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THE MICROBIOLOGICAL HYDROXYLATION OF 3,16-DISUBSTITUTED ANDROSTANES BY *CEPHALOSPORIUM APHIDICOLA*

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Key Word Index—*Cephalosporium aphidicola*; microbiological hydroxylation; steroid; 5α-androstane-3, 16-dione.

Abstract—5α-Androstane-3, 16-dione has been prepared by hydroboration and oxidation of 3β-hydroxy-5α-androst-16-ene. Reduction with sodium borohydride gave the 3β, 16β-diol. The fungus *Cephalosporium aphidicola* has been shown to hydroxylate 5α-androstane-3, 16-dione predominantly at the C-6β position with minor hydroxylation occurring at the C-7α and C-14α positions. In contrast hydroxylation of the 3β, 16β-diol took place at C-11α. The 3β-hydroxy-16α, 17α-epoxide was hydroxylated at the C-6β position.

INTRODUCTION

Predictive models for the microbiological hydroxylation of steroids have been based on the results of the transformation of a large number of steroids by relatively few organisms. In studies with 5α-androstanes it has been observed [1, 2] that when the substrate possessed oxygen functions at two of the 3-, 11- (6 or 7) or 16-positions, substantial monohydroxylation occurred at the third position. When a substrate possessed an oxygen function in each terminal ring the 'normal' mode [1] of binding led to hydroxylation at position 11 and the 'reverse' mode of binding to hydroxylation on ring B. The microbiological hydroxylation of 3, 16-disubstituted 5α-androstanes was of particular interest in this context since this disubstitution pattern has a quasi-symmetry in which the C-3 position in the 'normal' binding mode and the C-16 position in the 'reverse' binding mode can be superimposed.

5α-Androstane-3, 16-dione and the 16-hydroxy-3-ketone were hydroxylated by *Aspergillus ochraceus* and *Rhizopus nigricans* at C-11α. The 3-hydroxy-16-ketone was hydroxylated by *Aspergillus ochraceus* at C-11α and by *Rhizopus nigricans* at C-7α [2, 3] We have been studying [4, 5] the microbiological hydroxylation of steroids by *Cephalosporium aphidicola*. This fungus is capable of hydroxylating progesterone firstly at C-11α and then at C-6β whilst testosterone is hydroxylated at the C-6β position with minor hydroxylation occurring at the C-11α and C-14α positions. It was therefore of interest to examine the microbiological hydroxylation of 5α-androstane-3, 16-dione and the 3β, 16β-diol by *C. aphidicola* to see if this idea of quasi-symmetry might be extended to hydroxylations by this organism.

RESULTS AND DISCUSSION

The 16-ketone was prepared by the transposition of an oxygen function from C-17. The literature method [6, 7] based on the formation of a 16-benzylidene derivative did not give a satisfactory yield. An alternative method (Scheme 1) involved the conversion of the 17-ketone (3) via the 17-hydrazone (4) to a 17-vinyliodide (5) by oxidation with iodine and triethylamine [8]. Reduction of the vinyliodide with sodium in ethanol gave the 16-ene (6). Hydroboration of the alkene gave a separable mixture of the 16α- and 17α-alcohols, (7) and (8). The stereochemistry of the alcohols followed from nOe studies based on irradiation of the 13β-methyl group signal (δ_H 0.70 and 0.65 in 7 and 8 respectively). This gave an enhancement of 3.5% at δ_H 4.45 in the 16α-alcohol (7) and 6.2% at δ_H 3.73 in the 17α-alcohol (8). The 16α-alcohol was oxidized with chromium trioxide to the 3, 16-diketone (9) and on reduction with sodium borohydride this gave the 3β, 16β-diol (10) [7]. Epoxidation of the 16-ene (6) with m-chloroperbenzoic acid gave the 16α,17α-epoxide (11).

The substrates were incubated with *C. aphidicola* for 7 days in shake culture. The metabolites were

