Evidence for a New Pathway in the Microbial Conversion of 3β-Acetoxycholest-5-en-19-ol into Estrone

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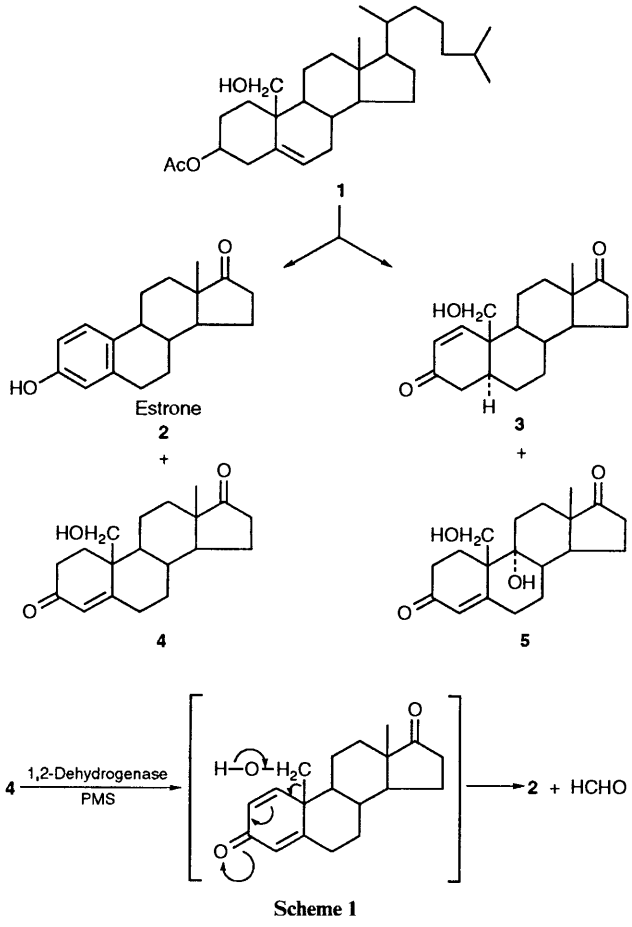
Incubation of 3β-acetoxycholest-5-en-19-ol **1** with *Moraxella* sp. gave three neutral metabolites 19-hydroxy-5α-androst-1-ene-3,17-dione **3**, 19-hydroxyandrost-4-ene-3,17-dione **4** and 9α,19-dihydroxyandrost-4-en-3,17-dione **5** besides estrone **2**. Hitherto, the metabolite **3** was unknown. Acidic metabolites were not formed. Time course, resting cell and cell-free experiments clearly suggest: (a) complete removal of the C_{17} side chain takes place prior to the aromatisation of A ring in **2**. (b) The non-involvement of C_{22} phenolic acids as intermediates in the degradative sequence from **1** to **2**. (c) Partially purified steroid 1,2-dehydrogenase readily converts the neutral metabolite **4** into estrone **2** and formaldehyde in the presence of phenazine methosulfate, an artificial electron acceptor.

It was shown earlier that modified sterols such as 19-hydroxycholest-4-en-3-one and 3β-acetoxycholest-5-en-19-ol **1** readily undergo microbiological conversion into estrone **2**.[1-3] These studies have clearly demonstrated that degradation of the C_{17} side-chain proceeds *via* C_{22} phenolic acid intermediates.[1-3] The three-carbon side-chain of C_{22} phenolic acid is cleaved to **2** and propionic acid, suggesting that the complete removal of C_{17} side-chain takes place only after aromatisation of the A ring. In contrast to microorganisms, mammalian systems convert sterols into 17-oxo steroids by way of C(20)–C(22) bond cleavage followed by C(17)–C(20) bond cleavage.[4] Further, conversion of a 17-oxo steroid into estrone **2** is catalysed by microsomal cyt P-450 enzyme aromatase during which reaction 3 mol equiv. of each of NADPH and oxygen are used.[5]

