amount in the latter. One of the sterol trienes ($R_f \times 100 = 22$ in system II) was identified as ergosterol (UV spectrum: λ_{max} 271, 282, 294 nm, Lit.[7] 271, 282, 293 nm). The other C_{28} triene showed no absorption in the UV and therefore did not contain conjugated double bonds. Its retention time and MS were similar to those of 'lichesterol' (ergosta-5,8,22-trien-3β-ol) previously isolated from another lichen species.[4b] Both C_{28} and C_{29} sterols were present in the solvent-extractable and tightly bound sterol fractions but the proportion of C_{27} compounds was distinctly higher in the former. The configuration of the C-24 methyl or ethyl groups in the sterols isolated from *P. furfuracea* could not be deduced from the results obtained. Both 24-*R* and 24-*S* isomers are known to occur in living organisms but, so far, they cannot be separated by chromatography nor distinguished by MS.

The steryl ester fraction obtained from the acetone-chloroform–methanol extract was purified on silica gel plates (solvent system IV), saponified and both the sterol and fatty acid components isolated and investigated. Because of the small amount of material available the sterol components were not separated but directly analysed by means of combined GLC–MS. It is possible that some minor components of this fraction could not be detected

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OF THE STEROLS ISOLATED FROM *Pseudevernia furfuracea*

Mass spectrum (*m/e*)‡				
A	B	C	D	Other prominent ions
370(76)	257(8)	230(40)	215(100)	276(48)
384(67)	257(13)	230(40)	215(100)	276(47)
398(71)	257(18)	230(45)	215(100)	276(41)
382(6)	255(52)	299(16)§	213(20)	315(10), 273(10)
396(14)	255(61)	229(12)§	213(18)	315(12), 273(9)
368(100)	255(15)	228(11)	213(11)	260(11), 247(19)
382(100)	255(16)	228(3)	213(11)	274(11), 261(14)
396(100)	255(15)	228(3)	213(9)	288(8), 275(11)
394(100)	255(39)	228(4)	213(5)	296(4), 282(3)
380(100)	255(44)	228(8)	213(12)	296(4), 282(8)
378(90)	253(28)	—	211(12)	363(100), 337(10), 237(10)
380(8)	255(44)	228(8)	213(12)	356(58), 313(100)
378(100)	253(11)	—	211(6)	335(3), 280(3)

‡ A: M^+-acetate; B: M^+-side chain-acetate; C: M^+-side chain-27-acetate; D: M^+-side chain-part of ring D (42)-acetate. Relative abundance (%) is given in parenthesis.
§ M^+-side chain-27-MeCOO.[6]

in this case. The results are presented in Table 2. GLC of the methyl esters of the fatty acids of this fraction showed that the major component was linoleic acid. Only traces of other acids (oleic, palmitic, myristic and unidentified acids) were found. It is noteworthy that the fraction of free fatty acids isolated from this species also contained predominantly linoleic acid (see Experimental).

TABLE 2. STEROL COMPONENTS OF *Pseudevernia furfuracea*

Component	Amount (μg per 100 g of dry tissue) Direct solvent extract Free	Esterified	Extract from saponified lichen residue
Cholestan-3β-ol	70	—	—
24-Methylcholestan-3β-ol	20	—	—
24-Ethylcholestan-3β-ol	60	—	—
24-Methylcholest-7-en-3β-ol	280	—	—
24-Ethylcholest-7-en-3β-ol	20	—	—
Cholest-5-en-3β-ol	330	160	50
24-Methylcholest-5-en-3β-ol	670	190	610
24-Ethylcholest-5-en-3β-ol	360	100	340
24-Ethylcholesta-5,22-dien-3β-ol	1860	530	2000
24-Methylcholesta-5,22-dien-3β-ol	900	60	660
24-Methylcholesta-5,8,22-trien-3β-ol (lichesterol)	—	—	800
24-Methylcholesta-7,24 (28)-dien-3β-ol	—	—	90
24-Methylcholesta-5,7,22-trien-3β-ol (ergosterol)	—	—	760
5α,8α-Epidioxy-5α-ergosta-6,22-dien-3β-ol (ergosterol peroxide)	30800	—	—

In addition to the steryl esters and free sterols a more polar material showing a positive reaction with the Lieberman–Burchard reagent (deep green) was isolated by column chromatography from the acetone–chloroform–methanol extract. This material was purified by TLC and crystallization and identified as 5α,8α-epidioxy-5α-ergosta-6,22-dien-3β-ol

(ergosterol peroxide) on the basis of its m.p., IR, NMR and MS (see Experimental). Since the MS differed from those already published[7,8] an authentic sample of ergosterol peroxide was prepared by photosensitized oxygenation of ergosterol.[9] The compound obtained was identical in all respects with that isolated from the lichen. It is possible, that ergosterol peroxide is not a natural lichen sterol but an artefact formed from ergosterol during drying and/or isolation as proved in the case of some pigmented fungi.[7,10,11] On the other hand it has been suggested that ergosterol peroxide might be a natural intermediate in the biosynthesis of ergosterol[12] and ergosta-4,6,8(14),22-tetraene-3-one.[13]

