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A COMPARISON OF THE TYPES OF STEROL FOUND IN SPECIES OF THE SAPROLEGNIALES AND LEPTOMITALES WITH THOSE FOUND IN SOME OTHER PHYCOMYCETES*

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Abstract—The sterols produced by a number of Phycomycetes have been studied by several gas chromatographic techniques and in some cases by mass spectroscopy. Fungi of the Orders Saprolegniales and Leptomitales contain varying proportions of cholesterol, desmosterol, 24-methylenecholesterol and fucosterol. Species of the Mucorales contain varying proportions of cholesterol, ergosterol and 22-dihydroergosterol. No detectable amount of sterol was found in three species of the Peronosporales.

INTRODUCTION

In previous investigations of the types of sterol occurring in fungi, the species examined contained ergosterol and closely related steroids rather than those commonly found in green plants.[1] These species wall have typical cell walls of fungal chitin,[2] however, and we have seen no record of investigations of species having cellulose walls. We have therefore examined the types of sterol in six species from the Saprolegniales, three from the Leptomitales (cellulose walls) and compared them with the types found in twelve other species of Phycomycetes having chitin walls.

RESULTS

The amount of sterol obtained from the mycelium of various species of Phycomycetes grown under standard conditions is given in Table 1. Four species of Saprolegniales and one of Leptomitales gave the best yields (0·1–0·25 per cent of the dry weight of mycelium), while three species of Peronosporales did not give detectable amounts. It has been reported that sterols could not be detected in the mycelium of another species of the Peronosporales (*Phytophthora cactorum*).[3]

The composition of the sterol mixtures obtained from the mycelia of different Phycomycetes is shown in Table 2, the individual sterols being identified by GLC relative retention data[4] and using combined GLC–mass spectroscopy[5] in four cases.

* Part I in the series "Fungal Sterols".

[1] L. J. Goad, in *Terpenoids in Plants* (edited by J. B. Pridham), p. 160, Academic Press, New York, (1967); L. F. Fieser and M. Fieser, *Steroids*, Reinhold, New York (1959).

[2] J. M. Aronson, in *The Fungi* (edited by G. S. Ainsworth and A. S. Sussman), Chapter 3, p. 49, Academic Press, New York (1965).

[3] C. G. Elliott, M. R. Hendrie, B. A. Knights and W. Parker, *Nature* **203**, 427 (1964).

[4] Cf., e.g., B. A. Knights, *Phytochem.* **4**, 857 (1965).

[5] B. A. Knights, *Advances in Gas Chromotography*, Vol. 4, p. 135, Academic Press, New York (1967); B. A. Knights, *J. Gas Chromatogr.* **5**, 273 (1967).

Table 1. Amount of sterols isolated from the mycelia of various phycomycetes as percentage dry weight of mycelium

Saprolegniales*	
0·1–0·25%	*Saprolegnia ferax* (No. 204b)
	S. megasperma (No. 208a)
	Leptolegnia caudata (No. 351a)
	Aplanopsis terrestris (No. 312a)
0·01–0·025%	*Achlya caroliniana* (No. 152b)
	Pythiopsis cymosa (No. 361a)
Leptomitales*	
0·025–0·05%	*Apodachlya minima* (No. 503a)
	Apodachlyella completa (No. 504a)
0·01–0·025%	*Apodachlya brachynema* (No. 501a)
Mucorales	
0·01–0·025%	*Mucor hiemalis* (+) (CMI 21216)
	M. hiemalis (−) (CMI 21217)
	M. dispersus (CMI 89318)
	Rhizopus stolonifer (−) (CMI 57762)
	Phycomyces blakesleeanus (+) (CMI 21156)
	P. blakesleeanus (−) (CMI 44142)
0·005–0·01%	*Absidia glauca* (+) (CMI 15405)
	A. glauca (−) (CMI 15406)
	Mortierella rammaniana (CvS, Baarn)
	Thamnidium elegans (CMI 43624)
	Zygorhynchus moelleri (CMI 82702)
0–0·005%	*Syncephalastrium racemosum* (CMI 35039)
	Cunninghamella echinulata (CMI 53585)
Peronosporales	
Negligible	*Phytophthora infestans* (Glasgow University)
	Pythium ultimum (Glasgow University)
	P. debaryanum (CMI 50465)

* Aquatic Phycomycetes Culture Collection, University of Reading, Department of Botany (Dr. M. W. Dick).

