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THE IDENTIFICATION OF 24-METHYLENE-24,25-DIHYDROLANOSTEROL AND OTHER POSSIBLE ERGOSTEROL PRECURSORS IN *PHYCOMYCES BLAKESLEEANUS* AND *AGARICUS CAMPESTRIS*

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Key Word Index—*Phycomyces blakesleeanus*; Mucoraceae; *Agaricus campestris*; Agaricaceae; mushroom; biosynthesis; sterols; ergosterol; lanosterol; 24-methylene-24,25-dihydrolanosterol.

Abstract—The following sterols have been isolated from the fungi, *Phycomyces blakesleeanus* and *Agaricus campestris*: ergosterol, lanosterol, 24-methylene-24,25-dihydrolanosterol and episterol. 4,4-Dimethyl-5α-ergosta-8,24(28)-dien-3β-ol and 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol have been tentatively identified. Evidence for the incorporation of label from L-methionine-[methyl-^{14}C] into some of these sterols in *P. blakesleeanus* has been obtained. The significance of these sterols in ergosterol biosynthesis is discussed.

INTRODUCTION*

Ergosterol is synthesized in fungi by a route essentially the same as that by which cholesterol is synthesized in animal tissues; acetate, mevalonate, squalene and lanosterol have all been shown to be ergosterol precursors [1–5]. The 24-methyl group is derived from methionine [1] via *S*-adenosylmethionine [6]. The transmethylation reaction involves loss of one of the hydrogen atoms of the methionine methyl group [7] and transfer of a hydrogen atom from C-24 to C-25 [8] so implicating the participation of a 24-methylene sterol intermediate. It is clear that the sterol undergoing alkylation has a Δ^{24} double bond [9] but the point in the biosynthetic sequence at which transmethylation takes place appears to be different in different fungi. The characterization of eburicol [10, 11], eburical [11], eburicodiol [11], eburicoic acid [10–12] and the polyporenic acids [12] in several wood-rotting fungi (e.g. *Fomes officinalis*, *Polyporus* spp) shows that a 24-methylene group can be introduced at the lanosterol level. However 24-methylene-24,25-dihydrolanosterol (eburicol) could not be found in an exhaustive survey of *Saccharomyces cerevisiae* sterols [13] suggesting that in this organism alkylation does not normally take place at the lanosterol level in spite of the fact that 24-methylene-24,25-dihydrolanosterol has been shown to be converted into ergosterol by yeast [14]. In this paper we report the isolation and identification of 24-methylene-24,25-dihydrolanosterol and other possible ergosterol precursors in

* *Sterol nomenclature and abbreviations*: The trivial names of the sterols used in the text have the following systematic names: ergosterol = (24R)-24-methylcholesta-5,7,22-trien-3β-ol; episterol = 5α-ergosta-7,24(28)-dien-3β-ol; eburicol = 24-methylene-24,25-dihydrolanosterol = 4,4,14α-trimethyl-5α-ergosta-8,24(28)-dien-3β-ol; eburical = 4,4,14α-trimethyl-5α-ergosta-8,24(28)-dien-3β-ol-21-al; eburicodiol = 4,4,14α-trimethyl-5α-ergosta-8,24(28)-dien-3β,21-diol; eburicoic acid = 3β-hydroxy-4,4-14α-trimethyl-5α-ergosta-8,24(28)-dien-21-oic acid; polyporenic acid A = 3α,12α-dihydroxy-4,4,14α-trimethyl-5α-ergosta-8,24(28)-dien-26-oic acid; polyporenic acid C = 16α-hydroxy-3-oxo-4,4,14α-trimethyl-5α-ergosta-7,9(11),24(28)-trien-21-oic acid. The following abbreviations are used in the textual description of the MS data: SC = side chain; 27 = [C − 16 + C − 17 + 3H]; 42 = [C − 15 + C − 16 + C − 17 + 6H]; 43 = [C − 24 to C − 27]; 56 = [C − 15 to C − 17 + C − 32 + 8H]; 59 = [C − 1 to C − 3 + OH + 6H]; 84 = [C − 23 to C − 28]; [C − 22→ + H] = H plus C − 22 to end of SC.

the fungi *Phycomyces blakesleeanus* and *Agaricus campestris*. This work extends previous communications [15, 16] and shows that in these organisms alkylation at C-24 takes place at the lanosterol level.

