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INCORPORATION OF METHIONINE-[METHYL-$^2H_3$] AND MEVALONIC ACID-[2-^{14}C,(4*R*)-4-$^3H_1$] INTO *PHYCOMYCES BLAKESLEEANUS* STEROLS

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Key Word Index—*Phycomyces blakesleeanus*; Mucoraceae; biosynthesis; lanosterol; 24-methylene-24,25-dihydrolanosterol; 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol; episterol; ergosta-5,7,24(28)-trien-3β-ol; ergosterol; methionine-[methyl-$^2H_3$]; mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$].

Abstract—Ergosterol, episterol, 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol and 24-methylene-24,25-dihydrolanosterol, isolated from *Phycomyces blakesleeanus* grown in the presence of methionine-[methyl-$^2H_3$], each contained two deuterium atoms; lanosterol, however, was unlabelled. The ^{14}C:^{3}H atomic ratio of the following sterols isolated from *P. blakesleeanus* grown in the presence of mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$], was: ergosterol, 5:3; episterol, 5:4; ergosta-5,7,24(28)-trien-3β-ol, 5:3; 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol, 5:4; 24-methylene-24,25-dihydrolanosterol, 6:5; lanosterol, 6:5. The significance of these results in terms of ergosterol biosynthesis is discussed.

INTRODUCTION

We have recently shown that in *P. blakesleeanus* [1], as in *Saccharomyces cerevisiae* [2, 3], the C-24 methyl group of ergosterol arises from the *S*-methyl group of methionine in a process which involves the loss of one of the methyl hydrogen atoms and the transfer of hydrogen from C-24 to C-25. This indicates that a 24-methylene sterol is a precursor of ergosterol in both organisms. The presence of 24-methylene-24,25-dihydrolanosterol in *P. blakesleeanus* [4, 5], in contrast to *S. cerevisiae* [6], suggests that alkylation at C-24 takes place at the lanosterol level [4]. Here we show that the labelling patterns of some sterols isolated from *P. blakesleeanus* grown in the presence of either methionine-[methyl-$^2H_3$] or mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$] are consistent with the route proposed [4] for the conversion of lanosterol into ergosterol in this fungus.

RESULTS

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

The ionic compositions of the molecular ion (M^+) clusters of peaks in the MS of the sterols of *P. blakesleeanus* grown in the presence of methionine-[methyl-$^2H_3$] are shown in Table 1. Each sterol also had a M^+–Me and a M^+–H_2O fragment ion cluster of almost identical composition to that of its M^+ cluster. The most abundant ion in the M^+, M^+–Me and M^+–H_2O clusters in the MS of all the sterols, except that of lanosterol, was two mass units greater than normal. 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 expected for the equivalent ion in the MS of the unlabelled sterol. Apart from the differences in the M^+, M^+–Me and M^+–H_2O ion clusters mentioned above the MS of all the sterols were identical with those obtained previously [4, 5], thus confirming their identity.

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

The results of the radioassay of the squalene and sterols isolated from *P. blakesleeanus* grown in the presence of mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$], along with that of a sample of the doubly-labelled mevalonic acid, are shown in Table 2. The dimedone derivative of 2,3-dimethylbutanal, derived from the side chain of a sample of the ergosterol (52900 dpm ^{14}C) by ozonolysis, contained 10500 dpm of ^{14}C.

Table 1. 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	33·5	5·6	0	0
24-Methylene-24,25-dihydrolanosterol	*m/e* Value	440	441	442	443	444
	Abundance*	27·8	36·6	100	33·1	5·5
4α-Methyl-5α-ergosta-8,24(28)-dien-3β-ol	*m/e* Value	412	413	414	415	416
	Abundance*	27·0	36·5	100	31·9	6·4
Episterol	*m/e* Value	398	399	400	401	402
	Abundance*	100	51·5	81·2	23·2	4·6
Ergosterol	*m/e* Value	396	397	398	399	400
	Abundance*	25·6	30·8	100	28·4	5·9

* 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.