Sterols of Saprolegniales and Leptomitales

The sterol mixtures from the nine different species of Saprolegniales and Leptomitales consisted almost entirely of various proportions of cholesterol, desmosterol, fucosterol and 24-methylenecholesterol. The presence of the latter was initially seen from the chromatographic retention data which were close to but distinguishable from, those from ergosterol, campesterol and stigmasterol as indicated in Table 3. It had a slightly longer retention time on a P.V.P.–C.H.D.M.S. column than campesterol although the retention times on F-60 were similar, behaviour which would be expected for an unsaturated analogue of campesterol such as 24-methylenecholesterol (cf. the pairs desmosterol–cholesterol and fucosterol–β-sitosterol on F-60 and P.V.P.–C.H.D.M.S. columns in Table 3).

Similarities in its breakdown pattern with that of fucosterol also suggested that this sterol might be 24-methylenecholesterol. The high intensity of the ions at *m/e* 314, 299, 296

and 271 are particularly significant (cf. Table 4). Finally, comparison of its retention data and mass spectrum with those of an authentic sample of 24-methylenecholesterol synthesized[6] from 24-ketocholesterol* showed them to be identical. Addition of this sterol to the sterol mixture from *Saprolegnia ferax* resulted in enhancement of the main GLC peak using several columns and derivatives.

TABLE 2. PERCENTAGE COMPOSITION OF STEROL MIXTURES FROM PHYCOMYCETES

Species	Sterol* 1	2	3	4	5	6	GLC†
Saprolegniales							
Saprolegnia ferax	4	13		68		15	abcdef MS
S. megasperma	23	1		47		29	acdef
Leptolegnia caudata	2	14		70		14	adef
Aplanopsis terrestris	34			6		60	acdef MS
Achlya caroliniana	72	27		1			aef
Pythiopsis cymosa	23			73		4	a
Leptomitales							
Apodachlya minima	85	0·5		6		1	adef
A. brachynema	68	2		8		22	adef
Apodachlyella completa	3			41		56	ab
Mucorales							
Mucor hiemalis (+)	1		7		85		a MS
M. hiemalis (−)	15		6		52		a
M. dispersus			80		10		a MS
Rhizopus stolonifer (−)			96		2		a
Phycomyces blakesleeanus (+)			60		20		a
P. blakesleeanus (−)			80		10		a
Absidia glauca (+)			12		83		ab
A. glauca (−)	1		3		93		ab

* 1. Cholesterol. 2. Desmosterol. 3. Ergosterol. 4. 24-Methylenecholesterol. 5. 22-Dihydroergosterol. 6. Fucosterol.

† Indices refer to GLC conditions used as in Table 3. Combined GLC–mass spectroscopic studies are denoted by MS.

Sterols of Mucorales

In view of the low yields of sterols obtained from these fungi, examination of the sterol composition was limited in most cases to GLC of the free sterols, using 1 % F-60 as stationary phase. The sterol mixtures from *Mucor dispersus*, from a (−) strain of *Rhizopus stolonifer* and from (+) and (−) strains of *M. hiemalis*, *Phycomyces blakesleeanus* and *Absidia glauca* contains two main components, one corresponding in retention time to ergosterol or 5-dihydroergosterol (retention times 2·8 and 2·85 respectively), the other having relative retention time 3·3 (22-dihydroergosterol). Smaller amounts of cholesterol (retention time 2·2) were also present in some cases.

* Kindly provided by Dr. G. Wood, Organon Laboratories.

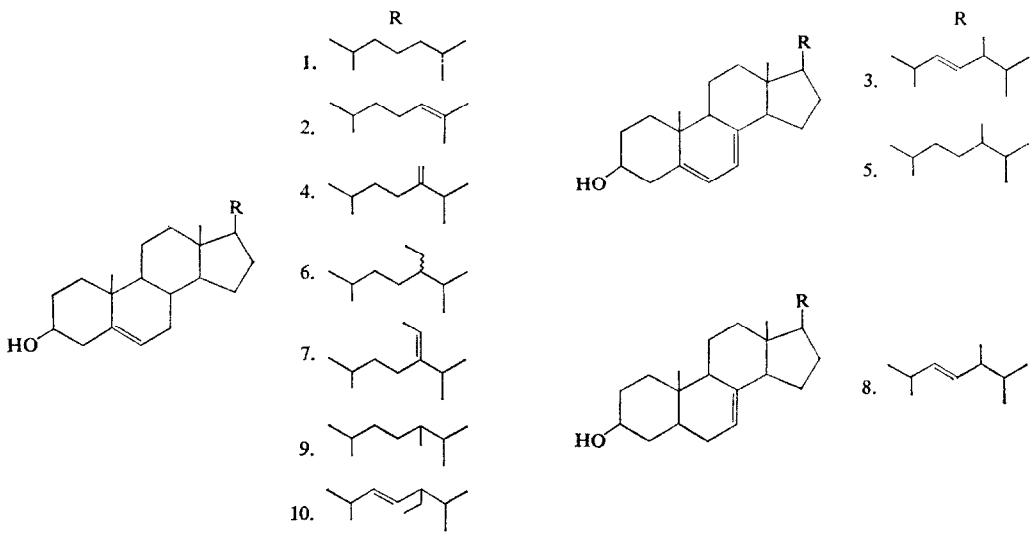
[6] D. R. IDLER and U. H. M. FAGERLUND, *J. Am. Chem. Soc.* **79**, 1988 (1957).