During our attempts to develop a microbial process to selectively eliminate the C-17 side-chain of cholesterol or its derivatives, a soil microorganism belonging to the genus *Moraxella* was isolated by an enrichment-culture technique using the model compound, isooctylcyclopentane as the sole source of carbon.[6,7] The organism also accepted cholesterol and 3β-acetoxycholest-5-en-19-ol **1** as the sole source of carbon. Although earlier, it was shown that this organism efficiently transforms **1** into **2**, no attempts were made to establish the degradative sequence in the conversion. The present study was initiated to gain further information regarding A-ring aromatisation, the steroid nucleus remaining intact and the C-17 side-chain being cleared. We report here, the isolation and identification of neutral metabolites from **1** and propose a new pathway for the microbial conversion of **1** into **2** where a neutral metabolite **4** acts as a key intermediate (Scheme 1). It is of interest to note that the organism produced no C-22 phenolic acid intermediates during the transformation. Even after a shortening of the incubation period to 48 h or incubation in the presence of inhibitors such as 2,2′-bipyridyl (0.6 mol dm^{-3}) and propanol (2%) did not result in the formation of acidic metabolites and thus the degradative sequence of **1** appears to be different from that reported earlier.[1-3]



Scheme 1

Results and Discussion

Fermentation of **1** with *Moraxella* (see Experimental section) gave the neutral metabolites **3**, **4** and **5** besides **2**. In fact, **3**, **4** and **5** have never been shown to be metabolites of **1** and, moreover, **3** is a hitherto unknown compound. From the total transformation products formed (*ca.* 1 g), metabolites **2**, **3**, **4** and **5** were isolated pure as judged by TLC and HPLC analyses. Metabolites **2** (712 mg) and **4** (52 mg) were identified as estrone and 19-hydroxyandrost-4-ene-3,17-dione by comparison (IR, NMR, MS and HPLC) with authentic samples. The spectral analyses of metabolite **3** (33 mg) indicated that it was 19-hydroxy-5α-androst-1-ene-3,17-dione. The stereochemistry of the A/B ring junction was established as *trans* by X-ray analysis (Fig. 1). Metabolite **5** (12 mg) was identified as 9α,19-dihydroxyandrost-4-ene-3,17-dione on the basis of spectral analysis. The ^1H NMR of this compound is similar to that of **4**. However both IR and mass spectral analyses indicated the

presence of one more hydroxy group. The ^{1}H NMR pattern suggests that the second hydroxy group is tertiary in nature and the possible positions are 8β, 9α and 14α. Absence of deshielding of the 18-CH_3 protons rules out the possibility of a hydroxy group at the 14α position. The logical conclusion is, therefore, that the hydroxy group may be either at the 8β or 9α position. X-Ray analysis[8] of **5** conclusively established a tertiary hydroxy group at the 9α position. A time-course plot (Fig. 2a) of the fermentation of **1** carried out for 7 days showed that although in the early stages (2 days) only small amounts of **4** were formed, with increasing time (3 days) significant accumulation of **4** occurred (*ca.* 80% of the total metabolites formed, by HPLC analyses) and low levels of **2** were noted. After 3 days and up to 7 days gave a decrease in the amount of **4** present and a gradual increase in the amount of **2**. The presence of small quantities of the metabolites **3** and **5** could be seen only after 3 days (together they constitute nearly 10% of the total metabolites formed, by HPLC analyses, not shown in Fig. 2a). This study suggests that there is a precursor–product relationship between **4** and **2**, an observation supported by carrying out the transformation of **4** into **2** using resting cells and also with partially purified 1,2-dehydrogenase (see Experimental section). In the resting cells experiment, transformation products formed at different time intervals (12, 24 and 48 h) were analysed by HPLC (Fig. 2b). It was noticed that the level of **4** dropped gradually with a concomitant increase in the level of **2** and at the end of 24 h, nearly 80% of **4** had been converted into **2**. During this period, small amounts of the metabolite **5** were also formed (10–15% of the total products formed). In the *in vitro* experiment, partially purified 1,2-dehydrogenase isolated from steroid-induced 48 h-old *Moraxella* cells readily converted **4** into **2** and HCHO in the presence of phenazine methosulfate, an artificial electron acceptor.

