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STEREOCHEMISTRY OF C-4 DEMETHYLATION DURING CONVERSION OF OBTUSIFOLIOL INTO PORIFERASTEROL BY *OCHROMONAS MALHAMENSIS*

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Key Word Index—*Ochromonas malhamensis*; Chrysophyceae; obtusifoliol; poriferasterol; sterol biosynthesis; C-4 demethylation.

Abstract—Obtusifoliol-[2,2,4-$^3H_3$] was synthesised and incubated with the chrysophyte alga *Ochromonas malhamensis* which converted it into poriferasterol. A reaction sequence applied to poriferasterol showed that the tritium retained at C-4 occupied the 4α-position. This demonstrates that biological C-4 demethylation of a 4α-methylsterol precursor by *O. malhamensis* results in the axial 4β-hydrogen being inverted into the equatorial 4α-position of the 4-desmethyl sterol product.

INTRODUCTION

The C-4 demethylation of triterpene precursors during sterol formation has received considerable attention [1]. Demethylation proceeds by stepwise oxidation of the Me group (Me → CH_2OH → CHO → COOH) [2, 3], formation of the 3-ketone [4, 5] and then decarboxylation of the resulting β-keto acid [6]. It is now recognised that the 4α-Me group of lanosterol is the first to be removed during sterol biosynthesis in animals [7–9] and that the original 4β-Me group is epimerised into the 4α-position in the 4-mono Me sterol before it, in turn, is lost by oxidative decarboxylation.

In photosynthetic plants it has been established that the demethylation of the 4,4-di Me precursor cycloartenol, also proceeds with loss first of the 4α-Me group with epimerisation of the 4β-Me group to the 4α-position [10]. The present investigation was undertaken in an attempt to determine the stereochemical fate of the axial 4β-H of the 4α-Me sterol intermediate during the second C-4 demethylation sequence. The 4α-Me sterol obtusifiol (1) is a suggested intermediate in phytosterol biosynthesis [11]. In this paper we describe the conversion of obtusifoliol-[2,2,4-$^3H_3$] into poriferasterol (2) by the chrysophyte alga *Ochromonas malhamensis* and the determination of the configuration of the C-4 T atom in the poriferasterol. A preliminary report of this work has already been published [12].

RESULTS AND DISCUSSION

In order to investigate the stereochemistry of the demethylation of a 4α-mono Me sterol it was necessary to prepare a suitable precursor sterol labelled with tritium (T) at the 4β-position. For this purpose the phytosterol precursor obtusifoliol (1) was chosen. Oxidation using Jones reagent gave the 3-ketone; basic exchange with tritiated water and subsequent reduction then gave obtusifoliol-[2,2,4β-$^3H_3$]. Incubation of the chrysophyte alga *O. malhamensis* with the labelled obtusifoliol for 6 days resulted in a 4.5% incorporation into poriferasterol (2) which is the major sterol of this alga [13].

To determine the configuration of the T retained at C-4 of poriferasterol, a reaction was required to specifically remove one of the C-4 hydrogens. The simplest route envisaged was the *trans*-diaxial dehydration to a 5α-OH compound. Conversion of poriferasterol (2) to the 5α, 6α-epoxide (3) and opening of the epoxide by nucleophilic attack of chloride at C-6 gave the *trans*-diaxial chlorohydrin (4). The secondary 3β-OH group of 4 was then readily acetylated and the resulting chlorohydrin acetate (5) could undergo *trans*-diaxial dehydration by loss of the 4β-H [14] to give compound 6. Dehydration was not expected to yield the Δ^5-compound since the C-6 axial (β) position is blocked by the chloride substituent.

Because of the critical importance to the present work it was first necessary to establish unambiguously the stereochemistry of the dehydration of compound 5. It has been established by other workers [15, 16] that metal hydride reduction of compounds of type 6 proceeds by intramolecular attack at C-4 followed by rearrangement to yield the Δ^5-sterol (for example 2). Since hydride attack is on the β-face of the steroid ring system, this method has been utilised to prepare 4β-deuterated and and tritiated steroids, for example androst-5-en-3α, 17α-diol-[4β-$^2H_1$] [17]. In the present study stigmasta-5,22-dien-3β-ol-[4β-$^2H_1$] (C-24 epimer of 2) was prepared (see Experimental) and taken through the reaction sequence outlined above. Dehydration of the derived chlorohydrin acetate followed by conversion back to stigmasta-5, 22-dien-3β-ol resulted in the loss of the 4β-D thus confirming the *trans*-dehydration of compound 5.