RESULTS

The 4,4-dimethylsterol fractions of *P. blakesleeanus* and *A. campestris* were separated into three components, A C, by column chromatography on $AgNO_3$-impregnated alumina or by preparative argentation-TLC of their acetates.

Compound A and its acetate, from both fungi, had GLC retention times relative to cholestane (RR_t) identical to those of lanosterol and lanosteryl acetate (RR_t on QF-1, 5·05 and 6·6; RR_t on CHMDS, 12·3 and 10·1). The acetate of compound A co-chromatographed on argentation-TLC with lanosteryl acetate. Crystallization of the compound A isolated from *P. blakesleeanus* from $CHCl_3$–MeOH gave crystals of m.p. 130° (reference 12, 139°). Treatment with the Liebermann–Burchard reagent produced a clear yellow colour (λ_{max} 460 nm) which is characteristic of lanosterol [17] and other sterols containing a 14α-methyl group [18]. The MS of compound A acetate had a molecular ion at *m/e* 468 and fragment ions at *m/e* values of 453 (M^+-Me) and 393 (M^+-[Me + AcOH]) which are consistent with its being the acetate of a C_{30} sterol with two double bonds. An ion at *m/e* 255 (M^+-[SC + 42 + AcOH]) represents a characteristic sterol fragmentation [19] and shows that the side chain has eight carbons and one double bond. The ions at *m/e* values of 301 (M^+-[SC + 56]) and 241 (M^+-[SC + 56 + AcOH]) indicate the presence of a 14α-methyl group [18] in a sterol devoid of a 9,19-cyclopropane ring [20]. The MS is therefore consistent with the identification of compound A acetate as lanosterol acetate. The NMR spectrum, which included signals at τ values of 9·33 (C-18 protons), 9·13 (4α, 4β and 14α-methyl protons), 9·01 (C-19 protons), 8·43 (C-26 protons) 8·34 (C-27 protons) 8·00 (3β-*O*-acetyl protons), 5·5 (3α-proton) and 4·92 (C-24 proton), is also consistent with this identification.

Compound B and its acetate, from both fungi, had longer GLC retention times than those of lanosterol and its acetate (RR_t on QF-1, 5·9 and 8·0; RR_t on CHMDS, 13·7 and 11·7). Treatment with the Liebermann–Burchard reagent produced a clear yellow colour (λ_{max} 460 nm) indicating the presence of a 14α-methyl group [18]. The MS of compound B acetate had a molecular ion at *m/e* 482 and fragment ions at *m/e* values of 467 (M^+-Me), 422 (M^+-AcOH) and 407 (M^+-[Me + AcOH]) indicating an empirical formula $C_{33}H_{54}O_2$ consistent with its being the acetate of a C_{31} triterpene alcohol. Ions at *m/e* values of 315 (M^+-[SC + 42]) and 255 (M^+-[SC + 42 + AcOH]) show that the side chain has nine carbon atoms and one double bond. Ions at *m/e* values of 383 (M^+-[Me + 84]) and 323 (M^+-[Me + 84 + AcOH]) indicate the presence of a 24-methylene group [20–22]. Ions at *m/e* values of 301 (M^+-[SC + 56]) and 241 (M^+-[SC + 56 + AcOH]) indicate the presence of a 14α-methyl group [18] in a sterol devoid of a 9,19-cyclopropane ring [20]. The MS is therefore consistent with the identification of compound B acetate as 24-methylene-24,25-dihydrolanosterol acetate. The NMR spectrum, which included signals at τ values of 9·31 (C-18 protons), 9·12 (4α, 4β and 14α-methyl protons), 9·02 (C-19 protons), 9·00 (C-26 protons), 8·95 (C-27 protons), 7·98 (3β-*O*-acetyl protons), 5·35 (C-28 protons) and 5·50 (3α-proton), is also consistent with this identification. The presence of a methylene group was also indicated by peaks at 887 and 1640 cm^{-1} in the IR spectrum. The m.p. of compound B (150–151°) isolated from *P. blakesleeanus* was also in reasonable agreement with the literature value (154°) for synthetic 24-methylene-24,25-dihydrolanosterol [14, 23].