Cursory examination of the metabolites formed from **1** and time–course experiments, provide information on the sequence of reactions taking place during estrone **2** formation and suggests that A-ring aromatisation takes place only after the cleavage of the C-17 side-chain. Time-course experiments on the fermentation of **1** (Fig. 2a) and resting-cell experiments carried out with metabolite **4** (Fig. 2b) clearly show the precursor–product relationship between the metabolites **4** and **2**. We have, therefore, clearly demonstrated using partially purified enzyme that introduction of a 1,2-double bond into the metabolite **4** facilitates a spontaneous nonenzymatic retro aldol type reaction to yield estrone **2** and formaldehyde (Scheme 1). Earlier it was reported that C-22 phenolic acids are the intermediates in the degradative sequence from **1** to **2** and cleavage of the three-carbon side-chain in a C-22 acid takes place only after A ring aromatisation.[1–3] It was also known that addition of propanol or 2,2′-bipyridyl to a cholesterol-decomposing microorganism caused marked accumulation of C-22 acid intermediates.[9,10] The mode of degradation of the hydrocarbon side-chain of cholesterol in the microbial system differs from that of the mammalian system which involves the cleavage of the C(20)–C(22) and C(17)–C(20) bonds.[4] Our results appear to be different from either of these two modes of fission. We were not able to demonstrate the formation of acidic metabolites from **1** when the transformation was carried out for a shorter duration (up to 48 h) or in the presence of specific inhibitors suggesting that acidic intermediates are not involved in the degradative sequence. Thus, we have demonstrated a new pathway in *Moraxella* where a neutral metabolite **4** plays a key role in the conversion of **1** into **2**.

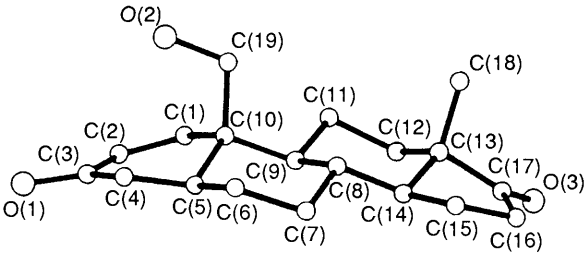


Fig. 1 Crystal structure of compound **3**

Experimental

3β-Acetoxycholest-5-en-19-ol **1** was synthesised following the procedure of Kalvoda *et al.*[11] UV spectra were measured using a Hitachi 557 spectrometer. IR spectra were taken using a Perkin–Elmer spectrometer. NMR studies were carried out at 90 MHz using a JEOL FT-90 spectrometer or at 270 MHz using a Bruker WH270 spectrometer. *J* Values are given in Hz. Mass spectra were determined using a JEOL JMS-DX 303 spectrometer. HPLC analyses were carried out on a Waters Associate ALC/GPC 244 series instrument. The analysis was performed on a normal phase micro Porosil column using chloroform–methanol (95:5, v/v) as the solvent system and monitoring with a UV detector at 254 nm. TLC analyses were performed on silica gel G plates (0.5 mm) developed with ethyl acetate–hexane (50:50, v/v, system I). $[\alpha]_D$ Values are given in 10^{-1} deg cm^2 g^{-1}.

Microorganism and Growth Media.—The microorganism used in this study was isolated from the garden soil and identified as *Moraxella*.[7] It was maintained on Seubert's

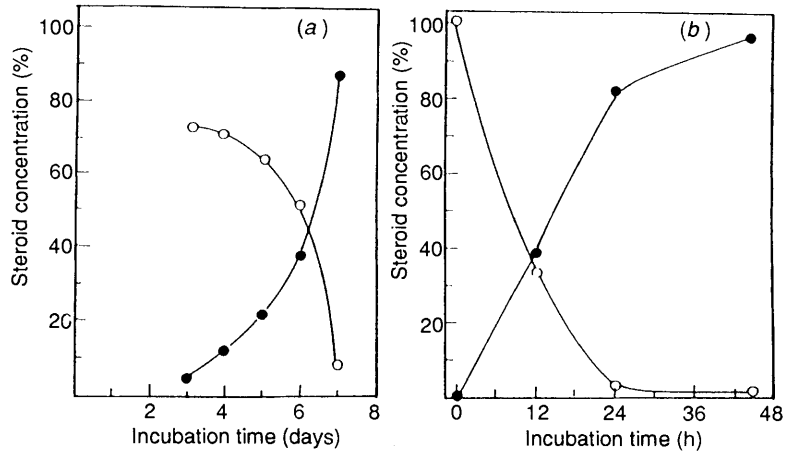


Fig. 2 Time–course experiments: (*a*) metabolism of 3β-acetoxycholest-5-ene-19-ol by *Moraxella sp.*; (b) transformation of 19-hydroxyandrost-4-ene-3,17-dione by resting cells. ○ = 19-hydroxyandrost-4-ene-ol-3,17-dione and ● = estrone.

medium[12] containing 0.05% cholesterol and 2% agar (pH 7.2). It was regularly maintained in a liquid mineral salts medium containing 0.05% cholesterol. Whenever starter culture was required, an aliquot (5 cm^3) from the maintenance culture was transferred to a 100 cm^3 sterilised liquid mineral salts medium (pH 7.2) containing 0.05% cholesterol and incubated on a rotary shaker (220 rpm) for 3 days. The present studies were carried out with a 3-day growth culture as the inoculum. Although the organism accepts **1** as the sole source of carbon, the growth rate is slow. Hence to enhance the growth rate, 0.2% glucose was added to the medium when the metabolism of **1** was carried out.

