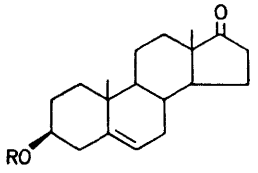
Microbiological Transformations. Part 3.[1,2] The Oxidation of Androstene Derivatives with the Fungus *Cunninghamella elegans*

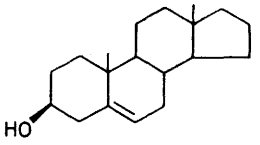
By **Trevor A. Crabb,* Philip J. Dawson,** and **Roger O. Williams,** Department of Chemistry, Portsmouth Polytechnic, Portsmouth, Hampshire PO1 2DT

The products obtained from the incubation of some Δ^4- and Δ^5-androstene derivatives with *Cunninghamella elegans* are largely those arising from allylic oxidation or epoxidation of the double bond, but some 9-, 12-, 14-, and 16-hydroxylation also occurs.

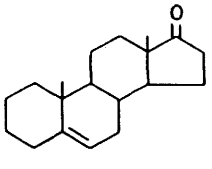
THE paucity of data on the microbiological transformation of steroidal derivatives by *Cunninghamella elegans* [1-3] prompted this investigation into the transformations of 3β-hydroxyandrost-5-en-17-one acetate (1), androst-5-en-3β-ol (3), androst-5-en-17-one (4), and androst-4-ene-3,17-dione (5). It was hoped that results on these compounds would provide some indication of the effect of structural modifications in the substrate on the outcome of the microbiological transformations.



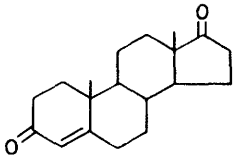
(1) R = Ac
(2) R = H



(3)



(4)



(5)

Structural assignments were based largely on the ^{1}H n.m.r. spectral data (Tables 1—4), particularly on the changes in chemical shifts ($\Delta\delta$) of the angular methyl protons with substituent,[4] and the C_xHOH parameters. Assignments are only discussed below when either less common ^{1}H n.m.r. parameters were utilised, or when marked deviations from additivity of $\Delta\delta$ values were noted.

TABLE 1

^{1}H N.m.r. data of the products from the action of *C. elegans* on 17-oxoandrost-5-en-3β-yl acetate (1)[a]

	δ (*J* or $W_{\frac{1}{2}}$)					Δδ Values [b,c]			
						Observed		Calculated	
Compound	3α-H	6-H	18-H	19-H	C_xHOH	18-H	19-H	18-H	19-H
(1)	4.62	5.42 (d, *J* 5.0)	0.89	1.05					
(2)	3.54	5.39 (d, *J* 5.0)	0.89	1.04					
3β-Hydroxyandrost-5-ene-7,17-dione	3.70	5.76 ($W_{\frac{1}{2}}$ 4)	0.90	1.23		0.01	0.19	0.01	0.28
3β,14α-Dihydroxyandrost-5-ene-7,17-dione	3.70	5.76 ($W_{\frac{1}{2}}$ 4)	1.00	1.25		0.11	0.21	0.13	0.28
3β,7β-Dihydroxyandrost-5-en-17-one	3.57	5.32 ($W_{\frac{1}{2}}$ 5)	0.90	1.08	3.97 (d, *J* 8)	0.01	0.04	0.03	0.03
3β,7α-Dihydroxyandrost-5-en-17-one	3.58	5.66 (d, *J* 5.0)	0.89	1.03	3.98 ($W_{\frac{1}{2}}$ 11)	0.00	−0.01	0.01	0.00
5,6β-Epoxy-3β,12α-dihydroxy-5α-androstan-17-one	3.76	3.15 (d, *J* 2.8)	0.86	1.04	4.21 ($W_{\frac{1}{2}}$ 10)				

[a] [^{2}H]Chloroform solutions. [b] Δδ Values relative to (2). [c] Minus sign (Δδ) represents an upfield shift.

Incubation of 17-*Oxoandrost*-5-*en*-3β-*yl Acetate* (1).—The broth extract for the incubation of (1) was chromatographed over alumina and appropriate fractions were crystallised. The residues from the mother-liquors (from the crystallisations) were also chromatographed (p.l.c.). Five compounds (in order of decreasing R_F value) were isolated, 5.4% of 3β-hydroxyandrost-5-ene-7,17-dione, 1.1% of 3β,14α-dihydroxyandrost-5-ene-7,17-dione, 9% of 3β,7β-dihydroxyandrost-5-en-17-one, 26.8% of 3β,7α-dihydroxyandrost-5-en-17-one, and 4.1% of 5,6β-epoxy-3β,12α-dihydroxy-5α-androstan-17-one.

In the n.m.r. spectrum of 3β,14α-dihydroxyandrost-5-ene-7,17-dione, Δδ values of +0.10 (18-H) and +0.02 (19-H) relative to 3β-hydroxyandrost-5-ene-7,17-dione were consistent with hydroxylation at the 14α-position,[4] as was simplification of the 15α-multiplet at δ 2.76 [relative to the corresponding signal (δ 2.82, $W_{\frac{1}{2}}$ 28 Hz) in the spectrum of 3β-hydroxyandrost-5-ene-7,17-dione] to a doublet of triplets. This 14α substitution permitted observation of a doublet at δ 2.61 ($J_{8\beta,9\alpha}$ 13.7 Hz) for the 8β-proton.

