Sterol Biosynthesis in Fungi

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The extra methyl group at C-24 of the ergosterol side chain is derived by transmethylation from *S*-adenosylmethionine (Alexander & Schwenk, 1958; Parks, 1958) and possible mechanisms have been suggested (Castle, Blondin & Nes, 1963; Lederer, 1964; Clayton, 1965*a,b*; Goad, Hamman, Dennis & Goodwin, 1966; Akhtar, Hunt & Parvez, 1966*a*). The observation that lanosterol but not zymosterol is converted into ergosterol by yeast preparations (Schwenk & Alexander, 1958) indicates that transmethylation occurs at an early stage in the biosynthetic pathway. Some wood-rotting fungi contain acidic triterpenes (e.g. eburicoic acid and the polyporenic acids; see Fieser & Fieser, 1959) that have a methylene group at C-24, showing that transmethylation occurs, at least in these organisms, at the lanosterol level. The possibility that transmethylation also occurs immediately after squalene cyclization in ergosterol biosynthesis is supported by recent reports of the conversion of synthetic labelled 24-methylenelanosterol into ergosterol by yeast (Akhtar, Parvez & Hunt, 1966*b*; Barton, Harrison & Moss, 1966).

The present communication reports the isolation from two moulds of 4,4-dimethylsterols and 4 α -methyl sterols that contain a methylene group at C-24.

Methods. *Aspergillus fumigatus* extracts were kindly provided by Dr F. W. Hemming and Mr K. J. Stone, Department of Biochemistry, University of Liverpool. *Phycomyces blakesleeanus* was cultured in 10l. batches in a New Brunswick Microform Laboratory Fermentor. The medium contained (per l.): 25g. of glucose, 1.5g. of KH₂PO₄, 1.25g. of L-asparagine, 1.25g. of L-leucine, 500mg. of MgSO₄·7H₂O, 500mg. of yeast extract and 0.25mg. of thiamine hydrochloride. The mycelium was harvested after 5 days' culture, washed and extracted by homogenization in ethanol followed by addition of KOH solution to give a final concentration of 15% (w/v) KOH in 85% (v/v) ethanol. After refluxing for 90min. the non-saponifiable lipid was extracted and separated into the hydrocarbon, 4,4-dimethyl sterol, 4 α -methyl sterol and 4-demethyl sterol fractions by chromatography on alumina (Brockmann grade III) (Goad & Goodwin, 1966). The sterols were

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purified where necessary by preparative thin-layer chromatography on silica gel (Avigan, Goodman & Steinberg, 1963). The 4,4-dimethyl sterols were separated on a column of alumina impregnated with 15% (w/v) AgNO₃ with increasing concentrations of diethyl ether in light petroleum (b.p. 40–60°) as eluent (Fig. 1). A Varian Aerograph 1522 instrument with flame ionization detectors was used for gas-liquid-chromatographic analysis. On column injection was employed with columns (6ft. × $\frac{1}{8}$ in.) of 1% QF-1 or 1% SE-30 on 80–100-mesh silane-treated Chromosorb W at 226° and a nitrogen flow of 40ml./min. Retention times are given relative to cholestane. Radioactivity was determined by liquid-scintillation counting.

Results. Evidence for 4,4-dimethyl sterols and 4 α -methyl sterols with a methylene group at C-24 was first obtained with a crude sterol sample from *A. fumigatus*. Preparative thin-layer chromatography yielded small amounts of 4,4-dimethyl sterols and 4 α -methyl sterols. Gas-liquid chromatography of the 4 α -methyl sterols showed two

Fig. 1. Chromatography on 15%-AgNO₃-impregnated alumina of the 4,4-dimethyl sterols isolated from *P. blakesleeanus* after incubation with [*Me*-¹⁴C]methionine. ●, Concn. of sterol; ×, radioactivity.

