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$\Delta^{8(14)}$-Steroids in the Bacterium *Methylococcus capsulatus*

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The 4,4-dimethyl and 4α-methyl sterols of the bacterium *Methylococcus capsulatus* were identified as 4,4-dimethyl- and 4α-methyl-5α-cholest-8(14)-en-3β-ol and 4,4-dimethyl- and 4α-methyl-5α-cholesta-8(14),24-dien-3β-ol. Sterol biosynthesis is blocked at the level of 4α-methyl $\Delta^{8(14)}$-sterols.

Among the various classes of bacteria that have been investigated for their steroid content, most have none (or very low amounts) of these products (Goldfine, 1972); it cannot be excluded that, when present, these products might have arisen from contamination (Bouvier, 1974).

A striking exception is the bacterium *Methylococcus capsulatus*, in which Bird *et al.* (1971) reported the presence of 4,4-dimethyl-5α-cholest-8(9)-en-3β-ol, 4,4-dimethyl-5α-cholesta-8(9),24-dien-3β-ol, 4α-methyl-5α-cholest-8(9)-en-3β-ol and 4α-methyl-5α-cholesta-8(9),24-dien-3β-ol, using g.l.c. and g.l.c.–mass spectrometry, but the location of the nuclear double bond still remained to be proven. In the present work we show that this double bond is located in the $\Delta^{8(14)}$ position.

Experimental

Physical methods

Melting points were determined on a Reichert microscope and are corrected. $[\alpha]_D$ values were measured at 589nm in chloroform, on a Perkin–Elmer 141 polarimeter. G.l.c. was carried out as described by Rohmer *et al.* (1972) with either 1% SE-30 or 1% OV17 as stationary phase. Combined g.l.c.–mass spectroscopy was performed on a LKB 9000 apparatus with a glass column packed with 1% Dexsil-impregnated Gas-Chrom Q. The carrier gas was helium; the injector block was maintained at 250°C, the molecular separator at 270°C, the ion source at 290°C, and the temperature of the oven was raised at a rate of 6°C/min from 230° to 300°C; the accelerating potential was 70eV and the filament current was 60μA. N.m.r. (nuclear-magnetic-resonance) spectra were measured at 90MHz on a Brucker WH 90 instrument with [^{2}H]chloroform as solvent.

Culture of Methylococcus capsulatus (strain F.D.)

The organism was grown on methane as sole carbon source, in the mineral salts medium proposed by Harwood & Pirt (1972). For the preliminary experiments, cells were grown in sealed Erlenmeyer flasks of 1 litre capacity. Larger amounts of cells were obtained from a Biotec fermenter in the Milstead Laboratories of Shell Research Ltd., Sittingbourne, Kent, U.K.

Extraction and separation of steroids from Methylococcus capsulatus

The cells (0.5g–5g dry wt.) were harvested, freeze-dried and extracted once with acetone and twice with chloroform/methanol (40ml) (2:1, v/v). The organic extract was hydrolysed under reflux in 6% (w/v) methanolic KOH and the non-saponifiable lipids were separated by preparative t.l.c. on silica-gel plates, with methylene chloride as eluent (two migrations in the same direction). Three fractions were scratched off the plates, corresponding to the 4,4-dimethyl sterols (fraction 1, R_F 0.25), 4α-methyl sterols (fraction 2, R_F 0.20) and sterols (fraction 3, R_F 0.14). This last fraction was analysed by g.l.c. and was negligible (5μg/g of dried cells, and the same sterols were found in a control sample isolated by the same extraction procedure without cells, and therefore are probably not synthesized by *M. capsulatus*).

