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THE MECHANISM OF ALKYLATION AT C-24 DURING CLIONASTEROL BIOSYNTHESIS IN *MONODUS SUBTERRANEUS*

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Key Word Index—*Monodus subterraneus*; Xanthophyceae; biosynthesis; clionasterol; alkylation at C-24; methionine-[methyl-$^2H_3$]; mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$].

Abstract—Clionasterol isolated from *Monodus subterraneus* grown in the presence of methionine-[methyl-$^2H_3$] contained four ^2H atoms showing the participation of a 24-ethylidene sterol intermediate in its biosynthesis. Clionasterol isolated from *M. subterraneus* grown in the presence of mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$] had a ^{14}C:^3H atomic ratio of 5:3 indicating that the 24-ethylidene sterol intermediate is reduced directly to clionasterol and not isomerized to a Δ^{24}-sterol which is then reduced.

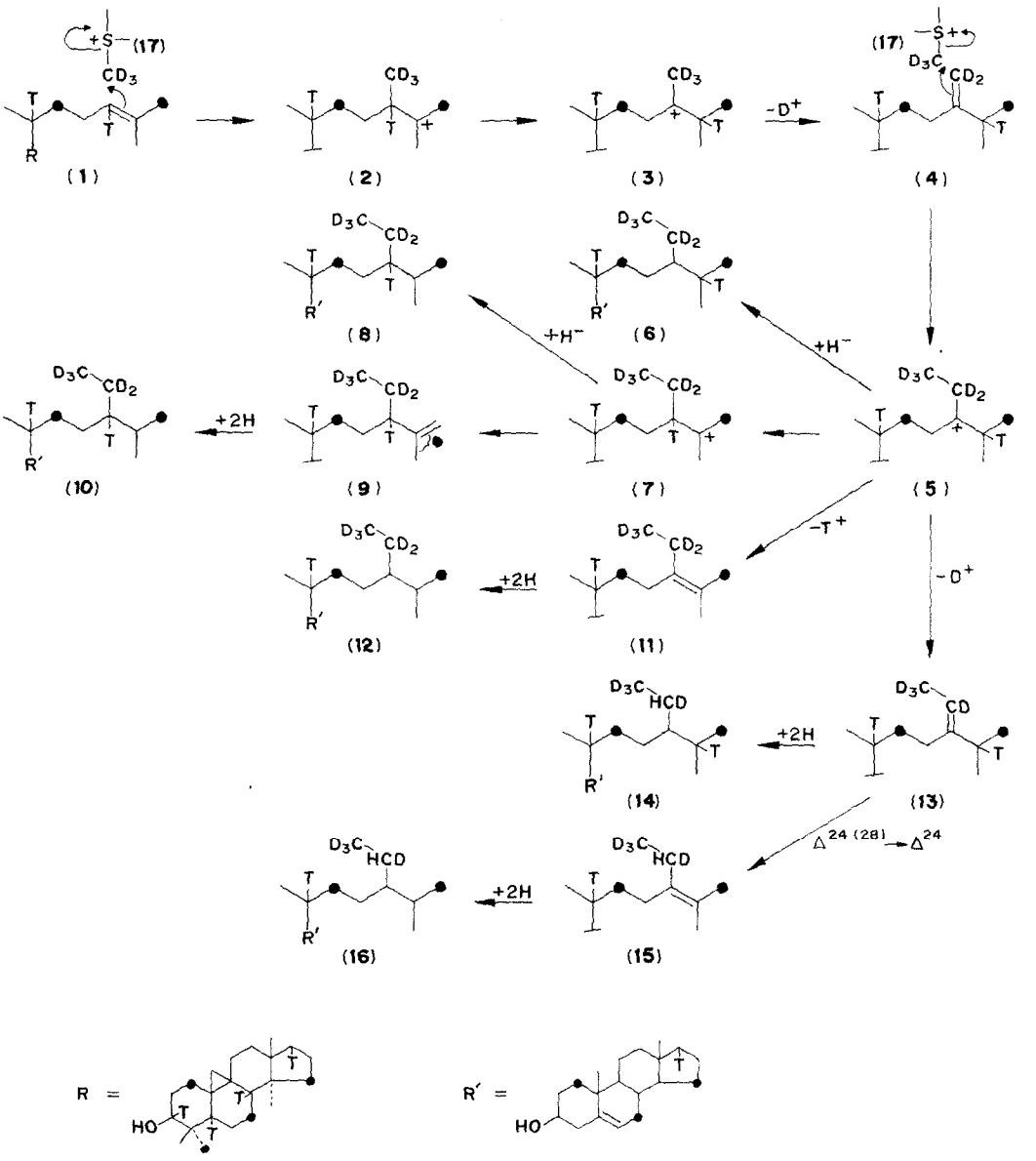
INTRODUCTION*

The most abundant sterols of the yellow-green alga, *Monodus subterraneus* are clionasterol and cholesterol which constitute about 67 and 33% of the 4-demethyl sterol fraction [1]. We now report the results of an investigation into the mechanism of the introduction of the 24-ethyl group of clionasterol in this alga utilizing methionine-[methyl-$^2H_3$] [2–4] and mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$] [5–9]. Scheme 1 shows the several alternative routes by which alkylation may take place and the expected labelling in clionasterol when biosynthesized in the presence of the two labelled substrates; it is based upon several previously published schemes [3, 4, 9–12]. It is likely that cycloartenol (**1**), a compound present in *M. subterraneus* [1] and considered [3] to be the first cyclic precursor of sterols in higher plants and algae, is transmethylated from *S*-adenosylmethionine (**17**) since 24-methylenecycloartanol (side chain = **4**; nucleus = R) has been detected in this alga [1]. Cycloartenol