Examination of the sterols from *M. hiemalis* (+) and *M. dispersus* by combined GLC–mass spectroscopy confirmed the presence of ergosterol in both fungi and of cholesterol in *M. hiemalis* (+). The sterol of retention time 3·3 was found to have molecular weight 398.

TABLE 3. RELATIVE RETENTION DATA OF STEROLS AND THEIR DERIVATIVES (CHOLESTANE = 1).

	Molecular weight:	386	384	396	398	398	414	412	398	400	412
3β Substituent	Stationary phase	Sterol* 1	2	3	4	5	6	7	8	9	10
a. OH	F-60	2·2	2·35	2·8	2·85	3·3	3·85	3·8	2·85	2·95	3·25
b. OH	P.V.P./C.H.D.M.S.	5·85	7·55	8·85	8·3	10·45	10·05	10·55	7·6	7·85	8·4
c. $OCOCH_3$	F-60	3·2	3·4	4·3	4·3	4·6		5·7			4·8
d. $OSiMe_3$	NGS	2·1	2·7	3·3	3·1		3·55	3·8		2·9	3·0
e. $OCOCF_3$	NGS	2·1	2·7		2·9		3·4	3·6			2·85
f. $OCOCF_3$	F-60	1·6	1·75		2·1		2·75	2·75		2·1	2·3

* 1. Cholesterol. 2. Desmosterol. 3. Ergosterol. 4. 24-Methylenecholesterol. 5. 22-Dihydroergosterol. 6. 24-Ethylcholesterol. 7. Fucosterol. 8. 5-Dihydroergosterol. 9. Campesterol. 10. Stigmasterol.



This retention time is longer than that of either 24-methylenecholesterol or 5-dihydroergosterol (both 2·85) and the mass spectra were distinct. However, saturation of the double bond at C_{22}, as in 22-dihydroergosterol would be expected to result in a longer retention time than the unsaturated counterpart (cf. the pair, stigmasterol/β-sitosterol in Table 3). This assignment was shown to be correct by comparison (retention time and mass spectrum) with

an authentic sample of 22-dihydroergosterol prepared from ergosterol acetate via the maleic anhydride adduct.[7]

TABLE 4. ABUNDANCE OF SOME IONS IN THE MASS SPECTRA OF STEROLS RELATIVE TO THAT OF THE PARENT ION ($M^+=100$)

Molecular weight:	386	384	396	398	398	414	412	398	400	412
	Sterol*									
Fragment ion†	1	2	3	4	5	6	7	8	9	10
m/e 314	5	18	—	910	—	23	1250	—	—	18
m/e 299	2	91	—	350	—	15	326	20	—	44
m/e 296	—	5	—	150	—	10	190	—	—	—
m/e 271	5	455	—	246	250	26	230	150	—	70

* 1. Cholesterol. 2. Desmosterol. 3. Ergosterol. 4. 24-Methylenecholesterol. 5. 22-Dihydroergosterol. 6. 24-Ethylcholesterol. 7. Fucosterol. 8. 5-Dihydroergosterol. 9. Campesterol. 10. Stigmasterol.

† These ions can be considered to arise by losses of ZH, ZH+15, ZH+18 and ZH+43 mass units respectively from the parent ion, where McLafferty type elimination of the side-chain fragment Z together with a hydrogen atom as in Fig. 1 would give an isopropenyl group at C_{17}. A facile loss of 43 mass units from this has a parallel in the breakdown of monoterpenes having an isopropenyl grouping.[8]

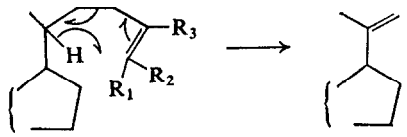


FIG. 1.

