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SHORT COMMUNICATION

CYCLIZATION OF SQUALENE-2,3-OXIDE TO LANOSTEROL IN A CELL-FREE SYSTEM FROM *PHYCOMYCES BLAKESLEEANUS*

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Abstract—[^{14}C]squalene was formed biosynthetically from [2-^{14}C]mevalonic acid by a rat-liver enzyme preparation and converted via its 2,3-monobromohydrin into [^{14}C]squalene-2,3-oxide. A cell-free enzyme preparation from *Phycomyces blakesleeanus* is described which catalysed the cyclization of the [^{14}C]squalene-2,3-oxide to [^{14}C]lanosterol.

INTRODUCTION

THE CONVERSION of squalene into lanosterol in animal tissues proceeds in two stages. In the first, squalene is oxidized to squalene-2,3-oxide in the presence of O_2 and NADPH; in the second, squalene-2,3-oxide is cyclized to lanosterol.[1–4] The cyclization of squalene-2,3-oxide to the pentacyclic triterpene, β-amyrin, has been demonstrated with a cell-free system from peas.[5] Similarly a cell-free system from French bean leaves was shown to catalyse the cyclization of squalene-2,3-oxide to the tetracyclic triterpene cycloartenol.[6] This was particularly significant since there is mounting evidence that cycloartenol rather than lanosterol is the first cyclized intermediate in the biosynthesis of sterols in higher plants[7–9] and algae.[10] The fungi, unlike the higher plants and algae, appear to utilize lanosterol as the first cyclic precursor of sterols.[11] It is of interest, therefore, to see whether fungi can utilize squalene-2,3-oxide for lanosterol synthesis. Whole cells and a cell-free preparation of *Saccharomyces cerevisiae*, an Ascomycete, have been shown to be capable of converting squalene-2,3-oxide into lanosterol.[12] This paper reports the ability of a cell-free preparation of *Phycomyces blakesleeanus*, a Phycomycete, to convert squalene-2,3-oxide into lanosterol and confirms the ability of fungi to utilize squalene-2,3-oxide for this purpose.

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RESULTS AND DISCUSSION

^{14}C-Squalene was prepared by incubating a cell-free enzyme system from rat liver with [2-^{14}C]mevalonic acid under anaerobic conditions. The [^{14}C]squalene was then converted into racemic [^{14}C]squalene-2,3-oxide. Two batches of [^{14}C]squalene-2,3-oxide were prepared with specific activities of $1{\cdot}134 \times 10^5$ and $1{\cdot}148 \times 10^6$ dis./min/mg. They were used in two separate experiments. In the first, 520 μg (= 59,000 dis./min) of squalene-2,3-oxide were incubated in each of two flasks under anaerobic conditions with a cell-free system (10 mg protein/ml) prepared from *Phycomyces* mycelium. The lanosterol isolated from the bulked incubation mixture contained 3055 dis./min, representing a 5·18 per cent conversion of one enantiomer of squalene-2,3-oxide into lanosterol. In the second experiment 140 μg (= 160,700 dis./min) of squalene-2,3-oxide were incubated in each of ten flasks under anaerobic conditions, with the *Phycomyces* cell-free system (6·5 mg protein/ml) prepared from a different batch of mycelium. The lanosterol isolated from the bulked incubation mixture contained 18,250 dis/min, representing a 2·27 per cent conversion of one enantiomer of the squalene-2,3-oxide into lanosterol.

These results show that squalene-2,3-oxide is converted in the fungus *P. blakesleeanus* into lanosterol presumably by a proton-initiated cyclization reaction catalysed by a specific cyclase.

EXPERIMENTAL

Preparation of [^{14}C]*Squalene*

[^{14}C]Squalene was prepared by anaerobic incubation of [2-^{14}C]mevalonic acid with a rat-liver cell-free enzyme system prepared by the procedure of Bucher and McGarrahan.[13] The [^{14}C]squalene isolated from the incubation mixtures was purified by chromatography on thin layers of silica gel G impregnated with Rhodamine 6G using light petroleum (b.p. 40–60°) for development; markers of authentic squalene were run on each thin-layer plate.