To enable the loss of T to be more easily determined during the reaction sequence, the labelled poriferasterol obtained from the incubation with obtusifoliol-[2,2,

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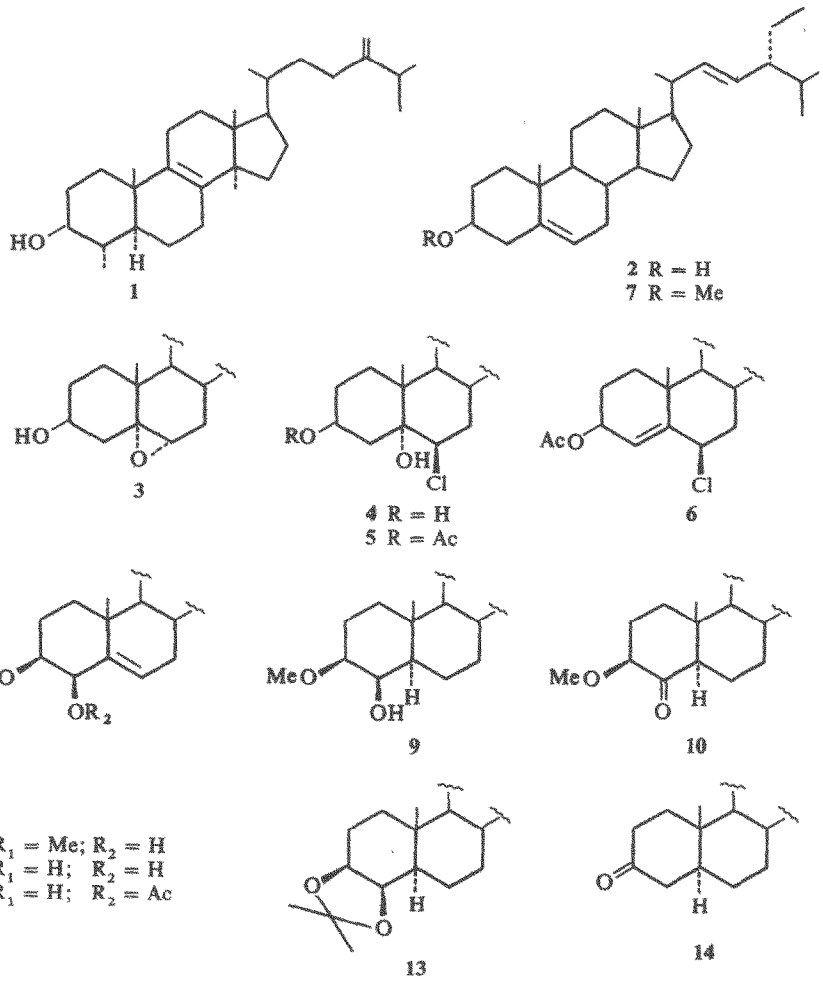


4-$^3H_3$] was mixed with poriferasterol-[1,7,15,22, 26-$^{14}C_5$]; any loss of T then being detectable by a change in the $^3H:^{14}C$ ratio. When the reactions outlined above were performed on the dual labelled poriferasterol the $^3H:^{14}C$ ratios of the products did not change (Table 1). These results can be interpreted in two ways: either the T originally present at C-4 on the precursor (1) was now all at C-4α in compound 2, or alternatively T was no longer at C-4 of the biosynthesised poriferasterol (2). To determine the presence or absence of T at C-4 of the poriferasterol (2) several routes were investigated in an attempt to functionalise C-4. Those approaches that involved initial oxidation at C-3 were avoided because of the possibility of enolisation and thus loss of T at C-4. Conversion of the labelled poriferasterol to the Me ether (7) followed by SeO_2 oxidation gave the 4β-OH derivative (8). Reduction of the $\Delta^{5,22}$ bonds of compound 8 to give 9, followed by oxidation, yielded the desired 4-oxo compound (10). These reactions produced a fall in the $^3H:^{14}C$ ratio (Table 2) thus confirming the retension of T at C-4 in the biosynthesised poriferasterol (2). The same SeO_2 oxidation of stigmasta-5,22-dien-3β-ol (2, 24α-) gave a

Table 1. Degradation of poriferasterol [(24*R*)-24-ethylcholesta-5,22-dien-3β-ol] to eliminate the 4β-hydrogen atom

	Experiment 1		Experiment 2	
	Specific radioactivity ^{14}C dpm/μmol	$^3H:^{14}C$ ratio	Specific radioactivity ^{14}C dpm/μmol	$^3H:^{14}C$ ratio
Poriferasterol (2)	226	4.27	174	9.18
5α,6α-Epoxy-(24*R*)-24-ethylcholest-22-en-3β-ol (3)	225	4.40	153	9.03
6β-Chloro-(24*R*)-24-ethylcholest-22-en-3β,5α-diol (4)	218	4.54	144	9.02
5α-Hydroxy-6β-chloro-(24*R*)-24-ethylcholest-22-en-3β-yl acetate (5)	202	4.36	157	8.91
6β-Chloro-(24*R*)-24-ethylcholesta-4,22-diene-3β-yl acetate (6)	—	—	159	9.00
(24*R*)-24-Ethylcholesta-5,22-diene-3β-ol (2) (regenerated from 6)	209	4.10	169	9.11

