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STEROLS OF THE MYCOBIONT AND PHYCOBIONT ISOLATED FROM THE LITCHEN *XANTHORIA PARIETINA*

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(Dedicated to Professor E. LEDERER on the occasion of his sixty-fifth birthday)

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Abstract—The mycobiont, *Xanthoria parietina*, and the phycobiont, *Trebouxia decolorans*, of the lichen *X. parietina* have been cultured separately and their sterols analysed. *X. parietina* contained ergosterol and lichesterol as the major constituents together with lower levels of three other C_{28} sterols. Culture of the mycobiont in the presence of [CD_3]-methionine resulted in the incorporation of two deuterium atoms into the C-24 methyl group of these sterols demonstrating that a 24-methylene intermediate was produced as occurs in other fungi. The phycobiont, *T. decolorans* contained predominantly poriferasterol with lower levels of clionasterol, ergost-5-en-3β-ol, brassicasterol and cholesterol. Two other *Trebouxia* spp. (213/3 and 219/2) contained similar sterol mixtures.

INTRODUCTION

TWO STEROL 'pools' were demonstrated[1] in the lichen *Xanthoria parietina*, one was readily extracted with organic solvents while the other was tightly bound and only released by saponification of the tissue remaining after solvent extraction. The main components of the solvent-extractable sterols were a novel C_{28} triene, ergosta-5,8,22-trien-3β-ol (I, lichesterol) and ergosta-5,7,22-trien-3β-ol (II, ergosterol) whereas the main component of the sterols obtained by saponification was (24*R*)-24-ethylcholesta-5,22-dien-3β-ol (III, poriferasterol). Since ergosterol is regarded as the typical sterol of fungi[2] whilst poriferasterol is more usually thought of as an algal sterol[3] their distribution between the two lichen sterol fractions suggested that the sterols of the mycobiont were extracted readily with organic solvent but that the release of the phycobiont sterols required saponification of the lichen tissue. To test this possibility we have now examined the composition of the sterols of the mycobiont (*X. parietina*) and the phycobiont (*Trebouxia decolorans*) isolated from the lichen *X. parietina*.

RESULTS AND DISCUSSION

The common lichen phycobionts are *Trebouxia* spp. which can be divided into two morphological groups.[4] Cultures of *Trebouxia* sp. 213/3 (Group I), 219/2 (Group II) and *T. decolorans* (Group II) were examined and all found to contain sterol mixtures in which the C_{29} sterols predominated (Table 1). The identities of the various sterols were determined by combined GC–MS which showed that the three major constituents in all three

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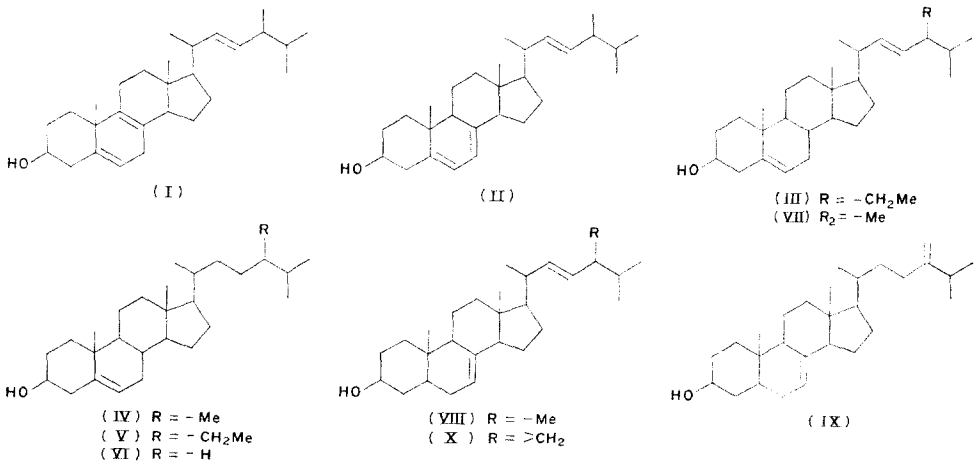
[1] LENTON, J. R., GOAD, L. J. and GOODWIN, T. W. (1973) *Phytochemistry* **12**, 1135.
[2] GOODWIN, T. W. (1973) in *Lipids and Biomembranes of Eucaryotic Micro-organisms* (ERWIN, J. A., ed.), p. 1, Academic Press, London.
[3] PATTERSON, G. W. (1971) *Lipids* **6**, 120.
[4] AHMADJIAN, V. (1967) *The Lichen Symbiosis*, Blaisdell, Massachusetts.