components (relative retention times: on SE-30, 3.52 and 3.76; on QF-1, 4.70 and 5.20), of which one component (40%) corresponded to 24-methylenelophenol (relative retention times: on SE-30, 3.76; on QF-1, 5.20) (Osske & Schreiber, 1965). The i.r. spectrum, with peaks at 887 and 1640 cm⁻¹, indicated the presence of a methylene group, and the mass spectrum showed a parent ion at *m/e* 412 and fragments at *m/e* 397, 379, 328, 285 and 227, which are in accord with 24-methylenelophenol fragmentation (Benveniste, Hirth & Ourisson, 1966). The second 4 α -methyl sterol is probably an isomer ($\Delta^8?$) of 24-methylenelophenol. Gas-liquid chromatography of the 4,4-dimethyl sterols separated four compounds [relative retention times: on SE-30, 3.87 and 4.64; on QF-1, 4.80, 5.70, 5.90 and 6.30 (very minor)]; one component (approx. 8%) corresponded to lanosterol (relative retention times: on SE-30, 3.80; on QF-1, 4.80). Peaks at 887 and 1640 cm⁻¹ in the i.r. spectrum indicated the presence of a methylene group in one or more of the components. The mass spectrum of the 4,4-dimethyl sterol fraction had a major peak at *m/e* 426 and a minor peak at *m/e* 440. Thin-layer chromatography of the 4,4-dimethyl sterol acetates on 10% AgNO₃-impregnated silica gel revealed three components, the most mobile corresponding to lanosterol acetate.

Separation of the non-saponifiable lipid from *P. blakesleeanus* on alumina provided a 4,4-dimethyl sterol fraction, which according to gas-liquid chromatography contained three components. Chromatography of this material (47.8 mg.) on 5g. of alumina impregnated with 15% AgNO₃ (Fig. 1) produced three compounds, A, B and C respectively. Compound A (16 mg.) had relative retention times identical with those of lanosterol on SE-30 (3.80) and on QF-1 (4.80), and the i.r. spectrum also agreed with that of lanosterol. Crystallization from methanol-CHCl₃ gave crystals, m.p. 130°. A clear yellow colour ($\lambda_{\max}$ 460 m μ) was produced with the Liebermann-Burchard reagent that reached a maximum intensity after 33 min. This is characteristic of lanosterol (Clayton, Nelson & Frantz, 1963). The mass spectrum of compound A acetate gave a mass peak at *m/e* 468 (lanosterol acetate has mol.wt. 468). Compound B (13 mg.) after crystallization had m.p. 150–151°, and the i.r. spectrum showed peaks at 887 and 1640 cm⁻¹, indicating a methylene group. On gas-liquid chromatography it had longer relative retention times than lanosterol (on SE-30, 4.35; on QF-1, 5.70), but gave a colour response identical with that of lanosterol with the Liebermann-Burchard reagent. These observations suggested that compound B was 24-methylenelanosterol, and this was confirmed by the mass spectrum of compound B acetate, which gave

a mass peak at *m/e* 482 (24-methylenelanosterol acetate has mol.wt. 482). Compound C (8 mg.) had a relative retention time of 5.4 on QF-1 and m.p. 152–154°. The i.r. spectrum showed peaks at 887 and 1640 cm⁻¹. With the Liebermann-Burchard reagent this sterol gave a different response to that of lanosterol or compound B. A weak blue colour was first produced that rapidly faded (1–2 min.) to be replaced by a yellow colour, maximal after about 15 min. The mass spectrum of compound C acetate showed a mass peak at *m/e* 468. These findings are consistent with the structure 4,4-dimethylergosta-8,24(28)-dien-3 β -ol (14-demethyl-24-methylenelanosterol) or a closely related sterol.

Gas-liquid chromatography of the 4 α -methyl sterol fraction from *P. blakesleeanus* revealed one major compound (relative retention time on QF-1: 4.70) that gave a fast blue colour with the Liebermann-Burchard reagent (Δ^7 - or Δ^8 -sterol), and it was apparently identical with one of the 4 α -methyl sterols isolated from *A. fumigatus*.