Compound C has been tentatively identified as 4,4-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol. It was found to be very unstable in the small amounts isolated and consequently purification was difficult. The purest sample isolated from *P. blakesleeanus* had, after crystallization from $CHCl_3$–MeOH, a m.p. of 152–154°. With the Liebermann–Burchard reagent it first gave a weak blue colour which rapidly faded with the concomitant appearance of yellow coloration. However, the possibility that the yellow colour may have been due to small amounts of contaminating compound B cannot be excluded. This suggested that compound C may lack a 14α-methyl group. This conclusion was supported by its more polar behaviour on argentation chromatography and GLC on CHMDS (RR_t of C and its acetate, 15·9 and 13·3) than 24-methylene-24,25-dihydrolanosterol (compound B); possibly

the absence of a 14α-methyl group permits greater interaction of an adjacent nuclear double bond (e.g. Δ^8) with silver ions or CHMDS. The MS of compound C acetate was also consistent with the identity suggested above. The molecular ion at *m/e* 468 and fragment ions at *m/e* values of 453 (M^+-Me), 408 (M^+-AcOH) and 393 (M^+-[Me + AcOH]) indicate that compound C is a triterpene alcohol. Ions at *m/e* values of 341 (M^+-[SC + 2H]), 281 (M^+-[SC + AcOH + 2H]), 316 (M^+-[SC + 27]) and 241 (M^+-[SC + 42 + AcOH]) show that the side chain has nine carbon atoms and one double bond. Ions at *m/e* values of 384 (M^+-84), 369 (M^+-[Me + 84]) and 309 (M^+-[Me + 84 + AcOH]) indicate the presence of a 24-methylene group [20–22]. The absence of ions lacking the [SC + 56] fragment from the MS supports the contention that compound C has no 14α-methyl group. The IR spectrum of compound C had peaks at 887 and 1640 cm^{-1} characteristic of a methylene group. The foregoing data are therefore consistent with the structure 4,4-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol for compound C but the position of the nuclear double bond remains to be established unequivocally.

The main component of the 4-demethylsterol fraction of *P. blakesleeanus* and *A. campestris* was shown to be ergosterol. The UV spectrum (λ_{max} at 272, 282, 293 nm; shoulder at 262 nm) was characteristic of $\Delta^{5,7}$-sterols [24]. The IR spectrum showed a peak at 975 cm^{-1} characteristic of a *trans*-Δ^{22} double bond [25]. The MS had a molecular ion at *m/e* 396 and fragment ions at *m/e* values of 381 (M^+-Me), 378 (M^+-H_2O), 363 (M^+-[Me + H_2O]), 337 (M^+-59), 271 (M^+-SC), 253 (M^+-[SC + H_2O]) and 211 (M^+-[SC + 42 + H_2O]) in accord with that of ergosterol [7]. The NMR spectrum had unresolved multiplets at τ values of 4·7–4·85 (integration for three protons) for the olefinic protons at C-7, C-22 and C-23, and at 4·57 for the olefinic proton at C-6 as would be expected for ergosterol.

