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ERGOSTA-5,7,24(28)-TRIEN-3β-OL, A NEW INTERMEDIATE IN ERGOSTEROL BIOSYNTHESIS IN *PHYCOMYCES BLAKESLEEANUS*

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Abstract—Ergosta-5,7,24(28)-trien-3β-ol (I) has been isolated from *Phycomyces blakesleeanus*. A cell-free preparation of *P. blakesleeanus* catalyses the conversion of episterol (ergosta-7,24(28)-dien-3β-ol) into I and ergosterol.

INTRODUCTION

ERGOSTEROL and episterol (ergosta-7,24(28)-dien-3β-ol) have been identified as the two major 4-demethyl-3β-hydroxysterols of the fungus, *Phycomyces blakesleeanus* Burgeff.[1] This paper reports the nature of the next most abundant component, a sterol more polar than ergosterol, and its biosynthesis from episterol.

RESULTS AND DISCUSSION

Chromatography of the 4-demethyl-3β-hydroxysterol mixture extracted from *Phycomyces blakesleeanus* mycelium on thin layers of silver nitrate-impregnated silica gel G using 5 % (v/v) acetone in chloroform for development resolved an unknown sterol (R_f 0·05) from ergosterol (R_f 0·13) and episterol (R_f O-34). This sterol, when subjected to gas-liquid chromatography (GLC), had a retention time relative to cholestane (RRT) on 3 % OV-1 of 2·73 (RRT ergosterol 2·40; RRT episterol 2·90) and on 1 % QF-1 of 4·0 (RRT ergosterol 3·48; RRT episterol 4·16). Its Liebermann-Burchard colour response was typical of a $\Delta^{5,7}$-sterol, an immediate transient pink flush changing rapidly through purple and blue to a blue-green whose intensity is maximal about 90 sec after the addition of the reagent. Its u.v. spectrum had absorption maxima and minima at wavelengths identical with those of ergosterol confirming the presence of a $\Delta^{5,7}$ double-bond system; the absorption maxima, however, had slightly greater intensities than those of ergosterol (Table 1). The i.r. spectrum

TABLE 1. MOLAR EXTINCTION COEFFICIENTS FOR ERGOSTEROL AND ERGOSTA-5,7,24(28)-TRIEN-3β-OL

	Molar extinction coefficients at absorption maxima			
	262·6 nm	271.3 nm	281.9 run	293.5 nm
Ergosterol	7,800	11,130	11,820	6,780
Ergosta-5,7,24(28)-trien-3β-ol	9,750	13,720	14,120	8,500

[1] G. GOULSTON, E. I. MERCER, L. J. GOAD and T. W. GOODWIN, in press.

had peaks at 887 cm^{-1} (strong) and 1650 cm^{-1} which are indicative of a terminal methylene group,[2] and at 800 cm^{-1} and 843 cm^{-1} which are characteristic of nuclear tri-substituted double bonds in $\Delta^{5,7}$-sterols.[3]

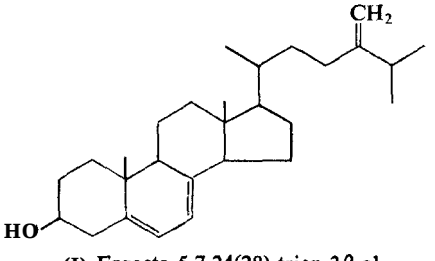
A mass spectrum of the sterol gave a molecular ion at *m/e* 396 with fragment ions as shown in Table 2. The molecular ion suggests a C_{28} sterol with three double bonds. The ion at ***m/e*** 337 is produced by the splitting of ring A and appears to be characteristic of $\Delta^{5,7}$-sterols,[4] whilst that at ***m/e*** 211 suggests a side-chain of molecular weight 125, therefore having one centre of unsaturation.

