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MECHANISM OF ALKYLATION AT C-24 DURING ERGOSTEROL BIOSYNTHESIS IN *PHYCOMYCES BLAKESLEEANUS*

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Key Word Index—*Phycomyces blakesleeanus*; Mucoraceae; biosynthesis; ergosterol; alkylation at C-24; methionine-[methyl-$^2H_3$]; mevalonic acid-[2-^{14}C, (4R)-4-$^3H_1$]; hydrogen transfer from C-24 to C-25.

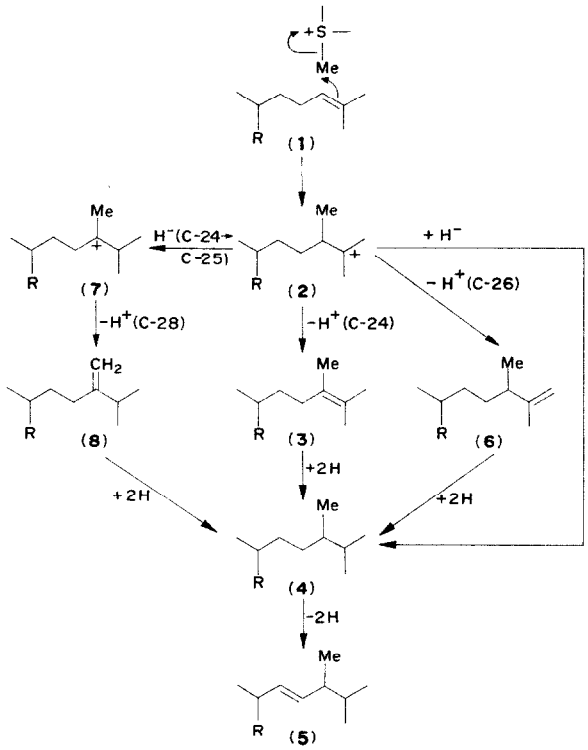
Abstract—Ergosterol isolated from *Phycomyces blakesleeanus* grown in the presence of methionine-[methyl-$^2H_3$] contained two ^2H atoms showing that one ^2H atom is lost during transmethylation. Ergosterol isolated from *P. blakesleeanus* grown in the presence of mevalonic acid-[2-^{14}C,(4R)-4-$^3H_1$] had a ^{14}C:^3H atomic ratio of 5:3. Chemical degradation of 2,3-dimethylbutanal obtained by ozonolysis of the doubly-labelled ergosterol showed that the ^3H atom originally at C-24 of lanosterol is transferred to C-25 of ergosterol during transmethylation. The mechanism of formation of the ergosterol side chain in *P. blakesleeanus* is presented.

INTRODUCTION*

The incorporation of the methyl carbon of methionine into the methyl group on C-24 of ergosterol was first shown, in yeast (*Saccharomyces cerevisiae*), by Alexander *et al.* [1]. The direct participation of *S*-adenosylmethionine in this alkylation process was first demonstrated by Parks [2].

Ergosterol biosynthesized by a methionine-less mutant of *Neurospora crassa* from methionine-[methyl-$^2H_3$] was found to contain only two ^2H atoms in the 24-methyl groups [3]. Moreover ergosterol isolated from *Gliocladium roseum* and *Ochromonas danica* grown in the presence of methionine-[methyl-$^2H_3$] was similarly labelled [4]. However Δ^5-ergostenol isolated from *Chlorella elliposidea* [5] and *Trebouxia* species 213/3 [6] and Δ^7-ergostenol from *Chlorella vulgaris* [7] grown in the presence of methionine-[methyl-$^2H_3$] contained three ^2H atoms in their 24-methyl groups. Clearly different organisms introduce the 24-methyl group by different mechanisms. Figure 1 illustrates the more likely of these (see also Akhtar *et al.* [8], Lederer [4], and Goad *et al.* [6]). Russell *et al.* [9] have shown that the sterol undergoing alkylation at C-24 must have a Δ^{24}-double bond (**1**, Fig. 1), which initiates a nucleophilic attack on the methyl carbon attached to the positively-charged sulphur atom of *S*-adenosylmethionine [8]. This results in the formation of a carbonium ion (**2**, Fig. 1) which may be transformed into the side chains characteristic of ergosterol (**5**, Scheme 1) and Δ^5- and Δ^7-ergostenol (**4**, Fig. 1) by several possible routes. The route involving intermediates **1**, **2**, **7**, and **8** (Scheme 1) is generally accepted as that by which the 24-methyl group of ergosterol is introduced in *N. crassa* and *S. cerevisiae* because (i) it involves elimination of a proton from the newly-introduced 24-methyl group, which would explain the retention of only two deuterium atoms from methionine-[methyl-$^2H_3$] [3] and (ii) it involves a 1,2-hydride shift (C-24 to C-25) which has been experimentally demonstrated [8]. This route, however, cannot be operating in the biosynthesis of Δ^5- and Δ^7-ergostenol since these sterols do not lose any ^2H atoms from methionine-[methyl-$^2H_3$] [5–7]. The other three routes shown in Scheme 1 do not however involve ^2H loss and are therefore possible. Goad *et al.* [6] favoured the route involv-