A minor component of the 4-demethylsterol fraction, compound D, running just ahead of ergosterol on argentation-TLC was isolated and identified as episterol. With the Lieberman–Burchard reagent, compound D gave a blue–green coloration whose intensity was maximal after about 90 sec; however, unlike ergosterol, this coloration was not preceded by a transient pink flush. This behaviour, allied with lack of UV absorption in the 260–300 nm region, suggests that compound D has a single nuclear double bond at Δ^7 or Δ^8. The MS had a molecular ion at *m/e* 398 and fragment ions at *m/e* values of 383 (M^+-Me), 314 (M^+-84), 299 (M^+-[Me + 84]), 271 (M^+-[SC + 2H]), 231 (M^+-[SC + 42]) and 213 (M^+-[SC + 42 + H_2O]) which indicates that compound D is a C_{28} sterol containing one nuclear double bond and a 24-methylene group. The presence of the latter was confirmed by the IR spectrum (peaks at 890 and 1640 cm^{-1}). The olefinic region of the NMR spectrum of compound D integrated for three protons, two of which formed a multiplet centred at τ 5·3 characterizing them as the 24-methylene protons. That the remaining olefinic proton is at C-7, indicating a Δ^7 double bond, is indicated by a shift to τ 9·22 of the C-19 methyl protons [26].

Very small amounts of a 4-monomethylsterol were isolated from both *P. blakesleeanus* and *A. campestris*. This has been tentatively identified as 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol, a sterol previously reported in yeast [27]. Its GLC RR_t values on SE-30 (3·1) and QF-1 (3·85) compared closely with several authentic 4α-methyl sterols. With the Liebermann–Burchard reagent it gave a rapid blue–green coloration with no preceding transient pink flush. It had no UV absorption in the 260–300 nm region. The MS had a molecular ion at *m/e* 412 and fragment ions at *m/e* values of 397 (M^+-Me), 379 (M^+-[Me + H_2O]), 328 (M^+-84), 313 (M^+-[Me + 84]), 285 (M^+-[SC + 2H]), 269 (M^+-[SC + H_2O]), 245 (M^+-[SC + 42]) and 227 (M^+-[SC + 42 + H_2O]).

In order to obtain evidence for the ergosterol-precursor role of the 24-methylenesterols isolated from these fungi, a culture of *P. blakesleeanus* was incubated with L-methionine-[methyl-^{14}C]. Radioactivity was incorporated into the 4,4-dimethyl- and 4-demethylsterol fractions. Chromatography of the 4,4-dimethylsterol fraction on a column of $AgNO_3$-impregnated alumina (Fig. 1a) showed that radioactivity co-chromatographed with 24-methylene-24,25-dihydrolanosterol (peak B) and also with the material tentatively identified as 4,4-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol (peak C). Lanosterol (peak A), however, was completely devoid of radioactivity showing that label from L-methionine-[methyl-^{14}C] does not become randomized and thus indicating that the radioacti-

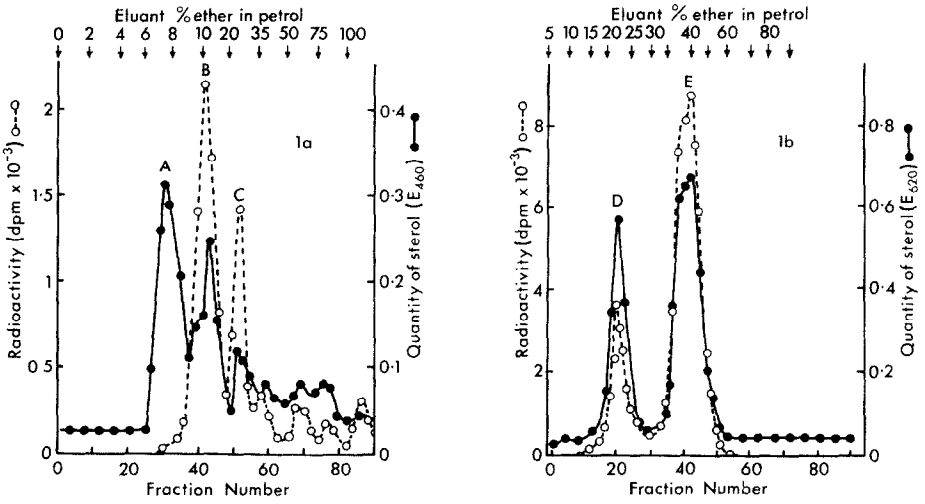


Fig. 1. Chromatography of sterols isolated from *Phycomyces blakesleeanus* following incubation with L-methionine-[methyl-^{14}C].

