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STEROL BIOSYNTHESIS VIA CYCLOARTENOL IN *SAPROLEGNIA*

JOHN D. BU'LOCK and ANTHONY U. OSAGIE*
Weizmann Microbial Chemistry Laboratory, Department of Chemistry, University of Manchester, Manchester M13 9PL, England

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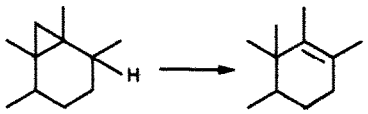
Key Word Index—*Saprolegnia*; Oomycetes; sterol biosynthesis; cycloartenol; 24-methylenecholesterol.

Abstract—The Oomycete *Saprolegnia ferax* incorporates ^3H from both cycloartenol-[2-^3H] and lanosterol-[2-^3H] into its normal sterols cholesterol, fucosterol, desmosterol, and 24-methylenecholesterol. It is concluded that sterol biosynthesis in this organism is *via* cycloartenol and the taxonomic implications are discussed.

INTRODUCTION

It is accepted that in sterol biosynthesis the cyclization of squalene 2,3-oxide affords lanosterol in animals and cycloartenol in plants. Biosynthesis of typical sterols by the latter pathway therefore requires at some stage the participation of an additional enzyme, cyclosterol isomerase, which converts a 9β,19-cyclosteroid to the isomeric 10-methyl-Δ^8-steroid:



As a general principle (see e.g. Gibbons *et al.* [1]), while systems lacking this isomerase can only produce their typical sterol from lanosterol, systems containing the isomerase (e.g. a maize preparation) can incorporate both lanosterol and cycloartenol. Since the occurrence of these alternate pathways potentially defines a major taxonomic boundary, its more precise delineation is desirable.

Various studies (mainly by the Liverpool school) based on direct identifications of the cyclization product of squalene 2,3-oxide have roughly located this boundary within the Protista. The formation of cycloartenol has been demonstrated in the Phaeophyte alga *Fucus* [2] and the Chrysophyte *Ochromonas* [3], direct cyclization to lanosterol is well known in yeast and has also been established in the Zygomycete *Phycomyces* [4]. However, there are no data for the other divisions of the lower (aseptate) fungi, the so-called Phycomycetes. Within this group, which in any case is no longer considered as a valid taxon [5], the Zygomycotina are obviously allied to all the higher fungi in containing sterols of the ergosterol series. In contrast the Mastigomycotina (filamentous organisms with motile zoospores) either contain no sterols (in the parasitic Peronosporales) or contain a range of sterols strikingly similar to those of the red and brown algae, as was first shown by McCorkindale *et al* [6]. This situation [7] makes it particularly desirable to know whether such organisms, though normally classed as fungi, utilize the 'algal' pathway of sterol synthesis via cycloartenol. We have therefore investigated the *in vivo* conversions of lanosterol and of cycloartenol into typical sterols of the Oomycete *Saprolegnia ferax*.

RESULTS

The published identification of the major sterols of *S. ferax* as cholesterol, desmosterol, 24-methylenecholesterol and fucosterol was fully confirmed by GLC, and GC–MS of both the free sterols and of their derived acetates; the 4,4-dimethylsterol fraction separated during the work-up was unfortunately too small for useful analysis. The four major sterols were usefully separated by $AgNO_3$ chromatography, either as the acetates on silica gel TLC or as free sterols on an alumina column.

Incorporation of precursors

Labelled 4,4-dimethylsterol precursors (obtained via ^3H exchange into the corresponding 3-keto-derivatives, see Experimental) were fed to *S. ferax* cultures in 6 separate pairs of experiments. Between 4 and 25 g fr. wt of mycelium was worked up in each case (either from labelled plus unlabelled cultures or from labelled cultures alone) and on each occasion the 4-desmethylsterol fraction (*ca* 0.5–1 mg/g mycelium) was rechromatographed at least once, and usually twice, with excess of unlabelled precursor. In one experiment the 4-desmethylsterol fraction was further purified by chromatography as the benzoates.