Comparison of the olefinic resonances (Table 1) of the

epimeric 3β,7ξ-dihydroxyandrost-5-en-17-ones with those of the epimeric cholest-5-ene-3β,7ξ-diols (3β,7β-diol, 6-H δ 5.30, 7α-H δ 3.83, $J_{6.7\alpha}$ 1.5 Hz; 3β,7α-diol 6-H δ 5.60, 7β-H 3.85, $J_{6.7\beta}$ 5.5 Hz) [5] supported the assignments. Confirmation of these came from partial synthesis. Reaction between 17-oxoandrost-5-en-3β-yl acetate (1), t-butyl perbenzoate, and acetic acid, in the presence of cuprous bromide,[6] gave a mixture of the expected [7] 3β,7ξ-diacetates which, on subsequent alkaline hydrolysis, yielded a mixture of the 3β,7ξ-diols, separated by p.l.c. The i.r. and n.m.r. spectra and R_F values of the synthesised compounds were identical with those of the compounds derived from the microbiological transformation. The physical constants of 3β-hydroxyandrost-5-ene-7,17-dione, 3β,14α-dihydroxyandrost-5-ene-7,17-dione, 3β,7β-dihydroxyandrost-5-en-17-one, and 3β,7α-dihydroxyandrost-5-en-17-one were in agreement with the reported values.[8,9]

The β-configuration of the 5,6β-epoxy-3β,12α-dihydroxy-5α-androstan-17-one was determined by comparison of the 6-H n.m.r. parameters with those in the isomeric 5,6ξ-epoxy-3β-hydroxy-5ξ-androstan-17-ones prepared from 3β-hydroxyandrost-5-en-17-one (2) and *m*-chloroperbenzoic acid.[10] The assignments were based on the lower field absorption of 6-H and smaller $J_{6.7}$ in the β-epoxide.[8,11,12]

The narrow line width ($W_{\frac{1}{2}}$ 10 Hz) of the C$_x$*H*OH absorption in the spectrum of 5,6β-epoxy-3β,12α-dihydroxy-5α-androstan-17-one indicated axial hydroxylation. Comparison of the chemical shifts of the angular methyl protons with those of 5,6β-epoxy-3β-hydroxy-5β-androstan-17-one gives incremental Δδ values, attributable to the inserted hydroxy-group, of +0.01 (18-H) and and +0.02 (19-H). Literature Δδ values [4] show that only an (axial) hydroxy-group at positions 1α-, 12α-, or 16α- satisfy these shift requirements. The chemical shift (δ 4.21) of the C*H*OH proton eliminates 16α-hydroxylation, and 1α-hydroxylation was ruled out since a 1α-OH would exert an unobserved *syn*-axial deshielding of the 3α-proton.

An observation was made [13] that results obtained with 17-oxo-17a-aza-D-homoandrost-5-en-3β-yl acetate and 17-oxoandrost-5-en-3β-yl acetate (1)[2], where allylic oxidation had occurred, may have been due, in whole or in part, to auto-oxidation. Cholesterol has been oxidised by passing air through aqueous sodium stearate dispersions [14] and through γ-irradiated methanolic solutions,[15] to give mixtures of 7ξ-hydroperoxides which subsequently yield the isomeric 5ξ,6ξ-epoxides and the 7-oxygenated products.

17-Oxoandrost-5-en-3β-yl acetate (1) was incubated for three days with (a) no fungus and (b) sterilized fungus, under otherwise standard conditions. In another experiment a continuous stream of air was passed through an ethanolic dispersion of the steroid (1), for six days. In all three experiments, auto-oxidation products were absent (t.l.c.).

A monitored incubation of 17-oxoandrost-5-en-3β-yl acetate (1) with *C. elegans* showed the presence of allylic-oxidation products after *ca.* 3 h. In contrast, reaction between cholesterol and hydrogen peroxide in an aqueous sodium stearate dispersion, did not give any allylic-oxidation products at an identical temperature (25 °C), within the time studied (6 h).[16] Consequently the fear of auto-oxidation [13] proved unfounded.

Incubation of Androst-5-en-3β-ol (3).—Androst-5-en-3β-ol (3) was synthesised [17] from 17-oxoandrost-5-en-3β-yl acetate (1), and incubated in the usual way. T.l.c. of the extract indicated a large number (*ca.* 15) of products but, subsequently, many of the compounds were isolated only in trace amounts (or as complex mixtures) such as to preclude identification. Only three products, 3β,16β-dihydroxyandrost-5-en-7-one, 3β,12β-dihydroxyandrost-5-en-7-one, and androst-5-ene-3β,7β,12β-triol were identified, and the ^{1}H n.m.r. spectra of these, on which the structural assignments were largely based, are summarised in Table 2. Although the 18-Me and 19-Me Δδ values in the spectrum of the product assigned as 3β,16β-dihydroxyandrost-5-en-7-one are more in agreement with 15β-hydroxylation than with 16β-hydroxylation, the actual chemical shift and the multiplicity (Table 2) of the C*H*OH proton signals favours 16β-hydroxylation.

The structure of 3β,12β-dihydroxyandrost-5-en-7-one was confirmed by acetylation to the diacetate, the angular methyl resonances of which were δ 0.80 (18-Me)

TABLE 2

^{1}H N.m.r. data of the products from the action of *C. elegans* on androst-5-en-3β-ol (3)[a]

Compound	δ (J or $W_{\frac{1}{2}}$)					Δδ Values [b,c]			
						Observed		Calculated	
	3α-H	6-H	18-H	19-H	C$_x$*H*OH	18-H	19-H	18-H	19-H
(3)	3.53	5.37 ($W_{\frac{1}{2}}$ 10)	0.72	1.02					
3β,16β-Dihydroxyandrost-5-en-7-one	3.72	5.80 (d, J 2.0)	1.02	1.20	4.67 (J 6.0, 3.0)	0.30	0.18	0.26	0.30
3β,12β-Dihydroxyandrost-5-en-7-one	3.68	5.72 (d, J 1.6)	0.74	1.22	3.41 (J 10.5, 4.5)	0.02	0.20	0.03	0.30
Androst-5-ene-3β,7β,12β-triol	3.55	5.31 (t, J 1.5)	0.75	1.08	3.84 (dt, J 1.5, 7.9) 3.42 (dd, J 11, 4.5)	0.03	0.06	0.05	0.05

[a] [^{2}H]Chloroform solutions. [b] Δδ Values relative to (3). [c] Minus sign (Δδ) represents an upfield shift.

and δ 1.23 (19-Me). The downfield shift [4] of the 18-Me (and not of the 19-Me) confirmed the site of further hydroxylation as 12β.