Table 2. Degradation of poriferisterol [(24*R*)-24-ethylcholesta-5,22-dien-3β-ol] to remove the C-4 hydrogen atoms

	Experiment 2				Experiment 3*	
	Specific radioactivity ^{14}C dpm/μmol		^{3}H:^{14}C ratio		Specific radioactivity ^{14}C dpm/μmol	^{3}H:^{14}C ratio
Poriferasterol (2)	174		9.18		835	1.52
(24*S*)-24-Ethylcholestan-3β-ol	163		9.51		—	—
(24*S*)-24-Ethylcholestan-3-one (14)						
(a) Initial	181		9·28		—	—
(b) After basic equilibration	156		1.30		—	—
	(a)	(b)	(a)	(b)		
(24*R*)-24-Ethylcholesta-5,22-diene-3β-ol-methyl ether (7)	187	178	8.82	8.92	828	1.53
(24*R*)-24-ethylcholesta-5,22-diene-3β,4β-diol-3β-methyl ether (8)	172	167	8.95	8.99	697	1.54
(24*S*)-24-ethylcholestane-3β,4β-diol 3β-methyl ether (9)	181	—	8.86	8.79	752	1.48
3β-Hydroxy-(24*S*)-24-ethylcholestan-4-one-3β-methyl ether (10)	154	—	7.87	7.82	715	1.28

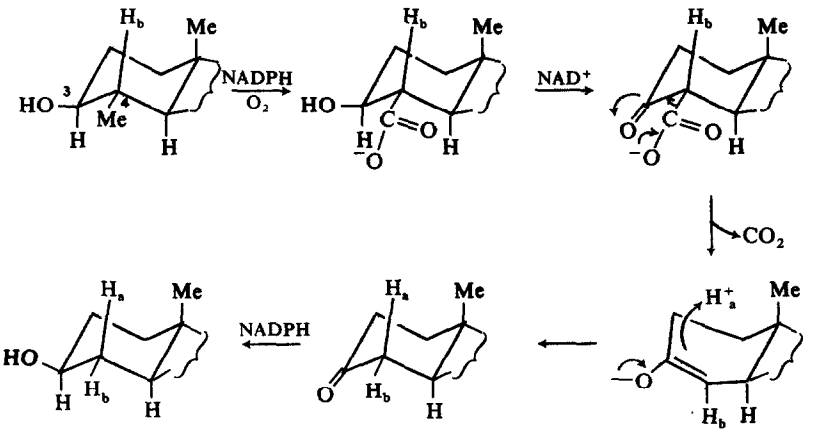
*The poriferasterol (2) used in this experiment was obtained from an incubation of *O. malhamensis* with 24-ethylidenelophenol-[2,2,4-$^{3}H_3$] [see Ref. 21].

product which was identical with the diol (11) formed by hydrolysis of the 4β-acetoxy-stigmasta-5,22-dien-3β-ol (12) produced by bromine–silver acetate treatment of stigmasta-5,22-dien-3β-ol (2, 24α-). The C-4 OH group of 11 was established as axial (4β-) by PMR and by the formation of an acetonide (13). The 4β- configuration for the C-4 OH group introduced by SeO_2 oxidation of the cholestane series has also been reported [18]. The fact that the 4β-hydroxy-(24*R*)-24-ethylcholesta-5,22-dien-3β-methyl ether (8) had the same ^{3}H:^{14}C ratio as the starting poriferasterol (2) provides further confirmation that the T at C-4 occupies the 4α-position.

A portion of the labelled poriferasterol (2) was reduced to the 5α-stanol followed by oxidation to the 3-oxo compound (14) and equilibrated with base (Table 2). The recovered stanone (14) had lost *ca* 85% of its initial T thus showing that the bulk of the T of the precursor obtusifoliol-[2, 2, 4-$^{3}H_3$] retained its location at C-2 and C-4 rather than entering newly synthesised sterols by a degradation-randomisation process.

