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THE CONVERSION OF (24*S*)-24-ETHYLCHOLESTA-5,22,25-TRIEN-3β-OL INTO PORIFERASTEROL, BOTH *IN VIVO* AND WITH A CELL-FREE HOMOGENATE OF THE ALGA *TREBOUXIA* SP.

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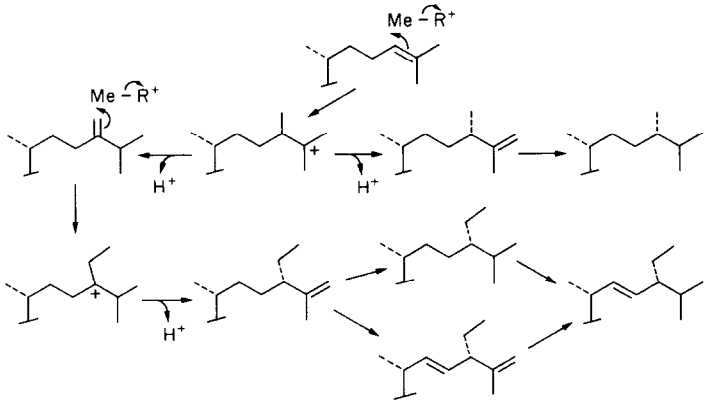
Key Word Index—*Trebouxia* sp.; Chlorophyta; Chlorococcales; alga; sterol biosynthesis; Δ^{25}-sterol reductase; (24*S*)-24-ethylcholesta-5,22,25-trien-3β-ol; poriferasterol.

Abstract—[6-$^3H_1$](24*S*)-24-Ethylcholesta-5,22,25-trien-3β-ol added to the growth medium of a culture of *Trebouxia* sp. 213/3 was efficiently taken-up by the cells and converted into (24*R*)-24-ethylcholesta-5,22-dien-3β-ol (poriferasterol) which is one of the major sterols of this alga. A cell-free homogenate was obtained from *Trebouxia* which catalysed the NADPH-dependent reduction of [6-$^3H_1$](24*S*)-24-ethylcholesta-5,22,25-trien-3β-ol to yield poriferasterol. The Δ^{25}-sterol reductase was found to be mainly localized in the microsomal fraction of the homogenate.

INTRODUCTION

Investigations on the mechanism of introduction of the C-24 alkyl group into the phytosterol side chain have established that *S*-adenosylmethionine is the methyl donor for these reactions [1–3]. The sterol substrates for these transmethylation reactions are Δ^{24}- or $\Delta^{24(28)}$-compounds and they yield the cations shown in Scheme 1. It is now established that stabilization of the cations can proceed with loss of protons from various carbons as indicated in Scheme 1 and that particular pathways operating in an organism may have some phylogenetic significance [3, 4]. In most higher plants 24α-ethyl sterols, such as sitosterol, tend to predominate and these are believed to arise via a 24-ethylidene intermediate which is isomerized to a $\Delta^{24(25)}$-sterol prior to stereospecific reduction to yield the 24α-ethyl group [4–11].

In most algae the major 24-ethyl sterols have the 24β-configuration. In the chrysophyte, *Ochromonas malhamensis*, the major sterol is poriferasterol (**1**) and this originates by a stereospecific reduction of a 24-ethylidene group, probably after introduction of a Δ^{22}-bond to give a $\Delta^{22,24(28)}$-diene intermediate [12, 13]. In algae belonging to the Order Chlorococcales, an alternative route to 24β-ethyl sterols has evolved. This involves production of the 24β-ethyl-25-methylene sterol intermediate from the transmethylation generated cation (Scheme 1). Evidence for the involvement of a 25-methylene intermediate in poriferasterol (**1**) biosynthesis in these latter algae derives from studies on the incorporation of [C^2H_3]methionine [14, 15], identification of 25-methylene sterols in *Chlorella* species grown in the presence of sterol synthesis inhibitors [16–18] and the demonstration that (24*R*)-24-ethylcholesta-5,25-dien-3β-ol (clerosterol) is converted into poriferasterol by a species of *Trebouxia* [19].



Scheme 1. Possible routes for the production of the poriferasterol and ergost-5-en-3β-ol side chains in *Trebouxia* sp.