EXPERIMENTAL

General procedures. GLC of steryl acetates was performed using a Pye 104 instrument fitted with a hydrogen flame ionization detector. All-glass columns (150 cm × 8 mm) packed with 3% SE 30 on 80–100 mesh Gaschrom Q were employed. The carrier gas was argon (60 ml/min) and the column temp. was 270°. MS were determined by Mrs. A. Holcroft on an AEI MS-12 instrument. M.ps were determined using a Reichert Micro Hot Stage apparatus; IR spectra: Perkin-Elmer Infracord 237 spectrometer in KBr pellets; UV spectra; Unicam SP-800 spectrophotometer in EtOH; NMR spectra (100 Hz): Varian HA-100D spectrometer in $CDCl_3$, with tetramethylsilane as internal reference. TLC was carried out on plates coated with 0·5 mm silica gel G or $AgNO_3$ (15% w/w) impregnated silica gel G using the following solvent systems: $CHCl_3$ (I), $CHCl_3$–Et_2O (19:1) (II), $CHCl_3$–MeOH (19:1) (III) and cyclohexane–C_6H_6 (1:1) (IV). For TLC commercial $CHCl_3$ was washed several times with dist. H_2O to remove EtOH, dried over anhyd. Na_2SO_4 and distilled immediately before use. Column chromatography was performed on alumina (Woelm, neutral, Brockmann grade III) using light petrol. and mixtures of light petrol containing increasing quantities of Et_2O for elution.

Extraction and fractionation. Pseudevernia furfuracea was collected in July 1971 near Newcastleton, Roxburghshire, Scotland. Air dried lichen tissue (200 g) was powdered and extracted under reflux 2× with boiling acetone (1·2 l., 3 hr) and once with boiling $CHCl_3$–MeOH mixture (1·2 l., 3 hr). The combined extracts were evaporated to dryness and the residue extracted with Et_2O (3 × 50 ml). The material insoluble in Et_2O gave after three recrystallisations from acetone 0·7 g of atranorin; m.p. 195–7° (Lit.[14] 196°) MS. *m/e* 374 (M^+), 213, 196, 179, 164, 150, 136, 121, 107. The Et_2O solution containing free sterols and steryl esters was successively washed with 5% (w/v) aq. $NaHCO_3$ and 5% (w/v) aq. NaOH. From the NaOH extract after acidification to pH 4, extraction with Et_2O and TLC on silica gel (solvent system III) a fatty acid mixture was obtained (oil, ~0·7 g). Methylation with CH_2N_2 and GLC showed that linoleic acid was the predominant component. The lichen residue left after the extraction of the tissue with organic solvents was saponified with 10% (w/v) KOH in 80% (v/v) EtOH (1·5 l., 3 hr). The hydrolysate was filtered, diluted with H_2O and extracted with Et_2O. From this extract a second portion of sterols was obtained (20·1 mg).

Identification of sterols. Separation of the components of the acetone–$CHCl_3$–MeOH extract on alumina gave the steryl ester (21·9 mg) and free sterol (28·4 mg) fractions which were further examined by TLC, GLC and MS (see Results). A more polar material (76·1 mg) identified as ergosterol peroxide was eluted with Et_2O and purified by TLC and crystalization from aq. EtOH (needles, 61·6 mg) m.p. 175–7° (Lit.,[2,8,9] 177–82°, 178°); ν_{max} (KBr, cm^{-1}) 3525, 1465, 1375, 1045, 970. MS. *m/e* 428 (M^+, 5%), 410 (5%), 396 (100%), 376 (12%), 363 (24%), 253 (10%), 251 (20%). NMR (δ, ppm) 0·75, 0·82, 0·88, 0·94, 0·96, 1·03 (18H), 3·93 (1H, *bm*), 5·10–5·23 (2H, *m*), 6·14–6·56 (2H, *AB*-quartet centred at 6·35); acetate: m.p. 193–7° (Lit.[8] 201–3°). MS. *m/e* 470 (M^+, 4%), 452 (3%), 436 (10%), 410 (6%), 378 (100%), 299 (6%), 253 (8%), 251 (16%); TMS ether. MS. *m/e* 500 (M^+, 2%), 482 (2%), 468 (6%), 428 (24%), 410 (30%), 396 (100%), 376 (42%), 363 (26%), 337 (10%), 253 (26%), 251 (56%). An authentic sample of ergosterol peroxide prepared from ergosterol as described by Windaus and Brunken[9] had m.p. 175–9° and all spectra were identical to the isolated compound.

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