The results of the present experiments demonstrate that the T atom retained at C-4 in poriferasterol (2) was in the equatorial 4α-position. Since this atom was in the axial 4β-position of the precursor obtusifoliol (1) there must have been an inversion, most probably during the C-4 demethylation step. Qualitatively there appeared to be less T present at C-4 of the poriferasterol (2) than was theoretically expected. Assuming uniform labelling at C-2 and C-4 during the preparation of the obtusifoliol-[2,2,4-$^{3}H_3$], up to 33% of the label in obtusifoliol (1), and hence, poriferasterol (2), would be expected at C-4. However, in the degradations of labelled poriferasterol (2) only 10–16% of the T was lost on conversion of compound 9 into compound 10. Two possibilities can be advanced to explain this observation. First some of the C-4 H may have been exchanged during the biological demethylation reaction at the enolate or 3-ketone stage. Secondly, the incorporation of less T at C-4 than at C-2α and C-2β may have resulted from the incomplete enolisation towards the C-4 position during the initial labelling of the obtusifolione to prepare obtusifoliol-[2,2, 4-$^{3}H_3$]. This might in fact be expected since the C-4 position is more substituted and thus it is the less stable carbanionic centre, thus perhaps directing enolisation more towards C-2.

The H atoms introduced at C-4 during sterol demethy-



Scheme 1. Suggested mechanism for the demethylation of a 4α-methylsterol. H_b was labelled with tritium in the experiments described using obtusifoliol-[2,2,4β-$^{3}H_3$],

lation originate from the protons of the medium [19]. A mechanism is presented in Scheme 1 for 4α-demethylation which is compatible with the intermediate formation of the 3-ketone required for C-4 demethylation, loss of the Me group as CO_2 and introduction of a proton from the medium with inversion of configuration of the existing C-4 H atom.

EXPERIMENTAL

General methods. Mp's were determined on a Reichart hot-stage apparatus and are uncorr. IR spectra were obtained in KBr discs. PMR spectra were measured in $CDCl_3$ with TMS as internal standard. TLC was on Si gel developed with the following. Solvent 1, $CHCl_3$–EtOH (49:1); Solvent 2, petrol–Et_2O (93:7); Solvent 3: $CHCl_3$–EtoAc (13:7). Spots were detected with 20% aq. H_2SO_4 spray followed by heating to 120°. For PLC, bands were located by spraying with Rhodamine 6G in Me_2CO and viewing under UV light. GLC employed 1.52 m × 0.6 cm columns of 3% OV-17 or 3% SE-30 on Gas Chrom Q, column temp. 245° or 260°, Ar 60 ml/min. For smaple trapping, a splitter with 1:25 split ratio was used. Samples were trapped at 1 min intervals in capillary tubes at room temp. and washed into scintillation counting vials with Et_2O. Radioactivity was measured by scintillation counting using 5% PPO in toluene.

Preparation of obtusifoliol-[2,2,4-$^3H_3$] (1). Obtusifoliol (**1**, 24 mg) was oxidised with Jones reagent [20] and the obtusifolione purified by TLC (solvent 1) and crystallised from Me_2CO to give needles, IR ν_{max}^{KBr} cm^{-1}: 1740 (ketone); MS *m/e* (rel. int.): 424 (M^+, 60), 409 (100), 395 (9), 381 (20), 325 (36), 311 (26), 257 (42), 243 (94) and 231 (62). A portion of the ketone was refluxed for 30 min with D_2O (1ml) and dioxan (4 ml) containing a small pellet of Na. After TLC purification MS showed the exchange of 3 H atoms at C-2α, C-2β and C-4β with D.MS *m/e* (rel. int.): 427 (M^+, 57°), 412 (100), 398 (8), 383 (12), 328 (19), 314 (14) and 260 (16). T labelled material was prepared by basic exchange of the ketone (7 mg) in ^3H_2O (1 Ci) and dioxan (3 ml) as in ref. [21]. The labelled ketone was reduced with $NaBH_4$ in MeOH and purified by TLC (Solvent 1) to give obtusifoliol-[2,2,4-$^3H_3$] (67μCi/mg). Radiochemical purity was established by GLC with sample trapping, which showed only one radioactive compound that co-chromatographed with the mass-peak of obtusifoliol (**1**). There was no change in sp. act. during crystallisation of a portion of the tritiated material with carrier obtusifoliol (6780, 6980, 6590 dpm/mg for successive crystallisations).

Administration of obtusifoliol-[2,2,4-$^3H_3$] to Ochromonas malhamensis. Obtusifoliol-[2,2,4-$^3H_3$] (30μCi) in 0.3 ml EtOH was added to 3 × 100 ml cultures of the alga [21]. After 6 days the nonsaponifiable lipid was extracted [21] and the poriferasterol (**2**, 22 mg, 3.00×10^6 dpm) purified by PLC on Si gel (Solvent 1) followed by PLC on 10% $AgNO_3$-Si gel developed with hexane–C_6H_6 (1:1). The radiochemical purity of the labelled poriferasterol (**2**) was determined by GLC with sample trapping. Most of the radioactivity (92%) co-chromatographed with the mass peak of poriferasterol (**2**). There was a small amount of radioactivity (4%) which chromatographed in the region of brassicasterol (ergosta-5,22-dien-3β-ol).