We now demonstrate the presence of a microsomal 25-methylene-sterol reductase in *Trebouxia* and present

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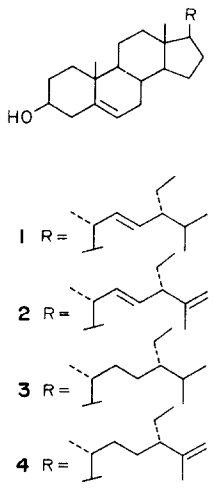

evidence suggesting that a $\Delta^{22,25}$-diene may be the preferred intermediate in poriferasterol biosynthesis.

RESULTS AND DISCUSSION

After *in vivo* incubation of *Trebouxia* cells with [6-$^3H_1$](24*S*)-24-ethylcholesta-5,22,25-trien-3β-ol (**2**) for 14 days the sterols (2.41×10^6 dpm) were isolated, acetylated and separated by silver nitrate–Si gel TLC to yield fractions corresponding to unchanged [6-$^3H_1$] (24*S*)-24-ethylcholesta-5,22,25-trien-3β-yl acetate, poriferasteryl acetate and a mixture of clionasteryl acetate and ergost-5-en-3β-yl acetate (Table 1). In each of the three cultures the major labelled sterol was identified as poriferasterol (**1**) and the incorporation of the precursor [6-$^3H_1$](24*S*)-24-ethylcholesta-5,22,25-trien-3β-ol (**2**) was 25–30%. The poriferasterol (**1**) also had a much higher sp. act. (6.15×10^5 dpm/mg in culture 1) than the clionasterol (**3**) fraction (9.72×10^3 dpm/mg). The labelling of the poriferasterol (**1**) was confirmed by crystallization of samples to constant sp. act. after addition of unlabelled carrier poriferasterol (culture 1: 1.78×10^4, 1.71×10^4, 1.83×10^4, 1.73×10^4, 1.80×10^4 dpm/mg; culture 2: 1.04×10^4, 1.17×10^4, 1.13×10^4, 1.18×10^4, 1.21×10^4 dpm/mg for successive crystallizations).

The reduction of the 25-methylene group of [6-$^3H_1$](24*S*)-24-ethylcholesta-5,22,25-trien-3β-ol was demonstrated using a cell-free homogenate of *Trebouxia* (Table 2). Although the homogenate showed reductase activity to yield poriferasterol without any added cofactors the reduction was significantly enhanced by addition of NADPH or a NADPH generating system. There was insignificant reduction of the Δ^{22}-bond as evidenced by the very low recovery of radioactivity in clionasterol (**3**).

Assay of the mitochondrial, microsomal and soluble protein fractions obtained from a *Trebouxia* cell-free homogenate revealed that the enzyme was located mainly in the microsomal material and it is presumably a membrane-bound enzyme (Table 3). The 24-methylene sterol reductase of yeast has previously been reported to be a microsomal enzyme [20].

In the assay system developed for the measurement of *Trebouxia* microsomal 25-methylene reductase the production of poriferasterol from **1** was proportional to protein concentration up to 300–350 μg protein/ml (Fig. 1) and it was linear with incubation time up to *ca* 100–120 min. The microsomal reductase showed a pH optimum at *ca* pH 7.4 (Fig. 2).

The very efficient incorporation of (24*S*)-24-ethyl

Table 1. Incorporation of [6-$^3H_1$](24*S*)-24-ethylcholesta-5,22,25-trien-3β-ol into *Trebouxia* sterols

Steryl acetate	Culture 1 (dpm)	Culture 2 (dpm)	Culture 3 (dpm)
[6-$^3H_1$](24*S*)-24-Ethylcholesta-5,22,25-dien-3β-yl acetate	871 000	715 000	903 000
Poriferasteryl acetate	1 593 000	1 393 000	1 542 000
Clionasteryl acetate	10 500	1230	5750

Cultures of *Trebouxia* were grown for 14 days in the presence of [6-$^3H_1$](24*S*)-24-ethylcholesta-5,22,25-trien-3β-ol (**1**) and the steryl acetates recovered as described in the Experimental. The clionasteryl acetate fraction also contained the acetate of ergost-5-en-3β-ol which is a constituent of the *Trebouxia* sterol mixture [15], but which would not be expected to be labelled from **1**.