* *Abreviations used*: 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; Δ^5-ergostenol = (24S)-24-methylcholest-5-en-3β-ol; Δ^7-ergostenol = (24S)-24-methylcholest-7-en-3β-ol; lanosterol = 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol; norcyclolaudenol = 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-25-en-3β-ol; episterol = 5α-ergosta-7,24(28)-dien-3β-ol; 24-methylene-24,25-dihydrolanosterol = 4,4,14α-trimethyl-5α-ergosta-8,24(28)-dien-3β-ol; E/P = Et_2O in petrol.



Scheme 1. Possible routes for the biosynthesis of the ergosterol side chain. R = sterol nucleus.

ing intermediate **6** in Δ^5-ergostenol formation in *Trebouxia* because they were able to demonstrate the exclusive conversion of norcyclolaudenol, a Δ^{25}-24-methylsterol, into Δ^5-ergostenol in this organism. However they did not rule out the possible intermediacy of a Δ^{24}-24-methyl intermediate (**3**, Fig. 1). The route involving this intermediate (**3**, Scheme 1) can be distinguished from the route involving the Δ^{25}-24-methyl intermediate (**6**, Scheme 1) and that involving direct stabilization of intermediate **2** (Scheme 1) by H^- addition by the loss of the C-24 hydrogen atom of intermediate **1** (Scheme 1). Akhtar *et al.* [10] have shown that the introduction of the Δ^{22}-double bond of ergosterol in *S. cerevisiae* is mechanistically unrelated to alkylation at C-24 and can occur after alkylation; in fact the balance of evidence indicates that alkylation precedes desaturation at C's 22 and 23 [4].

Methionine-[methyl-^{14}C] has been shown to label the 24-methyl group of ergosterol and other sterols in *Phycomyces blakesleeanus* [11, 12] but the mechanism of alkylation has not, to date, been studied in detail. In this paper we have carried out such a study. We have been able to show that alkylation proceeds via route **1**→**2**→**7**→**8**→**4**→**5** (Scheme 1) by demonstrating (i) the loss of one ^{2}H atom during ergosterol biosynthesis from methionine-[methyl-$^2H_3$] and (ii) the transfer of hydrogen from C-24 to C-25 during ergosterol formation.

RESULTS

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

A 100 ml culture of *P. blakesleeanus* was grown from spores on a medium containing 100 mg of methionine-[methyl-$^2H_3$]. The mycelium (wet wt 3·57 g) was harvested after 5 days and saponified. The unsaponifiable material (30·85 mg) was chromatographed on alumina and the 20% *E/P** fraction (6·28 mg), containing most of the ergosterol, was further purified by TLC (system 1). The zone cochromatographing with authentic ergosterol was removed and eluted. GLC showed it to be composed of ergosterol (RRT, 2·35) with a smaller amount of episterol (RRT, 2·72) and trace of ergosta-5,7,24(28)-trien-3β-ol (RRT, 2·58) [13].

Table 1. Radioassay of squalene, ergosterol and the chemical degradation products of ergosterol biosynthesized from 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†	5184	51956	1:10·02	1:1
Squalene	562	5613	1: 9·98	6:5·98
Ergosterol	179	1144	1: 6·38	5:3·18
2,3-Dimethylbutanal‡ (before tautomerization)	266	2610	1: 9·81	1:0·98
2,3-Dimethylbutanal‡ (after tautomerization)	157	1479	1: 9·42	1:0·94
2,3-Dimethylbutanoic acid§	175	1614	1: 9·22	1:0·92
Acetic acid§	247	0	/	1:0

* Each sample, along with ^{14}C, ^{3}H and Blank standards, was counted, for a period sufficient to give a statistical accuracy of 95%, nine times. The figures given are the mean values of these counts.

† 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 *P. blakesleeanus* culture medium.