Preparation of [^{14}C]*Squalene-2,3-oxide*

The [^{14}C]squalene was converted into racemic [^{14}C]squalene-2,3-oxide by the method of Corey *et al.*[1] It was purified by TLC on Rhodamine 6G impregnated silica gel G using 5% (v/v) ethyl acetate in hexane for development;[6] markers of authentic squalene-2,3-oxide were run on each plate. The R_f of squalene-2,3-oxide in this chromatographic system is about 0·42.

Preparation of the Phycomyces Cell-free System

Batches (10 l.) of *Phycomyces* mycelium were grown in a New Brunswick Microferm Laboratory Fermentor by inoculating the medium with spores from a parent culture grown on Sabouraud dextrose agar medium. The mycelium was harvested in the logarithmic phase of growth (36 hr). It was washed thoroughly with 0·1 M phosphate buffer, pH 7, and squeezed dry. The yield from each batch was 50–60 g wet weight. The mycelium (50–60 g) was then placed in a mortar precooled to −20° and just enough liquid N_2 to cover it added. The frozen mycelium was then ground with a pestle into a fine powder. The powder was then allowed to thaw at +2°; this took about 5 hr and left a spongy mass. The material was then transferred to a 250 ml centrifuge tube and 10 ml 0·1 M phosphate buffer, pH 7, at +2° added. The mixture was allowed to stand at +2° for 30 min and then centrifuged at $16{,}300 \times g$ for 20 min. The supernatant, 15–20 ml of a slightly turbid, pale yellow liquid, was decanted off and used as the cell-free enzyme preparation.

Estimation of Protein

The protein content of the *Phycomyces* cell-free preparations was estimated by the Biuret method.[14]

Incubation of the Phycomyces Cell-free System with [^{14}C]*Squalene-2,3-oxide*

An appropriate quantity of [^{14}C]squalene-2,3-oxide in cyclohexane was transferred to each of a number of Warburg flasks and the solvent removed by evaporation under N_2 at 25°. It was then dissolved in 1 ml acetone and 5 mg Tween 80 added in 1 ml acetone followed by 0·5 ml 0·1 M phosphate buffer, pH 7; the acetone was

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then removed by evaporation under N_2 at 25°.[6] Finally 2 ml of the *Phycomyces* cell-free preparation were added to each flask. The flasks were then flushed with oxygen-free N_2 for 15 min, stoppered and incubated in darkness at 25° for 3 hr. At the end of the incubation period the contents of the flasks were bulked, saponified and the unsaponifiable lipid extracted.

Isolation and Purification of the Lanosterol

The unsaponifiable lipid was chromatographed on Rhodamine 6G impregnated silica gel G thin-layer plates using 20% (v/v) ethyl acetate in hexane for development;[6] markers of authentic squalene-2,3-oxide (R_f 0·73) and lanosterol (R_f 0·40) were run on each plate. The zone chromatographing at the same R_f as the marker lanosterol was scraped off, eluted with peroxide-free diethyl ether and assayed for radioactivity. It was then diluted with 50 μg pure, non-radioactive lanosterol crystallized to constant specific activity and subjected to GLC analysis using a Packard Gas Chromatograph Series 7500 fitted with a glass column (6 ft × 2 mm i.d.) packed with 3% OV-1 supported on 80–100 mesh silane-treated Gas Chrom Z. The chromatograms were developed at 225° with an argon flow rate of 40 ml/min. The column effluent was led through a stream splitter; $\frac{1}{40}$th passed through a hydrogen flame detector whilst $\frac{39}{40}$th passed through a precooled U-tube. The latter allowed sterols to be collected with an efficiency in excess of 95 per cent. A preliminary chromatogram without the stream splitter showed that the lanosterol fraction contained no major component apart from lanosterol and no peaks near to the lanosterol peak. This enabled the whole of the lanosterol to be collected in one U-tube when the lanosterol fraction was chromatographed with the stream splitter incorporated in the system. The lanosterol was washed out of the U-tube with diethyl ether and assayed for radioactivity.

Radioassay

All radioactive materials were assayed with a Packard Tricarb Scintillation Spectrometer Series 314E. They were dissolved in 10 ml scintillator fluid (0·3 g dimethyl POPOP (1,4-*bis*-(4-methyl-1-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 68·5 per cent and a background of 25 counts/min.

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