5α,6α-Epoxy-(24R)-24-ethycholest-22-en-3β-ol (**3**). Poriferasterol-[1,7,15,22,26-$^{14}C_5$], biosynthesised by *O. malhamensis* cultured in the presence of mevalonic acid-[2-^{14}C] (kindly provided by Dr. G. Beastall) was added to the tritiated poriferasterol to obtain the desired ^3H:^{14}C ratio (Experiment 1, 4.27:1; Experiment 2, 9.19:1). Additional carrier poriferasterol (200 mg) was added to give a sp. act. of 200 dpm ^{14}C/mol. The 5α,6α-epoxide was formed by stirring a portion of the dual-labelled poriferasterol (49 mg) with *m*-chloroperbenzoic (20 mg) in $CHCl_3$ (10 ml) at 27° for 2 hr. Dilution with $CHCl_3$, followed by successive washes with 10% aq. $NaHCO_3$ and H_2O, drying and removal of solvent gave **3** (31 mg, 61%), after purification by TLC (Solvent 3), needles from MeOH, mp 153–154°; MS *m/e* (rel. int.): 428 (M^+, 100), 413 (8), 410 (40), 395 (8), 385 (14), 367 (70), 349 (19), 316 (40), 287 (40), 271 (64) and 253 (30); PMR ($CDCl_3$): δ 1.06 (3H, *s*, C-19, calculated [22] for 5α,6α-oxide 1.075, for 5β,6β-oxide 0·867), 2.88 (1H, *d*, *J* = 4 Hz), 5.05 (2H, *m*, C-22, C-23).

6β-Chloro-(24R)-24-ethycholest-22-en-3β,5α-diol (**4**). The epoxide (**3**, 31 mg) was dissolved in $CHCl_3$ and dry HCl gas passed through the soln for 30 min [23]. The soln was diluted with $CHCl_3$, washed with 10% aq. $NaHCO_3$ and H_2O and solvent removed to give the *trans*-diaxial chlorohydrin (**4**), fine needles from Et_2O–petrol (21 mg), mp 176–177° decomp.); IR ν_{max}^{KBr} cm^{-1}: 3560 (–OH); MS *m/e* (rel. int.): 466 (M^+ for ^{37}Cl, 3), 464 (M^+ for ^{35}Cl, 8), 428 (100), 410 (44), 385 (18), 376 (77), 323 (12), 316 (68) 287 (58), 271 (94), 253 (53); PMR ($CDCl_3$); 3.79 (1H, *m*, C-6), 5.05 (2H, *m*, C-22, C-23).

5α-Hydroxy-6β-chloro-(24R)-24-ethycholest-22-en-3β-yl acetate (**5**). The chlorohydrin (**4**, 20 mg) was dissolved in C_5H_5N (1 ml) and Ac_2O (1 ml) and warmed at 100° for 30 min. The chlorohydrin acetate (**5**) was obtained in the usual manner and crystallised as needles from MeOH (19 mg), mp 203–206° (decomp.); IR ν_{max}^{KBr} cm^{-1}: 3430, 1742, 1710; MS *m/e* (rel. int.); 508 (M^+, 8); 506 (M^+ 18), 470 (42), 452 (10), 410 (100), 394 (30), 392 (48), 385 (32), 367 (52), 349 (84), 329 (35), 289 (20), 271 (24), 253 (64).

6β-Chloro-(24R)-24-ethylcholesta-4,22-diene-3β-yl acetate (**6**). The chlorohydrin acetate (**5**, 19 mg) was dissolved in 5 ml C_5H_5N and cooled to 0°. 0.5 ml $SOCl_2$, freshly purified [24], was added dropwise over a 5 min period. The yellow soln was stirred for an additional 15 min and then ice was carefully added. The soln, was partitioned between Et_2O and 10% aq. HCl and the Et_2O layer washed with H_2O, dried and evaporated to dryness. TLC (Solvent 2) gave **6** (11·4 mg). Although other workers [25] have reported crystallisation of similar allylic chlorides from MeOH we found that **6** was unstable and even mild treatment with pure MeOH gave 6β-methoxy-(24*R*)-24-ethylcholesta-4,22-dien-3β-yl acetate and a small amount of 4ξ-methoxy-(24*R*)-24-ethylcholesta-5,22-dien-3β-yl acetate. Compound **6** had MS *m/e* (rel. int.); 490 (M^+, 1) 488 (M^+, 4), 465 (1), 463 (3), 452 (35) 392 (100), 349 (30), 253 (49).