‡ Assayed as their dimedone derivatives.

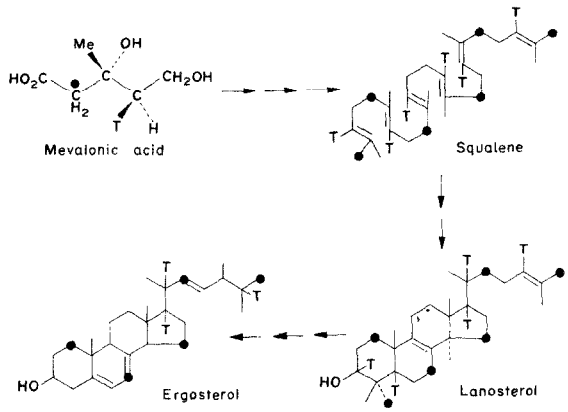
§ Assayed as their sodium salts.

This material was subjected to GC–MS. The molecular ion cluster of peaks in the MS of ergosterol was composed of ions at *m/e* values of 396, 397, 398, 399 and 400 with relative intensities of 25·57, 30·77, 100, 28·38 and 5·86 respectively. Similar clusters for the M-Me, M-H_2O and M-[CH_3 + H_2O] ions were present, having their most abundant ions at *m/e* values of 383, 380 and 363 respectively. The most abundant ion in the M-59 cluster which is characteristic of $\Delta^{5,7}$-sterols [3, 14] was at *m/e* 339. The most abundant ions in the M-[side chain (SC) + 2H] and M-[SC + 2H + H_2O] clusters were at *m/e* values of 269 and 253 respectively.

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

A 100 ml culture of *P. blakesleeanus* was grown from spores on a medium containing mevalonic acid-[2-^{14}C, (4R)-4-$^{3}H_1$] (2·5 μCi ^{14}C; 25 μCi ^{3}H). The mycelium (wet wt 4·11 g) was harvested after 5 days and saponified. The unsaponifiable material (75·11 mg, 252280 dpm ^{14}C) was chromatographed on alumina and the 20% *E/P* (5·25 mg, 181100 dpm ^{14}C) and ether (3·32 mg, 48240 dpm ^{14}C) fractions bulked and purified by TLC (system 1). The zone cochromatographing with ergosterol was removed and eluted. This material was then separated by argentation TLC [13] into an ergosterol zone (R_f 0·13), an episterol zone (R_f 0·34) and a minor zone of ergosta-5,7,24(28)-trien-3β-ol (R_f 0·05). After two further passes through the argentation-TLC system the resulting ergosterol was shown to be 95% pure by GLC. Aliquots of this ergosterol sample were then subjected to preparative GLC and the peak corresponding to ergosterol collected. Analytical GLC of an aliquot of this ergosterol showed it to be uncontaminated by any other sterol. The remainder of this ergosterol was then radioassayed along with a sample of the doubly-labelled mevalonate used in the culture medium and a sample of squalene isolated from the petrol fraction of the alumina chromatography of the unsaponifiable material and purified by TLC (system 2) and preparative GLC. The results are shown in Table 1.

The major part of the argentation-TLC-purified ergosterol (1·97 mg, 52900 dpm ^{14}C) was diluted with 503·9 mg of non-radioactive ergosterol and subjected to ozonolysis. About a quarter of the resulting 2,3-dimethylbutanal was oxidized to 2,3-dimethylbutanoic acid which was then radioassayed. A further quarter of the 2,3-dimethylbutanal was tautomerized by alkali and then trapped as the dimedone derivative which was radioassayed. The residual 2,3-dimethylbutanal was converted into the dimedone derivative immediately. About half of this was radioassayed whilst the other half was subjected to the Kuhn Roth [15] oxidation procedure. The resulting acetic acid was radioassayed as the sodium salt. The results of the radioassay of these ergosterol degradation products are shown in Table 1.



Scheme 2. Labelling pattern of ergosterol biosynthesized by *Phycomyces blakesleeanus* from mevalonic acid-[2-^{14}C, (4R)-4-^{3}H$_1$]. T = tritium; ● = ^{14}C.

DISCUSSION

The major ion of the molecular ion cluster of the ergosterol isolated from the methionine-[methyl-^{2}H$_3$]-grown *P. blakesleeanus* has 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-[CH_3 + H_2O] and M-59 clusters are also two mass units greater than the equivalent ions in the MS of unlabelled ergosterol. However the major ions of the M-[SC + 2H] and M-[SC + 2H + H_2O] were at *m/e* values characteristic of unlabelled ergosterol, showing that the two ^{2}H atoms are in the side chain. There is little doubt that they are located in the 24-methyl group which therefore has the structure $CH^{2}H_2$.

