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ERGOSTEROL BIOSYNTHESIS IN *MUCOR PUSILLUS*

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Key Word Index—*Mucor pusillus*; Mucoraceae; lanosterol; 24-methylene-24,25-dihydrolanosterol; obtusifoliol; 22-dihydroergosterol; ergosterol; methionine-[methyl-$^2H_3$]; mevalonic acid-[2-^{14}C, (4R)-4-$^3H_1$].

Abstract—Ergosterol, 22-dihydroergosterol, obtusifoliol and 24-methylene-24,25-dihydrolanosterol, isolated from *Mucor pusillus* grown in the presence of methionine-[methyl-$^2H_3$], each contained two deuterium atoms; lanosterol, however, was unlabelled. Ergosterol and 22-dihydroergosterol, isolated from *M. pusillus* grown in the presence of mevalonic acid-[2-^{14}C, (4R)-4-$^3H_1$] had ^{14}C:^3H atomic ratios of 5:3. The significance of these results in terms of sterol biosynthesis in this organism in general and alkylation at C-24 in particular is discussed.

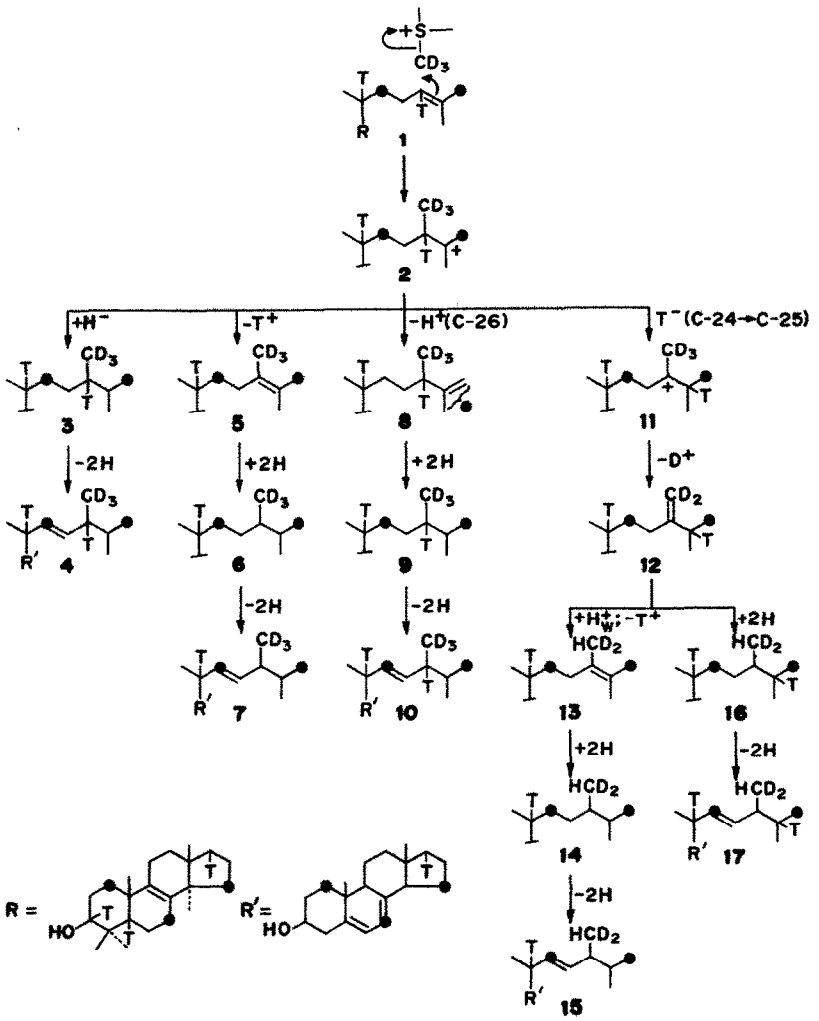
INTRODUCTION

Although it is well established[1] that ergosterol and 22-dihydroergosterol are the major sterols of at least two species of *Mucor*, little is known of the details of their biosynthesis. The present work was undertaken to elucidate the mechanism of alkylation at C-24 in *Mucor pusillus* and the stage in the sterol biosynthetic pathway at which it occurs. Following the rationale of Goad *et al.*[2], this has been accomplished by (i) utilizing methionine-[methyl-$^2H_3$] to determine how many of the 24-methyl hydrogens are derived from the methyl group of methionine, (ii) utilizing mevalonic acid-[2-^{14}C, (4R)-4-$^3H_1$] to determine the fate of the C-24 hydrogen of the precursor Δ^{24}-sterol and (iii) examining the minor sterols of the fungus for those which are alkylated at C-24. Scheme 1, based upon several previously published schemes [2–6], shows several alternative routes by which alkylation may take place and the labelling patterns which would result from each route if ergosterol were biosynthesized from methionine-[methyl-$^2H_3$] and mevalonic acid-[2-^{14}C, (4R)-4-$^3H_1$]; the latter are summarized in Table 1.

RESULTS

Identification of minor sterols

Mycelium of *M. pusillus* was saponified and the resulting unsaponifiable lipid chromatographed on alumina using increasing percentages of Et_2O in petrol (*E/P*) for development. TLC of the 20% *E/P* and Et_2O fractions revealed a zone co-chromatographing with authentic ergosterol. The sterols extracted from this zone exhibited the UV absorption characteristic of $\Delta^{5,7}$-sterols (peaks at 272, 282, 293 nm; shoulder at 262 nm) [7] and separated into two peaks A (RR_t 2·32; co-chromatographing with authentic ergosterol) and peak B (RR_t 2·71) which were present in the ratio 2·2:1. GC-MS showed that peaks A and B had molecular ions and fragmentation patterns characteristic of ergosterol (M^+ 396) and 22-dihydroergosterol (M^+ 398) respectively. Ergosterol and 22-dihydroergosterol together constituted about 95% of the total sterols of *M. pusillus*. TLC of the 6% *E/P* fraction revealed a zone running just behind authentic lanosterol. GC-MS of the material extracted from this zone gave two main peaks C and D in the ratio 1·5:1. The MS of peak C had a molecular ion at *m/e* 414 and fragment ions at *m/e* values of 399 ($M^+ - Me$), 396 ($M^+ - H_2O$), 381 ($M^+ - [Me + H_2O]$), 287 ($M^+ - SC$), 285 ($M^+ - [SC + 2]$), 245 ($M^+ - [SC + 42]$) and 227 ($M^+ - [SC + 42 + H_2O]$). This indicates a C_{29} sterol with one nuclear double bond and a saturated C_9H_{19} side chain. The absence of fragment ions at *m/e* values of 231 ($M^+ - [SC + 56]$) and 213 ($M^+ - [SC + 56 + H_2O]$) indicate that the extra nuclear methyl group is at C-4 rather than C-14. A small amount of sterol C was isolated by preparative GLC and, with the Liebermann–Burchard reagent, gave a rapid blue–green coloration with no preceding transient pink flush. This indicates that the nuclear double bond is either Δ^7 or Δ^8. Sterol C is thus tentatively identified as 4α-methyl-5α-ergosta-7 (or 8)-en-3β-ol. The MS of peak D had a molecular ion at *m/e* 426 and fragment ions at *m/e* values of 411 ($M^+ - Me$), 408 ($M^+ - H_2O$), 393 ($M^+ - [Me + H_2O]$), 327 ($M^+ - [84 + Me]$), 309 ($M^+ - [84 + Me + H_2O]$), 283 ($M^+ - [SC + H_2O]$), 259 ($M^+ - [SC + 42]$), 245 ($M^+ - [SC + 56]$), 241 ($M^+ - [SC + 42 + H_2O]$) and 227 ($M^+ - [SC + 56 + H_2]$). This fragmentation pattern matches that of obtusifoliol [8,9] indicating that this is the probable identity of sterol D. TLC of the 4% *E/P* fraction revealed a zone co-chromatographing with authentic lanosterol. GLC of the sterols extracted from this zone gave two peaks E (RR_t 3·10; co-chromatographing with authentic lanosterol) and F (RR_t 3·72) in the ratio 1:2·4. GC–MS showed that peaks E and F had molecular ions and fragmentation patterns characteristic of lanosterol (M^+ 426) and 24-methylene-24,25-dihydrolanosterol (M^+ 440) respectively [10].



Scheme 1. Possible mechanisms of alkylation at C-24 during ergosterol biosynthesis in *Mucor pusillus* from methionine-[methyl-$^2H_3$] and mevalonic acid-[2-^{14}C, (4R)-4-$^3H_1$]. D = deuterium; T = tritium; ● = ^{14}C; H_w^+ = proton from water; CD_3–$S^+<$ = S-adenosylmethionine-[methyl-$^2H_3$].