biosynthesized from mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$] in higher plants and algae has a ^{14}C:^3H atomic ratio of 6:6 and the labelling pattern shown in (**1**) [5, 9] and yields on transmethylation 24-methylenecycloartanol with an identical labelling pattern save that the C-24 tritium atom has migrated to C-25. The second transmethylation may take place at the 24-methylenecycloartanol level or at a different stage of nuclear transformation. The resulting carbonium ion (**5**) could then be converted into clionasterol by several different routes all of which would result in the labelling pattern shown in (R′) in the nucleus of clionasterol biosynthesized from mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$]. However these routes would result in different labelling patterns in the side chain when clionasterol is biosynthesized from mevalonic acid-[2-^{14}C,(4*R*)-4-$^3H_1$] and methionine-[methyl-$^2H_3$]; these are summarized in Table 1.

* *Sterol nomenclature.* The trivial names of the sterols used in the text have the following systematic names: clionasterol = (24*S*)-24-ethylcholest-5-en-3β-ol; cholesterol = cholest-5-en-3β-ol; sitosterol = (24*R*)-24-ethylcholest-5-en-3β-ol; cycloartenol = 4,4,14α-trimethyl-9,19-cyclo-5α-cholest-24-en-3β-ol; 24-methylenecycloartanol = 4,4,14α-trimethyl-9,19-cyclo-5α-ergost-24(28)-en-3β-ol; isofucosterol = Z-24-ethylidenecholest-5-en-3β-ol; stigmasterol = (24*S*)-24-ethylcholesta-5,22-dien-3β-ol; α-spinasterol = (24*S*)-24-ethyl-5α-cholesta-7,22-dien-3β-ol.

RESULTS AND DISCUSSION

The MS of clionasterol isolated from *M. subterraneus*, grown in the presence of methionine-[methyl-$^2H_3$], had a molecular ion (M^+) cluster composed of ions with *m/e* values at 414, 415, 416,



Scheme 1. Possible mechanisms of alkylation at C-24 during clionasterol biosynthesis in *Monodus subterraneus* from methionine-[methyl-$^{2}H_3$] and mevalonic acid-[2-^{14}C,(4*R*)-4-$^{3}H_1$]. D = deuterium; T = tritium; ● = ^{14}C.