DISCUSSION

The major ion in the M^+, M^+–Me and M^+–H_2O ion clusters of 24-methylene-24,25-dihydrolanosterol, 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol, episterol and ergosterol is two mass units greater than normal indicating the presence of two ^2H atoms. Since the major ions of the fragment ion clusters which have lost the side chain are at normal *m/e* values, the extra ^2H atoms must be in the side chain in each of these sterols. Moreover, since it is known that the *S*–methyl carbon atom of methionine is incorporated exclusively into C-28 of these sterols [4], there is little doubt that the ^2H atoms are located on C-28. The mole per cent of each ionic species in the M^+ ion cluster, calculated from relative peak heights and taking account of the natural abundance of ^{13}C, is almost the same for 24-methylene-24,25-dihydrolanosterol and 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol as it is for ergosterol (undeuterated, 18·3%; monodeuterated, 16·2%; dideuterated, 65·5% and trideuterated, 0%), where the relatively large quantity of the monodeuterated species ($-CH_2{}^2H$) has been considered [7] to arise by the reversibility of reaction (1) operating at a late stage in ergosterol biosynthesis, rather than by random loss of ^2H from the *S*–

Table 2. Radioassay of squalene and the sterols isolated from *Phycomyces blakesleeanus* grown in the presence of mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$]

	^{14}C dpm*	^3H dpm*	^{14}C:^3H dpm ratio	^{14}C:^3H atomic ratio†
Mevalonic acid‡	5184	51956	1:10·02	1:1
Squalene	562	5613	1:9·98	6:5·98 (6:6)
Lanosterol	247	2107	1:8·50	6:5·08 (6:5)
24-methylene-24,25-dihydrolanosterol	353	2923	1:8·28	6:4·95 (6:5)
4α-Methyl-5α-ergosta-8,24(28)-dien-3β-ol	160	1289	1:8·06	5:4·02 (5:4)
Episterol	373	2905	1:7·78	5:3·88 (5:4)
Ergosta-5,7,24(28)-trien-3β-ol	223	1321	1:5·92	5:2·95 (5:3)
Ergosterol	179	1144	1:6·38	5:3·18 (5:3)

* Each sample, along with ^{14}C, ^3H and Blank standards, was counted for a period sufficient to give a statistical accuracy of at least 95%, nine 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.

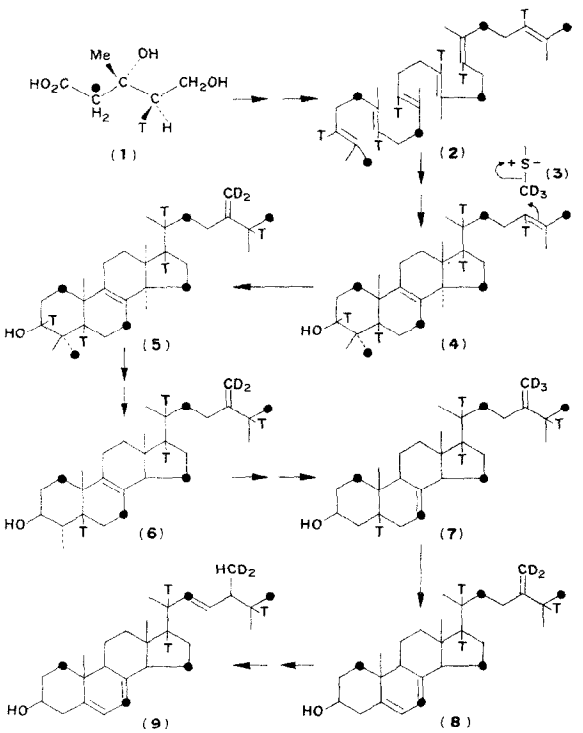
‡ Mevalonic acid-[3*R*,2-^{14}C + 3*S*,2-^{14}C] + mevalonic acid-[3*R*,4*R*-$^3H_1$ + 3*S*,4*S*-$^3H_1$] mixture used in the *P. blakesleeanus* culture medium.

methyl of methionine due to metabolic turnover. It is unlikely that reaction (1) accounts for the

$$=CH_2 \underset{-2H}{\overset{+2H}{\rightleftharpoons}} -Me \qquad (1)$$

presence of significant quantities of the monodeuterated species ($=CH^2H$) of the two 24-methylene sterols; this, therefore, suggests that random loss of ^2H from methionine has taken place. The mole per cent of each ionic species in the M^+ ion cluster of episterol, another 24-methylene sterol, is different (undeuterated, 52·6%; monodeuterated, 10·4%; dideuterated, 37·0%) and more like that found in eburicoic acid-[methylene-$^2H_2$] [8] and pachymic acid-[methylene-$^2H_2$] [7] where the quantity of the $=CH^2H$ species was very low. Since episterol is known to be an ergosterol precursor [9] and, from structural considerations, closer to ergosterol in the biosynthetic pathway than the other two 24-methylene sterols discussed above, it seems likely that the relatively low level of its monodeuterated species is indicative of the operation of reaction (1) in *P.* blakesleeanus in addition to the random loss of ^2H at the methionine level.