DISCUSSION

As indicated above, cholesterol was found in species of Saprolegniales, Leptomitales and Mucorales. This sterol has previously been found in the fungus *Penicillium funiculosum*,[9] in red algae,[10,11] in date palm pollen[12] and also in several higher plants.[13]

The sterols found only in the Saprolegniales and Leptomitales, namely desmosterol, 24-methylenecholesterol and fucosterol, have not previously been found in fungi but occur in red and/or brown algae[11] in and plant pollens.[14] On the other hand, ergosterol and its

[7] P. SINGH and S. RANGASWAMI, *Current Sci.* **35**, 515 (1966).
[8] H. BUDZIKIEWICZ, C. DJERASSI and D. H. WILLIAMS, *Structural Elucidation of Natural Products by Mass Spectrometry*, Vol. 2, p. 145, Holden-Day, London (1964).
[9] Y. S. CHEN and R. H. HASKINS, *Can. J. Chem.* **41**, 1647 (1963).
[10] K. TSUDA, S. AKAGI and Y. KISHIDA, *Pharm. Bull. (Japan)* **6**, 101 (1958).
[11] K. TSUDA, S. AKAGI, Y. KISHIDA, R. HAYATSU and K. SAKAI, *Pharm. Bull. (Japan)* **6**, 724 (1958).
[12] R. D. BENNETT, S. T. KO and E. HEFTMANN, *Phytochem.* **5**, 231 (1966).
[13] C. DJERASSI, J. C. KNIGHT and H. BROCKMANN, *Chem. Ber.* **97**, 3118 (1964); D. F. JOHNSON, R. D. BENNETT and E. HEFTMANN, *Science* **140**, 198 (1963).
[14] M-F. HUGEL, W. VELLER, H. AUDIER, M. BARBIER and E. LEDERER, *Phytochem.* **3**, 7 (1964); M. BARBIER, M-F. HUGEL and E. LEDERER, *Bull. Soc. Chim. Biol.* **42**, 91 (1960).

22-dihydro derivative which were found in the Mucorales have previously been isolated from a variety of fungi, reported sources of ergosterol including Basidiomycetes,[15] Fungi Imperfecti,[1] yeasts[1] and also one Zygomycete, *Blakeslea trispora*,[16] while 22-dihydroergosterol has been obtained from the Basidiomycete *Polyporus paragamenus*.[8]

These findings support the concept of the species of Saprolegniales and Leptomitales as fungi which are uniquely different from those of other orders, and they support obvious possible phylogenetic implications.

EXPERIMENTAL

Organisms

The cultures of Mucorales were obtained from the Commonwealth Mycological Institute and maintained on potato-dextrose agar. The latter was prepared with the liquor obtained by boiling 200 g of potato, expressing through muslin and making up to 1000 ml with distilled water after the addition of dextrose (20 g) and agar (20 g).

Cultures of Saprolegniales and Leptomitales were kindly supplied by Dr. M. W. Dick. These were maintained on hemp seeds (2–3 seeds in 15 ml of distilled water), and were subcultured every 3 weeks by growing out on Petri dishes containing some of the following medium: glucose (10 g), soluble starch (5 g), yeast extract (2 g), Na_2HPO_4 (0·6 g), K_2TeO_3 (0·1 g), agar (30 g), made up to 1 l. with distilled water.

Culture Methods

Cultures were grown at 25° under artificial illumination on peptone–glucose broth [glucose (25 g) and "Difco" Bacto-peptone (0·5 g) dissolved in deionized water (1000 ml) and dispensed into 200 ml aliquots in 1000 ml Roux bottles with cotton-wool plugs]. After autoclaving at 117° for 30 min, each culture was inoculated as follows.

In the case of the Saprolegniales and Leptomitales, small discs of tellurite-agar were taken from the perimeter of the Petri dish. This resulted in the introduction into 20 l. of medium of up to 0·5 mg of yeast extract and, assuming complete diffusion of the hemp seed constituents throughout the agar, of up to 0·14 mg of hemp seed constituents. This in fact did not result in the introduction of detectable amounts of sterol.

