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THE CONVERSION OF 5α-LANOST-24-ENE-3β,9α-DIOL AND PARKEOL INTO PORIFERASTEROL BY THE ALGA *OCHROMONAS MALHAMENSIS*

M. A. PALMER†, L. J. GOAD†, T. W. GOODWIN†, D. B. COPSEY‡ and R. B. BOAR‡

† Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool, L69 3BX, U.K. ‡ Department of Chemistry, Chelsea College, University of London, Manresa Road, London, S.W.3, U.K.

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Key Word Index—*Ochromonas malhamensis*; Chrysophyceae; sterol biosynthesis; 5α-lanost-24-ene-3β,9α-diol; parkeol; poriferasterol; cycloartenol; obtusifoliol.

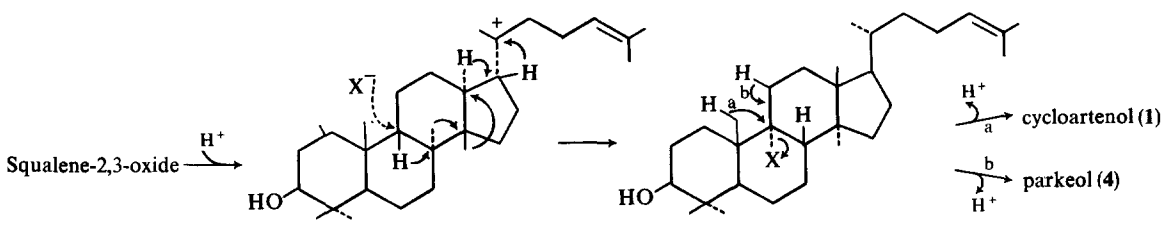
Abstract—The 4,4-dimethylsterols 4α-lanost-24-ene-3β,9α-diol-[2-$^3H_2$] and parkeol-[2-$^3H_2$] were synthesized from lanosterol and subsequently incubated with cultures of *Ochromonas malhamensis*. 5α-Lanost-24-ene-3β,9α-diol was converted into poriferasterol with three times the efficiency of parkeol. Clionasterol was also found to be labelled from both parkeol and 5α-lanost-24-ene-3β,9α-diol. No significant incorporation of radioactivity into sterols was obtained after feeding 5α-lanost-24-ene-3β,9α-diol to higher plants, the chlorophyte alga *Trebouxia*, yeast or a cell free homogenate of rat liver.

INTRODUCTION

The chrysophyte alga *Ochromonas malhamensis* has been shown [1] to transform exogenous cycloartenol (**1**) and lanosterol (**2**) into poriferasterol (**3**). However in the algae and higher plants so far examined it has been established that cycloartenol and not lanosterol is the normal 4,4-dimethylsterol precursor [2]. Also parkeol (**4**) does not appear to be a phytosterol precursor [3] although both parkeol and lanosterol are present in plant species [4, 5]. The mechanism for the cyclisation of (3*S*)-squalene-2,3-oxide [6] to produce cycloartenol (**1**) in phytosterol biosynthesis has been discussed [7]. In order to conform to the biogenetic isoprene rule it is suggested that an intermediate cation is produced which is stabilised at C-9 by a hypothetical electron donating group X^-. The subsequent removal of the X^- group together with a C-19 or a C-11 proton from the protosterol would give either cycloartenol (**1**) or parkeol (**4**) respectively [8, 9]. Similarly, lanosterol (**2**) could be produced as a result of the simultaneous loss of the C-8 hydrogen. The X^- may be any electron donating group such as a specific amino acid residue located on the cyclase (Scheme 1). The protosterol 5α-lanost-24-ene-3β,9α-diol (**5**) is an analogue of the proposed intermediate cation stabilized by a hydroxyl group on C-9. We now report the conversion of both 5α-lanost-24-ene-3β,9α-diol-[2-$^3H_2$] and parkeol-[2-$^3H_2$] into poriferasterol (**3**) by *Ochromonas malhamensis*.