The incorporation of [*Me*-¹⁴C]methionine into the sterols of *P. blakesleeanus* has also been demonstrated. *P. blakesleeanus* was cultured in 250 ml. conical flasks containing 100 ml. of medium for 15 hr. before introduction of [*Me*-¹⁴C]methionine into two flasks (5 μ C and 10 μ C per flask respectively). The incubation was then continued for a further 17 hr. with continual shaking. The non-saponifiable lipid (3 230 000 disintegrations/min.) was extracted and separated on alumina to give the hydrocarbon (640 disintegrations/min.), 4,4-dimethyl sterol (69 600 disintegrations/min.), 4 α -methyl sterol (260 000 disintegrations/min.) and 4-demethyl sterol (2 278 000 disintegrations/min.) fractions. The 4,4-dimethyl sterols were purified by thin-layer chromatography to give a sterol fraction containing 34 800 disintegrations/min. Unlabelled 4,4-dimethyl sterols (approx. 2 mg.) obtained from another *P. blakesleeanus* incubation were added and the mixture was chromatographed on 5g. of AgNO₃-impregnated alumina. Fractions (5 ml.) were collected and assayed for radioactivity and for sterol by the Liebermann-Burchard method by measuring *E*₄₆₀ after 33 min. The three compounds A, B and C were separated (Fig. 1). No radioactivity was associated with compound A (lanosterol), demonstrating that the [*Me*-¹⁴C]methionine had not been incorporated into sterols after randomization of the label. Compounds B and C, however, were radioactive, indicating that they contain an alkyl group that has arisen by transmethylation from methionine.

P. blakesleeanus and *A. fumigatus* both contain ergosterol as one of their main sterols. Therefore the present identification of lanosterol, 24-methylenelanosterol and possibly 14-demethyl-24-methy-

lenelanosterol, and also the evidence for the presence of 4 α -methyl sterols containing a methylene group at C-24, is of considerable significance. The occurrence of such compounds in fungi can be postulated on biosynthetic grounds (Akhtar *et al.* 1966b; Barton *et al.* 1966; Goad, 1966), and we believe that trans-methylation does occur at the lanosterol level to produce 24-methylenelanosterol, which is subsequently modified by loss of the 4 α -, 4 β - and 14 α -methyl groups and double-bond rearrangements to give ergosterol.

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The Iodination of Super-reactive and Essential Tyrosine Residues in a Microbial Aminopeptidase

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Uhlig, Lehmann, Salmon, Jollès & Jollès (1965) isolated an aminopeptidase from micro-organisms that has mol.wt. 60 000. The present communication reports on an investigation of the role of tyrosine residues in the catalytic properties of this enzyme by the active-site labelling technique. It has been previously discovered that the aminopeptidase isolated from pig-kidney particles was inactivated when some of its tyrosine residues were iodinated (Wachsmuth, 1966b, 1967), and it was considered that the microbial aminopeptidase might behave similarly. This investigation seemed more worthwhile since the tyrosine residues of chymotrypsin do not appear to be important for the

catalysis (Filmer & Koshland, 1964) whereas those of carboxypeptidase A do appear to be involved (Roholt, Radzinski & Pressman, 1965).

It was shown that the enzymic activity of microbial aminopeptidase was decreased by incubation with iodine. At first therefore it was necessary to demonstrate whether the enzyme contains free SH groups, which could be oxidized by the iodine. For this purpose 3mg. of aminopeptidase was dissolved in 2ml. of 10M-urea in 0.6M-phosphate buffer, pH 7.2, and 0.05ml. of 15mM-5,5'-dithiobis-(p-nitrobenzoic acid) was added. The difference in E_{412} (Ellman, 1959) between this solution and control, containing no enzyme, was measured. The increase in extinction (0.004) corresponded to only 0.01 SH group/protein unit of mol.wt. 60 000. Thus

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