Biotransformation Procedure.—Metabolism of **1**. A batch of 120 flasks containing 100 cm^3 of sterile mineral salts medium,[12] 0.2% glucose and 0.05% of **1** were inoculated from a 5-day old culture (5 cm^3) and incubated at 29–30 °C on a rotary shaker (220 rpm) for 7 days. At the end of the incubation period, the contents were pooled, acidified to pH 3–4 and then extracted with an equal volume of dichloromethane (×3). The pooled extracts were washed well with 2% saline solution and were concentrated to a small volume. The concentrated organic phase was washed with 10% aqueous sodium hydrogen-carbonate and distilled water, dried (Na_2SO_4) and evaporated to yield neutral steroid metabolites. The hydrogen carbonate layer was acidified and extracted with dichloromethane and the extract shown to contain no acidic metabolites; it was not, therefore, processed further. Fermentation of **1** was also carried out for a shorter duration (48 h) and also in the presence of inhibitors like 2,2′-bipyridyl (0.6 mol dm^{-3}) and propanol (2%) and the culture media was processed as described above.

Isolation and Analysis of the Products.—The crude neutral fraction (4.8 g) was subjected to column chromatography on silica gel (140 g) and unmetabolised compound **1** (3.4 g) was eluted using 5% ethyl acetate in hexane. Elution of the column with 7% ethyl acetate in hexane yielded the major metabolite **2** (712 mg). Subsequently, the column was eluted with methanol to afford all the polar metabolites. This fraction (Fraction I; 370 mg) was further subjected to column chromatography on silica gel (7 g) and the column was eluted with 20% ethyl acetate in hexane. The initial eluate contained the pure polar metabolite **3** (37 mg). From the latter fraction pure polar metabolite **4** was also isolated (58 mg). Finally, the column was eluted with ethyl acetate to give the most polar metabolite **5** in pure form (12 mg). All the metabolites isolated were subjected to spectral analysis. Other very minor neutral metabolites could not be isolated in pure form and were not, therefore, characterised.

Metabolite **3** (R_f 0.26, system I): m.p. 182–183 °C (from CH_2Cl_2–hexane); $[\alpha]_D^{24}$ +15 (*c* 0.6 in MeOH); λ_{max}(MeOH)/nm 228; ν_{max}(Nujol)/cm^{-1} 3440 (OH), 1730 (C=O) and 1680 (α,β-unsaturated ketone); δ_H(270 MHz; $CDCl_3$) 0.92 (3 H, s, 18-CH_3), 3.83 (1 H, d, *J* 11.25, 19-CH_2), 4.1 (1 H, d, *J* 11.25, 19-CH_2), 6.13 (1 H, d, J_{cis} 10.13, 2-H) and 7.06 (1 H, d, J_{cis} 10.13, 1-H); δ_C(90 MHz, $CDCl_3$) 14.18, 21.34, 21.86, 27.45, 30.58, 32.0, 35.78, 36.04, 41.90, 43.98, 44.50, 48.14, 50.36, 52.05, 61.42, 131.03, 153.66, 201.16 and 221.06; *m/z* 302 (M^+, 42%), 284 (9), 272 (100) and 108 (81) (Found: M^+, 302.1884 $C_{19}H_{26}O_3$ requires M^+, 302.1882). On the basis of spectral analyses, compound **3** was identified as 19-hydroxyandrost-1-ene-3,17-dione. The AB ring junction was established as *trans* by X-ray analysis. Thus **3** was identified as 19-hydroxy-5α-androst-1-ene-3,17-dione **3**.

X-Ray Analysis of **3**.—*Crystal data.* $C_{19}H_{26}O_3$, M = 302.4. Orthorhombic, a = 7.598(2), b = 12.268(2), c = 17.316(2) Å, V = 1614.1(4) $Å^3$, space group $P2_12_12_1$, Z = 4, D_X = 1.24 g cm^{-3}, μ = 0.769 cm^{-1}, reflections for lattice parameters obtained and refined using 25 reflections in the range $10 < \theta < 16°$; $F(000)$ = 656, crystal size: 0.22 × 0.10 × 0.32 mm. Atomic coordinates, bond lengths and angles, and thermal parameters have been deposited as supplementary material with Cambridge Crystallographic Data Centre.*

Data collection and processing. CAD4 diffractometer, graphite monochromator, λ = 0.7107 Å, ω–2θ scan, 3 standard reflections monitored every hour, 770 unique reflections with $I \geqslant 3\sigma(I)$.

