Mechanism of the Transmethylation Reaction by *S*-Adenosylmethionine: Stereochemistry of Hydride Migration from C-24 to C-25 in the Biosynthesis of Poriferasterol in the Crysophyte *Ochromonas malhamensis*

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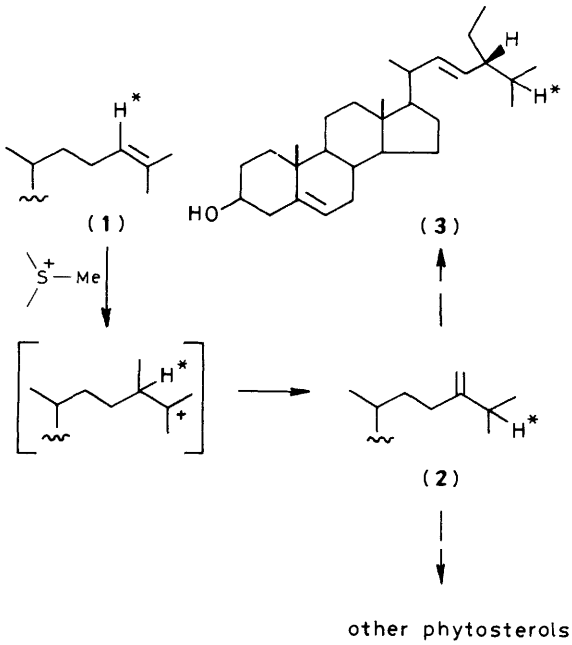
Administration of sodium [2-$^2H_3$]acetate to the crysophyte *Ochromonas malhamensis* yielded [$^2H_{22}$]poriferasterol, from which, by chemical degradation, the C(24)–C(29) fragment of the side chain was obtained as (2*S*,3*R*)-2-[$^2H_2$]methyl[1,1,1,2-$^2H_4$]pentan-3-ol. ^2H N.m.r. analysis of this deuteriated compound shows that the 1,2-hydride migration from C-24 to C-25 engages the *si*-face of the Δ^{24}-sterol double-bond, and suggests that *S*-adenosylmethionine approaches the same double bond from the *re*-face.

The mechanism of the most significant step in phytosterol biosynthesis, that is the introduction of the 24-alkyl group into the sterol side-chain, implies attack by *S*-adenosylmethionine on a double-bond sterol intermediate (**1**).[1-3]

The stereochemistry of the attack by *S*-adenosylmethionine can be directly deduced from the configuration at C-24 only in the case of those 24-methylphytosterols in which the asymmetric centre at C-24, created by the introduction of the methyl group, is not further involved in the following biosynthetic steps.

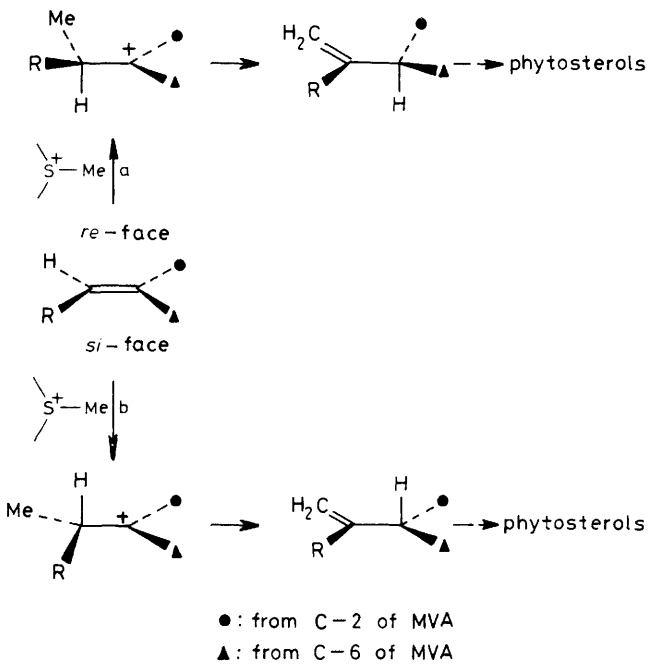
However, most phytosterols are formed through the intermediacy of a 24-methylene compound (**2**), which originates, after the electrophylic methylation, by 1,2-hydride migration from C-24 to C-25 and proton loss from C-28 (Scheme 1). In