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

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

417, 418, 419 and 420 with relative intensities of 100, 45·6, 15·7, 25·3, 27·4, 7·6 and 1·8 respectively. The ion at *m/e* 414 was due to undeuterated clionasterol which constituted the major ionic species. Relatively large peaks at *m/e* values of 415, 417 and 418 were due to clionasterol containing 1,3 and 4 deuterium atoms per molecule respectively. The peaks at *m/e* values of 416, 419 and 420 were of the order expected for the ^{13}C isotope content of the peaks at 414, 415, 417 and 418. The fragment ion clusters at M^+–Me and M^+–H_2O had approximately the same proportion of component ions as that of the M^+. This was, however, not the case with the fragment ion clusters devoid of the side chain, where the proportions of the component ions were identical with those of the equivalent clusters in the MS of unlabelled clionasterol, thus indicating that the deuterium atoms were located in the side chain. Since the MS of cholesterol isolated from the same culture of *M. subterraneus* was identical with that of unlabelled cholesterol it is evident that the label from methionine-[methyl-$^{2}H_3$] did not become randomized and indicated that the deuterium atoms in clionasterol were located solely in the 24-ethyl group. The presence of clionasterol labelled with four deuterium atoms but not with five, establishes that 24-alkylation in *M. subterraneus* proceeds via a 24-ethylidene intermediate (**13**).

The ^{14}C:^{3}H atomic ratios of both clionasterol and cholesterol isolated from *M. subterraneus* grown in the presence of mevalonic acid-[2-^{14}C,(4*R*)-4-$^{3}H_1$] were 5:3 (see Table 2). This indicates that the tritium atom at C-24 in cycloartenol (**1**) which is transferred to C-25 during the formation of the 24-methylene sterol intermediate (**4**) is not lost during the second methylation leading to the production of the 24-ethyl group of clionasterol. This, taken with the result of the deuterium-labelling experiment, shows that alkylation in *M. subterraneus* proceeds by route 5 (Table 1) (**1 → → 5 → 13 → 14**, Scheme 1).

The mechanism of alkylation in *M. subterraneus* is therefore identical with that operating in the closely related chrysophyte, *Ochromonas malhamensis* [8, 11, 13] but contrasts with that in *Chlorella vulgaris* [14], *C. ellipsoidea* [15], *Trebouxia* sp. 213/3 [16] and the slime mould, *Dictyostelium discoideum* [12] where 24-ethyl sterols contain five deuterium atoms after synthesis from methionine-[methyl-$^{2}H_3$]. The situation in higher plants is less clear and present evidence indicates that different

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

	^{14}C dpm*	^{3}H dpm*	^{14}C:^{3}H dpm ratio	^{14}C:^{3}H atomic ratio†
Mevalonic acid‡	5199	51848	1:9·97	1:1
Cholesterol	563	3505	1:6·22	5:3·12 (5:3)
Clionasterol	1005	6049	1:6·01	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 at least 95%, 22 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*-$^{3}H_1$ + 3*S*,4*S*-$^{3}H_1$] mixture used in the *M. subterraneus* culture medium.