In the ^{1}H n.m.r. spectrum of androst-5-ene-3β,7β,12β-triol two C*H*(OH) resonances were readily assigned to 3α-H and 7α-H. The third C*H*(OH) proton absorbed at for 8β-H and the doublet of triplets at δ 2.79 (*J* 13.1, 5.1, 5.1 Hz) for 15α-H (deshielded by C=O) confirmed 14α-hydroxylation in 14α-hydroxyandrost-5-ene-7,17-dione, and 9α-hydroxylation in 9α-hydroxyandrost-5-ene-7,17-dione was confirmed by the doublet (*J* 11.5 Hz) observed for the 8β-proton (δ 2.81).

TABLE 3

^{1}H N.m.r. data of the products from the action of *C. elegans* on androst-5-en-17-one (4)[a]

	δ (*J* or $W_{\frac{1}{2}}$)					Δδ Values [b,c] Observed		Calculated	
Compound	6-H	8β-H	15α-H	18-H	19-H	18-H	19-H	18-H	19-H
(4)	5.30 (d, *J* 5.0)			0.88	1.02				
14α-Hydroxyandrost-5-ene-7,17-dione	5.72 ($W_{\frac{1}{2}}$ 3)	2.61 (d, *J* 13.3)	2.79 (dt, *J* 13.1, 5.1)	0.99	1.23	0.11	0.21	0.13	0.28
9α-Hydroxyandrost-5-ene-7,17-dione	5.78 ($W_{\frac{1}{2}}$ 4)	2.81 (d, *J* 11.5)	2.84 (*W* 28)	0.90	1.36	0.02	0.34	0.04	0.41

[a] [^{2}H]Chloroform solutions. [b] Δδ Values relative to (4). [c] Minus sign (Δδ) represents an upfield shift.

δ 3.42 as a doublet of doublets (*J* 11.0, 4.5 Hz), indicative of a 1β- or 12β-hydroxy-group. Subtraction of the Δδ contributions [4] for a 7β-hydroxy-group left values of Δδ 0.00 (18-Me) and Δδ +0.03 (19-Me). These Δδ values did not permit a conclusive assignment of structure but the spectrum of the derived triacetate showed methyl shifts of δ 0.81 (18-Me) and δ 1.11 (19-Me). Comparison of these values with those of the trihydroxy-compound (δ 0.75, 1.08) indicated 12β-hydroxylation.

Incubation of Androst-4-ene-3,17-dione (5).—Androst-4-ene-3,17-dione (5) was incubated in the usual way. Chromatography of the broth extract, over alumina, yielded androst-4,6-diene-3,17-dione, 14α-hydroxyandrost-4-ene-3,17-dione, 5-hydroxy-5β-androstane-3,6,17-trione, 4α,5-epoxy-3α-hydroxy-5α-androstan-17-one, and 4α,5-epoxy-3α,11α-dihydroxy-5α-androstan-17-one, listed in decreasing order of R_F value in Table 4.

It is proposed that androst-4,6-diene-3,17-dione ($\lambda_{max.}$

TABLE 4

^{1}H N.m.r. data of the products from the action of *C. elegans* on androst-4-ene-3,17-dione (5)[a]

	δ (*J* or $W_{\frac{1}{2}}$)			Δδ Values [b,c] Observed		Calculated	
Compound	4-H	18-H	19-H	18-H	19-H	18-H	19-H
(5)	5.75 ($W_{\frac{1}{2}}$ 4)	0.93	1.22				
Androsta-4,6-diene-3,17-dione	5.70 ($W_{\frac{1}{2}}$ 3)	0.98	1.15	0.05	−0.07	0.05	−0.03
14α-Hydroxyandrost-4-ene-3,17-dione	5.74 ($W_{\frac{1}{2}}$ 3)	1.04	1.22	0.11	0.00	0.12	0.00
5-Hydroxy-5β-androstane-3,6,17-trione		0.91	0.86				
4α,5-Epoxy-3α-hydroxy-5α-androstan-17-one	3.21 (d, *J* 4.0)	0.90	1.06				
4α,5-Epoxy-3α,11α-dihydroxy-5α-androstan-17-one	3.57 (d, *J* 3.7)	0.90	1.22				

	C_xHOH
	4.05 ($W_{\frac{1}{2}}$ 14) 3β-H
	4.12 (dt, *J* 8.2, 3.7) 11β-H
	3.93 (*J* 10.5, 9.0, 5.5)

[a] [^{2}H]Chloroform solutions. [b] Δδ Values relative to (5). [c] Minus sign (Δδ) represents an upfield shift.

Incubation of Androst-5-en-17-one (4).—Androst-5-en-17-one (4) was synthesized from 3β-hydroxyandrost-5-en-17-one (2) and incubated in the usual way. T.l.c. of the broth extract showed the presence of some 16 (or more) transformation products and six of these were isolated. However, only 14α-hydroxyandrost-5-ene-7,17-dione and 9α-hydroxyandrost-5-ene-7,17-dione (initial fractions) were identified, the remaining fractions containing only trace amounts of complex mixtures. The structures were assigned largely on the basis of the Δδ values (Table 3). The doublet at δ 2.61 (*J* 13.3 Hz)

282 nm, ε 27 200, lit.,[18] $\lambda_{max.}$ 283 nm, ε 27 000[8]) is not a microbial product but results from the dehydration of 6β-hydroxyandrost-4-ene-3,17-dione during chromatography over alumina. [6β-Hydroxy-derivatives are known transformation products of steroids containing the Δ^4-3-one moiety (with *C. elegans*).[19]] This was supported by a qualitative u.v. spectral analysis of the broth extract; this showed only $\lambda_{max.}$ *ca.* 238 nm and no absorbance at 282 nm. Further evidence came from t.l.c. of the broth extract which showed a compound (R_F = 0.41) which was not isolated, whilst androsta-4,6-diene-

3,17-dione (R_F = 0.67) was not observed in the original extract. The structures of 3β-hydroxyandrost-5-ene-7,17-dione and 3β-14α-dihydroxyandrost-5-en-17-one were based largely on ^{1}H n.m.r. comparisons (Table 4).