TABLE 2. MASS SPECTRUM FRAGMENTATION PATTERN OF ERGOSTA-5,7,24(28)-TRIEN-3β-OL

Fragmentation ion	*m/e*	Relative intensity (%)
Molecular ion, M^+	**396**	100
M^+-CH_3	**381**	**6**
M^+-H_2O	**378**	**6**
M^+-[CH_3+H_2O]	**363**	**72**
M+-[C-1 to C-31	**337**	**28**
M+-[side chain+H_2O]	**253**	**13**
M+-[side chain+C-15 to C-171	**229**	**5**
M^+-[side **chain+** *C-15* **to C-17+** H_2O]	**211**	**17**

The NMR spectrum of the sterol showed that the chemical shifts of C-18 and C-19 methyl protons at δ0·65 and 0·96 respectively were similar to the values calculated for a $\Delta^{5,7}$-sterol (δ0·64 and 0·95).[5] The ring olefinic protons, which integrated for two, were deshielded to δ5·5–5·65, suggesting conjugation. A two-proton signal at δ4·7 was assigned to protons of a methylene group, whilst C-26 and C-27 methyl protons resonated at δ0·99 and 1·08 (J = 7 c/s), deshielded by 0·1 ppm compared with the signals for the corresponding methyl groups of ergosterol.

On the basis of these observations the sterol has been identified as ergosta-5,7,24(28)-trien-3β-ol (I).



(I) Ergosta-5,7,24(28)-trien-3β-ol

A cell-free extract of ***P.*** *blakesleeanus* catalysed the conversion of episterol into ergosta-5,7,24(28)-trien-3β-ol and ergosterol. This was demonstrated initially with unlabelled

[2] L. C. CROSS and E. R. H. JONES, *J. Chem. Soc.* 1491 (1940).
[3] T. J. SCALLEN and W. KRUEGER, *J. Lipid Res. 9, 120 (1968).*
[4] F. R. SMITH and E. D. KORN, *J. Lipid Res. 9,405 (1968).*
[5] R. F. ZÜRCHER, *Helv. Chim. Acta* **46**, 2054 *(1963).*

episterol which was incubated with the cell-free extract for various periods of time. The 4-demethyl sterols were isolated from each incubation mixture and separated by argentation TLC. The quantity of I and ergosterol produced during each incubation period was based on the increase in extinction at 282 nm found in the TLC zones over that found in the equivalent zones of the control incubation. After 11- and 25-min incubation the E_{282} (corrected)[6]/3 ml cyclohexane in the ergosta-5,7,24(28)-trien-3β-ol zone increased from zero to 0·13 and 0·44 respectively, representing the formation of 0·03 and 0·1 μmole of this sterol. The extent of conversion of episterol into ergosterol could not be accurately determined in this experiment due to the high level of ergosterol already present in the cell-free extract. However, experiments with ^{14}C-labelled episterol confirmed that both ergosta-5,7,24(28)-trien-3β-ol (I) and ergosterol were formed. [^{14}C]Episterol, labelled in the 24-methylene group, was formed biosynthetically by incubating spores of *P. blakesleeanus* for 4 days with 5 μc of L-[methyl-^{14}C]methionine; its purity was confirmed by GLC using a 3% OV-1 column. [^{14}C]Episterol (46,400 disintegrations/min; 50 μg), dispersed in Tween 80, in two 2-hr incubations, gave conversions of 1·5 per cent to ergosterol and 1·4 per cent to ergosta-5,7,24-(28)-trien-3β-ol; a component cochromatographing with 5-dihydroergosterol (R_f 0·43) contained 0·3 per cent of the total radioactivity whilst the remainder was associated with unchanged episterol.

The apparent precursor relationship of episterol to ergosta-5,7,24(28)-trien-3β-ol and the concomitant formation of ergosterol suggests that in *P. blakesleeanus* ergosterol is formed by a route involving these two sterols.

EXPERIMENTAL

Extraction of 4-Demethyl-3β-hydroxysterols

Phycomyces blakesleeanus was cultured in 10 1. batches in a New Brunswick Microferm Laboratory Fermentor.[7] The mycelium was harvested after 5 days, washed and extracted by homogenization in ethanol; KOH solution was then added to give a final concentration of 15 % (w/v) KOH in 85 % (v/v) ethanol. The mixture was then refluxed under N_2 for 90 min and the unsaponifiable lipid extracted and chromatographed on Brockmann Grade III, acid-washed alumina (Woelm, anionotropic). The 4-demethyl sterol fraction was collected and purified via the digitonides.