species may utilize different alkylation mechanisms. A route involving a 24-ethylidene sterol intermediate (**13**) appears to operate in *Pinus pinea* seeds where alkylation involves the migration of hydrogen from C-24 to C-25 [17] and 24-methylenecholesterol is converted into isofucosterol [18] and the latter into sitosterol [18]. Similarly in *Euphorbia plepus* the time course of incorporation of mevalonic acid-[2-^{14}C] into sterols indicates that isofucosterol is the precursor of sitosterol. Moreover the occurrence of 24-ethylidene sterols in higher plants [4] and the incorporation of mevalonic acid-[2-^{14}C] into isofucosterol in the leaves of *Larix decidua* [19] and *Pisum sativum* [19] are consistent with the 24-ethylidene sterol route. In contrast a route via a Δ^{24}-sterol intermediate (**11** or **15**) appears to operate in *Nicotiana tabacum* and *Dioscorea tokoro* where stigmasterol biosynthesized from mevalonic acid-[2-^{14}C,(4*R*)-4-$^{3}H_1$] had a ^{14}C:^{3}H atomic ratio of 5:2 with no tritium located at C-24 or C-25 [6]. Similarly α-spinasterol biosynthesized from the same precursor in *Medicago sativa* and *Spinacea oleraceae* had a ^{14}C:^{3}H atomic ratio of 5:3 with no tritium located at C-24 or C-25 [9]. Whether the Δ^{24}-sterol intermediate is derived by proton elimination from the carbonium ion (**5**→**11**) or by isomerization of a 24-ethylidene side chain (**13**→**15**) has yet to be determined but evidence of the operation of the latter has been obtained in *Hordeum vulgare* [20]. A third route involving the transfer of hydrogen from C-25 to C-24 and the elimination of a proton from C-26 (**5**→**7**→**9**) appears to operate in the formation of (24*S*)-24-ethylcholest-5,22,25-trien-3β-ol (side chain, **9**) in *Clerodendrum campbelli* [21].

EXPERIMENTAL

Two 100 ml cultures (A and B) of *Monodus subterraneus* Peterson 848/1 Lewin, U.S.A., were grown on Bold's Basal Medium [22] containing 100 mg methionine-[methyl-$^{2}H_3$] (A) and mevalonic acid-[2-^{14}C,(4*R*)-4-$^{3}H_1$] (B) at 23° on a gyrotary shaker under constant illumination (3750 lx) from 'warm white' fluorescent tubes. The labelled mevalonic acid (Radiochemical Centre, Amersham) consisted of a mixture of mevalonic acid-[3*R*,2-^{14}C + 3*S*,2-^{14}C] (5 μCi) and mevalonic acid-[3*R*,4*R*-$^{3}H_1$ + 3*S*,4*S*-$^{3}H_1$] (50 μCi) with a ^{14}C:^{3}H dpm ratio of 1:9·97. Since only the 3*R* isomers can be utilized for terpenoid biosynthesis [23], the effective quantities of the ^{14}C and ^{3}H species present in growth medium B were 2·5 μCi and 25 μCi respectively. The cells (wet wt: 1·24 g, A; 2·82 g, B) were harvested after 3 weeks and the lipid (10·04 mg, A; 50·06 mg, B) extracted as described previously [1]. The lipid was saponified [1] and the unsaponifiable material (6·60 mg, A; 32·74 mg, B) chromatographed on alumina (Brockmann Grade 3) and the 8–12% Et_2O in petrol fractions collected and bulked (1·36 mg, A; 6·33 mg, B). The 4-demethyl sterols were isolated from these fractions by TLC on silica-gel G impregnated with Rhodamine 6G [24] developed with $CHCl_3$ and shown to be composed of 2 components with retention times relative to cholestane (RR_f) identical with those of cholesterol (RR_f 1·83) and sitosterol (RR_f 3·03) (the 24-epimer of clionasterol) by GLC on 1% SE-30.

The 4-demethyl sterol fraction from the methionine-[methyl-$^{2}H_3$] experiment was then subjected to GC MS whilst that from the mevalonic acid-[2-^{14}C,(4*R*)-4-$^{3}H_1$] experiment was separated into its 2 components by preparative-GLC on 1% SE-30. The two sterol components were checked for purity by analytical GLC, then dissolved in 10 ml NE 260 liquid scintillator and repetitively counted (22×). The cholesterol had a ^{14}C:^{3}H dpm ratio of 1:6·22, calculated ^{14}C:^{3}H atomic ratio 5:3·12; the clionasterol had a ^{14}C:^{3}H dpm ratio of 1:16·01, calculated ^{14}C:^{3}H atomic ratio 5:3·01.

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