RESULTS AND DISCUSSION

The 4,4-dimethylsterols 5α-lanost-24-ene-3β,9α-diol-[2-$^3H_2$] and parkeol-[2-$^3H_2$] were prepared from the corresponding 3-ketone derivatives by alumina catalysed exchange with tritiated water [10]. The starting material for the synthesis of both compounds was lanosterol (**2**). The tritiated sterols were added to the growth media of two-day-old cultures as ethanolic solutions and incubated for a further 3 days. The results of triplicate incubations of *Ochromonas malhamensis* 933/1A with 5α-lanost-24-ene-3β,9α-diol (**5**) and parkeol (**4**) (Table 1) showed that the diol was very efficiently incorporated into poriferasterol (14.9%). The greater polarity of 5α-lanost-24-ene-3β,9α-diol may have facilitated the uptake and transport of this sterol into the developing cells. Poriferasteryl acetate from each of the incubations was recrystallized to constant specific activity as a check on radiochemical purity (Table 1). Samples of poriferasteryl acetate were also saponified and subjected to Oppenauer oxidation to give (24*R*)-24-ethylcholesta-4,22-dien-3-one. Under the conditions of this reaction, basic exchange of the C-2 tritium occurred and the radioactivity of the original poriferasterol was almost completely lost. The possible randomisation of the C-2 tritium label during incubation was therefore eliminated. A mixture of poriferasterol (85%) and clionasterol (15%) was isolated from *Ochromonas malhamensis* 933/1A in the present study. Previous analyses of this species have demon-



Scheme 1. Suggested mechanism for the cyclization of squalene-2,3-oxide to produce cycloartenol (**1**) or parkeol (**4**).

Table 1. Incubation of *O. malhamensis* with 5α-lanost-24-ene-3β,9α-diol [2-$^3H_2$] and parkeol-[2-$^3H_2$]

	5α-Lanost-24-ene-3β,9α-Diol (dpm × 10^{-3})			Parkeol (dpm × 10^{-3})		
Expt	1	2	3	1	2	3
Added precursor	3380	3380	3380	2486	2486	2486
Recovered in non-saponifiable lipid	1453 (75)*	1738 (75)	1683 (68)	1984 (68)	1144 (72)	1012 (76)
Unconverted diol	393	394	320	—	—	—
4-Demethylsterols	1034 (37)	870 (38)	706 (29)	297 (27)	206 (21)	224 (28)
4,4-Dimethylsterols	27 (t)	18 (t)	22 (t)	425 (*t*)	300 (t)	396 (t)
Poriferasteryl acetate	621 (20)	388 (18)	504 (20)	137 (23)	96 (17)	122 (23)
Clionasteryl acetate	133 (3)	160 (3)	136 (2.5)	32 (2.5)	35 (2)	33 (1.2)
Sp. act. of poriferasteryl acetate after addition of carrier (dpm/mg × 10^{-3})	19.5	12.7	15.8	3.2	4.6	3.5
1st recrystallisation	18.9	13.2	16.2	3.0	5 0	3.2
2nd recrystallisation	18.7	10.1	16.5	2 9	4.6	3.1
3rd recrystallisation	18.7	11.2	15.8	2.9	4.4	3.2

* Values in parentheses are the weights (mg) of the various fractions obtained.

strated the presence of poriferasterol (98%) and brassicasterol (1%) [11].