The ^{14}C:^3H atomic ratio of 5:3 in ergosterol biosynthesized from mevalonic acid-[2-^{14}C, (4*R*)-4-$^3H_1$] was identical to that found in *Aspergillus fumigatus* [10]. The possibility of erroneus ^{14}C:^3H atomic ratios being calculated for sterols biosynthesized from doubly-labelled mevalonic acid has been recently pointed out [11] after the non-symmetrical labelling of monoterpenes biosynthesized from radioactive mevalonic acid had been observed in several higher plants [12]. This arises, particularly at low levels of incorporation, when labelled isopentenyl pyrophosphate, derived from the administered mevalonic acid, condenses with unlabelled dimethylallyl pyrophosphate from an endogenous pool [11] and leads to sterols which are unlabelled in those isoprenoid units which were derived from the terminal isoprene units of squalene. Non-symmetrical labelling can, therefore, be guarded against by checking that one or other of these isoprene units contain the expected fraction of the total ^{14}C label in the sterol under examination. Since the ergosterol was found to have one-fifth of its ^{14}C in 2,3-dimethylbutanal removed from its side chain by ozonolysis it was clearly labelled in a symmetrical manner. It follows, therefore, that the squalene from which it was formed was also symmetrically labelled. Since all the other sterols were isolated from the same *P. blakesleeanus* culture as the ergosterol and were consequently derived from the same pool of squalene, it follows that they also must have been symmetrically labelled. Thus the ^{14}C:^3H atomic ratios calculated for ergosterol and the other sterols are correctly based. Furthermore the fact that the observed ^{14}C:^3H dpm ratio of 1:10 for squalene (which is equivalent to a ^{14}C:^3H atomic ratio of 6:6 as previously shown in this organism [13]) is identical to that of the mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$] from which it was derived, provided an internal check on the veracity of the ^{14}C:^3H dpm ratios (and therefore the calculated atomic ratios) of the sterols. The ^{14}C:^3H atomic ratio of 6:5 in lanosterol is identical with that observed in animal tissues [14], the loss of ^3H being due to removal of a proton from C-9 during the cyclization of squalene-2,3-oxide [14, 15], a process known to occur in *P. blakesleeanus* [16]. The ^{14}C:^3H atomic ratio of 6:5 in 24-methylene-24,25-dihydrolanosterol is what would be expected if lanosterol, which has the necessary Δ^{24} double bond [17], were transmethylated from *S*-adenosylmethionine. However transmethylation would cause the transfer of a ^3H atom from C-24 to C-25, as has been shown to occur in ergosterol formation in *P. blakesleeanus* [1] and *S. cerevisiae* [3]. The ^{14}C:^3H atomic ratio of 5:4 in 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol is explicable in terms of loss of the 4α-methyl group and 3α-H of its immediate precursor which is probably 4,4-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol [4]. If this is the case *P. blakesleeanus* is like *Polypodium vulgare* where the 4α-methyl group of cycloartenol, which originates from C-2 of mevalonic acid, is lost along with the 3α-H atom, which originates from the 4 pro-R hydrogen of mevalonic acid, during the formation of its 4α-methyl derivative, 31-norcycloartanol, with the remaining methyl group (originally 4β) taking up the 4α configuration [18]. The ^{14}C:^3H atomic ratio of 5:4 in episterol is what would be expected since loss of the remaining 4-methyl group and isomerization of the Δ^8-double bond to the Δ^7 position causes no further loss of ^{14}C or ^3H. However the conversion of episterol into ergosta-5,7,24(28)-trien-3β-ol and ergosterol, processes known to occur in *P. blakesleeanus* [9], will cause the loss of



Scheme 1. Biosynthetic sequence and labelling patterns of sterols isolated from *Phycomyces blakesleeanus* grown in the presence of methionine-[methyl-$^2H_3$] and mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$]. D = deuterium; T = tritium; ● = ^{14}C; **1** = mevalonic acid-[2-^{14}C, (4*R*)-4-$^3H_1$]; **2** = squalene; **3** = S-adenosylmethionine; **4** = lanosterol; **5** = 24-methylene-24,25-dihydrolanosterol; **6** = 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol; **7** = episterol; **8** = ergosta-5,7,24(28)-trien-3β-ol; **9** = ergosterol.

the ^3H atom on C-5 as the Δ^5-double bond is introduced and therefore explain their ^{14}C:^3H atomic ratios of 5:3.