The last fraction eluted from the chromatographic separation of the incubation products of (5) was shown by ^{1}H n.m.r. spectroscopy to be a mixture (*ca.* 2 : 1) of two compounds. The spectra of both components were indicative of 4ξ,5ξ-epoxy-3ξ-ol systems. Comparison of the data with those of 3ξ-hydroxy-5ξ-cholestane-4ξ,5-epoxides [20] suggested either 4α,5-epoxy-3α-hydroxy- or 4β,5-epoxy-3β-hydroxyandrostan-17-ones. The narrow line width of the C(3)*H*OH signals supported the 4α,5-epoxy-3α-hydroxyandrostan-17-one structure for the minor isomer.

The doublet nature (*J* 3.7 Hz) of the 4-proton signal in the spectrum of the major isomer suggested either the 3α-OH, 5α epoxide or the 3β-OH, 5β epoxide,[20] but the absorption (δ 3.57, *cf.* 3.21 in the minor isomer) is at unusually low field. The C*H*OH absorption associated with the introduced OH was observed at δ 3.93 as an eight-line multiplet consistent with 7β- or 11α-hydroxylation. Relative to 4α,5-epoxy-3α-hydroxyandrostan-17-one, the C-19 methyl protons in this epoxide are deshielded by 0.16 p.p.m. whereas the C-18 shifts are unaffected. This is consistent with 11α-hydroxylation but not with 7β-hydroxylation. The deshielding of the 4-proton may be a result of distortion of ring A caused by interaction between the 11α-OH and C(1)-H_{eq}.

EXPERIMENTAL

^{1}H N.m.r. spectra were recorded using a Brüker Spectrospin WH-270 spectrometer (Fourier transform) at 270.06 MHz. Samples were measured as dilute solution (≤1% w/v, dependent upon solubility), in [^{2}H]chloroform (unless otherwise stated), with tetramethylsilane (*ca.* 0.01% v/v) as internal standard. Solvent deuterium was used as the internal lock.

I.r. spectra were recorded, with Perkin-Elmer 457 or 297 spectrophotometers, in solution ([^{2}H]chloroform). U.v. spectra were recorded as solutions, in absolute ethanol, with a Pye-Unicam SP 800 spectrophotometer. Mass spectra were obtained using an A.E.I. MS 20 spectrometer with a source temperature of 200 °C (±10 °C).

R_F Values were determined by analytical t.l.c. (silica gel 60 F_{254}, 0.25 mm thickness, on glass plates, supplied by E. Merck, Darmstadt), with chloroform–absolute ethanol (9 : 1) as eluant. Spots were located by firstly inspecting the plates with u.v. radiation and secondly, by spraying the plates with aqueous sulphuric acid (25% v/v) and placing them in a warm oven (80—90 °C) for a few minutes, until characteristic colours were observed. [In general, starting materials gave orange–pink spots, enones were u.v.-detectable, epoxides gave yellow spots, and (allylic) alcohols gave blue–purple spots].

M.p.s were recorded in sealed tubes and are uncorrected. Microanalyses were performed by either the Analytical Section, Chemistry Department, Portsmouth Polytechnic, or the Butterworth Microanalytical Consultancy, Teddington, Middlesex.

Steroid starting materials were supplied by Koch-Light Laboratories, Colnbrook, Bucks.

Microbiological Transformations (*Large-scale*).—The liquid culture (nutrient) medium [21] was prepared and divided into portions (200 ml) in 500 ml flasks, plugged (non-absorbent cotton wool) and sterilized (121 °C for 15 min). The fungus, *Cunninghamella elegans*, obtained from Centraalbureau Voor Schimmelcultures, Baarn, Holland, was added and grown for three days at 25 °C. The substrate (10—12.5 mg ml^{-1}) in ethanol (maximum 4 ml per flask), was then added and the incubation was continued for a further three days. At the end of the incubation period the contents of the flasks were combined and filtered through Kieselguhr.

The mycelial growth was extracted with acetone (3 × 50 ml l^{-1} of filtrate) by prolonged soaking (overnight) and decantation. Removal of the acetone left a residue which was partitioned between chloroform and water. The organic layer was separated, dried (sodium sulphate), and the chloroform was distilled off, under reduced pressure. The residual mycelial extract was mixed with light petroleum (b.p. 100—120 °C) and allowed to stand in the cold. For the steroids studied there was usually no precipitate and the extracts which were steroid-free (t.l.c.) were not examined further.

The filtrate was saturated with sodium chloride and extracted with dichloromethane (6 × 150 ml l^{-1} of filtrate), which was then itself washed with saturated sodium chloride solution (1 × 100 ml l^{-1} of filtrate) and dried (sodium sulphate). Removal of the dichloromethane, under reduced pressure, left a residue which was dried (*in vacuo* at 60 °C) to give the broth extract.

Where appropriate, the extracts were subjected to column (alumina) and/or preparative layer chromatography (p.l.c., silica gel, 2-mm thickness). Silica gel 60 PF_{254} was obtained from E. Merck, Darmstadt. Normally, extracts were chromatographed over alumina: where separation of compounds was incomplete, p.l.c. was used (same solvents as for t.l.c.). P.l.c. plates were prepared and activated at 130 °C for 2 h before use. Compounds were applied at the rate of 500 mg m^{-1} (maximum). Fractions from p.l.c. were located by u.v. and/or careful spraying (edges) and extracted by refluxing three times with acetone–methanol–water (18 : 1 : 1) for 5 min. The ratios of alumina : extract were 80—120 : 1 (w/w), whilst the column ratios (height: diameter) were *ca.* 20 : 1. Column fractions were 200 ml.