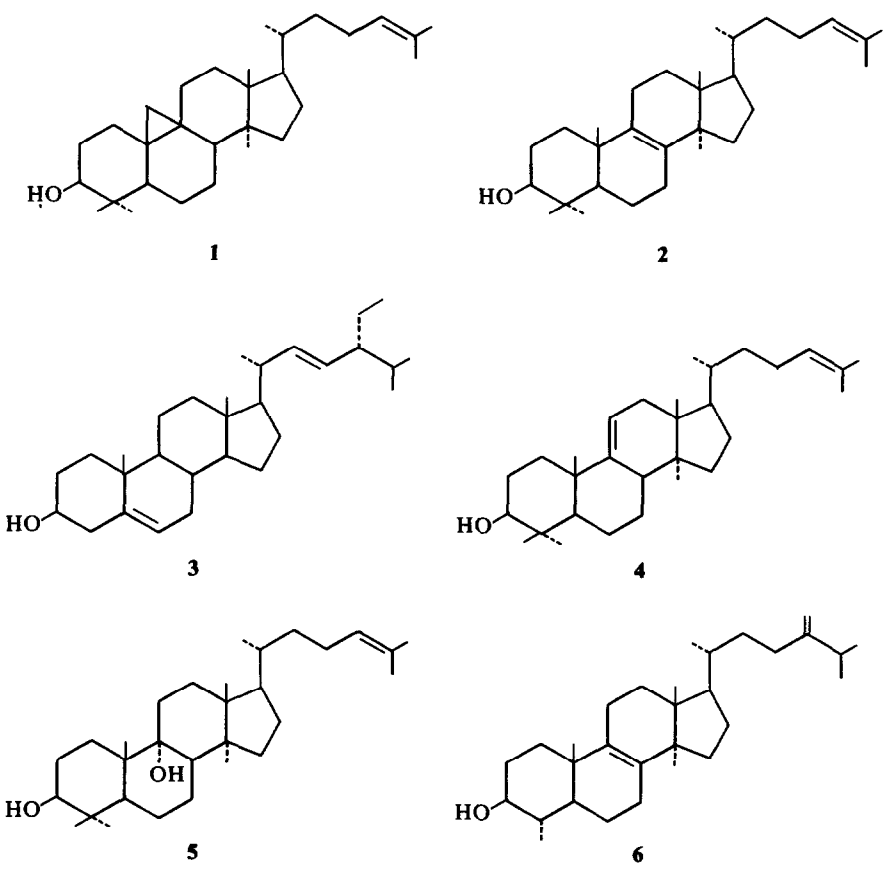
Duplicate experiments with 6 l. cultures of *Ochromonas malhamensis* allowed the recovery of the radioactivity from 5α-lanost-24-ene-3β,9α-diol [2-$^3H_2$] in the 4,4-dimethyl- and 4-monomethylsterols of *O. malhamensis* (Table 2). Fractions containing cycloartenyl acetate and 24-methylenecycloartanyl acetate incorporated 0.15% and 0.4% of the original radioactivity respectively (Table 2). An examination of the residual radioactivity recovered in the growth medium suggested that about one-third of the added 5α-lanost-24-ene-3β,9α-diol-[2-$^3H_2$] was taken up by the cells. The 4-monomethylsterol, obtusifoliol (**6**), was found to be labelled from the diol (0.02% conversion). The conversion of added obtusifoliol into poriferastol in *O. malhamensis* has been previously reported [12]. The acetates of obtusifoliol, cycloartenol and 24-methylenecycloartanol obtained in the present study were characterized by GLC and MS. Parkeyl acetate can be separated from the acetates of cycloartenol and 24-methylenecycloartanol by $AgNO_3$–Si gel PLC but lanosterol and cycloartenol cochromatograph. The possibility exists therefore that 5α-lanost-24-ene-3β,9α-diol-[2-$^3H_2$] may have undergone dehydration at C-9 and loss of the C-8 proton giving lanosterol of high specific radioactivity. However, *in vitro* dehydration of 9α-hydroxy-5α-lanost-24-ene-3β-yl acetate by means of thionyl chloride in pyridine gives as the sole product parkeyl acetate, and no detectable (by TLC) amount of 1a nosteryl acetate [13]. There is unequivocal evidence that *O. malhamensis* cyclises squalene-2,3-oxide to cycloartenol [14, 15]. A comparison between the incorporation of parkeol and 5α-lanost-24-ene-3β,9α-diol with lanosterol and cycloartenol suggests that both 5α-lanost-24-ene-3β,9α,diol and cycloartenol are incorporated with equal efficiency whereas parkeol and lanosterol are less efficiently utilized. It is likely that the nuclear rearrangements and side chain C-24 alkylation necessary to convert exogenous sterol precursors into poriferasterol need not occur in an ordered sequence but may vary with the structure of the substrate added.

In additional experiments, the chlorophyte alga *Trebouxia* sp. 913/3 showed negligible incorporation of 5α-lanost-24-ene-3β,9α,diol-[2-$^3H_2$] into poriferasterol although other experiments with desmosterol-[24-$^3H_2$] gave a 30% conversion [16]. The sterol requiring yeast mutant FK. erg 1 [17] and the FL 100 wild type strain which normally produce ergosterol via the intermediate lanosterol failed to incorporate 5α-lanost-24-ene-3β,9α-diol-[2-$^3H_2$] into lanosterol or ergosterol. Nor was any conversion of the diol into cholesterol observed after incubation of the diol with a rat liver homogenate. A C-20 cation is implicated as the intermediate product of squalene-2,3-oxide cyclisation in animal tissue [18] and