The 4,4-dimethylsterols (1a) and 4-demethylsterols (1b) were chromatographed on columns of 15% (w/w) $AgNO_3$-alumina (5 g) developed with increasing percentages of Et_2O in petrol as indicated. Alternate 5 ml eluent fractions were assayed for sterol content (●——●) by the Liebermann Burchard reaction and for radioactivity (O - - - O).

vity in the sterols of peaks B and C was localized in a 24-methylene group which has arisen directly by transmethylation. The radioactivity in the 4-demethylsterol fraction was found (Fig. 1b) to be distributed between materials which co-chromatographed with episterol (peak D) and ergosterol (peak E).

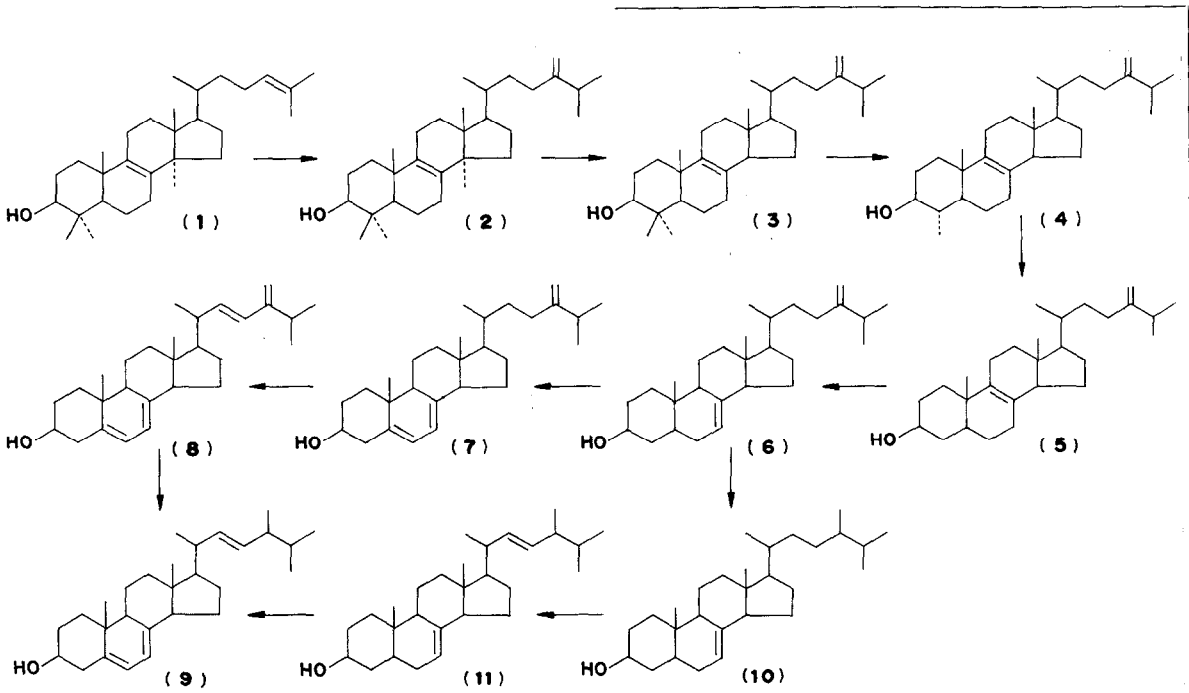
DISCUSSION

The isolation of 24-methylene-24,25-dihydrolanosterol from *P. blakesleeanus* and *A. campestris* shows that in these fungi, whose main sterol is ergosterol, alkylation at C-24 of lanosterol can occur. The demonstration that label from L-methionine-[methyl-^{14}C] is rapidly incorporated into 24-methylene-24,25-dihydrolanosterol in *P. blakesleeanus* under conditions where ergosterol also becomes labelled suggests that C-28 of ergosterol is introduced by transmethylation at the lanosterol level. Subsequent reduction of the $\Delta^{24(28)}$ double bond and introduction of a *trans*-Δ^{22} double bond would give the ergosterol side chain [28]. The presence of 4,4-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol, 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol and episterol in these fungi along with ergosta-5,7,24(28)-trien-3β-ol [29] and small amounts of 5α-ergosta-7-en-3β-ol [30] and 5α-ergosta-7,22-dien-3β-ol [30] in *P. blakesleeanus* indicates that these changes in the side chain normally occur after most of the changes attendant upon the formation of the ergosterol nucleus have taken place. Scheme 1 indicates the most likely biosynthetic routes from lanosterol to ergosterol in these fungi; fecosterol and ergosta-5,7,22,24(28)-tetraen-3β-ol, sterols known to be present in *S. cerevisiae* [13], are postulated as intermediates even though they have not yet been demonstrated in *P. blakesleeanus* or *A. campestris*. The first part of this biosynthetic pathway contrasts with that which operates in *S. cerevisiae* where failure to detect 24-methylene-24,25-dihydrolanosterol or any 24-alkylated 4,4-dimethyl sterols [13] coupled with the presence of 14-norlanosterol, 4α-methyl-5α-cholesta-8,24-dien-3β-ol and 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol [13] indicates that transmethylation at C-24 normally takes place at the 4-demethyl- or 4α-methylsterol level rather than at the 4,4-dimethylsterol level. This is supported by the fact that a soluble Δ^{24}-sterol methyltransferase from *S. cerevisiae* preferred 5α-cholesta-8,24-dien-3β-ol as its