species were ergost-5-en-3β-ol (IV), poriferasterol (III) and (24*S*)-24-ethylcholest-5-en-3β-ol (V, clionasterol). However *T. decolorans* contained in addition small amounts of cholest-5-en-3β-ol (VI, cholesterol) and ergosta-5,22-dien-3β-ol (VII, brassicasterol) which were previously observed[1] in the intact lichen *X. parietina*. Confirmation of the identification of poriferasterol (III) was obtained by the isolation of this compound as its acetate by silver nitrate–silica gel TLC of the acetates of the sterol mixture obtained from *Trebouxia* sp. 213/3. The purified material had an NMR spectrum identical to authentic poriferasteryl acetate but clearly differing from the NMR spectrum of its C-24 epimer stigmasteryl acetate.[5]



The ratio of C_{28}:C_{29} sterols in the *Trebouxia* sp. 213/3 (Group I) was very similar (Table 1) to the ratio noted previously[6] for both autotrophic and heterotrophic cultures of this alga. By contrast the Group II algae, *Trebouxia* sp. 219/2 and *T. decolorans* contained a significantly higher proportion of C_{28} sterol (Table 1). Verification of this difference in C_{28}:C_{29} sterol ratio for other Group I and Group II *Trebouxia* spp. is required and may possibly be a useful additional criterion for differentiating the two Groups. The significance of this apparent difference between the two groups is uncertain but may perhaps be related to the degree of control exerted by the Δ^{24} sterol: *S*-adenosyl-methionine transmethylase[6,7]

TABLE 1. PERCENTAGE COMPOSITION OF THE STEROL MIXTURES OBTAINED FROM *Trebouxia* SPECIES

Sterol	GLC RR_T*	*Trebouxia* sp. 213/3†	*Trebouxia* sp. 219/2†	*Trebouxia decolorans*‡
Cholesterol (VI)	1·00	—	—	0·7
Brassicasterol (VII)	1·12	—	—	1·1
Ergost-5-en-3β-ol (IV)	1·30	23·1	34·7	31·4
Poriferasterol (III)	1·42	70·4	57·8	64·0
Clionasterol (V)	1·63	6·5	7·5	2·8

* GLC analysis was on 1% SE-30, retention times are given relative to cholesterol.
† Grown heterotrophically.
‡ Grown autotrophically.

[5] THOMPSON, M. J., DUTKY, S. R., PATTERSON, G. W. and GOODEN, E. L. (1972) *Phytochemistry* **11**, 1781.
[6] GOAD, L. J., KNAPP, F. F., LENTON, J. R. and GOODWIN, T. W. (1972) *Biochem. J.* **129**, 219.
[7] WOJCIECHOWSKI, Z. A., GOAD, L. J. and GOODWIN, T. W. (1973) *Biochem. J.* in press.

in producing either a Δ^{25} sterol required for C_{28} sterol production or a $\Delta^{24(28)}$ compound which can then undergo a second transmethylation to yield the C_{29} sterols.

The sterols of *T. decolorans* were solvent extractable whereas the corresponding sterols of the intact lichen, *X. parietina*, were present mainly in the tightly bound pool released by saponification.[1] The reasons for this inconsistency are obscure but are presumably related to the vastly different cultural conditions experienced by the algae when grown in liquid media compared with algae developing in the whole lichen in the wild.