Table 2. Reduction of [6-$^3H_1$](24*S*)-24-ethylcholesta-5,22,25-trien-3β-ol by a cell-free 4000 *g* homogenate of *Trebouxia* to yield poriferasterol and clionasterol

	Poriferasterol	Clionasterol
Homogenate alone	890*	50*
Homogenate + NADPH	1240	40
Homogenate + NADPH generating system	1940	0
Boiled homogenate	0	9

Details are given in the Experimental. Incubations were for 90 min.
*Dpm in the steryl acetates recovered after silver nitrate–Si gel TLC.

Table 3. Incorporation of [6-$^3H_1$](24*S*)-24-ethylcholesta-5,22,25-trien-3β-ol into poriferasterol by sub-cellular fractions of a homogenate of *Trebouxia* cells

Incubation	20000 *g* pellet (Mitochondria)	20000 *g* supernatant	105000 *g* pellet (Microsomes)	105000 *g* supernatant
− NADPH	300*	460	450	110
+ NADPH	380	910	1420	17
+ NADPH generating system	100	1180	1280	0

Experimental details are given in the Experimental.
*Dpm recovered in poriferasteryl acetate obtained after silver nitrate–Si gel TLC.

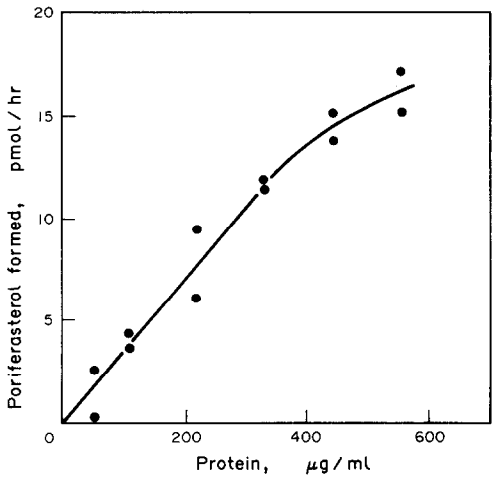

Fig. 1. Relationship between the concentration of microsomal protein and the production of poriferasterol (**1**) from [6-$^3H_1$](24*S*)-24-ethylcholesta-5,22,25-trien-3β-ol (**2**). The incubations were for 90 min and contained increasing amounts of microsomal suspension with 2 mg NADPH and 4 × 10^5 dpm of **1** in a final volume of 5 ml 0.1 M sodium phosphate buffer, pH 7.6.

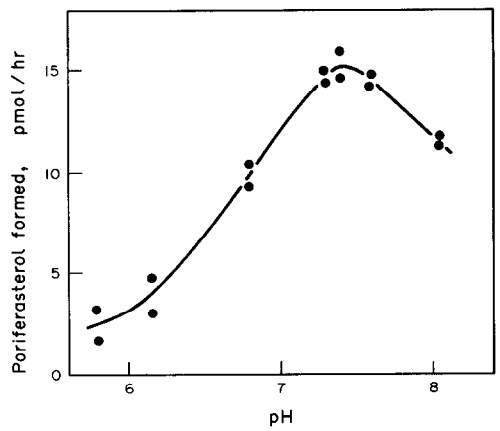

Fig. 2. Relationship between pH and *Trebouxia* microsomal 25-methylenene sterol reductase activity. The incubations contained microsomes (1.5 mg protein), 4 × 10^5 dpm of **2**, 2 mg NADPH and 0.1 M sodium phosphate buffer in a final volume of 5 ml. pH values were checked using a pH meter. Incubations were for 90 min.

cholesta-5,22,25-trien-3β-ol (**2**) into poriferasterol (**1**) *in vivo* by *Trebouxia* cells contrasts with the much lower transformation of (24*R*)-24-ethylcholesta-5,25-dien-3β-ol (**4**, clerosterol) into poriferasterol (**1**) observed in a previous study [19]. These results suggest that introduction of the Δ^{22}-bond precedes the reduction of the Δ^{25}-bond in the conversion of a 24-ethyl-25-methylene intermediate as suggested previously [18] and that clionasterol is not an obligatory precursor of poriferasterol. In this respect it is notable that a $\Delta^{22,24(28)}$-diene is the preferred substrate for the 24-methylene sterol reductase in yeast [20, 21].