Fractions 1 and 2 were acetylated with pyridine/acetic anhydride (2:1, v/v) at room temperature (25°C) overnight, and purified by preparative t.l.c. with benzene/ethyl acetate (9:1, v/v) as eluent (R_F 0.60). The total amount of 4,4-dimethyl and 4α-methyl sterol acetates was about 2mg/g of dried cells. In every culture, 4,4-dimethyl sterols were present in smaller amounts (15–30%) than 4α-methyl sterols. G.l.c. examination of each fraction showed two

components. The retention times of the two peaks of material in fraction 2 were similar to those of authentic samples of 4α-methyl-5α-cholest-8-en-3β-yl and 4α-methyl-5α-cholesta-8,24-dien-3β-yl acetates, on both 1% SE 30 and 1% OV 17 columns. The Δ^7 isomers have distinctly longer retention times. In both fractions 1 and 2, the mono-unsaturated compound was always the major component.

These fractions 1 and 2 were separated by preparative t.l.c. on $AgNO_3$-impregnated silica-gel plates developed continuously with benzene/cyclohexane (7:3, v/v) for 16h, into two bands. For the 4,4-dimethyl sterol acetates these were the acetate derivative of product (V) (R_F value of 24,25-dihydrolanosteryl acetate, II) and that of product (III) (R_F value of lanosteryl acetate, I). For the 4α-methyl steryl acetates these were the acetate derivative of product (VI) (R_F value of 31-norlanostenyl acetate) and of product (IV) (R_F value of 31-norlanosteryl acetate). These four fractions were shown by g.l.c. and g.l.c.–mass spectrometry to be pure compounds. They were analysed by n.m.r. and crystallized from methanol before determination of m.p. and $[\alpha]_D$.

Physical properties of the described products

Attribution of the n.m.r. peaks was made after comparison with theoretical chemical shifts (Zürcher, 1963) and published spectra (see the Results section). The chemical shifts are given in p.p.m. with reference to tetramethylsilane.

4,4-*Dimethyl-5α-cholesta*-8(14),24-*dien*-3β-*yl acetate* (*III acetate*). This had m.p. 103.5–104.5°C; mass spectrum, see Table 1; n.m.r.: 0.77 (3H, s, C-19), 0.83 (3H, s, C-18), 0.88 (6H, s, C-30 and C-31), 0.94 (3H, d, $J = 6$ Hz, C-21), 1.60 and 1.69 (6H, 2s, C-26 and C-27), 2.05 (3H, s, CH_3CO_2–), 4.51 (1H, dd, $J_1 = 9$ Hz, $J_2 = 5$ Hz, 3α-H), 5.05 (1H, t, $J = 6$ Hz, 24-H).

4,4-*Dimethyl-5α-cholest*-8(14)-*en*-3β-*yl acetate* (*V acetate*). This had m.p. 114–116°C (literature value m.p. 115–116°C, Gautschi & Bloch, 1958); $[\alpha]_D$ +28° (literature value $[\alpha]_D$ +28°; Gautschi & Bloch, 1958); mass spectrum, see Table 1; n.m.r.: 0.78 (3H, s, C-19), 0.83 (3H, s, C-18), 0.86 (6H, d, $J =$ 6 Hz, C-26 and C-27), 0.88 (6H, s, C-30 and C-31), 0.92 (3H, d, $J = 6$ Hz, C-21), 2.05 (3H, s, CH_3CO_2–), 4.52 (1H, dd, $J_1 = 9$ Hz, $J_2 = 5$ Hz, 3α-H).

4α-*Methyl-5α-cholesta*-8(14),24-*dien*-3β-*yl acetate* (*IV, acetate*). For mass spectrum, see Table 1; n.m.r.: 0.71 (3H, s, C-19), 0.83 (3H, s, C-18), 0.84 (3H, d, $J = 6$ Hz, C-30), 0.94 (3H, d, $J = 6$ Hz, C-21), 1.60 and 1.69 (6H, 2s, C-26 and C-27), 2.04 (3H, s, CH_3CO_2–), 4.39 (1H, dt, $J_1 = 11$ Hz, $J_2 = 5$ Hz, 3α-H), 5.08 (1H, t, $J = 6$ Hz, 24-H).