The isolated mycobiont, *X. parietina*, is characterized by a very slow growth rate in defined liquid media and the gross morphology is considerably different from the lichenised state.[4] Sterols were isolated from both the solvent-extractable and tightly bound pools of actively growing cultures of the mycobiont (Table 2). Both fractions contained the C_{28} trienes lichesterol (I) and ergosterol (II), which were isolated and fully characterised by GC, MS and NMR spectrometry. It was noticeable that the solvent extractable and tightly bound pools of sterol contained differing proportions of lichesterol and ergosterol. Also the quantity of C_{28} trienes in the solvent-extractable and tightly bound pools varied with the age of the culture and the tightly bound sterols were absent in older cultures which may be related to a possible change in membrane characteristics with the ageing of the mycobiont. A number of minor sterols were present in both actively growing and aged cultures and were shown by silver nitrate TLC of the acetates followed by GC and MS analysis to be ergosta-7,22-dien-3β-ol (VIII), ergosta-7,24(28)-dien-3β-ol (IX, episterol) and ergosta-7,22,24(28)-trien-3β-ol (X) which was recently identified in yeast.[8] The identification of these various sterols in the mycobiont establishes that it has the capacity for *de novo* sterol production and it is not dependent upon the symbiotic alga to provide either preformed ergosterol and lichesterol or a suitable C_{28} sterol for modification to these compounds.

TABLE 2. PERCENTAGE COMPOSITION OF THE STEROL MIXTURES ISOLATED FROM CULTURES OF THE MYCOBIONT *Xanthoria parietina*

Sterol	GLC RR_t*	Experiment 1 Solvent extractable	Experiment 1 Tightly bound	Experiment 2 Esters	Experiment 2 Free
Lichesterol (I)	1·12	46·6	43·8	15·3	16·0
Ergosterol (II)	1·22	27·7	42·5	4·9	5·8
Ergosta-7,22-dien-3β-ol (VIII)	1·25	—†	—†	7·8	19·6
Ergosta-7,22,24(28)-trien-3β-ol (X)	1·32	14·1	5·6	28·6	32·2
Ergosta-7,24(28)-dien-3β-ol (IX)	1·42	11·6	8·1	43·4	26·4

In Experiment 1 the total solvent-extractable and tightly bound sterols were isolated from cultures harvested after 3 weeks. In Experiment 2 the steryl esters and free sterols were obtained from solvent-extracts of cultures harvested after 4–6 weeks.

* GLC analysis was on 1% SE 30, retention times are given relative to cholesterol.

† Not quantitated, since this sterol was only present at low levels and appeared as a shoulder on the ergosterol (II) peak.

The presence of sterols IX and X in the mycobiont suggested that the C-24 alkylation mechanism leading to the 24-methyl sterols, lichesterol (I) and ergosterol (II) probably involves the intermediate production of 24-methylene compounds as observed in other fungi.[9,10] This was confirmed by the addition of [CD_3]-methionine to the culture medium of the mycobiont followed by MS analysis of the sterols isolated after 3 weeks culture.

[8] BARTON, D. H. R., KEMPE, U. M. and WIDDOWSON, D. A. (1972) *J. Chem. Soc. Perkin* **I**, 513.

[9] JAURÉGUIBERRY, G., LAW, J. H., MCCLOSKEY, V. A. and LEDERER, E. (1965) *Biochemistry* **4**, 347.

[10] VARENNE, J., POLONSKY, J., CAGNOLI-BELLAVITA, N. and CECCHERELLI, P. (1971) *Biochimie* **53**, 261.

In all the sterols examined only two deuterium atoms were incorporated (Fig. 1) thus confirming the intermediacy of 24-methylene compounds. This contrasts with the production of C_{28} sterols in the phycobiont, *Trebouxia* sp. 213/3, which was shown previously[6] to incorporate three deuterium atoms into the C_{28} sterol that was produced via a 25-methylene intermediate.[6,7] The various sterols identified in the mycobiont suggest the possible operation of the biosynthetic sequence IX → X → VIII → II similar to that recently indicated for ergosterol (II) production in yeast.[11] A non-enzymic origin for lichesterol (I), for example by photoisomerization of ergosterol (II) induced by the rather extreme environmental conditions to which lichens are exposed, was previously considered.[1] However, the present identification of lichesterol (I) in the isolated mycobiont grown on liquid media in the dark now suggests that it may be produced enzymically, possibly by the action of a reversible $\Delta^8 \rightleftharpoons \Delta^7$ isomerase[12] on ergosterol (II).