substrate to its 4α-methyl- or 4,4-dimethyl derivatives [31]. However, the ability of *S. cerevisiae*, when presented with 24-methylene-24,25-dihydrolanosterol, to convert it efficiently into ergosterol [14] shows the lack of specificity of the enzymes in the first part of the pathway. The *S. cerevisiae* pathway is, on the other hand, similar to that in *P. blakesleeanus* and *A. campestris* in two respects: (a) saturation of the $\Delta^{24(28)}$ double bond and introduction of the trans-Δ^{22} double bond occur after most of the ergosterol nuclear changes have taken place and (b) there appear to be several routes from episterol to ergosterol (although it is clear that one of these is preferred [32]).

Extraction procedures. A. campestris (1·5 kg), purchased locally, were homogenized and extracted twice with 1·25 l. Et_2O–EtOH (1:1). The bulked extracts were filtered, diluted with H_2O and extracted with Et_2O–petrol to yield 7·67 g lipid which, after saponification, gave 0·95 g unsaponifiable lipid. *P. blakesleeanus* Burgeff, (−) strain, was cultured in 10 l. batches as described previously [15]. The mycelium was harvested after 5–6 days (349 g wet wt/10 l.) and saponified directly to yield 0·882 g unsaponifiable lipid.

Chromatography of the unsaponifiable lipids from both fungi on alumina (Brockmann Grade 3) gave fractions containing 4,4-dimethyl- and 4α-methylsterols (36 mg, *A. campestris*; 42·8 mg, *P. blakesleeanus*) and 4-demethylsterols (594 mg, *A. campestris*; 559 mg, *P. blakesleeanus*). Acetylation of the former fractions followed by preparative TLC on 10% (w/w) $AgNO_3$-silica gel G developed with 40% C_6H_6 in hexane gave in both cases three sterol-containing zones, designated A, B and C. Zones A and B co-chromatographed with lanosteryl- and 24-methylene cycloartanyl acetates respectively; zone C, the least abundant, ran just behind zone B. The sterols were extracted from the three zones and characterized in the manner described in the results section. Preparative TLC of the 4-demethylsterol fractions from both fungi on 10% (w/w) $AgNO_3$-silica gel G developed with $CHCl_3$ yielded a major zone co-chromatographing with ergosterol (R_f 0·05), a zone designated D (R_f 0·13) and a minor zone (R_f 0·21). Zone D in *P. blakesleeanus* was quite large, representing about 40–50% of the total fraction; however in *A. campestris*



Scheme 1. Possible biosynthetic routes from lanosterol to ergosterol in *A. campestris* and *P. blakesleeanus*. **1** = lanosterol; **2** = 24-methylene-24,25-dihydrolanosterol; **3** = 4,4-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol; **4** = 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol; **5** = fecosterol; **6** = episterol; **7** = ergosta-5,7,24(28)-trien-3β-ol; **8** = ergosta-5,7,22,24(28)-tetraen-3β-ol; **9** = ergosterol; **10** = ergosta-7-en-3β-ol; **11** = ergosta-7,22-dien-3β-ol.