4α-*Methyl-5α-cholest*-8(14)-*en*-3β-*yl acetate* (*VI, acetate*). This had m.p. 76.5–79°C (literature value for m.p. 78–78.5°C, Kandutsch & Russell, 1959); $[\alpha]_D$ +38° (literature value $[\alpha]_D$ +37°, Kandutsch & Russell, 1959); mass spectrum, see Table 1; n.m.r.: 0.71 (3H, s, C-19), 0.84 (3H, s, C-18), 0.84 (3H, d, $J = 6$ Hz, C-30), 0.87 (6H, d, $J = 6$ Hz, C-26 and C-27), 0.93 (3H, d, $J = 6$ Hz, C-21), 2.05 (3H, s, CH_3CO_2–), 4.41 (1H, dt, $J_1 = 11$ Hz, $J_2 = 5$ Hz, 3α-H).

5α-*Cholest*-8(14)-*en*-3β-*yl acetate*. For mass spectrum, see Table 1; n.m.r.: 0.71 (3H, s, C-19), 0.84 (3H, s, C-18), 0.87 (6H, d, $J = 6$ Hz, C-26 and C-27), 0.93 (3H, d, $J = 6$ Hz, C-21), 2.02 (3H, s, CH_3CO_2–), 4.73 (1H, m, 3α-H).

Results

G.l.c.–mass spectrometry of the acetate derivatives showed that products (III) and (V) were C_{29} sterols

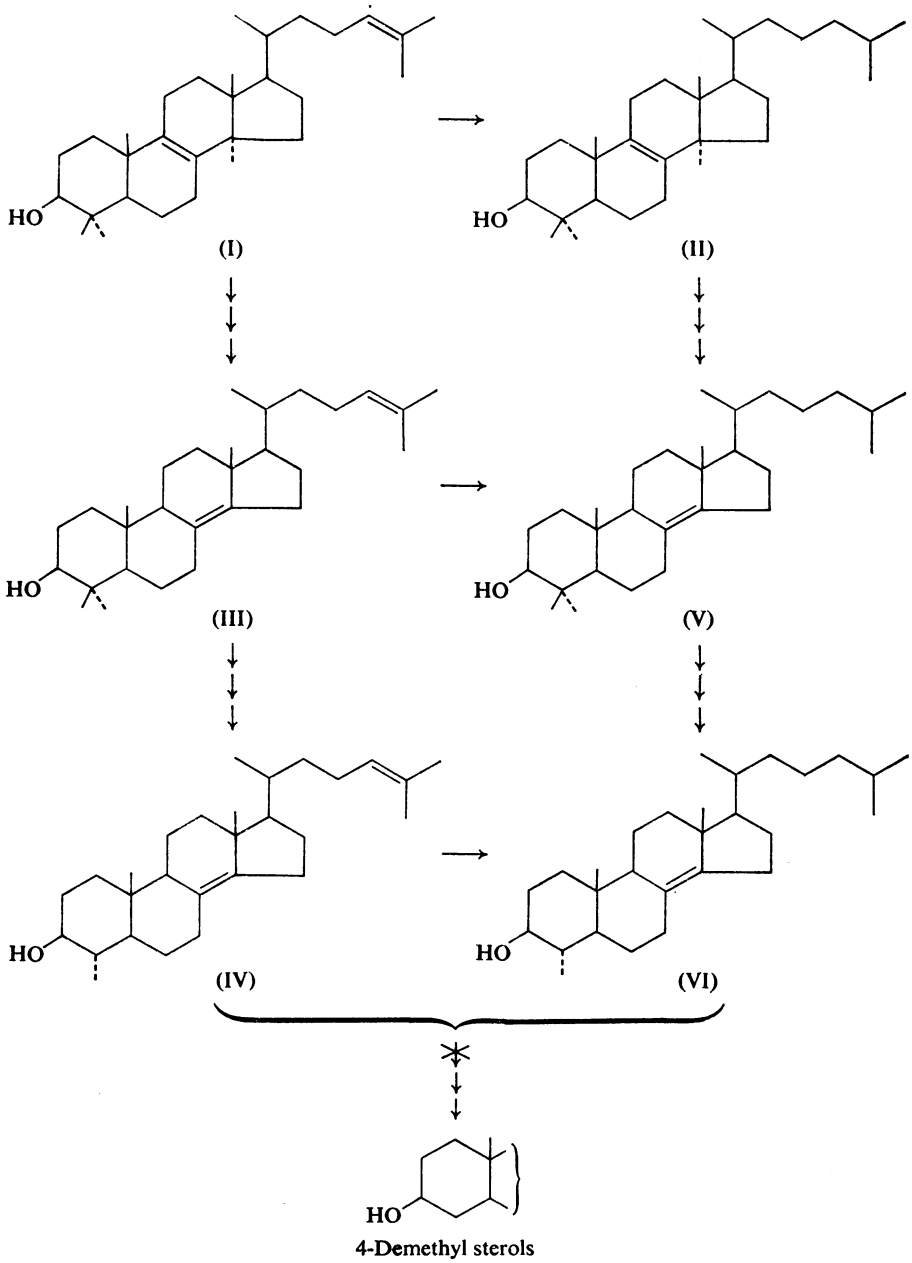
Table 1. *Mass-spectral data for the acetates of the* 4,4-*dimethyl and* 4α-*methyl sterols from Methylococcus capsulatus and for* 5α-*cholest*-8(14)-*en*-3β-*yl acetate*