Table 2 Distribution of radioactivity in the sterol precursors of *O. malhamensis* after the incorporation of 5α-lanost-24-ene-3β,9α-diol

	Expt 1		Expt 2	
	(mg)	(dpm × 10^{-3})	(mg)	(dpm × 10^{-3})
Precursor added to 6 l. of culture		49031		49031
Radioactivity recovered from growth medium		35788		33374
from non-saponifiable lipid	433	16276	360	12652
Total sterol faction*	215	5232	188	4003
4,4-Dimethylsterols†	17	444	11	346
4-Monomethylsterols†	8	26	5	33
4-Demethylsterols†	167	7795	142	6096
Cycloartenyl acetate‡	1	84	1	66
24-Methylenecycloartanyl acetate‡	2	206	1.5	183
Obtusifoliol acetate‡	1.5	8	2	12

* Isolated by column chromatography on alumina eluted with 40% Et_2O in petrol; † Obtained by PLC of the total sterol mixture; ‡ Separated by $AgNO_3$–Si gel PLC of the 4-monomethyl- and 4,4-dimethylsterol fractions.



an analogue has been synthesized in radioactive form as the stabilized C-20 hydroxy sterol (20*R*)-protost-24-en-3β,20-diol [19]. However, no significant incorporation of this compound into lanosterol was reported [19]. Enzyme systems for opening the 9β,19-cyclopropane ring are not operative in yeast or rat liver [20, 21] and this fact may possibly explain the lack of any conversion of 5α-lanost-24-ene-3β,9α-diol into sterols. 5α-Lanost-24-ene-3β,9α-diol-[2-$^3H_2$] was fed to a succulent plant, *Senecio articulata*, via an incision in the stem and to the pitcher plant, *Sarracenia purpurea*, by direct injection into the immature pitchers. No radioactivity was recovered in the stigmasterol isolated from either plant although a low by significant conversion of 28-isofucosterol-[7-$^3H_2$] into stigmasterol was obtained with the pitcher plant.

EXPERIMENTAL

General methods. IR spectra were obtained in CS_2. GLC analyses were carried out at 250° with an argon flow rate of 40 ml/min. Glass columns 1.5 m × 0.64 cm were used packed with either 3% OV-17 or 3% SE-30 on Gaschrom Q (100–120 mesh). MS were obtained by direct probe insertion. Radioactivity was determined by liquid scintillation counting with butyl-PBD in toluene (0.7%).

Preparation of 5α-lanost-24-ene-3β,9α-diol-[2-$^3H_2$]. 5α-Lanost-24-ene-3β,9α-diol, mp 127–128°, $[\alpha]_D$ + 8.5° (*c* 0.9 in chloroform) was prepared by lithium–ethylamine reduction of 8α,9α-epoxy-5α-lanost-24-en-3β-yl acetate [22] as previously described for the corresponding 24,25-dihydro compound [23]. Oxidation of the diol with Jones' chromic acid gave 9α-hydroxy-5α-lanost-24-en-3-one, mp 144–145°, $[\alpha]_D$ + 14.7° (*c* 0.8 in $CHCl_3$). Labelling with tritium at C-2 was carried out by equilibration on a basic alumina column with T_2O [10]. The tritiated ketone was finally reduced with $NaBH_4$ to 5α-lanost-24-ene-3β,9β-diol-[2-$^3H_2$] (130 μCi/mg sp. act.) which was recrystallized from MeOH.

Preparation of parkeol-[2-$^3H_2$]. 5α-Lanost-24-ene-3β,9α-diol was monoacetylated (Py–Ac_2O, 2:1) and dehydrated at C-9 with $SOCl_2$ in Py to give parkeyl acetate. Removal of the 3β-acetoxy group with $LiAlH_3$ followed by Jones' oxidation gave 5α-lanost-9(11)-en-3-one which was labelled at C-2 with tritium by base catalysed exchange on alumina as described above. The 3-ketone was finally reduced with $NaBH_4$ and recrystallized from MeOH giving parkeol-[2,$^3H_2$] (sp. act. 70 μCi/mg). Aliquots of tritiated 5α-lanost-24-ene-3β,9α-diol and parkeol were mixed with cold carrier and subjected to TLC on Si gel developed in $CHCl_3$–EtOH (98:2). Zones corresponding to marker poriferasterol, parkeol and 5α-lanost-24-ene-3β,9α-diol were eluted. Radioassay showed that 99.3% of the radioactivity from parkeol-[2-$^3H_2$] was associated with the parkeol band and 99.8% of the radioactivity from 5α-lanost-24-ene-3β,9α-diol-[2-$^3H_2$] was associated with the diol band. GLC analysis of the parkeol and 5α-lanost-24-ene-3β,9α-diol on 3% OV-17 gave a single peak for each compound and showed them to be about 99.5% pure.