In the case of the Mucorales, cultures were inoculated with portions of the cultures on potato-dextrose agar. This resulted in the introduction into 20 l. of medium of about 75 ml of potato-dextrose agar, or the equivalent of the aqueous extract from 15 g of potato. If it is assumed that the total amount of sterol occurring in potato is 0·012 per cent[17] this could result in the introduction into the culture medium of 1·8 mg of potato sterol, the principal constituent of which is β-sitosterol.[18] However, control extractions of potato broths suggested that the actual amount introduced was less than 0·2 mg. Although the mycelia of several Mucorales cultured in this way did not afford detectable amounts of sterol, nevertheless in some cases where sterols were found (*Mucor hiemalis*, *M. dispersus*), small amounts of a sterol corresponding in GLC properties and mass spectrum to β-sitosterol were detected and it cannot be discounted that these were derived from medium trapped in the mycelium or by adsorption from the medium. Accordingly, this has not, in the present work, been considered as one of the sterols produced by these Mucorales.

The optimal conditions for accumulation of any particular sterol or for maximum total accumulation may well be different for each species, e.g. when *M. hiemalis* (+), *Saprolegnia ferax* and *Aplanopsis terrestris* were grown in shake flasks of the same medium at 20°, the same range of sterols were produced, but ergosterol became the major component of the mixture accumulated by *M. hiemalis*, forming a much higher proportion relative to 22-dihydroergosterol. Variation in the degree of saturation of ergosterol derivatives by yeasts has previously been found to be affected by cultural conditions.[19]

The above conditions were therefore chosen arbitrarily after a few preliminary tests in which they were found to support sufficient accumulation of sterols to permit comparison of the species examined. The results for each species were confirmed by at least two consistent replicate experiments.

Extraction of Mycelium and Isolation of Sterols

The dried mycelium of each species examined was extracted with petroleum ether (b.p. 60–80°) in a Soxhlet for 24 hr. The resulting extract was adsorbed on a column of Woelm neutral alumina (Grade III, 40 g/g of

[15] F. H. Milazzo, *Can. J. Botany* **43**, 1347 (1965).
[16] L. J. Goad, A. S. A. Hammam, A. Dennis and T. W. Goodwin, *Nature* **210**, 1322 (1966).
[17] T. Galliard, *Phytochem.* **7**, 1907 (1968).
[18] M. Lepage, *J. Lipid Res.* **5**, 587 (1964).
[19] O. N. Breiwick, J. L. Owades and R. F. Light, *J. Org. Chem.* **19**, 1734 (1954).

extract) in petroleum ether–benzene (1:1). After elution with this solvent (200 ml/g of extract) in order to remove non-polar fatty material, elution with 5% ether in benzene and with 50% ether in benzene (100 ml of each mixture/g of extract) gave fraction 2 containing most of the sterols present, as indicated by TLC. No sterols were found in the remaining more polar fractions (TLC).

In order to separate the sterols from fatty material, fraction 2 was generally refluxed for 10 hr with 10% KOH in aqueous MeOH (1:9). The neutral fraction of the product consisted mainly of sterols. Purification was effected either via the digitonides[20] or by preparative TLC on silica.

Thin-Layer Chromatography

Sterols were detected on thin layers of silica gel G by the characteristic blue-black stain developed after spraying with ceric ammonium nitrate and heating to 100°. Where $\Delta^{5,7}$ sterols were present, coloration occurred rapidly on gentle warming. Using $CHCl_3$ as solvent, sterols had R_f 0·29. The convenient reference standards *p*-hydroxyazobenzene (R_f 0·2) and *p*-aminoazobenzene (R_f 0·4) were used as markers. Preparative TLC was carried out on 0·6 mm layers of silica gel H, impregnated with trisodium pyrene tricarboxylate in order that the sterol band should fluoresce in u.v. light. After elution three times with 10% ethyl acetate in petroleum ether (b.p. 60–80°), the sterols formed a band of R_f 0·2–0·3, from which they were recovered using ethyl acetate or ether (Soxhlet).

Gas–Liquid Chromatography

Gas chromatographic analysis was carried out at 225° on a Pye Argon Chromatograph equipped with a flash heater and arranged for septum injection into 4-ft columns. The columns were packed with acid-washed siliconized Gas-chrom P (100–200 mesh) coated with various stationary phases. Retention times were calculated relative to cholestane which was added as an internal standard in each chromatographic run. Combined gas–liquid chromatography/mass spectrometry of sterols were carried out using a 1% SE30 column at 225° in an L.K.B. 8000 Spectrometer.

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[20] R. SCHOENHEIMER and H. DAM, *Z. Physiol.* **215**, 59 (1933); W. BERGMANN, *J. Biol. Chem.* **132**, 471 (1940).