The labelling patterns of the sterols examined in this paper, biosynthesized in the presence of either methionine-[methyl-$^2H_3$] or mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$], are shown in Scheme 1 placed in a sequence consistent with the biosynthetic route from lanosterol to ergosterol in *P. blakesleeanus* proposed previously [4] largely on the basis of the sterols shown to be present in the fungus. This work therefore provides further evidence for the operation of this pathway.

EXPERIMENTAL

Two 100 ml cultures (A and B) of *Phycomyces blakesleeanus* Burgeff, (−) strain were grown from spores on a medium [19] containing 100 mg methionine-[methyl-$^2H_3$] (A) and mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$] (B). The labelled mevalonic acid consisted of a 1:10 mixture of mevalonic acid-[3*R*,2-^{14}C + 3*S*,2-^{14}C] (5 μCi) and mevalonic acid-[3*R*,4*R*-$^3H_1$ + 3*S*,4*S*-$^3H_1$] (50 μCi) obtained from the Radiochemical Centre, Amersham. Since only the 3*R* isomers can be utilized for terpenoid biosynthesis [20], the effective quantities of the ^{14}C and ^3H species present in growth medium B were 2·5 μCi and 25 μCi respectively. The mycelium (wet wt: 3·57 g, A; 4·11 g, B) was harvested after 5 days and saponified. The unsaponifiable lipid (30·85 mg, A; 35·11 mg, B) was chromatographed on alumina (Brockmann Grade 3) and the 9% (v/v) Et_2O in petrol (E/P) fraction (0·53 mg, A; 0·61 mg, B) and 20% E/P fraction (6·28 mg, A; 5·23 mg, B) collected. The petrol fraction (3·31 mg) of unsaponifiable lipid B was also collected. The 4,4-dimethyl sterols (R_f 0·20) and 4-demethyl sterols (R_f 0·40) were isolated from the 9 and 20% E/P fractions by TLC on silica gel G impregnated with Rhodamine 6G [21] and developed with $CHCl_3$. Squalene (R_f 0·4) was isolated from the petrol fraction by TLC on silica gel G impregnated with Rhodamine 6G and developed with petrol [22]. The 4,4-dimethyl sterol fractions were shown by GLC on 1% SE-30 [1] to contain 3 main components with retention times relative to cholestane (RR_t) of 3·05, 3·27 and 3·78; the latter 2 co-chromatographed with authentic lanosterol and 24-methylene-24,25-dihydrolanosterol and the former with a sterol previously identified [4] as 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol. The 4-demethyl fractions were shown by GLC on 1% SE-30 [1] to contain three components with RR_t values of 2·35, 2·58 and 2·72 which had previously been shown [4, 5, 9] to be ergosterol, ergosta-5,7,24(28)-trien-3β-ol and episterol.

The 4,4-dimethyl and 4-demethyl sterol fractions from the methionine-[methyl-$^2H_3$] experiment were then subjected to GC–MS [1]. However these fractions from the mevalonic acid-[2-^{14}C,(4*R*)4-$^3H_1$] experiment were separated into their individual sterol components by preparative GLC on 1% SE-30 [1] in the case of the 4,4-dimethyl sterols and argentation-TLC [9] followed by preparative GLC on 1% SE-30 [1] in the case of the 4-demethyl sterols. Squalene was also purified by preparative GLC on 1% SE-30. The purity of the samples collected after preparative GLC was confirmed by analytical GLC. A sample of the ergosterol purified by argentation-TLC (1·97 mg, 52900 dpm) was subjected to ozonolysis and the resulting 2,3-dimethylbutanal isolated as its dimedone derivative and radioassayed as described previously [1]. The samples (sterols, squalene and dimedone derivative of 2,3-dimethylbutanal) were then dissolved in 10 ml aliquots of NE 260 liquid scintillator and repetitively counted (9×) in an NE 8310 liquid scintillation counter along with ^{14}C, ^3H and Blank standards for a period sufficient to give at least 95% statistical accuracy for each count.

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