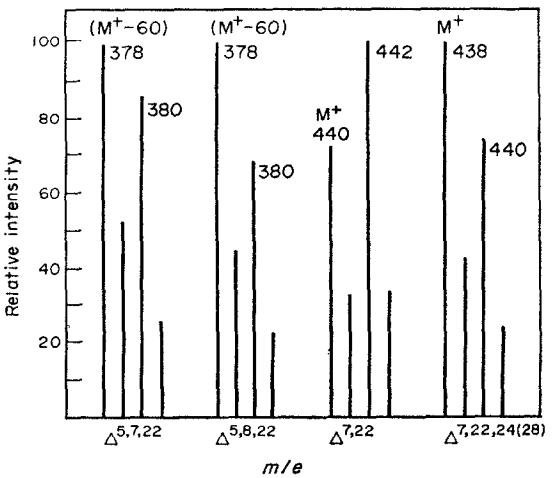


FIG. 1. MS OF THE STERYL ACETATES OBTAINED FROM *Xanthoria parietina* INCUBATION WITH [CD_3]-METHIONINE.

$\Delta^{5,7,22}$: ergosta-5,7,22-trien-3β-yl acetate; $\Delta^{5,8,22}$: ergosta-5,8,22-trien-3β-yl acetate; $\Delta^{7,22}$: ergosta-7,22-dien-3β-yl acetate; $\Delta^{7,22,24,28,}$: ergosta-7,22,24(28)-trien-3β-yl acetate.

EXPERIMENTAL

General procedures. These were as described previously.[1]

Culture of the mycobiont. Xanthiora parietina was grown in 100 ml cultures of autoclaved Lilly and Barnett's medium, pH 6·0 containing 2% (w/v) glucose[4] shaken at 20° in the dark.

Culture of the phycobiont. Trebouxia decolorans was grown autotrophically in 6 l. of Bold's basal medium, pH 6·0[4] at room temp. (~18°) under illumination and an atmosphere of 5% (v/v) CO_2 in air for 4 weeks. *Trebouxia* sp. 213/3 and *Trebouxia* sp. 219/2 were obtained from the Cambridge Culture Collection and grown heterotrophically in 20 l. of defined medium,[13] pH 5·2 containing 2% (w/v) glucose at room temperature (~18°) under illumination for 4 weeks.

Isolation of the sterols from the mycobiont. (a) Major sterols: *X. parietina* (76 g wet wt.) was harvested after 3 weeks culture and extracted with acetone (2 × 400 ml) and 2:1 (v/v) $CHCl_3$–MeOH (300 ml) each under reflux for 3 hr. Solvent was removed, Et_2O added (400 ml) and partitioned with 5% (w/v) $NaHCO_3$ (2 × 100 ml) and 5% (w/v) NaOH (2 × 100 ml) to remove strong (76·8 mg) and weak (68·7 mg) acids respectively. Neutral lipid (1·6 g) and also the fungal tissue (19·0 g) dry wt remaining after solvent extraction were saponified separately with 10% (w/v) KOH and 1% (w/v) pyrogallol in 80% (v/v) EtOH under reflux

[11] FRYBERG, M., OEHLSCHLAGER, A. C. and UNRAU, A. M. (1972) *Biochem. Biophys. Res. Commun.* **48,** 593.
[12] WILTON, D. C., RAHIMTULA, A. D. and AKHTAR, M. (1969) *Biochem. J.* **114,** 71.
[13] AARONSON, S. and BAKER, H. (1961) *J. Protozool.* **8,** 274.