Relative abundances of ions above *m/e* 200 are given in parentheses. For details, see the text.

	Product (III)	Product (IV)	Product (V)	Product (VI)	5α-Cholest-8(14)-en-3β-yl acetate
M^+	454 (84)	440 (100)	456 (100)	442 (100)	428 (100)
M^+-CH_3	439 (20)	425 (38)	441 (18)	427 (20)	413 (26)
M^+–acetate	394 (100)	380 (28)	396 (26)	382 (11)	368 (10)
M^+-CH_3–acetate	379 (60)	365 (32)	381 (16)	367 (15)	353 (16)
M^+–side chain	343 (6)	—	343 (13)	329 (11)	315 (14)
M^+–side chain–2H	341 (9)	327 (15)	—	—	—
M^+–side chain–42	301 (4)	287 (6)	301 (5)	287 (5)	273 (6)
M^+–side chain–acetate	283 (20)	269 (13)	283 (16)	269 (23)	255 (24)
M^+–side chain–acetate–2H	281 (12)	267 (6)	—	—	—
M^+–side chain–acetate–26	257 (40)	243 (38)	257 (20)	243 (30)	229 (44)
M^+–side chain–acetate–42	241 (44)	227 (55)	241 (32)	227 (36)	213 (44)
Other important ions	315 (6), 69	301 (8), 69	316 (4)	302 (5)	288 (6)

(molecular ion at *m/e* 456 and 454) and that products (IV) and (VI) were C_{28} sterols (molecular ion at *m/e* 442 and 440) (Table 1). Products (V) and (VI) contained one double bond, whereas products (III) and (IV) contained two double bonds. Fragments at *m/e* 343 and 329 arising from the loss of the side chain indicated that all compounds possessed an unalkylated C_8 chain.

Bird *et al.* (1971) located the additional double bond at the Δ^{24} position, from the predominance of



Scheme 1. *Possible pathway for the sterol metabolism in M. capsulatus*

ion at *m/e* 69 (Galli & Maroni, 1967); it was further substantiated by the peaks in the n.m.r. spectra at 1.60 and 1.69 (6H, 2s) for the C-26 and C-27 methyl protons, and by the broad n.m.r. peak at 5.05 or 5.08 p.p.m. (1H, t, $J = 6$ Hz), for the one olefinic proton at C-24.