EXPERIMENTAL

General methods. TLC and column chromatographic methods were generally as described previously [33]. GLC was carried out on 1·83 m × 3·2 mm i.d. columns packed with (a) 1% QF-1, (b) 1% or 0·7% CHDMS (cyclohexanedimethanolsuccinate; HiEFF8B) and (c) 1% SE-30, all on 80–100 mesh silanized Chromosorb W using operating parameters described previously [34]. NMR spectra were determined in $CDCl_3$. MS were determined on an AE1 MS9 instrument.

it constituted only a small part of the total fraction. The characterization of the sterols in zone D and the major zone is described in the results section. The identities of the sterols in the minor zone (R_f 0·21) were not completely established, largely because of the tiny amounts isolated. However GLC retention and MS data were consistent with the two main components being 5α-ergosta-7-en-3β-ol and 5α-ergosta-7,22-dien-3β-ol.

Incorporation of L-*methionine-[methyl-*^{14}*C] into* P. *blakesleeanus sterols.* After 54 hr growth two 100 ml cultures of *P. blakesleeanus* were inoculated with a total of 15 μCi L-methionine-[methyl-^{14}C] (56·8 mCi/mmol). After a further 17 hr growth the mycelia were harvested and the unsaponifiable lipid (20·1 mg; 3·23 × 10^6 dpm) extracted. Chromatography on alumina (Brockmann Grade 3) gave the 4,4-dimethylsterol (6·96 × 10^4 dpm) and 4-demethylsterol (2·28 × 10^6 dpm) fractions which were further purified by TLC on silica gel G developed with $CHCl_3$. The 4,4-dimethylsterols were then mixed with 1·6 mg of non-radioactive 4,4-dimethylsterols previously isolated from *P. blakesleeanus* and chromatographed on a column of 15% (w/w) $AgNO_3$ alumina (5g) [34] developed with petrol containing increasing percentages of Et_2O (see Fig. 1a). 5 ml fractions being collected. Aliquots of alternate fractions were assayed for radioactivity and for sterol by the Liebermann–Burchard reaction (E_{430} after 33 min at 25°) [35]. The apparent small mass peak for compound C (Fig. 1a) may possibly be attributed to the lower sensitivity of this compound to the Liebermann–Burchard reagent under the conditions used, which were designed to give a maximal response for compounds A and B. The GLC properties of peaks A, B and C were identical with those of lanosterol, the compound identified as 24-methylene-24,25-dihydrolanosterol and that identified as 4,4-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol respectively. A portion of the 4-demethylsterol fraction (4·7 mg) was mixed with 28 mg of unlabelled 4-demethylsterols previously isolated from *P. blakesleeanus* and chromatographed on 15% (w/w) $AgNO_3$ alumina as described above. Fractions of 8 ml were collected and aliquots assayed for radioactivity and for sterol by the Liebermann–Burchard reaction (E_{620} after 90 sec at 25°). The GLC properties of peaks D and E (Fig. 1b) were identical to those of episterol and ergosterol.