for 1 hr. The non-saponifiable lipid (solvent extract 174·8 mg; fungal residue 219·6 mg) was purified by TLC on silica gel G developed with 1% (v/v) EtOH–$CHCl_3$ to give the crude sterol containing fractions (solvent extract 37·1 mg; fungal residue 34·1 mg). These were acetylated (pyridine–Ac_2O), purified by TLC on silica gel G developed with 1:1 (v/v) cyclohexane–C_6H_6 (solvent extract 18·2 mg; fungal residue 13·4 mg) and analysed by GC and GC–MS on 1% SE-30. The steryl acetates from the two pools were then combined and resolved on 10% (w/w) $AgNO_3$–silica gel G TLC developed twice in 1:1 (v/v) cyclohexane:C_6H_6. Ergosteryl acetate (5·4 mg) and lichesteryl acetate (6·0 mg) were eluted and fully identified by UV, MS and NMR spectrometry as described previously.[1] (b) Minor sterols: *X. parietina* (33 g wet wt) was harvested after 4–6 weeks in culture and extracted exhaustively with EtOH (200 ml), acetone (2 × 200 ml) and 2:1 (v/v) $CHCl_3$–MeOH (150 ml) each under reflux for 2 hr. Solvent was removed, Et_2O added (300 ml) and the strong (15·2 mg) and weak (79·6 mg) acids removed by partition with 5% (w/v) $NaHCO_3$ and 5% (w/v) NaOH respectively. The neutral lipid (1·79 g) was eluted from an alumina column (100 g Woelm, neutral Brockmann, grade III) with increasing proportions of Et_2O in light petrol. (40–60°). The steryl ester fraction (306 mg) was saponified, the sterols purified by TLC (16·2 mg), acetylated (15·4 mg) and repurified by TLC (2·4 mg). The sterol containing fraction from the alumina column (244 mg) was purified by TLC (52 mg), acetylated (51 mg) and repurified by TLC (2 mg). The two steryl acetate fractions were analysed by GC on 1% SE-30 and then combined and resolved on 10% (w/w) $AgNO_3$–silica gel G developed with 1:19 (v/v) Et_2O–EtOH free $CHCl_3$. Four bands were eluted with Et_2O. Bands 1 and 2 were shown by combined GC–MS analysis to be mixtures of ergosteryl acetate, lichesteryl acetate and episteryl acetate. Band 3 was ergosta-7,22, 24(28)-trien-3β-yl acetate, MS: *m/e* 438 (5%, M^+), 342 (14%, M^+–part of side chain (SC)), 313 (100%, M^+–SC–2H), 255 (6%, M^+–SC–acetate), 227 (4%) 213 (3%, M^+–SC–acetate–part of ring D), UV: λ_{max}^{EtOH} 231·5, 225, 240 nm (lit.[8]). Band 4 was ergosta-7,22-dien-3β-yl acetate, MS: *m/e* 440 (90%, M^+), 425 (15%, M^+–Me), 397 (12%, M^+–43), 342 (27%, M^+–part SC), 313 (100%, M^+–SC–2H), 288 (24%), 255 (73%, M^+–SC–acetate), 241 (12%), 229 (38%), 213 (22%, M^+–SC–acetate–part ring D).

Isolation of sterols from the phycobiont. (a) *Trebouxia decolorans*: cells were harvested by centrifugation (2·65 g wet wt) and extracted with EtOH (100 ml), acetone (2 × 100 ml) and 2:1 (v/v) $CHCl_3$–MeOH (90 ml) each under reflux for 1·5 hr. The combined solvent extracts were reduced to dryness, saponified and the non-saponifiable lipid (5·2 mg) purified by TLC and the sterols (1·8 mg) analysed by combined GC–MS (Table 1). Cholesterol (*m/e* 386), brassicasterol (*m/e* 398), ergost-5-en-3β-ol (*m/e* 400), poriferasterol (*m/e* 412) and clionasterol (*m/e* 414) were identified by their characteristic fragmentation patterns.[1] No sterols were detected in the non-saponifiable lipid (483·6 mg) obtained from the algal tissue remaining after solvent extraction. (b) *Trebouxia* sp. 213/3(A) and *Trebouxia* sp. 219/2 (B): cells were harvested and freeze dried (A, 7·0 g; B, 3·5 g) and the non-saponifiable lipid (A, 134 mg; B, 85 mg) purified by alumina column and silica gel G TLC to give the sterol fractions (A, 8·6 mg; B, 6·0 mg) which were analysed by GC. After acetylation the steryl acetates (A, 8·0 mg) were resolved by TLC on 10% (w/w) $AgNO_3$–silica gel developed with EtOH–free redistilled $CHCl_3$ to give pure poriferasteryl acetate (4·1 mg, m.p. 145–146°, lit.[14] 146–147°).[3] The NMR spectrum was identical to that of authentic poriferasteryl acetate obtained from *Ochromonas malhamensis*[14] and reported in the literature.[5]

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