Scheme 1.

these cases, the stereochemistry of *S*-adenosylmethionine attack cannot be directly deduced from the configuration at C-24; indirect information can, however, be obtained from the stereochemistry of the 1,2-hydride migration, since these two processes are stereochemically linked.[2,3]

The 1,2-hydride migration from C-24 to C-25 can follow two opposite trajectories, leading to the two possible stereochemistries at C-25 (Scheme 2). The steric course actually followed seems different depending on the organism. In fact, in higher plants[2,4] the 24-H→25-H migration occurs on the *si*-face of the 24-double bond (case a in Scheme 2), whereas in the fungus



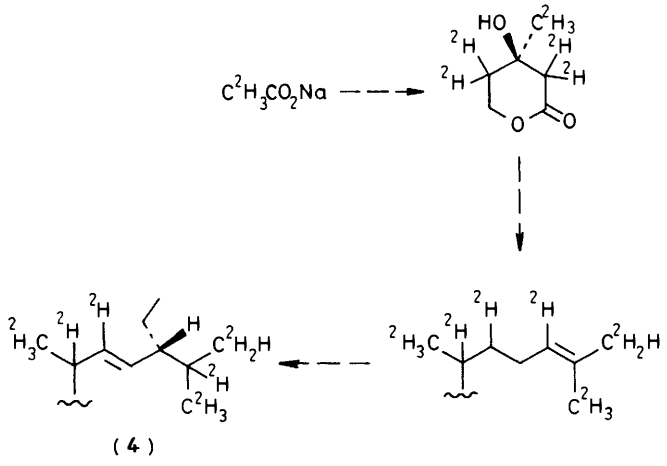
Scheme 2.

Claviceps paspali[3] the 24-H→25-H migration occurs on the *re*-face of the 24-double bond (case b in Scheme 2). These findings prompted us to study the stereochemical features of sterol biosynthesis in a further species.

We chose for our work the crysophyte *Ochromonas mal-*

hamensis, a phytoflagellate, which is an organism quite different from higher plants and fungi. Its major sterol is poriferasterol (**3**), the formation of which involves[5] the intermediacy of a 24-methylene compound (**2**) and the migration of the hydride from C-24 to C-25 (Scheme 1).

To study the biosynthesis of poriferasterol in *O. malhamensis*, we chose to effect the stereochemical analysis of the biosynthesized product by ^2H n.m.r. spectroscopy, in order to minimize the chemical manipulation of the products. Accordingly, we chose a biosynthetic precursor which would allow direct differentiation between the terminal isopropyl methyl groups, that is $C^2H_3CO_2H$. In fact, the deuteriated sterol (**4**) biosynthesized from this precursor (Scheme 3) should have,



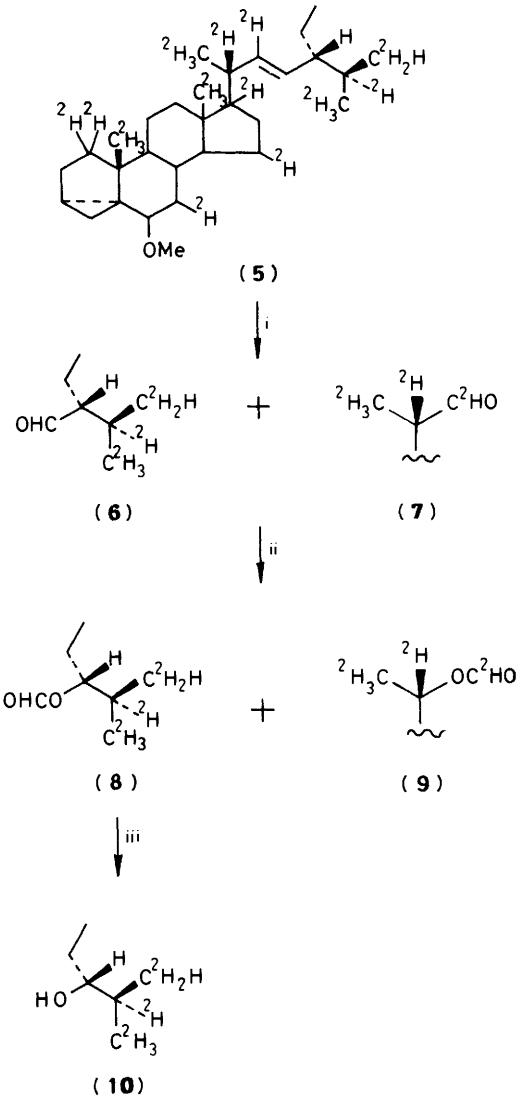
Scheme 3.

