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CONVERSION OF A 24β-ETHYL-25-METHYLENE INTERMEDIATE INTO PORIFERASTEROL BY *TREBOUXIA* SPECIES

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Key Word Index—*Trebouxia* sp.; Chlorococcales; chlorophyceae; clionasterol; poriferasterol; clerosterol; 25-methylene intermediate; sterol biosynthesis.

Abstract—Clerosterol-[26-^{14}C], a 24β-ethyl-25-methylene sterol [(24*S*)-24-ethylcholesta-5,25-dien-3β-ol], was incorporated into clionasterol and poriferasterol by cultures of the green algae *Trebouxia* sp. 213/3 and *Trebouxia* sp. 219/2. Degradation of the labelled poriferasterol showed that the ^{14}C retained its identity and was not incorporated as a result of metabolism of the clerosterol-[26-^{14}C] and randomisation of label. These results are consistent with the proposed production, and subsequent reduction, of a 24β-ethyl-25-methylene intermediate in 24β-ethyl sterol biosynthesis in algae of the order Chlorococcales.

INTRODUCTION

In the past 10 years much information has been obtained on the details of the transmethylation reactions responsible for the introduction of the 24-alkyl group into phytosterols [1–3]. From such studies it is now evident that the mechanisms of the reactions can vary in certain details in various classes of algae, fungi and higher plants thus leading to intermediates differing in the position of olefinic bonds and in the final configuration assumed by the C-24 alkyl group. For example in Chrysophyte algae and higher plants a 24-ethylidene sterol is produced which is then reduced to give either a 24β-ethyl or a 24α-ethyl sterol respectively [3–8]. In algae of the Order Chlorococcales the experimental evidence is consistent with the production of 24β-alkyl-25-methylene intermediates which are then reduced as outlined in Scheme 1 [9–12]. We have previously presented evidence for the methylation of a Δ^{24}-sterol (**1**) to give both a 25-methylene (**2**) and a 24-methylene (**3**) product using cell free preparations of *Trebouxia* sp. 213/3 and *Scenedesmus obliquus* [13]. The conversion of tritium labelled sterols with side chains **2** and **3** into 24β-methyl (**4**) and 24β-ethyl (**7** and **8**) sterols respectively, has also been demonstrated using cultures of *Trebouxia* sp. 213/3 and *Trebouxia* sp. 219/2 [10]. The involvement of a 24β-ethyl-25-methylene (**6**) sterol in the production of 24β-ethyl side chains (**7** and **8**) is based upon the observed incorporation of 5 deuterium atoms from methionine-[CD_3] in species of *Chlorella* and *Trebouxia* [9, 10]. To gain further support for the sequence **6** → **7** → **8** we now report the conversion of clerosterol-[26-^{14}C] (**9**)† into clionasterol (**10**) and poriferasterol (**11**) by cultures of *Trebouxia* sp. 213/3 and *Trebouxia* sp. 219/2.

RESULTS AND DISCUSSION

GLC analysis of the sterol mixtures isolated from the two cultures of *Trebouxia* used for the present work showed them to have sterol compositions similar to those reported previously [14]. The 3 major sterols were dihydrobrassicasterol (**12**), poriferasterol (**11**) and clionasterol (**10**) in the proportions of *ca* 35% of **12**, 55% of **11** and 10% of **10** in *Trebouxia* sp. 219/2 and *ca* 20% of **12**, 73% of **11** and 7% of **10** in *Trebouxia* sp. 213/3.

After culture of *Trebouxia* sp. 213/3 and *Trebouxia* sp. 219/2 with clerosterol-[26-^{14}C] the sterols were isolated, acetylated and separated by $AgNO_3$–Si gel TLC to give the acetates of poriferasterol (**8**), a mixture of clionasterol (**10**) and dihydrobrassicasterol (**12**), which co-chromatograph and unmetabolised clerosterol-[26-^{14}C] (**9**). Both the poriferasteryl acetate and clionasteryl acetate were labelled (Table 1); dihydrobrassicasteryl acetate, a 24β-methyl sterol, would not be expected to be labelled from clerosterol-[26-^{14}C]. In the 3 experiments performed the incorporation of ^{14}C into clionasterol (**10**) plus poriferasterol (**11**) was in the range 2–4% based upon the radioactivity recovered in the sterol fraction from the cells or 0.5–0.8% when based upon the clerosterol-[26-^{14}C] administered initially to the cultures. Addition of carrier poriferasteryl acetate to each of the labelled poriferasteryl acetate fractions recovered from the incubations followed by crystallisation to constant sp. radioact. confirmed the conversion of the clerosterol-[26-^{14}C] (**9**) into poriferasterol (**11**).