The mass spectra of the four steroids were compared with published or experimental spectra run on authentic samples of isomeric 4,4-dimethyl and 4α-methyl sterol acetates, and of 5α-cholest-8(14)-en-3β-yl acetate (Table 1; Galli & Maroni, 1967; Smith *et al.*, 1973, Beastall *et al.*, 1974). The acetate derivatives of products (V) and (VI) differed respectively from 4,4-dimethyl- and 4α-methyl-5α-cholest-7-en-3β-yl acetates with respect to the percentage of ion at *m/e* 283 and 269 (M^+–side chain–acetate); the acetate derivatives of products (III) and (IV) differed from 4,4-dimethyl- and 4α-methyl-5α-cholesta-7,24-dien-3β-yl acetates in the relative abundances of ions at *m/e* 454 and 341 (product III) and of ions at *m/e* 440 and 327 (product IV) (M^+ and M^+–side chain–2H). But the mass spectra of the four products did not differ significantly from the $\Delta^{8(9)}$ isomers.

The n.m.r. spectra confirmed the exclusion of Δ^7 structure by the absence of the broad peak at 5.1 p.p.m. integrating for the olefinic proton located at C-7. The chemical shifts of C-18 and C-19 methyl protons gave evidence for the $\Delta^{8(14)}$ structure. Δ^7 isomers have a C-18 methyl resonance at about 0.54 p.p.m., and Δ^8 isomers at about 0.60 p.p.m. (Zürcher, 1963; Scallen & Krueger, 1968; Scallen *et al.*, 1971; Smith *et al.*, 1973; Beastall *et al.*, 1974), whereas none of the four steroids of *Methylococcus* showed a peak of methyl protons with a chemical shift smaller than 0.71 p.p.m. Also the relative positions of the C-18 and C-19 methyl resonances confirm the $\Delta^{8(14)}$ structure; in the spectra of the four products, the peak of methyl protons with the smallest chemical shift (0.71 p.p.m. for the 4,4-dimethyl sterol acetates and 0.78 p.p.m. for the 4α-methyl sterol acetates) which is sensitive to the presence of the methyl group at C-4β, corresponds to the C-19 methyl protons; the theoretical data indicate for the $\Delta^{8(14)}$ isomer a C-19 methyl-proton resonance at higher field than that of C-18, but for the Δ^7 and Δ^8 isomers they indicate a C-18 methyl-proton resonance at higher field than that of C-19. Finally, the n.m.r. spectra are quite consistent with the spectrum of 5α-cholest-8(14)-en-3β-yl acetate, thus confirming the identifications.

In addition, the m.p and $[\alpha]_D$ of the acetate derivatives of products (V) and (VI) are in full agreement with the literature values observed on the corresponding synthetic products.

Discussion

Barton *et al.* (1970) have isolated 4α-methyl-5α-cholesta-8(14),24-dien-3β-ol from yeast, as a minor sterol. The $\Delta^{8(14)}$ sterols have been described as possible intermediates in sterol metabolism (Schroepfer *et al.*, 1972) after oxidation and decarboxylation at C-14. *M. capsulatus* is the first organism where 4,4-dimethyl and 4-methyl $\Delta^{8(14)}$-sterols are described as major sterols.

Traces of lanosterol (I) and 24,25-dihydrolanosterol (II) were detected in the cells from one experiment and identified as their acetates from their g.l.c. behaviour and their mass spectra. According to Schroepfer *et al.* (1972) they are the direct precursors of the 14-demethyl $\Delta^{8(14)}$-sterols, and their presence was therefore not unexpected.

No 14-demethyl $\Delta^{8(9)}$- or Δ^7-sterols could be detected, although these are the next intermediates in the sterol biosynthesis proposed by these authors. The 4-demethyl sterols, which are the usual end-products of this metabolic pathway, were also completely absent. On the basis of these findings we propose a scheme for the sterol biosynthesis in *M. capsulatus* (Scheme 1) which is unusually blocked at the level of the 4α-methyl sterols.

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