Incorporation of methionine-[methyl-$^2H_3$]

A 100 ml culture of *M. pusillus* was grown for 15 days on a medium containing 100 mg of methionine-[methyl-$^2H_3$]. Saponification of the mycelium (wet wt 3·89 g) yielded 17·9 ml of unsaponifiable lipid which was analysed as described in the previous section. The ionic compositions of the molecular ion clusters of peaks in the MS of the sterols are shown in Table 2. Each sterol also had $M^+ - Me$, $M^+ - H_2O$ and $M^+ - [Me + H_2O]$ fragment ion clusters of almost identical composition to that of its M^+ cluster and, with the exception of lanosterol, contained an ion two mass units greater than normal which was either the most abundant ion

Table 1. Labelling patterns in ergosterol biosynthesized from methionine-[methyl-$^2H_3$] and mevalonic acid-[2-^{14}C, (4R-4-$^3H_1$] assuming the operation of the alkylation routes shown in Scheme 1

Route No	Mechanism	^{14}C ^3H atomic ratio	No of ^2H atoms	^3H at C-24	^3H at C-25
1	1→2→3→4	5:3	3	Yes	No
2	1→2→5→6→7	5:2	3	No	No
3	1→2→8→9→10	5:3	3	Yes	No
4	1→2→11→12→13→14→15	5:2	2	No	No
5	1→2→11→12→16→17	5:3	2	No	Yes

Table 2. Ionic composition of the molecular ion cluster of peaks in the MS of sterols biosynthesized in the presence of methionine-[methyl-$^2H_3$]

Lanosterol	*m/e* Value	426	427	428	429	430
	Abundance*	100	34	5·5	0	0
24-Methylene-24,25 dihydrolanosterol	*m/e* Value	440	441	442	443	444
	Abundance*	28·3	37·6	100	32·4	4·6
4α-Methyl-5α-ergosta-7(or 8)-en-3β-ol	*m/e* Value	414	415	416	417	418
	Abundance*	100	39·7	66·2	19·9	0·6
Obtusifoliol	*m/e* Value	426	427	428	429	430
	Abundance*	100	36·9	57·1	18·7	1·5
22-dihydrolanosterol	*m/e* Value	398	399	400	401	402
	Abundance*	95·9	58·9	100	28·5	4·2
Ergosterol	*m/e* Value	396	397	398	399	400
	Abundance*	14·3	25·6	100	26·7	1·4

* The abundance of each ion is expressed as a percentage of the most abundant ion in the molecular ion cluster which is taken as 100.

Table 3. Radioassay of ergosterol and 22-dihydroergosterol isolated from *Mucor pusillus* grown in the presence of mevalonic acid-[2-^{14}C, (4R)-4-$^{3}H_1$]

	^{14}C dpm*	^{3}H dpm*	^{14}C:^{3}H dpm ratio	^{14}C:^{3}H atomic ratio†
Mevalonic acid‡	3928	38518	1:9·81	1·1
Ergosterol	784	4562	1:5·82	5:2·97 (5:3)
22-Dihydroergosterol	707	4179	1:5·91	5:3·01 (5:3)

* Each sample, along with ^{14}C, ^{3}H and Blank standards, was counted for a period sufficient to give a statistical accuracy of 95%, 20 times. The figures given are the mean values of these counts. † The experimentally observed ratio is given above the rounded off ratio which appears in parenthesis.

‡ Mevalonic acid-[3R,2-^{14}C + 3S, 2-^{14}C] + mevalonic acid-[3R,4R-$^{3}H_1$ + 3S,4S-$^{3}H_1$] mixture used in the *M. pusillus* culture medium.

or of very considerable abundance. This was, however, not the case in the clusters of fragment ions devoid of the side chain; here the most abundant ion was at the *m/e* value found for the equivalent ion in the MS of the unlabelled sterol.