17-*Oxoandrost-5-en-3β-yl Acetate* (1).—3β-Hydroxyandrost-5-en-17-one (2; R = H) (20.0 g) was dissolved in pyridine (200 ml) and acetylated with acetic anhydride (80 ml), at 80—90 °C, for 6 h. The reaction mixture was poured into water (2 l), and the resultant precipitate filtered off, washed (water), and dried (*in vacuo* at 80 °C). Crystallization from aqueous ethanol gave 17-oxoandrost-5-en-3β-yl acetate (1) 20.0 g (87%), m.p. 168—171 °C (lit.,[22] 168—169 °C; lit.,[23] 172—173 °C); $\nu_{max.}$ (solutions) 1 725 cm^{-1}; M^+ 330 ($C_{21}H_{30}O_3$); R_F 0.72

Incubation of 17-*Oxoandrost-5-en-3β-yl Acetate with* Cunninghamella elegans.—The acetate (1) (5.0 g), in ethanol (400 ml), was added to the fungus in the nutrient medium (20 l, 100 flasks), and incubated for three days. The broth extract (4.10 g) was chromatographed over alumina (Woelm neutral, activity II, 330 g) with ether–absolute ethanol (99 : 1) as eluant. Fractions were combined and crystallized, the residues from the mother-liquors also being chromatographed (p.l.c.), in order to maximise yields. This afforded the following fractions, in decreasing order of R_F value.

(i) Recrystallized from acetone to give 3β-*hydroxy-*

androst-5-ene-7,17-dione (245 mg, 5.4%), m.p. 237—238 °C (lit.,[8] 243—244.5 °C), $\lambda_{max.}$ 238 nm (ε 12 800) [lit.,[8] $\lambda_{max.}$ methanol) 238 nm (ε 13 500); lit.,[18] $\lambda_{max.}$ 235 nm (ε 12 300)]; $\nu_{max.}$ (solution) 3 600, 1 735, and 1 660 cm^{-1}; M^+, 302 ($C_{19}H_{26}O_3$); R_F 0.43.

(ii) Recrystallised from acetone to yield 3β,14α-*dihydroxyandrost-5-ene-7,17-dione* (54 mg; 1.1%), m.p. 244—246 °C (lit.,[9] 246—249 °C); $\lambda_{max.}$ 238 nm (ε 13 300); $\nu_{max.}$ (solutions) 3 600, 1 740, and 1 660 cm^{-1}; M^+ 318 ($C_{19}H_{26}O_4$); R_F 0.35.

(iii) Identified after crystallisation from acetone as 3β,7β-*dihydroxyandrost-5-en-17-one* (415 mg, 9.0%), m.p. 212—213 °C (lit.,[8] 215—216 °C), $\nu_{max.}$ 3 605 and 1 733 cm^{-1}; M^+ 304 ($C_{19}H_{28}O_3$); R_F 0.28.

(iv) Recrystallised from acetone to give 3β,7α-*dihydroxyandrost-5-en-17-one* (1.24 g, 26.8%), m.p. 179—182 °C (lit.,[8] 181.5—183.5 °C); $\nu_{max.}$ 3 600 and 1 732 cm^{-1}; M^+ 304 ($C_{19}H_{28}O_3$); R_F 0.26.

Further elution with ether–absolute ethanol (19 : 1) yielded fraction (v) which was crystallised from ethyl acetate to give 5,6β-*epoxy*-3β,12α-*dihydroxy*-5β-*androstan*-17-*one* (198 mg, 4.1%), m.p. 211—214 °C (Found: C, 71.4; H, 8.6. $C_{19}H_{28}O_4$ requires C, 71.2; H, 8.8%); $\nu_{max.}$ 3 610 and 1 734 cm^{-1}; M^+ 320; R_F 0.21, and fraction (vi) (24 mg), a mixture, the two components of which were not identified, R_F 0.08.

3β,7ξ-*Dihydroxyandrost-5-en-17-ones*.— 17-Oxoandrost-5-en-3β-yl acetate (1) (5.0 g) and cuprous bromide (5.0 g) in acetic acid (50 ml) were boiled under reflux in an atmosphere of nitrogen, whilst t-butyl perbenzoate (12 ml), in acetic acid (50 ml), was added during a period of 15 min. The mixture was boiled under reflux for a further 15 min, cooled, and then poured into chloroform (500 ml). The mixture was filtered and the chloroform layer in the filtrate was washed successively with water (3 × 100 ml), sodium carbonate solution (5%; 3 × 200 ml), and water (2 × 100 ml). The dried (Na_2SO_4) chloroform layer was evaporated and the residue was boiled under reflux with potassium hydroxide (2.0 g), in methanol (150 ml), for 2 h. Removal of the methanol and chromatography (p.l.c.) of the residual organic material (chloroform-soluble) over silica-gel, eluting once, yielded 3β,7β-*dihydroxyandrost-5-en-17-one* (120 mg, 2.6%), m.p. 213—214 °C (lit.,[8] 215—216 °C) from acetone and 3β,7α-*dihydroxyandrost-5-en-17-one* (330 mg, 7.2%), m.p. 180—183 °C (lit.,[8] m.p. 181.5—183.5 °C) from acetone. Both compounds were identical with the 3β,7β- and 3β,7α-dihydroxyandrost-5-en-17-ones isolated from the microbial transformation.