according to its biosynthetic origin, three deuterium atoms in the isopropyl methyl which originates from C-6 of MVA, and two deuterium atoms in the isopropyl methyl which originates from C-2 of mevalonic acid (MVA). Moreover, the utilization of the commercially available $C^2H_3CO_2H$ avoids a chemical synthesis of a stereospecifically labelled precursor.

Results and Discussion

Several preliminary experiments with *Ochromonas malhamensis* (933/A, Cambridge Culture Collection) in the medium of Aaronson and Baker[6] were effected, in order to find the best poriferasterol production, using different concentrations of cold sodium acetate and analysing the sterol, produced at different times, by g.l.c. Having found the best cultural conditions (Expt. 3b, Table 1), we evaluated the incorporation percentage and the dilution ratio administering $[2\text{-}^{14}C]CH_3CO_2Na$ (500 mg, 5.215×10^7 d.p.m./mg) to *O. malhamensis*. The labelled poriferasterol (1.81×10^7 d.p.m.) isolated in this experiment indicated a 5.7% incorporation of the labelled acetate; moreover, its specific activity (1.49×10^8 d.p.m./mmol) corresponded to a dilution ratio of 1:4.3. Using sodium $[2\text{-}^2H_3]$acetate (24 g, 99% ^2H, Merck, Sharp, and Dohme) under the preceding conditions, we obtained $[^2H_{22}]$poriferasterol (**4**) (2.23 g from 48 l of shaken culture), which showed, by mass spectral analysis, an average percentage of deuterium per labelled site of 5.4%.

The deuteriated sample was not analysed as such, owing to the complexity of its ^2H n.m.r. spectrum, but the unwanted part of the molecule was removed by chemical degradation of the side-chain, to give the C(24)–C(29) fragment as deuteriated 2-methylpentan-3-ol (**10**), the ^2H n.m.r. analysis of which is quite simple for the reasons which will appear later. The chemical degradation of the biosynthetic poriferasterol was effected according to Scheme 4: $[^2H_{22}]$poriferasterol (**4**) was trans-



Scheme 4. *Reagents:* i, O_3, CH_2Cl_2–1% pyridine, −78 °C; ii, Baeyer-Villiger, *m*-Chloroperbenzoic acid; iii, $LiAlH_4$, Et_2O

formed into the methyl ether (**5**), which was submitted to ozonolysis at −78 °C in CH_2Cl_2–1% pyridine. The reaction mixture, containing the aldehydes (**6**) and (**7**), after treatment with Zn–AcOH, was directly submitted to Baeyer-Villiger oxidation with *m*-chloroperbenzoic acid to afford the formates (**8**) and (**9**). The deuteriated formate (**8**) was recovered from the reaction mixture in the volatile phase by careful repeated distillation. The solvent (CH_2Cl_2) was exchanged with ether and the resulting ethereal solution was submitted to $LiAlH_4$ reduction to yield (2*S*,3*R*)-2-$[^2H_2]$methyl[1,1,1,2-$^2H_4$]pentan-3-ol (**10**) (for details see the Experimental part). The proton decoupled ^2H n.m.r. spectrum of (**10**) in the presence of $[Eu(dpm)_3]$ (0.43 mol) shows two resonances, at δ 5.89 and 6.29 p.p.m., for the prochiral methyl groups, which are sufficiently resolved to achieve a reliable integration. Since the above signals are in a 3:2 ratio, we assigned the upfield signal to C^2H_3 (coming from C-6 of MVA) and the downfield signal to $C^2H_2{}^1H$ (coming from C-2 of MVA). This assignment was