Since some radioactivity (<0.1%) was observed in materials of low polarity chromatographing on TLC in the region of squalene it was considered desirable to check for randomisation of the ^{14}C of the initial substrate (**9**) and reincorporation into the sterols by the *Trebouxia*

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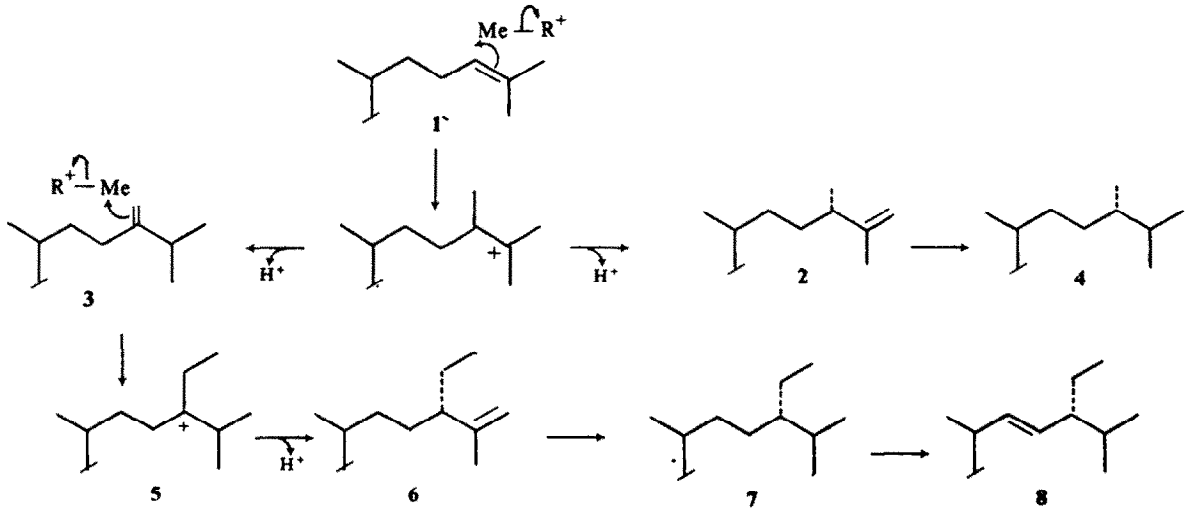
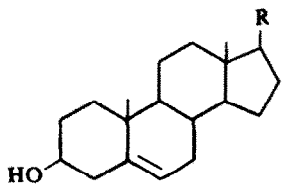
†Sterol nomenclature: clerosterol is (24*S*)-24-ethylcholesta-5, 25-dien-3β-ol; clionasterol is (24*S*)-24-ethylcholest-5-en-3β-ol; poriferasterol is (24*R*)-24-ethylcholesta-5, 22-dien-3β-ol.



Scheme 1. Proposed mechanisms for the elaboration of the side chains of sterols produced by algae of the Order Chlorococcales [3, 10].

cultures. Dilution of a sample of the labelled poriferasteryl acetate (14000 dpm) with stigmasteryl acetate, the 24α-epimer, followed by hydrolysis and Oppenauer oxidation [4] gave labelled 24-ethylcholesta-4,22-dien-3-one (10400 dpm) which was then treated with ozone to cleave the Δ^{22}-bond of the side chain. The recovered 3-oxo-bisnorchol-4-en-22-al was devoid of radioactivity thus indicating that only the side chain was labelled. It can be calculated from the chemical yield that *ca* 2400 dpm of ^{14}C would have been recovered in the aldehyde if the ^{14}C had been randomised and the poriferasterol uniformly labelled. It is therefore concluded that the label in the clerosterol-[26-^{14}C] retained its identity during the conversion of the sterol (**9**) into poriferasterol (**11**) and clionasterol (**10**).



9 R = side chain **6**
10 R = side chain **7**
11 R = side chain **8**
12 R = side chain **4**

Table 1. Specific radioactivities (dpm/mg) of clionasterol (**10**) and poriferasterol (**11**) recovered from *Trebouxia* spp. after culture with clerosterol-[26-^{14}C] (**9**)

		Clionasterol*	Porifera-sterol
Trebouxia sp. 213/3	Experiment 1	6280† (0.45)‡	3330 (1.8)
	Experiment 2	11900 (0.68)	5050 (2.4)
Trebouxia sp. 219/2		13800 (2.1)	2310 (1.7)

* The clionasterol (**10**), together with dihydrobrassicasterol (**12**) was separated from poriferasterol (**11**) by TLC of the acetates on $AgNO_3$–Si gel (Experimental). To determine the sp. act. of the clionasterol (**10**) the amount of this sterol in the mixture of **10** and **12** was estimated by GLC analysis.

† Dpm/mg.

‡ Values in parenthesis are the percentage incorporations of ^{14}C based upon the radioactivity recovered in the 4-desmethyl sterols.