Incorporation of mevalonic acid-[2-^{14}C, (4R)-4-$^{3}H_1$]

A 100 ml culture of *M. pusillus* was grown for 15 days on a medium containing mevalonic acid-[2-^{14}C, (4R)-4-$^{3}H_1$] (2·5 μCi ^{14}C; 25 μCi ^{3}H). Saponification of the mycelium (wet wt 4·39 g) yielded 24·1 mg of unsaponifiable lipid which was chromatographed on alumina as described earlier. The 20% *E/P* fraction (7·9 mg) was subjected to TLC and the 4-demethylsterol zone, co-chromatographing with authentic ergosterol, extracted. The sterol from this zone was then subjected to preparative GLC and the ergosterol and 22-dihydroergosterol peaks collected. Analytical GLC of aliquots of these two sterols showed that they were uncontaminated by any other sterol. The ergosterol and 22-dihydroergosterol were then radioassayed along with a sample of the doubly-labelled mevalonic acid, the results of which are shown in Table 3.

DISCUSSION

The major ion of the molecular ion cluster of ergosterol isolated from the methionine-[methyl-$^{2}H_3$]-grown *M. pusillus* had an *m/e* value of 398, two mass units greater than that of unlabelled ergosterol, and therefore indicating the presence of two ^{2}H atoms. The major ions of the M^+–Me, M^+–H_2O, M^+–[Me + H_2O] and M^+–59 clusters were also two mass units greater than the equivalent ions in the MS of unlabelled ergosterol. However the major ions of the fragment ion clusters which were devoid of the side chain were at *m/e* values characteristic of unlabelled ergosterol showing that the two ^{2}H atoms are in the side chain. Since it is known that the *S*-methyl carbon atom of methionine is incorporated exclusively into C-28 of ergosterol [10], there is little doubt that the two ^{2}H atoms are located on C-28 which therefore has the structure $-CH^2H_2$. This result is consistent with that found for ergosterol biosynthesis in *Neurospora crassa* [11], *Gliocladium roseum* [4], *Oospora virescens* [4], *Xanthoria parietina* [12] and *Phycomyces blakesleeanus* [6]. It is also clear from Table 2 that 22-dihydroergosterol, 4α-methyl-5α-ergosta-7 (or 8)-en-3β-ol, obtusifoliol and 24-methylene-24,25-dihydrolanosterol also have two ^{2}H atoms attached to C-28, the structure of which will be $-CH^2H_2$ in the case of the two former sterols and $=C^2H_2$ in the case of the latter two.

The mol % of each ionic species of ergosterol in the molecular ion cluster, calculated from relative peak heights and taking account of the natural abundance of ^{13}C is as follows: undeuterated, 11·1%; monodeuterated, 16·5%; dideuterated, 72·4% and trideuterated, 0%. This result is very similar to that obtained with ergosterol from *N. crassa* [11] and *P. blakesleeanus* [6]. The mol % of the ionic species of 24-methylene-24,25-dihydrolanosterol is almost the same as for ergosterol but those of 22-dihydroergosterol (non-D, 45·4%; mono-D, 13·6%; di-D, 41·0%; tri-D, 0%), 4α-methyl-5α-ergosta-7(or 8)-en-3β-ol (non-D, 60·3%; mono-D, 4·3%; di-D, 35·4%; tri-D, 0%) and obtusifoliol (non-D, 65·0%; mono-D, 2·1%; di-D, 32·9%; tri-D, 0%) are significantly different: The possible reasons for such differences and for the presence of significant quantities of the monodeuterated species have been discussed previously [4, 13].

The fact that only two of the three ^{2}H atoms of methionine-[methyl-$^{2}H_3$] are incorporated into ergosterol indicates that of the five possible alkylation mechanisms depicted in Fig. 1 only routes 4(1 → 2 → 11 → 12 → 13 → 14 → 15) and 5(1 – 2 → 11 → 12 → 16 → 17) (Table 1) are feasible. However it is clear that, since ergosterol biosynthesized from mevalonic acid-[2-^{14}C, (4R)-4-$^{3}H_1$] has been found to have a ^{14}C:^{3}H atomic ratio of 5:3 (Table 3), route 4 cannot be operative. Thus it is concluded that the 24-methyl group of ergosterol is introduced via route 5. This mechanism necessitates the transfer of hydrogen from C-24 to C-25; this has been proved to take place during ergosterol biosynthesis in *Saccharomyces cerevisiae* [14] and *P. blakesleeanus* [6].