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REFERENCES

1. Alexander, G. J. and Schwenk, E. (1958) *J. Biol. Chem.* **232,** 611.
2. Schwenk, E. and Alexander, G. J. (1958) *Arch. Biochem. Biophys.* **76,** 65.
3. Kodicek, E. (1959) *Biosynthesis of Terpenes and Sterols* (Wolstenholme, G. E. W. and O'Connor, M., eds.), Ciba Foundation Symposium, pp. 173–183, Churchill, London.
4. Clayton, R. B. (1965) *Q. Rev. Chem. Soc.* **19,** 201.
5. Frantz, I. D. and Schroepfer, G. J. (1967) *Ann. Rev. Biochem.* **36,** 656.
6. Parks, L. W. (1958) *J. Am. Chem. Soc.* **80,** 2023.
7. Jauréguiberry, G., Law, J. H., McCloskey, J. A. and Lederer, E. (1965) *Biochemistry* **4,** 347.
8. Akhtar, M., Hunt, P. F. and Parvez, M. A. (1967) *Biochem. J.* **103,** 616.
9. Russell, P. T., Van Aller, R. T. and Nes, W. R. (1967) *J. Biol. Chem.* **242,** 5802.
10. Epstein, W. W. and Van Lear, G. (1966) *J. Org. Chem.* **31,** 3434.
11. Anderson, C. G. and Epstein, W. W. (1971) *Phytochemistry* **10,** 2713.
12. Fieser, L. F. and Fieser, M. (1959) *Steroids* pp. 379–385. Reinhold, New York.
13. Barton, D. H. R., Kempe, U. M. and Widdowson, D. A. (1972) *J. Chem. Soc.* 513.
14. Akhtar, M., Parvez, M. A. and Hunt, P. F. (1969) *Biochem. J.* **113,** 727.
15. Goulston, G., Goad, L. J. and Goodwin, T. W. (1967) *Biochem. J.* **102,** 15C.
16. Goad, L. J. and Goodwin, T. W. (1967) *Proc. 9th Japanese Conference on the Biochemistry of Lipids* p. 37.
17. Clayton, R. B., Nelson, A. N. and Frantz, I. D. (1963) *J. Lipid. Res.* **4,** 166.
18. Goad, L. J., Williams, B. L. and Goodwin, T. W. (1967) *European J. Biochem.* **3,** 232.
19. Budzikiewicz, H., Djerassi, C. and Williams, D. H. (1964) *Structure Elucidation of Natural Products by Mass Spectrometry*, Vol. 2, Holden-Day, San Francisco.
20. Aplin, R. T. and Hornby (1966) *J. Chem. Soc.* (B), 1078.
21. Knights, B. A. (1967) *J. Gas Chromatog.* **5,** 273.
22. Beneveniste, P., Hirth, L. and Ourisson, G. (1966) *Phytochemistry* **5,** 31.
23. Barton, D. H. R., Harrison, D. M., Moss, G. P. and Widdowson, D. A. (1970) *J. Chem. Soc.* (C), 775.
24. Fieser, L. F. and Fieser, M. (1959) *Steroids* p. 93. Reinhold, New York.
25. Johnson, J. C., Grostic, M. F. and Jensen, A. O. (1957) *Anal. Chem.* **29,** 468.
26. Scallen, T. J. and Krueger, W. (1968) *J. Lipid Res.* **9,** 120.
27. Barton, D. H. R., Harrison, D. M. and Widdowson, D. A. (1968) *Chem. Commun.*, 17.
28. Akhtar, M., Hunt, P. F. and Parvez, M. A. (1968) *Biochem. J.* **106,** 623.
29. Goulston, G. and Mercer, E. I. (1969) *Phytochemistry* **8,** 1945.
30. Mercer, E. I. and Bartlett, K. (1974) *Phytochemistry* **13,** 1099.
31. Moore, J. T., Jr., and Gaylor, J. L. (1970) *J. Biol. Chem.* **245,** 4684.
32. Fryberg, M., Oehlschlager, A. C. and Unrau, A. M. (1972) *Biochem. Biophys. Res. Commun.* **48,** 593.
33. Goad, L. J. and Goodwin, T. W. (1966) *Biochem. J.* **99,** 735.
34. Goad, L. J. and Goodwin, T. W. (1967) *European J. Biochem.* **1,** 357.
35. Williams, B. L., Goad, L. J. and Goodwin, T. W. (1967) *Phytochemistry* **6,** 1137.