5,6α-*Epoxy*-3β-*hydroxy*-5α-*androstan*-17-*one*.— 3β-Hydroxyandrost-5-en-17-one (2) (5.0 g) was stirred in chloroform (250 ml), and the solution was cooled to 0 °C. This was treated with a solution of *m*-chloroperbenzoic acid (3.70 g) in chloroform (40 ml), precooled to the same temperature. The mixture was stirred and allowed to warm to room temperature. After 1.5 h the reaction mixture was further diluted with chloroform (100 ml). Excess of peracid was destroyed by successively washing with sodium sulphite solution (10% w/v, 200 ml), saturated sodium hydrogen carbonate solution (2 × 100 ml), and water (3 × 100 ml). The organic layer was dried (Na_2SO_4) and the solvent was removed, under reduced pressure. Analysis of the residue (^{1}H n.m.r.), gave the α : β epoxide ratio as *ca.* 5 : 1, the α-isomer predominating.[10] Crystallization from ethyl acetate yielded 5,6α-epoxy-3β-hydroxy-5α-androstan-17-one (3.40 g, 60%), m.p. 226—228 °C (lit.,[24] m.p. 225—226 °C; lit.,[25] 230—230.5 °C); ^{1}H n.m.r. ($CDCl_3$): δ 3.92 (sept, *J* 10.6, 5.3 Hz, 3α-C*H*OH), 2.95 (d, *J* 4.5 Hz, 6β-H), 1.09 (s, 19-H_3), and 0.82 (18-H_3); $\nu_{max.}$ (solution) 3 610, 1 732, 1 064, 1 050, and 1 030 cm^{-1}; M^+ 304 ($C_{19}H_{28}O_3$); R_F 0.47.

Evaporation of the mother-liquors from the crystallization, gave a mixture of the two epoxides, the β-isomer predominating (3 : 2); ^{1}H n.m.r. ($CDCl_3$) δ 3.70 (sept, *J* 10.0, 5.0 Hz, 3α-C*H*OH), 3.13 (d, *J* 2.5 Hz, 6α-H), 1.02 (s, 19-H_3), and 0.85 (s, 18-H_3).

Incubation of 17-*Oxoandrost-5-en-3β-yl Acetate* (1) *with Nutrient Medium*.—The steroid (1) (1.0 g) in ethanol (80 ml) was added to the nutrient medium (4 l, 20 flasks) and swirled for three days. Extraction with dichloromethane (2 × 1 000 ml) gave a residue (0.60 g), which was analysed (t.l.c.) and showed two spots. The major spot (R_F 0.72) corresponded to the starting material (1) and the minor spot (R_F 0.52) to the parent alcohol (2): no other spots were observed.

Incubation of 17-*Oxoandrost-5-en-3β-yl Acetate* (1) *with Sterilized* Cunninghamella elegans.—The above experiment was repeated, but with sterilized fungus, previously grown for three days. The extract (0.57 g) showed only one spot (R_F 0.72), corresponding to the starting material (1).

Prolonged Aerial Oxidation of 17-*Oxoandrost-5-en-3β-yl Acetate* (1).—The steroid (1) (0.75 g), in ethanol (60 ml), was added to sterilized nutrient medium (3 l, 1 flask) and the mixture was stirred for six days whilst a continuous stream of filtered air was passed through it. Samples (25 ml) were taken daily and extracted with dichloromethane (2 × 25 ml). Analyses of these extracts showed that no autoxidation had occurred and the experiment was discontinued.

Monitored Incubation of 17-*Oxoandrost-5-en-3β-yl Acetate* (1) *with* Cunninghamella elegans (*See Table* 1).—The steroid (1) (0.75 g), in ethanol (60 ml) was added to the fungus (three days growth) in the nutrient medium (3 l, 1 flask). Filtered air was passed continually over the surface and aliquots (25 ml) were taken every 2 h for 80 h. Each sample was saturated with sodium chloride (9.0 g) and heated to boiling. On cooling, each fraction was filtered and extracted with dichloromethane (2 × 25 ml). Evaporation of the solvent left a residue which was dissolved in chloroform (100 μl), aliquots (2 μl) of which were transferred to t.l.c. plates. Elution and development showed that all four of the allylic oxidation products were present after *ca.* 3 h, whilst the hydroxy-epoxide was present after *ca.* 15 h.

Incubation of Androst-5-en-3β-ol (3) *with* Cunninghamella elegans (*See Table* 2).—The steroid (3) (4.0 g), in ethanol (400 ml), was added to the fungus in the nutrient medium (40 l, 200 flasks), and incubated for three days. The broth extract (4.61 g) was chromatographed over alumina (Woelm, neutral, activity II, 460 g). Elution with ether–methanol (99 : 1) yielded two major fractions—a mixture (6 mg) of four components which were not identified, and a second fraction (26 mg) which was subject to p.l.c. This yielded three compounds which were identified as (i) 3β,16β-*dihydroxyandrost-5-en-7-one* (4 mg, 0.1%), m.p. 207—209 °C, $\lambda_{max.}$ 238 nm (ε 11 200); $\nu_{max.}$ (solution) 3 615 and 1 647 cm^{-1}; M^+ 304 ($C_{19}H_{28}O_3$); R_F 0.38; (ii) 3β,12β-*dihydroxyandrost-5-en-7-one* (8 mg, 0.2%), m.p. 148—150 °C (Found: C, 75.1; H, 9.1. $C_{19}H_{28}O_3$ requires C, 75.0; H, 9.3%); $\lambda_{max.}$ 238 nm (ε 10 900); $\nu_{max.}$ (solution) 3 620 and 1 672 cm^{-1}; M^+ 304; R_F 0.27.

Acetylation of the latter (acetic anhydride–pyridine) yielded 7-*oxoandrost-5-en*-3β,12β-*diyl diacetate*, ^{1}H n.m.r. δ 4.72 (sept, *J* 11.0, 5.5 Hz, 3α-C*H*OAc), 5.75 (s, $W_{\frac{1}{2}}$ = 3

Hz, 6-H), 0.80 (s, 18-H_3), 1.23 (s, 19-H_3), 4.62 (dd, J 11.0, 4.5 Hz, 12α-C*H*OAc), and 2.05 (s, 3β-OCOCH_3 and 12β-OCOCH_3); (iii) *androst-5-ene-3β,7β,12β-triol* (10 mg, 0.2%), m.p. 137—138 °C (Found: C, 74.8; H, 9.7. $C_{19}H_{30}O_3$ requires C, 74.5; H 9.9%); $\nu_{max.}$ (solution) 3 620 and 1 673 cm^{-1}; M^+ 306; R_F 0.19.