In all 3 cultures the clionasterol (**10**) had a significantly higher sp. act. than the poriferasterol (**11**) (Table 1). This is in accord with the postulated precursor–product relationship for side-chains **7** and **8** indicated in Scheme 1. The present results are therefore in accord with the postulated involvement [3, 10] of a 24β-ethyl-25-methylene sterol (**6**) in clionasterol (**10**) and poriferasterol (**11**) biosynthesis in *Trebouxia* species. The identifications of 25-methylene sterols in *Chlorella* species [11, 12] indicates the probable generality of the mechanisms outlined in Scheme 1 in members of the Order Chlorococcales.

EXPERIMENTAL

Methods were generally as described previously [10, 14, 15]. *Trebouxia* sp. 213/3 and 219/2 were obtained from the Cambridge Culture Collection and grown on Bold's mineral medium containing 1% peptone and 2% glucose [13, 16]. Clerosterol-[26-^{14}C] (0.75 μCi/mg) was synthesised as described [15] and administered to *Trebouxia* cultures as a soln in 0.2 ml EtOH. GLC used a column of 3% OV-17 as in previous work [15].

Culture of Trebouxia *sp. 213/3 with clerosterol-[26-^{14}C].* (i) Three 100 ml cultures of *Trebouxia* sp. 213/3 were each injected with clerosterol-[26-^{14}C] (0.6 μCi) and the cells harvested after 30 days growth at 19°. Saponification with 8% KOH in 85% EtOH and extraction with Et_2O gave the non-saponifiable lipid which was subjected to PLC on Si gel developed with $CHCl_3$–EtOH (49:1) to provide fractions from the solvent front containing low polarity material including squalene (1680 dpm), and 4-desmethyl sterol (1.4×10^6 dpm). Acetylation (C_5H_5N–Ac_2O) gave the 4-desmethyl steryl acetates (13 mg, 1.25×10^6 dpm) which were separated by PLC on 10% $AgNO_3$–Si gel developed with $CHCl_3$–Et_2O (97:3) to yield clionasteryl plus dihydrobrassicasteryl acetates (R_f = 0.53, 1.85 mg, 3910 dpm), poriferasteryl acetate (R_f = 0.44, 4.7 mg, 15720 dpm) and unchanged clerosteryl acetate (R_f = 0.27, 8.64×10^5 dpm). Carrier poriferasteryl acetate (20 mg) was added to the radioactive sample and the mixture crystallised several times from MeOH (660, 670, 650, 660, 660 dpm/mg for successive crystallisations). (ii) Four 100 ml cultures of *Trebouxia* sp. 213/3 were each inoculated with 0.7 μCi clerosterol-[26-^{14}C]. The cells were harvested after 11 days growth and the non-saponifiable lipid (3.3×10^6 dpm) extracted in the usual manner followed by isolation of the fractions containing squalene in the materials of low TLC polarity (1500 dpm) and the 4-desmethyl sterols

(2.5×10^6 dpm). Acetylation (C_5H_5N–Ac_2O) gave the 4-desmethyl steryl acetates (19 mg, 2.3×10^6 dpm) which were subjected to PLC on 10% $AgNO_3$–Si gel ($CHCl_3$–Et_2O, 97:3) to yield clionasteryl plus dihydrobrassicasteryl acetate (3.3 mg, 1.11×10^4 dpm), poriferasteryl acetate (7.9 mg, 3.97×10^4 dpm) and unchanged clerosteryl acetate (1.59×10^6 dpm). Poriferasteryl acetate (24 mg) was added to the labelled material and the mixture crystallised from MeOH (1540, 1530, 1520, 1550, 1550, dpm/mg for successive crystallisations).

To check for randomisation of the ^{14}C a sample of the labelled poriferasteryl acetate (9 mg, 14000 dpm) was mixed with stigmasteryl acetate (181 mg) and the mixture hydrolysed to give the 3β-hydroxy sterol (152 mg). Oppenauer oxidation with Al isopropoxide as described previously [4] gave 24-ethylcholesta-4,22-dien-3-one (121 mg, 10400 dpm) which was purified by chromatography on Al_2O_3, grade III, and eluted with petrol–Et_2O mixtures. Treatment of a CH_2Cl_2 soln of 24-ethylcholesta-4,22-dien-3-one with O_3 [4] gave 3-oxobisnorchol-4-en-22-al (31 mg) which was purified by chromatography on Al_2O_3 grade III and eluted with petrol–Et_2O mixtures, mp 152–153°. Radioassay showed that this aldehyde contained no ^{14}C.

Culture of Trebouxia *sp. 219/2 with clerosterol-[26-^{14}C].*

Four 100 ml cultures of *Trebouxia* sp. 219/2 were each inoculated with 0.6 μCi clerosterol-[26-^{14}C]. After 11 days culture the non-saponifiable lipid was extracted and the fractions obtained containing squalene in the low polarity material (950 dpm), 4,4-dimethyl sterol (2780 dpm) and 4-desmethyl sterol (8.37×10^5 dpm). The poriferasteryl acetate was crystallised after addition of 21 mg carrier from MeOH (390, 380, 380, 380, 380 dpm/mg for successive crystallisations).

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