Structure analysis and refinement. The structure was determined by direct methods using SHELX 86[13] and refined using SHELX 76.[14] 199 Refined parameters, $R = [\Sigma\Delta F/\Sigma F_o] = 0.085$, $R_w = [\Sigma W(\Delta F)^2/\Sigma W F_o^2]^{\frac{1}{2}} = 0.0984$, $w = 1$. $\Delta\rho$(residual electron density) = ±0.2. The geometry of the molecule **3** is shown in Fig. 1. Metabolite **4** (R_f 0.18, system 1): m.p. 168–169 °C (from MeOH) was identified as 19-hydroxyandrost-4-ene-3,17-dione by comparison (IR, NMR, MS and HPLC) with an authentic sample.

Metabolite **5**† (R_f 0.1, system I): m.p. 252–253 °C (from MeOH); $[\alpha]_D^{24}$ +13.28 (*c* 0.753 in MeOH); λ_{max}(MeOH)/nm 242; ν_{max}(Nujol)/cm^{-1} 3502 (OH), 3376 (OH), 1728 (C=O), 1647 (α,β-unsaturated ketone) and 1614 (C=C); δ_H(270 MHz; $CDCl_3$) 0.92 (3 H, s, 18-CH_3), 3.92 (1 H, d, *J* 11.25, 19-CH_2), 4.14 (1 H, d, *J* 11.25, 19-CH_2) and 6.09 (1 H, s, 4-H); *m/z* 318 (M^+, 10%), 300 (55), 288 (32), 282 (15), 270 (100) and 110 (67) (Found: M^+, 318.182 $C_{19}H_{26}O_4$ requires M^+, 381.1831). X-Ray analysis[8] conclusively established that the position of the tertiary hydroxy group is at 9α. Hence, the metabolite **5** was identified as 9α,19-dihydroxyandrost-4-ene-3,17-dione.

Metabolism of **4** *by Resting Cells.*—Cells grown (48 h) on 0.2% glucose and 0.05% of **1** were washed with saline and phosphate buffer (0.03 mol dm^{-3}, pH 7.2) aseptically and then suspended (1 g) in the same buffer (25 cm^3). To this cell suspension, **4** (25 mg) was added and incubated for 48 h. Transformation products formed were monitored by taking a known aliquot (5 cm^3) at different time intervals, extracted and analysed by HPLC.

Metabolism of **4** in vitro.—Sonic disruption of the steroid induced 48-h old cells was subjected to differential centrifugation to obtain the cytosolic fraction (105 000 × g supernatant). This fraction was purified on a DEAE Cellulose column by eluting with Tris HCl (0.03 mol dm^{-3}, pH 7.5, 10% glycerol) containing 0.2 mol dm^{-3} KCl to yield a 7-fold purified enzyme which was used as a source of 1,2-dehydrogenase. Partially purified enzyme (50 mg) was incubated with **4** (16.5 μmol) and phenazine methosulfate (20 μmol) in phosphate buffer (0.03 mol dm^{-3}, pH 7.5; 75 cm^3) at 30 °C for 1 h. The assay was terminated by the addition of 2 mol dm^{-3} HCl and centrifuged. One half of the assay mixture when extracted with dichloromethane yielded an enzymatic product, purified by PLC (system I) and identified as estrone **2**. To the other half of the assay mixture, 2,4-DNP was added and the hydrazone derivative formed was purified and identified as that of formaldehyde by mass spectral analysis and comparison with the chemically prepared sample.

Acknowledgements

The financial assistance for V. N. S. from CSIR, New Delhi, IISc. Bangalore, India is gratefully acknowledged.

* For the details of the Cambridge Crystallographic Data Centre deposition scheme, see *J. Chem. Soc., Perkin Trans. 1*, 1993, Issue 1, 'Instructions for Authors'.

† Although metabolite **5** is a known compound,[15] as far as we know, its NMR and MS data have not been reported.

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Paper 3/02487C
Received 30*th April* 1993
Accepted 12*th May* 1993