The mol per cent 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, 18·3%; monodeuterated, 16·2%; dideuterated, 65·5% and trideuterated, 0%. This result is very similar to that of Jauréguiberry *et al.* [3] which was: undeuterated, 16%; monodeuterated, 13%; dideuterated, 71% and trideuterated, 0%. The slightly lower incorporation of ^{2}H into ergosterol in *P. blakesleeanus* as compared with that in *N. crassa* [3] is probably due to the fact that the *N. crassa* was a methionine-less mutant which was therefore forced to utilize the methionine-[methyl-^{2}H$_3$] provided: this was not the case with the *P. blakesleeanus*. The relatively high proportion of monodeuterated ions, with a 24-methyl labelling pattern of $CH_2{}^{2}H_1$, appears to be characteristic of a biosynthetic pathway in which a methylene group is the precursor of the methyl group [4].

The result of this experiment indicates that the 24-methyl group of ergosterol arises via a 24-methylene group and that the ergosterol side chain is formed by route **1** → **2** → **7** → **8** → **4** → **5** (Scheme 1). Further support for this route has been obtained by providing evidence of the migration of hydrogen from C-24 to C-25 (**2** → **7**, Scheme 1). This has been accomplished by showing that the hydrogen on C-24 of the Δ^{24}-sterol intermediate (**1**, Scheme 1) ultimately resides on C-25 of ergosterol. Present evidence indicates that in *P. blakesleeanus* the Δ^{24}-sterol intermediate is lanosterol since 24-methylene-24,25-dihydrolanosterol is labelled by methionine-[methyl-^{14}C] [12] and methionine-[methyl-^{2}H$_3$] [16]. It is known that lanosterol biosynthesized from mevalonic acid-[2-^{14}C, (4R)-4-^{3}H$_1$] has a ^{14}C : ^{3}H atomic ratio of 6 : 5 with the labelling pattern shown in Scheme 2. This pattern shows a ^{3}H atom at C-24 and a ^{14}C atom at C-26. Ergosterol formed from lanosterol labelled in this way, via intermediates, **2**, **7**, **8**, and **4** (Scheme 1) should, therefore have a ^{3}H atom at C-25 and a ^{14}C at C-26 (Scheme 2). That this is so has been proved in the following way. 2,3-Dimethylbutanal isolated after ozonolysis of ergosterol, biosynthesized by *P. blakesleeanus* from mevalonic acid-[2-^{14}C, (4R)-4-^{3}H$_1$], had a ^{14}C : ^{3}H atomic ratio of 1 : 1 showing that one ^{3}H atom and ^{14}C atom were present in the C-23–C-28 fragment of ergosterol. Equilibration of the 2,3-dimethylbu-

tanal under basic conditions caused no loss of ^3H as would have been expected if the ^3H were located at C-24. The ability of the α-hydrogen of 2,3-dimethylbutanal to exchange freely with hydrogen ions in the medium under the conditions used has been demonstrated [17]. Thus the ^3H is not located at C-24. Oxidation of the 2,3-dimethylbutanal to 2,3-dimethylbutanoic acid also caused no change in the $^{14}C:^3H$ atomic ratio, showing that the ^3H is not located at C-23 of ergosterol. Kuhn–Roth oxidation of the dimedone derivative of 2,3-dimethylbutanal produced acetic acid with ^{14}C but no ^3H activity. The Kuhn–Roth procedure distinguishes between the methyl groups of 2,3-dimethylbutanal (26-,27- and 28-methyl groups of ergosterol) which remain unchanged, and the carbon atoms to which they are attached (C's 24 and 25 of ergosterol) which are oxidized to —COOH. Since no ^3H was present in the acetic acid the ^3H present in the C-23–C-28 fragment of ergosterol must, by elimination, have been located at C-25. Akhtar *et al.* [17] demonstrated a similar shift of hydrogen from C-24 to C-25 during ergosterol biosynthesis in *S. cerevisiae* using essentially the same degradative procedure but lanosterol-[24-$^3H_1$,26,27-$^{14}C_2$] as the substrate.