Table 1. Poriferasterol production by *O. malhamensis* in the presence of sodium acetate

Expt. no.	Sodium acetate added	Time (days)	Poriferasterol recovered (mg/flask)
1	0.7%		
(a)		2	Cell growth inhibited
(b)		4	Cell growth inhibited
(c)		6	Cell growth inhibited
2	0.1%		
(a)		2	Cell growth inhibited
(b)		4	Cell growth inhibited
(c)		6	Cell growth inhibited
3	0.05%		
(a)		2	0.5
(b)		4	6.0
(c)		6	0.5
4	0.025%		
(a)		2	0.5
(b)		4	2.5
(c)		6	6.2

confirmed by the broadening only of the lowfield resonance ($\Delta\nu_{\frac{1}{2}}$: 2.34→4.30 Hz) in the ^2H n.m.r. spectrum of the same sample, run in the ^1H coupled mode. To ascertain which of the two resonances has to be assigned to the *pro-R* and which to the *pro-S* methyl, we relied on the extensive work of Williamson *et al.*[7] on the conformational analysis of several aliphatic alcohols, which was achieved using an accurate ^1H and ^{13}C n.m.r. lanthanide induced shift (LIS) analysis. The outcome of major interest for our purpose was the assignment of ^{13}C chemical shifts of the two diastereotopic methyls in (3*R*)-2-methylpentan-3-ol, δ 18.81 for the *pro-S* and δ 17.05 for the *pro-R* methyl (see Table 2). Actually we needed to make similar assignments in the ^2H (and therefore in the ^1H) spectrum and in the presence of the optimal amount of Eu(dpm)$_3$. To do that we ran selective ^1H irradiations of the two prochiral methyl resonances during ^{13}C n.m.r. observations of a commercial racemic sample of 2-methylpentan-3-ol after the addition of increasing concentrations of Eu(dpm)$_3$ (see Table 2). In Table 2 a comparison is shown between the ^1H and the ^{13}C chemical shifts of the three methyl resonances of 2-methylpentan-3-ol upon addition of 0.28 mol of [Eu(dpm)$_3$]. Such a quantity of lanthanide salt was smaller than that used for the ^2H spectrum, but sufficient to induce an appreciable shift of the methyl proton signals without producing extreme line broadening in the ^{13}C resonances. In this way we could assign in the ^1H, and thence in the ^2H n.m.r. spectrum, of (3*R*)-2-methylpentan-3-ol both the higher field resonance of the *pro-S* methyl and the lower field resonance of the *pro-R*. It follows that in the deuteriated sample (**10**), the methyl $C^2H_2{}^1H$ occupies the *pro-R* position, and the methyl C^2H_3 occupies the *pro-S* position.

These data clearly indicate that, in poriferasterol biosynthesized by *Ochromonas malhamensis*, the isopropyl methyl group coming from C-2 of MVA is in the *pro-R* position, whilst the methyl coming from C-6 is in the *pro-S*. Owing to the known *pro-E* orientation of the isopropylidene methyl, *ex*-C-2 of MVA in the Δ^{24}-sterol precursor (**1**), we can conclude that the H-24→25 migration engages the *si*-face of the substrate double bond (path a, Scheme 2).

Therefore, the stereochemistry of the 1,2-hydride migration during the biosynthesis of poriferasterol in *O. malhamensis* is the same as in higher plants,[2,4] so indicating that, during the biological methylation which leads to phytosterols, *S*-adenosylmethionine approaches the sterol intermediate (**1**) from the 24-*re* face of the Δ^{24}-double bond.