Isolation of Ergosta-5,7,24(28)-trien-3β-ol (Z)

The 4-demethyl-3β-hydroxysterol mixture was further purified by preparative TLC on silica gel G using $CHCl_3$ (R_f 0.2). It was separated into four zones (R_fs 0·06, 0·13, 0·34 and 0.43) by TLC on silver nitrate-impregnated silica gel G (10% w/w) using 5% (v/v) acetone in $CHCl_3$. The zones chromatographing at R_fs 0·13 and 0·34 proved to be ergosterol and episterol, respectively, whilst that at R_f 0·43 co-chromatographed with 5-dihydroergosterol but separated into several components when subjected to GLC on a 3 % OV-1 column. The zone chromatographing at R_f 006 was purified by rechromatography on the same TLC system but with triple development. This yielded 11.1 mg of sterol of 95.5 per cent purity. This sterol was identified as ergosta-5,7,24(28)-trien-3β-ol by a combination of u.v., i.r., NMR and mass spectrometry.

Spectrometry

I.r. spectra were taken using KBr discs. Mass spectra were taken on an AEI MS9 spectrometer with a source temperature of 200° and a 70 eV beam. NMR spectra were taken on a Varian A60 spectrometer; samples were dissolved in $CDCl_3$ with tetramethylsilane as an internal reference.

Gas-Liquid Chromatography

Sterol samples were analysed with a Packard Gas Chromatograph Series 7500 with hydrogen flame detectors. Glass columns (6 ft long x 2 mm i.d.) packed with either 3 % OV-1 or 1 % QF-1 supported on 80–100

[6] W. H. C. Shaw and J. P. Jefferies, *Analyst* 78,509 (1953).
[7] G. Goulston, L. J. Goad and T. W. Goodwin, *Biochem. J.* 102, 15c (1967).

mesh silane-treated Gas Chrom Z and Gas Chrom Q respectively were used. The chromatograms were developed at 225" with an argon flow rate of 40 ml/min. Cholestane was chromatographed with each sample and retention times were determined relative to cholestane. Samples (approx. 1 μg) were injected on to the column dissolved in 1 μl cyclohexane.

Preparation of P. blakesleeanus ***Cell-free Extract***

Cells were harvested in the logarithmic phase of growth (36 hr). They were then washed with deionized water, freeze-dried and ground in a chilled mortar with their own weight of 0·15 mm dia. glass beads and twice their own weight of 0·1 M phosphate buffer, pH 7, until the mixture became a smooth paste. This was then centrifuged at 10,000 x *g* for 20 min in a Sorvall RC-2B centrifuge using the GSA rotor. The supernatant was used as the *Phycomyces* cell-free enzyme system.

Incubation Procedure

Episterol (^{14}C-labelled or unlabelled) was dispersed in 1 ml of a 1% (v/v) solution of Tween 80 in acetone; 1 ml of 0·1 M phosphate buffer, pH 7, was added and the acetone removed by evaporation under a stream of nitrogen. The cell-free enzyme system (2 ml; 50 mg protein/ml) and 1 ml of cofactor solution (containing 30 μmoles glutathione, 16 μmoles ATP, 2·4 μmoles NADPH, 1.5 μmoles $MnCl_2$ and 15 μmoles $MgCl_2$) were added to the aqueous suspension of episterol giving a final volume of 4 ml. Incubations were carried out in 25-ml conical flasks plugged with cotton wool, at 24°, in the dark and with shaking; they were stopped by addition of ethanol and KOH. The mixture was then refluxed under N_2 and the 4-demethyl-3β-hydroxysterols isolated and separated in the usual way.

Radioassay

The sterols were assayed for radioactivity with a Packard Tricarb Scintillation Spectrometer Series 314E. Suitable aliquots were introduced into the counting phials and dissolved in 10 ml scintillator fluid (0·3 g dimethyl POPOP (1,4-*bis*-(4-methyl-5-phenyloxazol-2-yl)-benzene) + 5 g PPO (2,5-diphenyloxazole) per l. toluene). The instrument was set to count ^{14}C with an efficiency of 50 per cent and a background of 25 counts/min.

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