In the 6 experiments, the percentage of labelling incorporated into 4-desmethylsterols ranged from 0.2 to 2.4% when cycloartenol-[2-^3H] was the precursor, and from 1.8 to 4.6% when lanosterol-[2-^3H] was fed; corresponding dilutions of the original sp act were 120–480 for cycloartenol and 26–150 for lanosterol. Thus lanosterol was always a "better" precursor but both triterpenes were quite significantly incorporated in every experiment.

Individual steroids

When $AgNO_3$–silica gel TLC plates on which the four major sterol acetates were well-resolved (see Experimental) were scanned with a radiochromatogram counter, labelling in all four major sterols was confirmed in each

*Present address: Department of Biochemistry, University of Benin, Nigeria.

case, although the patterns of labelling from the two precursors were different. In a typical experiment with cycloartenol-[2-^{3}H] cholesterol, fucosterol, desmosterol, and 24-methylenecholesterol were labelled in ratios of *ca* 8:7:10:8 where as in a parallel experiment with lanosterol-[2-^{3}H] the corresponding ratios were 3:3:2:10. In addition, however, there was appreciable activity in parts of the chromatograms where no major sterols were detectable.

To remove uncertainties arising from possible conversions into non-natural sterols, the 24-methylenecholesterol component from one pair of experiments was subjected to column chromatography on $AgNO_3$–alumina. The relevant peak was eluted in at least 12 successive fractions and alternate fractions were assayed for sterol (see Experimental) or counted for ^{3}H. Both the sample labelled from cycloartenol-[2-^{3}H] and that labelled from lanosterol-[2-^{3}H] showed sp acts constant to within ± 7% (2 × standard deviation) throughout the relevant series of samples. The dilution ratios for the sterols thus purified were 120 and 100 respectively.

DISCUSSION

The results show unambiguously that *S. ferax* is able to convert cycloartenol into its normal complement of sterols and that it contains a cyclosteroid isomerase. As we could not identify cycloartenol in the very small 4,4-dimethyl steroid fraction, the observations fall short of proving that this is the true biosynthetic intermediate. However, a cyclosteroid isomerase will only be functional in an organism which normally obtains its sterols *via* cycloartenol and in such organisms the ability to use lanosterol as a precursor, with equal or even greater efficiency, is an expected feature [1]. There is no reason to suppose that *Saprolegnia* differs significantly from the other Oomycetes which produce similar sterols [6,7] and so in respect of their mode of squalene oxide cyclization we conclude that these organisms should be grouped with the algae rather than with the Zygomycotina and the higher (septate) fungi.

Such a separation, effectively of the organisms with a motile phase, Mastigomycotina, from the true fungi generally, would be very acceptable on other grounds, and indeed these organisms seem to be retained by mycologists more from tradition and convenience than from conviction [5,8]. Quite apart from considerations of morphology, cytology, and life-cycle, the chemotaxonomic grounds for removing the Oomycetes away from the true fungi and towards the algae are strong because their cell-walls contain cellulose [9], they have a different pattern of enzymes for tryptophan synthesis [10], they synthesize lysine by the 'plant' (diaminopimelic) pathway [11,12] and their fatty acid dehydrogenases have distinctive specificity [13].

EXPERIMENTAL

Culture methods. Stock cultures of *Saprolegnia ferax* were maintained on hemp seeds and plated out on tellurite agar (glucose, 10; starch, 5; yeast extract, 2; Na_2HPO_4, 0.6; KH_2PO_4, 2; K_2TeO_3, 0.1; agar, 30 g/l.). Discs cut from the culture perimeter were inoculated into 1 l. conical flasks each containing 200 ml of glucose–peptone medium (glucose, 25; peptone, 0.5 g/l.), incubated for up to 7 days at 27° in the dark with shaking. For precursor-incorporation expts an inoculum was grown up in the same manner in 150 ml of medium in a 500 ml flask; after 60 hr the entire culture was briefly homogenized and dispensed to 5–10 flasks containing 200 ml of medium. The labelled precursor was added at this time and the incubation continued for 48 or 60 hr.