Culture of Ochromonas malhamensis *and administration of radioactive sterols. Ochromonas malhamensis* (933/1A) was obtained from the culture collection for algae and protozoa (Cambridge, U.K.). The cells were grown at 20° under constant illumination on a medium consisting of Bacto B_{12} *Ochromonas* medium (Difco) supplemented with vitamin B_{12}. Radioactive sterols were dissolved in 1.0 ml of EtOH and pipetted aseptically into 6 l. of 2-day-old culture. Large scale cultures were aerated with air–CO_2 (24:1). Small scale incubations were carried out using 200 ml cultures grown on an orbital shaker and inoculated with 0.2 ml of ethanolic sterol soln.

Administration of sterols to plant, liver and yeast tissue. Tritiated sterols were dissolved in 80% EtOH and injected through the epidermal wall into the developing pitchers of *Sarracenia*

purpurea while they were still closed. (Closed pitchers were used in an attempt to minimize contamination by microorganisms.) The incubation was stopped after 10 days.

A section of stem (*ca* 1 cm square) was cut out from the succulent plant *Senecio articulata* and an ethanolic soln of radioactive sterols was pipetted into the inner cavity. The section of stem was replaced and bound with Teflon tape. The incision was rapidly sealed by the growth of callus tissue. The incubation was for 10 days.

200 ml cultures of *Saccharomyces cerevisiae* were grown for 72 hr on an orbital shaker at 28°. 5α-Lanost-24-ene-3β,9α-diol-[2-$^3H_2$] was dissolved in EtOH and an aliquot added to fresh medium together with an inoculum of either the FK-erg 1 or FL 100 yeast strain. The yeast was harvested after 48 hr. Rat liver homogenates were prepared as previously described [24] and incubated with radioactive sterols for 6 hr at 37°.

Sterol extraction and purification. Algal cells were incubated for a further 3 days after inoculation and were harvested by centrifugation. Saponification was carried out by refluxing with 8% KOH in 80% MeOH under N_2 for 90 min. The unsaponifiable lipid from the 6 l. cultures was preliminary separated on a column of alumina (Woelm anionotropic, Brockmann grade III). Fractions containing hydrocarbons, pigments and quinones were eluted with petrol and 2% Et_2O in petrol respectively and were discarded. A sterol fraction was next eluted with 40% Et_2O in petrol and further separated by PLC on Si gel developed in $CHCl_3$–EtOH (98:2). The unsaponifiable lipid from higher plants and from 200 ml cultures of yeast and *O. malhamensis* were subjected to PLC directly. Bands corresponding to 4-demethylsterols, 4-monomethylsterols and 4,4-dimethylsterols were eluted. Sterols were further separated as their acetates (Py–Ac_2O, 1:1) by PLC on 10% $AgNO_3$ impregnated Si gel developed in pure $CHCl_3$. Labelled poriferasteryl acetate was mixed with carrier and crystallized from MeOH to constant sp. act. MS of obtusifoliolacetate *m/e* (rel. int.): 468 (66), 453 (100), 408 (14), 393 (61), 396 (7), 341 (9), 309 (5), 301 (8), 241 (13), 227 (15).

Preparation of (24R)-24-ethylcholesta-4,22-dien-3-one. In a typical experiment, tritiated poriferasteryl acetate was mixed with carrier (sp. act. 1131 dpm/mg) and saponified to give the free sterol. The poriferasterol was refluxed with aluminium isopropoxide (50 mg per 100 mg sterol) in toluene–cyclohexanone (6:1) yielding the 3-keto derivative [25]. After purification by PLC and crystallization from MeOH the (24R)-24-ethylcholesta-4,22-dien-3-one (sp. act. 26 dpm/mg) gave MS and IR spectra similar to those described elsewhere [26].

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