Reduction of **6** *to poriferasterol* (**2**). **6** (11 mg) was dissolved in Et_2O and $LiAlH_4$ in Et_2O added [15]. The product was purified by TLC (Solvent 1) to give poriferasterol (**2**, 6.6 mg), needles from MeOH, mp 153°, fully characterised by TLC, GLC, IR, MS and PMR

Preparation and enolisation of (24S)-24-ethylcholestan-3-one (**14**). Poriferasterol (**2**, 52 mg, ^3H:^{14}C = 9.18:1; 174 dpm of ^{14}C/μmol) was reduced with H_2 over PtO_2 in EtOAc and the product purified by TLC (Solvent 1) to give (24*S*)-24-ethylcholestan-3β-ol (43 mg), plates from MeOH, mp 124°; MS *m/e* (rel. int.): 416 (M^+; 100), 401 (14), 233 (32), 215 (18); PMR: no olefinic reasonance. The stanol was oxidised with Jones reagent [20] to give (24*S*)-24-ethylcholestan-3-one (**14**, 17.2 mg), long plates from MeOH, mp 154–156°; IR ν_{max}^{KBr} cm^{-1}: 1718 (ketone); MS *m/e* (rel. int.): 414 (M^+, 100) 399 (10), 246 (12) and 321 (90). The ketone (**14**, 8 mg) was refluxed for 30 min with a small piece of Na in dioxan (3 ml) and D_2O (1 ml). The exchanged ketone (**14**) was purified by TLC (Solvent 1), MS *m/e* (rel. int.): 418 (100), 417 (30), 416 (14), 415 (4) and 414 (1).

(24R)-24-Ethylcholesta-5,22-diene-3β-methyl ether (**7**). The dual labelled poriferasterol (**2**, 100 mg; ^3H:^{14}C = 9.19:1; 174 dpm of ^{14}C/μmol) was dissolved in C_5H_5N (5 ml) and freshly crystallised *p*-toluenesulphonyl chloride (400 mg) was then added. The mixture was left in the dark at 27° for 12 hr and then poured into Et_2O and washed successively with H_2O, 10% aq. HCl, 10% $NaHCO_3$ soln, H_2O and then dried and the solvent removed *in vacuo*. The crystalline steryl tosylate recovered was refluxed in dry MeOH for 2 hr. Crystallisation of the product from MeOH gave plates of **7** (58 mg), mp 118–119°; MS *m/e* (rel. int.): 426 (M^+, 100), 411 (8), 394 (34) 351 (32), 314 (24), 285 (20) and 255 (46); PMR ($CDCl_3$): δ 3.25 (3H, *s*, —OMe), 5.05 (2H, *m*, C-22, C-23) and 5.3 (1H, *m*, C-6).

(24R)-4β-Hydroxy-24-ethylcholesta-5,22-diene-3β-methyl ether (**8**). The Me ether (**7**, 87 mg) in C_6H_6 (5 m.) was added to a soln of SeO_2 (44 mg) in HOAc (10 ml) and H_2O (1 ml) at 80° [26, 27]. The mixture was refluxed for 1 hr then 300 mg NaOAc

added and the refluxing continued for an additional 15 min. The mixture was then filtered, the soln poured into NaCl soln and the product obtained by extraction into Et_2O. Purification by TLC (Solvent 3) gave the 4β-hydroxy compound (**8**, 16.4 mg), microcrystals from MeOH (10.5 mg); mp 170–172°; IR ν_{max}^{KBr} cm^{-1}: 3600 (—OH), 968 (*trans*-Δ^{22} bond); MS *m/e* (rel. int.): 442 (M^+, 100), 427 (22), 424 (13), 412 (18), 410 (38), 384 (60), 366 (10), 285 (8), 271 (12) and 253 (10); PMR ($CDCl_3$): δ 3.37 (3H, *s*, —OMe), 4.20 (1H, *d*, *J* = 4Hz, C-4), 5.09 (2H, *m*, C-22, C-23) and 5.65 (1H, *m*, C-6). Unchanged Me ether (**7**, 65 mg) was recovered from the reaction mixture and oxidised again by the same procedure to give a second sample of **8**.

(24S)-24-ethylcholestane-3β,4β-diol-3β-methyl ether (**9**). The diene (**8**, 16 mg) was hydrogenated in EtOAc with Pt_2O. Purification by TLC (Solvent 3) gave the satd compound **9**, 14 mg), plates from MeOH–H_2O; mp 151–153°; MS *m/e* (rel. int.): 446 (M^+, 100), 431 (12), 428 (18), 412 (26), 386 (44), 263 (32), 243 (18), 232 (18), 215 (20) and 213 (26).