Table 2. ^1H and ^{13}C Chemical shifts of the methyls of 2-methylpentan-3-ol in the absence and in the presence of [Eu(dpm)$_3$]

Me	δ(^1H)	δ(^1H)[c]	Δδ(^1H)	δ(^{13}C)	δ(^{13}C)[c]	Δδ(^{13}C)
pro-S[a]	0.89[b]	2.46	1.57	18.81	21.76	2.95
pro-R[a]	0.90[b]	2.60	1.70	17.05	20.43	3.38
CH_3CH_2	0.95	2.41	1.46	10.21	13.34	3.13

[a] For the (3*R*)-alcohol; these assignments must be reversed for the (3*S*)-alcohol. [b] Interchangeable values. [c] After addition of 0.28 mol [Eu(dpm)$_3$]

Experimental

N.m.r. spectra were recorded on a Varian XL-200 instrument operating at 50.308 MHz for ^{13}C, 200.057 for ^1H, and 30.710 for ^2H. T.l.c. was carried out on Merck 60 F_{254} plates and the spots were detected by spraying with 50% aqueous H_2SO_4 and heating at 110 °C for 5 min. G.l.c. was carried out on a Carlo Erba 4200 gas chromatograph equipped with a flame ionization detector, using N_2 as the carrier gas. 2-m Long columns were used, packed with (i) 2.5% SE-30, (ii) 1% OV-17, and (iii) 10% Carbowax 20 M. Compounds were identified by the peak enhancement technique with authentic samples and by g.l.c.–mass spectrometry. Radioactive samples were counted on a Packard Tri-Carb 3320 liquid scintillation counter as solutions in 10 ml of scintillation fluid containing 0.65% (w/v) 2,5-diphenyloxazole and 0.013% (w/v) 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene in toluene–dioxane (1:1, v/v).

Poriferasterol Production by Ochromonas malhamensis *Cultures in the Presence of Cold Sodium Acetate.—Ochromonas malhamensis* (933/1A) was obtained from the Culture Collection for Algae and Protozoa (Cambridge, U.K.).

Cultural conditions. O. malhamensis was grown in 500-ml Erlenmeyer flasks containing 200 ml of the medium of Aaronson and Baker[6] with different concentrations of sodium acetate (0.7, 0.1, 0.05, and 0.025% respectively, see Table 1). The flasks were inoculated with a rich inoculation from a 3-day old culture, previously grown on the same concentration of acetate. The cultures, maintained at 27 °C under constant illumination and continuous shaking, were harvested at different times (2, 4, and 6 days) and the poriferasterol (**3**), after work-up[8] was analysed by g.l.c. (column i, T_c 225 °C) (see Table 1) using the internal standard procedure. The conditions chosen for the following experiments were those of the Expt. 3b.

Administration of sodium [2-^{14}C]*acetate to* O. malhamensis *and isolation of labelled poriferasterol.* Sodium [2-^{14}C]acetate (500 mg, 3.18×10^8 d.p.m., The Radiochemical Centre, Amersham) was administered to 5 × 200-ml cultures of the alga, grown as described above. After 4 days the unsaponifiable fraction was extracted and the poriferasterol obtained (50 mg) purified as acetate, by SiO_2 H-60 column (hexane–ethyl acetate 8:2, v/v) followed by p.t.l.c. on 20% $AgNO_3$–SiO_2 (benzene–hexane 1:1, v/v, 2 elutions). After repeated crystallizations from $CHCl_3$–MeOH the product exhibited a specific activity of 1.49×10^8 d.p.m./mmol, corresponding to a dilution value of 19% (1:4.3).

Administration of sodium [2-$^2H_3$]*acetate to* O. malhamensis *and isolation of deuteriated poriferasterol* (**4**). Sodium [2-$^2H_3$]acetate (24 g, 99% ^2H, Merck, Sharp and Dohme) was added to 48-l of the culture of the alga, grown as described above. After 4 days the unsaponifiable fraction was extracted[8]

and the [$^2H_{22}$]poriferasterol (**4**) (2.23 g) was obtained by flash chromatography (hexane–etheyl acetate 7:3, v/v) and crystallization (97% pure by g.l.c., columns i, T_c 225 °C, and ii, T_c 260 °C); mass spectrometry $^2H_0$ 73.2%, $^2H_1$ 3.6%, $^2H_2$ 5.2%, $^2H_3$ 3.6%, $^2H_4$ 3.0%, $^2H_5$ 2.6%, $^2H_6$ 2.7%, $^2H_7$ 1.7%, $^2H_8$ 1.3%, $^2H_9$ 1.1%, $^2H_{10}$ 1.0%, $^2H_{11}$ 0.6%, $^2H_{12}$ 0.4%, corresponding to an average percentage of deuterium per labelled site of 5.4%.