Acetylation (acetic anhydride–pyridine) of the latter yielded *androst-5-ene-3β,7β,12β-triyl triacetate*, ^{1}H n.m.r. δ 4.61 (sept, J 10.0, 5.0 Hz, 3α-C*H*OAc), 5.26 (s, $W_{\frac{1}{2}}$ = 5 Hz, 6-H), 0.81 (s, 18-H_3), 1.11 (s, 19-H_3), 5.05 (dt, J 8.6, 1.6 Hz, 7α-C*H*OAc), 4.65 (dd, J 11.3, 5.0 Hz, 12β-C*H*OAc), 2.03 (s, 7β-OCOCH_3), 2.04 (s, 3β-OCOCH_3), and 2.05 (s, 12β-OCOCH_3).

Continued elution of the original broth extract with ether–methanol (19 : 1) yielded a fraction (54 mg), which was a complex mixture of four, or more, steroids, the components of which were not identified, R_F 0.10. Further elution with ether–methanol (9 : 1) gave a fraction (8 mg) which was a mixture of two unidentified steroids.

Androst-5-en-17-one (4).—3β-Hydroxyandrost-5-en-17-one (2) (20.0 g) was dissolved in pure dry pyridine (100 ml) and cooled to 0 °C. Toluene-*p*-sulphonyl chloride (20 g) was added and the mixture was left overnight at 10 °C. The mixture was poured into water (2 l) and the precipitate was filtered off and dried (*in vacuo*) over phosphorus pentaoxide at room temperature. Crystallization of a small amount, from methanol, gave needles, m.p. 137—138 °C, whilst crystallization from ethyl acetate gave prisms of 17-oxoandrost-5-en-3β-yl tosylate (27.6 g, 75%), m.p. 153—155 °C (lit.,[26] 157—158 °C).

The tosylate (27.0 g) was dissolved in ether (600 ml) and added dropwise to a refluxing solution of lithium aluminium hydride (13.5 g) in ether (400 ml). Refluxing was continued for a further 24 h, at which point excess of hydride was destroyed by the careful, successive additions of water (20 ml), sodium hydroxide (10% w/v; 30 ml) and water (100 ml). The precipitate was filtered off and washed thoroughly with ether. The filtrate and washings were combined and the ether was distilled off. The residual mixture (*ca.* 12 g) was stirred in acetone (1 000 ml) and treated rapidly with Jones' reagent (9.0 ml). After 5 min, the reaction mixture was poured into water (2 l) and extracted with ether (3 × 500 ml). The ether extract was dried (sodium sulphate) and the solvent removed. The residue was chromatographed over alumina (Spence Type H, 400 g), with light petroleum (b.p. 40—60 °C) as eluant. Elution yielded 3α,5-*cyclo*-5α-*androstan*-17-*one* (5.0 g, 30%), m.p. 142—143 °C (lit.,[27] 143—144 °C) from ethyl acetate. Further elution yielded *androst-5-en*-17-*one* (4) (1.5 g, 9.0%), m.p. 101—103 °C (lit.,[28] 105—107 °C) from aqueous ethanol, $\nu_{max.}$ 1 732 cm^{-1}; M^+ 272 ($C_{19}H_{28}O$); R_F 0.78.

Incubation of Androst-5-en-17-one (4) *with* Cunninghamella elegans.—The steroid (4) (1.4 g) in ethanol (105 ml), was added to the fungus in the nutrient medium (7 l, 35 flasks), and incubated for three days. The broth extract (0.80 g) was chromatographed over alumina (Woelm, neutral, activity II, 80 g), with ether as eluant. The first fraction was recrystallized from ether to give 14α-*hydroxyandrost-5-ene-7,17-dione* (5 mg, 0.3%), m.p. 216—219 °C, $\lambda_{max.}$ 239 nm (ε 13 200); $\nu_{max.}$ 3 580, 1 740, and 1 656 cm^{-1}; M^+ 302 ($C_{19}H_{26}O_3$); R_F 0.68.

The second fraction was purified by recrystallisation from ethyl acetate to give 9α-*hydroxyandrost-5-ene-7,17-dione* (4 mg, 0.3%), m.p. 228—230 °C, $\lambda_{max.}$ 239 nm (ε 13 500); $\nu_{max.}$ 3 620, 1 736, and 1 670 cm^{-1}; M^+ 302 ($C_{19}H_{26}O_3$); R_F 0.60.

Incubation of Androst-4-ene-3,17-dione (5) *with* Cunninghamella elegans.—The steroid (5) (5.0 g), in ethanol (400 ml), was added (no precipitate) to the fungus, in the nutrient medium (20 l, 100 flasks), and incubated for 3 days. The broth extract (4.47 g) was chromatographed over alumina (Woelm, neutral, activity II, 450 g), with ether as eluant. This yielded, in decreasing order of R_F value the following. (i) Starting material (5) (8 mg, 0.2%), R_F 0.72. Fraction (ii) recrystallised from ethyl acetate to give *androst-4,6-diene-3,17-dione* (150 mg, 3.0%), m.p. 164—166 °C (lit.,[8] 170.5—172.5 °C), $\lambda_{max.}$ 282 nm (ε 27 200) [lit.,[8] $\lambda_{max.}$ (methanol) 283 nm (ε 26 600)]; $\nu_{max.}$ 1 741 and 1 662 cm^{-1}; M^+ 284 ($C_{19}H_{24}O_2$); R_F 0.67.

Elution with ether–methanol (99 : 1) yielded fraction (iii) which was purified by recrystallisation from methanol to give 14α-*hydroxyandrost-4-ene-3,17-dione* (144 mg, 2.7%), m.p. 253—255 °C (lit.,[29] 256—259 °C), $\lambda_{max.}$ 235 nm (ε 12 800); $\nu_{max.}$ 3 615, 1 742, and 1 666 cm^{-1}; M^+, 302 ($C_{19}H_{26}O_3$); R_F 0.51.