3β-Hydroxy-(24S)-24-ethylcholestan-4-one-3β-methyl ether (**10**). The Me ether **9** (11 mg) was carefully oxidised with Jones reagent at 0° with stirring for 10 min [27]. Purification by TLC (Solvent 1) gave the 4-ketone (**10**), plates from MeOH–H_2O (9.6 mg), mp 59°; IR ν_{max}^{Nujol} cm^{-1}: 1730 (ketone); MS *m/e* (rel. int.): 444 (M^+, 100), 429 (6); 426 (8), 414 (34), 412 (10), 386 (8), 371 (22), 276 (8) and 261 (11).

4β-Acetoxy-(24S)-24-ethyl-cholesta-5,22-diene-3β-ol (**12**). Stigmasterol (412 mg) was dissolved in dry $CHCl_3$ (20 ml). Br_2 (0.6 ml) dissolved in $CHCl_3$ (1 ml) was then added and the mixture stirred for 10 min. After removal of the solvent *in vacuo*, the impure steryl dibromide was dissolved in Et_2O (3 ml) and C_5H_5N (2 ml) to which was then added Ag acetate (400 mg) [28]. The soln was left in the dark for 10 min, and the black ppt. was removed by filtration. After the addition of H_2O and Et_2O, the Et_2O layer was washed with 10% aq. HCl and H_2O then dried and solvent removed to leave a brown solid (232 mg). Purification by TLC (Solvent 1) gave **12**, fine needles from MeOH (221 mg), mp 142–145°; IR ν_{max}^{KBr} cm^{-1}: 1750 (acetate), 1720 (intramolecular H bonding); MS *m/e* rel. int.): 470 (M^+, 8), 452 (12), 410 (100), 367 (42), 298 (28), 271 (48), 269 (40) and 253 (14); PMR ($CDCl_3$): δ 2.04 (3H, *s*, acetate), 3.6 (1H, *m*, C-3α), 5.05 (2H, *m*, C-22, C-23), 5.35 (1H, *d*, *J* = 2Hz, C-4α) and 5.80 (1H, *m*, C-6).

(24S)-24-Ethylcholesta-5,22-diene-3β,4β-diol (**11**). (a) From **12**. The 4β-acetate (**12**, 210 mg) was hydrolysed by reflux with 12% KOH in 80% aq. EtOH. The diol was purified by TLC (Solvent 3) to give **11**, feathery needles from MeOH–H_2O, mp 182–184°; IR ν_{max}^{KBr} cm^{-1}: 3600 (—OH), 980 (*trans* Δ^{22}-bond); MS *m/e* (rel. int.): 428 (M^+, 100), 413 (22), 410 (44), 395 (9), 384 (54), 367 (42), 316 (22), 287 (38), 271 (72), 269 (24), and 253 (28); PMR $CDCl_3$): δ 3.55 (1H, *m*, C-3α), 4.15 (1H, *d*, *J* = 4Hz, -4α), 5.10 (2H, *m*, C-22, C-23), and 5.70 (1H, *m*, C-6). The diol (15 mg) was refluxed in 10 ml dry Me_2CO with 30 mg dry $CuSO_4$ for 1 hr. Purification of the product by TLC (Solvent 2) gave the acetonide (**13**), needles from petrol, mp 130°; IR ν_{max}^{KBr} cm^{-1}: 978 (*trans* Δ^{22}-bond); MS *m/e* (rel. int.): 486 (86), 453 (74), 410 (100), 366 (13), 312 (18) and 270 (14); PMR ($CDCl_3$): δ 1.38 and 1.55 (3H each, *s*, acetonide Me groups), 4.15 (1H, *m*, C-3α), 4.48 (1H, *d*, *J* = 6Hz, C-4α), 5.15 (2H, *m*, C-22, C-23) and 5.85 (1H, *m*, C-6). (b) From SeO_2 oxidation of stigmasterol. Stigmasterol (100 mg) in dry C_6H_6 (10 ml) was added to a refluxing soln of SeO_2 (50 mg) as described above. Purification of the diol by TLC (Solvent 3) gave **11**, needles (23 mg) from MeOH–H_2O; mp 182–184°. This compound was identical with that formed from **12** as shown by IR, MS and PMR and it also formed an identical acetonide (**13**).