EXPERIMENTAL

Trebouxia sp. 213/3, obtained from the Cambridge Culture Collection, was cultured in Bold's mineral medium containing 1% (w/v) peptone and 2% (w/v) glucose [22]. Flasks containing either 100 or 400 ml medium were inoculated with 4 or 6 ml *Trebouxia* sp. starter culture, respectively, and incubated for 10–14 days at 21° with continuous shaking and illumination.

Three 100 ml cultures of *Trebouxia* sp. 213/3 were inoculated with [6-$^3H_1$](24*S*)-24-ethylcholesta-5,22,25-trien-3β-ol in 0.2 ml EtOH (5.23 × 10^6 dpm/culture). After 14 days growth the cells were harvested and saponified by reflux with 8% (w/v) KOH in aq. 85% EtOH. Extraction with Et_2O gave the non-saponifiable lipid.

For preparation of the cell-free homogenate and cellular subfractions *Trebouxia* [23] cells were harvested by centrifugation, washed × 3 with ice-cold 0.1 M Na Pi buffer, pH 7.6, and resuspended in the same buffer at a concn of 70 ml/40 g wet cells. The suspension was mixed with 10 g glass beads (0.25–0.30 mm diam.) and shaken in a Braun Cell homogenizer (4000 oscillations/min) at *ca* 0° for 5 min. The resulting homogenate was then centrifuged at 4000 *g* for 20 min to provide a cell-free supernatant. For the preparation of the subcellular fractions this supernatant was centrifuged at 20000 *g* for 20 min to provide the mitochondrial pellet. The remaining supernatant was recentrifuged at 105000 *g* for 1 hr to give a microsomal pellet and the soluble protein fraction. The mitochondrial and microsomal pellets were resuspended, using a Potter–Elvehjem homogenizer, in 0.1 M Na Pi buffer, pH 7.6. For the investigations on pH-dependent activity the microsomal pellet was resuspended in a small vol. of 0.01 M Na Pi buffer, pH 7.6 and then diluted to final vol. and protein concn using 0.1 M Na Pi buffers of varying pH values.

The cell-free incubation mixtures contained homogenate (200–300 μg protein/ml), 4 × 10^5 dpm [6-$^3H_1$] (24*S*)-24-ethylcholesta-5,22,25-trien-3β-ol (**2**), 2 mg NADPH or a NADPH generating system (2 mg NADP, 5 mg glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase) and

10 μmol $MgCl_2$ in a final vol. of 5 ml 0.1 M Na Pi buffer. The incubation mixture was maintained at 28° for various times and the reaction was terminated by the addition of an equal vol. of 10% (w/v) KOH in aq. 75% EtOH. Saponification was achieved by heating to *ca* 70° for 30 min and then leaving the mixture to stand overnight at room temp. before dilution with H_2O and Et_2O extraction of the non-saponifiable lipid in the usual manner.

The sterols were obtained by TLC of the non-saponifiable lipid on Si gel developed with $CHCl_3$–EtOH (49:1). The sterols were acetylated (pyridine–Ac_2O) and separated into bands containing unchanged [6-$^3H_1$](24*S*)-24-ethylcholesta-5,22,25-trien-3β-yl acetate, poriferasteryl acetate and Δ^5-steryl acetate (clionasteryl and ergost-5-en-3β-yl acetates) by TLC on 10% $AgNO_3$–Si gel developed with EtOH free purified $CHCl_3$–Et_2O (99:1).

[6-$^3H_1$](24*S*)-24-Ethylcholesta-5,22,25-trien-3β-ol (**2**) (19.57 μCi/μmol) was prepared by the tritium labelling method described previously [24]. The (24*S*)-24-ethylcholesta-5,22,25-trien-3β-ol (**2**) required for this synthesis was isolated from the leaves of *Clerodendron campbellii* [25].

Protein concns were measured by the Lowry method [26].

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