Further elution with ether–methanol (49 : 1) gave fraction (iv) which on recrystallisation from methanol yielded 5-*hydroxy-5β-androstane-3,6,17-trione* (29 mg, 0.5%), m.p. 213—215 °C (Found: C, 71.9; H, 8.4. $C_{19}H_{26}O_4$ requires C, 71.7; H, 8.2%), $\lambda_{max.}$ 240 and 280 nm (ε 940 and 410); $\nu_{max.}$ (solution) 3 590, 1 738, and 1 708 cm^{-1}; $\nu_{max.}$ (Nujol) 3 445, 1 738sh, 1 723, and 1 707 cm^{-1}; M^+ 318; R_F 0.45.

A small amount (*ca.* 2 mg) of the last-named compound was treated with acetyl chloride (0.1 ml) overnight at room temperature. The residue after evaporation was washed with water (3 × 0.5 ml) and dried (*in vacuo* at 50 °C) to give 3,6,17-*trioxo-5β-androstan-5-yl acetate*; ^{1}H n.m.r. δ 0.91 (s, 18-H_3), 0.86 (s, 19-H_3), and 2.05 (s, 5β-OCOCH_3).

Pure methanol eluted fraction (v), 71 mg, which was a mixture of two steroids (*ca.* 2 : 1). The minor component (*ca.* 0.5%) was identified as 4α,5-*epoxy*-3α-*hydroxy*-5α-*androstan*-17-*one*, R_F 0.34 and the major component (*ca.* 0.9%) was identified as 4α,5-epoxy-3α,11α-dihydroxy-5α-androstan-17-one; R_F 0.32.

[9/1705 *Received*, 24*th October*, 1979]

REFERENCES

[1] Part 2, T. A. Crabb, J. A. Saul, and R. O. Williams, *J.C.S. Perkin I*, 1977, 2599; for Part 1 see ref. 2.
[2] T. A. Crabb, P. J. Dawson, and R. O. Williams, *Tetrahedron Letters*, 1975, 3623.
[3] W. Charney and H. L. Herzog, 'Microbial Transformations of Steroids,' Academic Press, New York, 1967.
[4] L. L. Smith, *Steroids*, 1964, **4**, 395; J. E. Bridgeman, P. C. Cherry, A. S. Clegg, J. M. Evans, E. R. H. Jones, A. Kasal, V. Kumar, G. D. Meakins, Y. Morisawa, E. E. Richards, and P. D. Woodgate, *J. Chem. Soc.* (*C*), 1970, 250; R. F. Zurcher, *Helv. Chim. Acta*, 1963, **46**, 2054.
[5] C. W. Shoppee and B. C. Newman, *J. Chem. Soc.* (*C*), 1968, 981.
[6] M. S. Kharasch, G. Sosnovsky, and N. C. Yang, *J. Amer. Chem. Soc.*, 1959, **81**, 5819.
[7] L. Starka, *Coll. Czech. Chem. Comm.*, 1961, **26**, 2452.
[8] R. M. Dodson, R. T. Nicholson, and R. D. Muir, *J. Amer. Chem. Soc.*, 1959, **81**, 6295.
[9] A. M. Bell, E. R. H. Jones, G. D. Meakins, J. O. Miners, and A. L. Wilkins, *J.C.S. Perkin I*, 1975, 2040.
[10] L. F. Fieser and M. Fieser, 'Reagents for Organic Synthesis,' J. Wiley and Sons, New York, 1967, p. 136.
[11] K. Tori, T. Komeno, and T. Nakagawa, *J. Org. Chem.*, 1964, **29**, 1136.
[12] A. D. Cross, *J. Amer. Chem. Soc.*, 1962, **84**, 3206.
[13] L. L. Smith, 1976, personal communication.
[14] L. L. Smith and M. J. Kulig, *Cancer Biochem. Biophys.*, 1975, **1**, 79.
[15] L. L. Smith, J. I. Teng, M. J. Kulig, and F. L. Hill, *J. Org. Chem.*, 1973, **38**, 1763.

[16] L. L. Smith and M. J. Kulig, *J. Amer. Chem. Soc.*, 1976, **98**, 1027.
[17] L. Norymberska, J. Norymberski, and A. Olade, *J. Amer. Chem. Soc.*, 1948, **70**, 1256.
[18] A. I. Scott, 'Interpretation of the Ultraviolet Spectra of Natural Products,' Pergamon Press, Oxford, 1964, p. 400.
[19] A. Schubert, K. Heller, D. Onken, K. Zetsche, and B. Kluger, *Z. Naturforsch.*, 1960, **156**, 269; Y. Nozaki, E. Masuo, H. Ishii, T. Okumura, and D. Satoh, *Ann. Rep. Shionogi Res. Lab.*, 1961, **11**, 9; A. Capek and O. Hanc, *Folia Microbiol.*, 1961, **6**, 237.
[20] D. J. Collins, J. J. Hobbs, and S. Sternhell, *Tetrahedron Letters*, 1963, 623.
[21] J. W. Blunt, I. M. Clark, J. M. Evans, E. R. H. Jones, G. D. Meakins, and J. T. Pinhey, *J. Chem. Soc.* (*C*), 1971, 1136.
[22] A. Butenandt, H. Dannenbaum, G. Henisch, and H. Kudzus, *Z. physiol. Chem.*, 1935, **237**, 57.
[23] T. Richstein and A. Lardon, *Helv. Chim. Acta*, 1941, **24**, 955.
[24] J. Borgna and M. Mousseron-Canet, *Bull. Soc. chim. France*, 1970, 2218.
[25] D. Fukushima and S. Teller, *Steroids*, 1963, **1**, 121.
[26] A. C. de Paulet and J. Bascoul, *Bull. Soc. chim. France*, 1966, 939.
[27] A. Kasal, V. Cerny, and F. Sorm, *Coll. Czech. Chem. Comm.*, 1965, **30**, 472.
[28] R. E. Marker, E. L. Wittle, and B. F. Tullar, *J. Amer. Chem. Soc.*, 1940, **62**, 223.
[29] F. Sondheimer, W. McCrae, and W. G. Salmond, *J. Amer. Chem. Soc.*, 1969, **91**, 1228.