(2S,3R)-2-[$^2H_2$]*Methyl*[1,1,1,2-$^2H_4$]*pentan*-3-*ol* (**10**) *from* [$^2H_{22}$]*Poriferasterol* (**4**).—[$^2H_{22}$]Poriferasterol (**4**) (2.23 g) was transformed into (24*R*)-[$^2H_{22}$]-6β-methoxy-3α,5α-cyclostigmast-22-ene (**5**) according to known procedures.[9] Compound (**5**) (1.44 g) was then dissolved in dry CH_2Cl_2–pyridine (1%) (140 ml) and submitted to ozonolysis at −78 °C. The reaction was monitored by t.l.c. (hexane–ethyl acetate 9:1, v/v) and g.l.c. (column iii, T_c 70 °C) of aliquots removed at intervals and stopped after 20 min by adding Zn dust (450 mg) and glacial acetic acid (4.5 ml). After being stirred for 1 h the reaction mixture was filtered, and the filtrate washed with 0.1M HCl,[10] and then water to neutrality, and finally dried (Na_2SO_4). Analysis by g.l.c. showed that the deuteriated 2-ethyl-3-methylbutanal (**6**) was formed in a 65% yield (column iii, T_c 70 °C, internal standard limonene).

The CH_2Cl_2 solution obtained from the work-up was directly submitted to Baeyer-Villiger oxidation by adding *m*-chloroperbenzoic acid [3 g in CH_2Cl_2 (15 ml)] and stirring for 48 h at room temperature. At the end of the reaction (monitored by g.l.c., column iii, T_c 70 °C) a solution of K_2CO_3 [2.5 g in water (2.5 ml)] was added and the stirring was continued for 2.5 h. Sodium sulphate was then added, the mixture was filtered through Celite, and the solids carefully washed with CH_2Cl_2. The solution obtained was concentrated to 35 ml under reduced pressure at room temperature and finally distilled on an oil-bath maintained at 150 °C, to separate the solids from the volatile phase containing the formate (**8**). When no more material distilled over, CH_2Cl_2 (30 ml) was added to the residue and the distillation effected again. This procedure was repeated (3 × 30 ml) until g.l.c. (column iii, T_c 70 °C) showed none of the formate (**8**) in the distillate. G.l.c. analysis (column iii, T_c 70 °C, limonene as internal standard) showed a 70% yield of the deuteriated formate (**8**). The CH_2Cl_2 solution was concentrated to 15 ml under reduced pressure at room temperature and then the CH_2Cl_2 was exchanged with Et_2O using a fractionating column packed with glass helices (*h* 40 cm, φ 1 cm). Dry Et_2O was repeatedly added to the distillation flask and the solution was distilled until no CH_2Cl_2 was detectable by g.l.c. During this operation no formate (**8**) was lost, as monitored by g.l.c. analysis.

To the ethereal solution of the formate (**8**) $LiAlH_4$ (110 mg) was added and the mixture was stirred at room temperature for 24 h. 10% HCl was then added until a precipitate was obtained. The solids were removed by filtration on Celite and carefully washed with ether. The deuteriated 2-methylpentan-3-ol (**10**) obtained was analysed by g.l.c. (column iii, T_c 70 °C, limonene as internal standard, 60% yield, 110 mg recovered): the ethereal solution was concentrated to 2 ml under reduced pressure at room temperature and the ether was exchanged with freshly distilled, acid-free, $CHCl_3$ as described above. The $CHCl_3$ solution recovered in the distilling flask was concentrated under reduced pressure at room temperature to 2.5 ml, and finally concentrated to 1 ml under a gentle flow of N_2 without loss of the deuteriated alcohol (**10**) (g.l.c. analysis). The resultant solution was used for the ^2H n.m.r. analysis.

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