Labelled precursors. Cycloartenol was isolated from potato haulms, and a generous sample was donated by Professor D. H. R. Barton; lanosterol was purchased. After re-chromatography both contained <2% of impurities (mostly 24-methylene homologues) by GLC. Each (300 mg) was oxidized to the corresponding ketone using CrO_3–pyridine [14], which after purification by TLC (Si gel) was passed through an Al_2O_3 column preloaded with 0.5 Ci of ^3H_2O following the procedures of ref. [15]. This gave cycloartenone (-[2-^{3}H] 190 mg, mp 110°, sp act 2.52 × 10^{10} dpm/g) and lanostadienone (-[2-^{3}H] 210 mg, mp 82°, sp act 2.63 × 10^{10} dpm/g). The ketones were reduced with excess $LiAlH_4$ in Et_2O and the products purified by TLC and recrystallisation (MeOH) to give the required cycloartenol (-[2-^{3}H] mp 108°; 2.32 × 10^{10} dpm/g) and lanosterol-[2-^{3}H] (mp 140°; 2.38 × 10^{10} dpm/g). For feeding, the precursor (3–5 mg) was dissolved in 2 ml Me_2CO; Tween 80 (2 drops) and H_2O (3 ml) were added and the Me_2CO was blown off in N_2; the aq emulsion was then dispensed into 2–4 culture flasks.

Extraction of sterols. The mycelium was pressed dry and (usually) mixed with 4 or 5 times the amount of fresh mycelium from an unlabelled incubation. For extraction of 20 g fr. wt, the mycelium was homogenized in MeOH (60 ml) containing 2% pyrogallol; aq KOH (60%, 30 ml) was added and the mixture was boiled for 45 min, filtered hot, and the filtrate diluted with H_2O (100 ml). The residue on the filter, and the diluted filtrate, were extracted with Et_2O (×4), and the combined extracts washed, dried and evaporated under N_2. The extracted 'non-saponifiable lipid' was then chromatographed on Woelm neutral Al_2O_3 (40 g/g extract; Brockmann grade 3) in petrol (bp 40–60°), first with 5% Et_2O and then with 25% Et_2O; the latter fractions afforded the 4-desmethylsterols. In the labelling expts this fraction (2–15 mg) was mixed with an equal wt of unlabelled cycloartenol or lanosterol and re-chromatographed once or twice before counting.

Chromatographic separations. The sterol acetates (prepared in Ac_2O–C_5H_5N, 18 hr) were separated by TLC, on Si gel F 254 plates previously impregnated with $AgNO_3$ [16] developing twice with C_6H_6–hexane mixtures; the ^{3}H distribution in the sterol acetates from the labelling expts could then be examined using a radiochromatogram scanner with integrator. Both free sterols and sterol acetates were also analysed by GLC [6], alone or in combination with MS using a variety of column packings (e.g. 1 or 1.5% Dexsil 300 on Gaschrom Q, 2 m × 3 mm, 240°, N_2 at 1.5 kg/sq cm. For detailed examination of the 24-methylenecholesterol component (see Results), the purified 4-desmethylsterol fraction (*ca* 25 mg) was applied in petrol to a column (5 g) of Al_2O_3 (Woelm, acid-washed) previously impregnated with $AgNO_3$ [17]. The column was eluted successively with 2,5, 10, 20, 30, 40, 60, and 80% Et_2O–petrol mixtures, collecting 5–6 ml fractions. From the 40% Et_2O eluate, alternate fractions were assayed for sterol (i.e. evaporated and taken up in 1 ml $CHCl_3$, 1 ml HOAc and 2 ml Ac_2O containing 5% H_2SO_4; A at 460 nm measured after 30 min in the dark, with cholesterol standards for calibration) and the remainder counted for ^{3}H content. Samples were counted in Bray's solution (dioxan) in a scintillation counter calibrated for ^{3}H and corrected for background.

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