Preparation and degradation of stigmasterol-[4β-$^2H_1$]. 6β-Chloro-(24S)-24-ethylcholesta-4, 22-dien-3β-yl acetate (103 mg, prepared from stigmasterol) was added to a slurry of $LiAl_2H_4$ (100 mg) in dry Et_2O. Purification of the product by TLC (Solvent 1) gave stigmasterol-[4β-$^2H_1$] (23 mg); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2450 (C—^{2}H stretch), 980 (*trans* Δ^{22}-bond; MS *m/e* 413 one D). The stigmasterol-[4β-$^2H_1$] was stirred with *m*-chloroperbenzoic acid in $CHCl_3$ and the 5α,6α-epoxide (13 mg) purified by TLC (Solvent 3), MS *m/e*: 427 (M^+). Formation of the *trans* diaxial chlorohydrin (*m/e* 429 and 431) and acetylation then gave the chlorohydrin acetate (14 mg, *m/e* 507 and 509). Dehydration with $SOCl_2$, followed by $LiAlH_4$ reduction and purification by TLC (Solvent 1), gave stigmasterol (5.3 mg). MS showed the absence of D (*m/e* 412) compatible with the *trans*-dehydration of the 4β-dueterochlorohydrin acetate. For final confirmation, *ca* equal amounts of the starting stigmasterol-[4β-$^2H_1$] and the recovered stigmasterol were combined and the mixture analysed by MS which showed ions of about equal intensity at *m/e* 412 and 413.

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REFERENCES

1. Gaylor, J. L. (1972) *Adv. Lipid Res.* **10**, 89.
2. Olsen, J. A., Lindberg, M. and Bloch, K. (1957) *J. Biol. Chem.* **226**, 941.
3. Miller, W. L., Kalafer, M. E., Gaylor, J. L. and Delwiche, C. V. (1967) *Biochemistry* **6**, 2673.
4. Lindberg, M., Gautschi, F. and Bloch, K. (1963) *J. Biol. Chem.* **238**, 1661.
5. Swindell, A. C. and Gaylor, J. L. (1968) *J. Biol. Chem.* **243**, 5546.
6. Rahimtula, A. and Gaylor, J. L. (1972) *J. Biol. Chem.* **247**, 9.
7. Sharpless, K. B., Snyder, T. E., Spencer, T. A., Maheshwari, K. K., Gahn, G. and Clayton, R. B. (1968) *J. Am. Chem. Soc.* **90**, 6874.
8. Sharpless, K. B., Snyder, T. E., Spencer, T. A., Maheshwari, K. K., Nelson, J. A. and Clayton, R. B. (1969) *J. Am. Chem. Soc.* **91**, 3394.
9. Rahman, R., Sharpless, K. B., Spencer, T. A. and Clayton R. B. (1970) *J. Biol. Chem.* **245**, 2667.
10. Ghisalberti, E. L., de Souza, N. J., Rees, H. H., Goad, L. J. and Goodwin, T. W. (1969) *Chem. Commun.* 1403.
11. Goad, L. J. and Goodwin, T. W. (1972) *Prog. Phytochemistry* **3**, 113.
12. Knapp, F. F., Goad, L. J. and Goodwin, T. W. (1973) *Chem. Commun.* 149.
13. Gershengorn, M. C., Smith, A. R. H., Goulston, G., Goad, L. J., Goodwin, T. W. and Haines, T. (1968) *Biochemistry* **7**, 1968.
14. Kirk, D. N. and Hartshorn, M. P. (1968) *Steroid Reaction Mechanisms*. Elsevier, Amsterdam.
15. Ireland, R. T., Wrigley, T. I. and Young, W. G. (1959) *J. Am. Chem. Soc.* **81**, 2818.
16. Collins, D. J. and Hobbs, J. J. (1964) *Australian J. Chem.* **17**, 677.
17. Malhotra, S. K. and Ringold, H. J. (1965) *J. Am. Chem. Soc.* **87**, 3228.
18. Campion, T. H. and Morrison, G. A. (1973) *Tetrahedron* **29**, 239.
19. Akhtar, M., Rahimtula, A. D. and Wilton, D. C. (1969) *Biochem. J.* **114**, 801.
20. Bowden, K., Heilbron, I. M., Jones, E. R. and Weedon, B. C. L. (1946) *J. Chem. Soc.* 39.
21. Lenton, J. R., Hall, J., Smith, A. R. H., Ghisalberti, E. L., Rees, H. H., Goad, L. J. and Goodwin, T. W. (1971) *Arch. Biochem. Biophys.* **143**, 664.
22. Bhacca, N. S. and Williams, D. H. (1964) *Applications for NMR spectroscopy in Organic Chemistry*. Holden Day, San Francisco.
23. Baxter, R. A. and Spring, F. S. (1943) *J. Chem. Soc.* 613.
24. Vogel, A. I. (1948) *Practical Organic Chemistry*. Longman Green, London.
25. Spring, F. S. and Swain, G. (1939) *J. Chem. Soc.* 1356.
26. Rosenheim, O. and Starling, W. W. (1937) *J. Chem. Soc.* 377.
27. Brown, B. R. and Sanbach, D. M. L. (1963) *J. Chem. Soc.* 5313.
28. Petrow, V. A., Rosenheim, O. and Starling, W. W. (1943) *J. Chem. Soc.* 135.