Squalene was isolated from the mevalonic acid-[2-^{14}C, (4R)-4-$^3H_1$]-grown *P. blakesleeanus* to act as an internal check on the $^{14}C:^3H$ atomic ratios of ergosterol and its degradation products since it was known to have a 6:6 ratio [18]. The $^{14}C:^3H$ atomic ratio of 5:3 in ergosterol was identical to that found in *Aspergillus fumigatus* [19]. Since we have proved that one ^3H atom occurs at C-25 and there is no reason to doubt that the other two are at C-17 and C-20, it follows that the ^3H atoms at C-5 and C-3 of lanosterol are lost during its conversion to ergosterol.

EXPERIMENTAL

Organism and cultural conditions. *Phycomyces blakesleeanus* Burgeff, (−) strain was cultured from spores under the condition described previously [20]. Labelled substrates were dissolved in the usual medium [11] and then sterilized by passage through a sterile, 0·2 μm membrane filter. They were then added aseptically to 100 ml of medium which had been sterilized by autoclave.

Isolation of ergosterol and squalene. The mycelium was harvested and saponified in the usual way. The unsaponifiable material was chromatographed on a 5 g column of acid-washed, Brockman Grade 3 alumina developed in a stepwise manner with successive 50 ml vol. of petrol, 2, 6, 9, 20% Et_2O in petrol, and Et_2O. Squalene was isolated from the petrol fraction by TLC (system 2) and purified by preparative GLC. Ergosterol was isolated from the 20% Et_2O in petrol and Et_2O fractions by TLC (system 1 followed by argentation TLC) and purified by preparative GLC.

TLC. System 1: silica gel G (0·25 mm thick) impregnated with Rhodamine 6G [21] developed with $CHCl_3$. System 2: silica gel G (0·25 mm thick) impregnated with Rhodamine 6G developed with petrol [22]. Argentation TLC: silica gel G (0·25 mm thick) impregnated with 10% (w/w) $AgNO_3$ developed with 5% Me_2CO in $CHCl_3$ [13].

GLC. Analytical and preparative GLC were carried out on 1·5 m × 4 mm i.d. coiled glass columns packed with 1% SE-30 on 80–100 mesh Gas-Chrom Q operating isothermally at 225–230°. 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 quantities of ergosterol and squalene for radioassay.

GC–MS. GC–MS was performed with a Pye 104 gas chromatograph linked, via a single-stage silicone rubber membrane separator, to an AEI MS-30 mass spectrometer. The GC was fitted with a 1·5 m × 4 mm i.d. glass column packed with 1% SE-30 on 80–100 mesh Gas-Chrom Q, operating isothermally at 225° and with a He flow rate of 40 ml/min. The MS were obtained with an electron energy of 24 eV, an emission current of 300 μA and a source temperature of 250°.

Mevalonic acid-[2-^{14}C, (4*R*)-4-$^3H_1$]. This consisted of mevalonic acid-[3R,2-^{14}C + 3S,2-^{14}C] and mevalonic acid-[3R,4R-$^3H_1$ + 3S, 4S-$^3H_1$], obtained from the Radiochemical Centre, Amersham, mixed in the ratio 1:10. In the uncubation with *P. blakesleeanus* 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 synthesis [23], the effective quantities of the ^{14}C and ^3H species present were 2·5 μCi and 25 μCi respectively.

Radioassay. Samples were dissolved in 10 ml NE-260 liquid scintillation fluid and repetitively counted in an NE8310 liquid scintillation counter, along with ^{14}C, ^3H and Blank standards, for periods sufficient to give 95% statistical accuracy.

Chemical degradation of the doubly-labelled ergosterol. Ozonolysis of ergosterol was carried out by the method of Hanahan and Wakil [24]. After neutralization with aq. NaOH, half the steam distillate containing the resulting 2,3-dimethylbutanal was treated with dimedone (200 mg) in EtOH (6 ml). The mixture was heated for 5 min and then left overnight at 0°. Crystals of the dimedone derivative of 2,3-dimethylbutanal (m.p. 158°) were filtered off and divided into two approximately, equal parts. One part was radioassayed and the other was subjected to Kuhn–Roth oxidation [15]. The resulting acetic acid was neutralized with NaOH and radioassayed.

The other half of the 2,3-dimethylbutanal-containing steam distillate was divided into two approximately equal parts. One part was made alkaline with NaOH (pH 12) and allowed to stand overnight at room temp. under N_2 [17]. It was then neutralized with AcOH and the dimedone derivative prepared as before and radioassayed. The other part was made slightly alkaline (pH 8·5) and treated with aq. $KMnO_4$ according to the method of Hanahan and Wakil [24]. The resulting 2,3-dimethylbutanoic acid was neutralized with NaOH and radioassayed.

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