The presence of 24-methylene-24,25-dihydrolanosterol in *M. pusillus* and the demonstration that label from methionine-[methyl-$^{2}H_3$] is incorporated into it under conditions where ergosterol also becomes labelled suggests that the 24-methyl group of ergosterol is introduced by transmethylation at the lanosterol level. In this respect *M. pusillus* is like *P. blakesleeanus* and *Agaricus campestris* [10] but differs from *S. cerevisiae* [15].

This work suggests that in outline the biosynthesis of ergosterol in *M. pusillus* follows the route: lanosterol → 24-methylene-24,25-dihydrolanosterol → obtusifoliol → 4α-methyl-5α-ergosta-7(or 8)-en-3β-ol → 22-dihydroergosterol → ergosterol. 24-Methylene-24,25-dihydrolanosterol has been shown to be capable of conversion into ergosterol in *S. cerevisiae* [16,17]. Obtusifoliol has been found to accumulate in *Monilinia fructigena* [8] and *Ustilago maydis* [9] when grown in the presence of the fungicides, S-1358 and Triarimol respectively and to be converted into ergosterol in cultures of *S. cerevisiae* [18].

EXPERIMENTAL

Organism and cultural conditions. Mucor pusillus was grown in static or shake culture at 37° in Yeast Dox medium adjusted to pH 6·5. Labelled substrates were dissolved in water and then sterilized by passage through a sterile, 0·2 μm membrane filter; 10 ml of sterilized solution were then added aseptically to 90 ml of Yeast Dox medium which had been sterilized by autoclave.

Isolation of sterols. The mycelium was harvested, washed and saponified in the usual way. The unsaponifiable material was chromatographed on acid-washed, Brockman Grade 3

alumina developed in a stepwise manner with successive, equal vols of petrol, 2, 4, 6, 9, 20% Et_2O in petrol, and Et_2O. Sterols were isolated from the 4, 6 and 20% Et_2O in petrol fractions by preparative TLC (Si gel G impregnated with Rhodamine 6G developed with $CHCl_3$).

GLC. Analytical and preparative GLC were carried out on 1·5 m × 4 mm i.d. glass columns packed with 1% SE-30 on 80-100 mesh Gas-Chrom Q operating isothermally at 230°. The carrier gas was O_2-free N_2 flowing at 40–50 ml/min. Detection was by FID. When preparative GLC was used the effluent gas was passed through a heated 10:1 splitter unit; the 9/10th fraction was passed through a pre-cooled U-tube. The material which condensed in the U-tube was washed out with Et_2O and an aliquot checked for purity by analytical GLC. Several preparative GLC runs were required to accumulate sufficient ergosterol and 22-dihydroergosterol for radioassay. GC–MS was performed as described previously [6].

Radioassay. Samples were dissolved in 10 ml NE-260 liquid scintillation fluid and repetitively counted, along with ^{14}C, ^3H and Blank standards for periods sufficient to give 95% statistical accuracy.

Mevalonic acid-[2-^{14}C, (4R)-4-^3H]. This consisted of mevalonic acid-[3R,2-^{14}C + 3S,2-^{14}C] (sp. ac., 17·5 mCi/mmol) and mevalonic acid-[3R,4R-$^3H_1$ + 3S,4S-$^3H_1$] (sp. act., 250 mCi/mmol) obtained from the Radiochemical Centre, Amersham, mixed in the nominal ratio of 1:10 (observed ratio 1:9·81, see Table 3). In the incubation with *M. pusillus* 5 μCi of the ^{14}C species and 50 μCi of the ^3H species were included in the 100 ml of medium. Since only the 3R isomers can be utilized for terpenoid biosynthesis [19], the effective quantities of the ^{14}C and ^3H species present were 2·5 μCi and 25 μCi respectively.

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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; 22-dihydroergosterol = (24S)-24-methylcholesta-5,7-dien-3β-ol; obtusifoliol = 4α,14α-dimethyl-5α-ergosta-8,24 (28)-dien-3β-ol; lanosterol = 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol. 24-methylene-24,25-dihydrolanosterol = 4,4,14α-trimethyl-5α-ergosta-8,24 (28)-dien-3β-ol. The following abbreviations are used in the textual description of the MS data: SC = side chain; 42 = [C-15 + C-16 + C-17 + 6H]; 56 = [C-15 to C-17 + C-32 + 8H]; 59 = [C-1 to C-3 + OH + 6H]; 84 = [C-23 to C-28].

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