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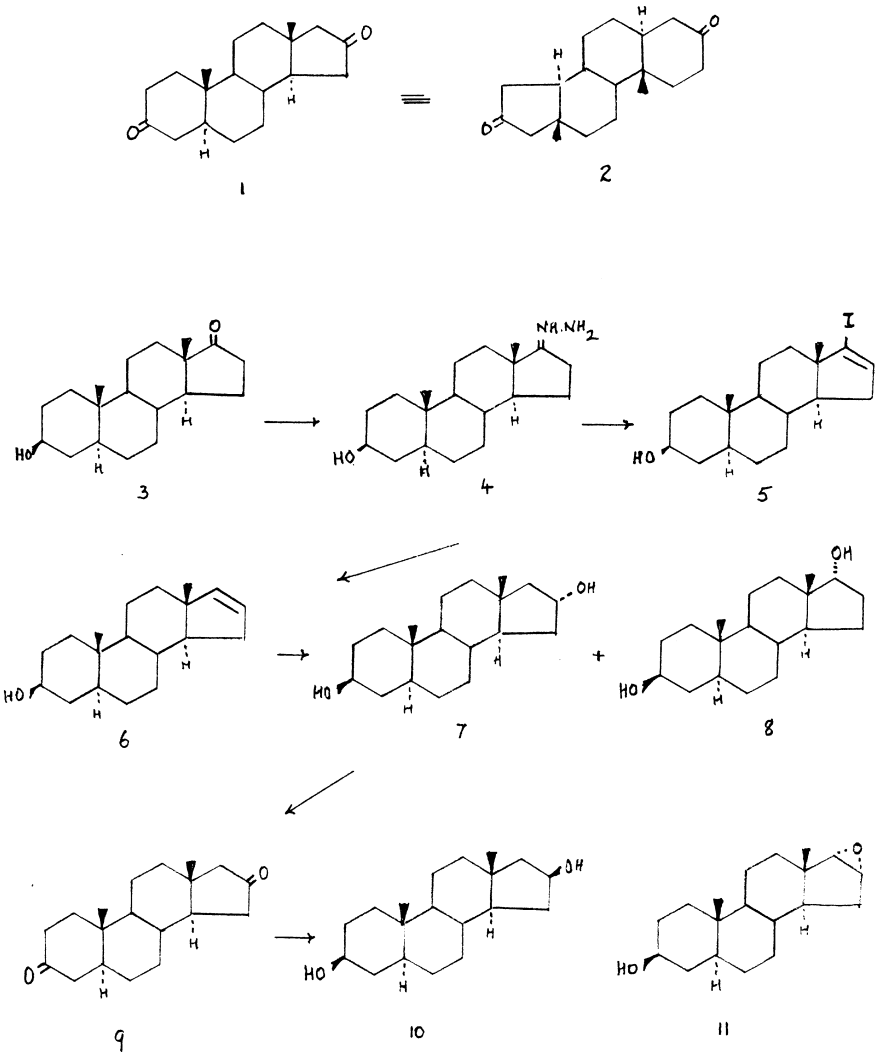


Scheme 1.

separated by chromatography. The results are given in Table 1. The sites of hydroxylation were established from changes in the ^{13}C NMR spectra (Tables 2 and 3). Hydroxylation at C-14 in 12, 13, and 14 was apparent from the replacement of a methine resonance at ca. 51 by a tertiary alcohol signal at ca. 81. The position of the tertiary alcohol was revealed by the β-carbon downfield shifts in the resonances assigned to C-8, C-13 and C-15 and by the γ-gauche upfield shifts for C-7, C-9, C-12 and C-17 when compared to the unsubstituted analogues. Hydroxylation at C-7α in 14 was revealed by the downfield shift of the signals assigned to C-6 and C-8 whilst there were γ-gauche upfield shifts for C-5, C-9 and C-14 when compared to 12. There was a strong hydrogen bond between the C-7 and C-14 hydroxyl groups. The hydrogen only exchanged slowly when a solution was treated with deuterium bromide [slow disappearance of a signal at δ_H 4.69 (OH).] The stereochemistry of this metabolite was confirmed by X-ray crystallography (Fig. 1).

Hydroxylation at C-6β in 15, 16 and 17 was revealed by the downfield shift in the ^1H NMR signal for H-19 and in the ^{13}C NMR signals assigned to C-5 and C-7. There were γ-gauche shieldings for C-4 and C-8. Hydroxylation at C-11α in 18 and 19 was revealed by the characteristic multiplicity of the ^1H NMR signal for H-11β (δ_H 4.20, triplet J 10 Hz, of doublets, J = 4.8 Hz). Irradiation of both the H-18 and H-19 signals produced significant one enhancements of this resonance (6.4% and 6.2% respectively in 18; 5.6% and 7.5% respectively in 19). Furthermore, the ^{13}C NMR signals assigned to C-9 and C-12 showed downfield shifts compared to the unsubstituted compound.

The hydroxylation patterns observed with this fungus fit the pattern of normal and reverse binding observed previously with other organisms [1, 2]. How-

Table 1. Hydroxylation of 3,16-dioxygenated androstanes by *C. aphidicola*

Substrate	Product	Yield%
5α-Androstane-3, 16-dione (9)		
	starting material	11
	14α-hydroxy-5α-androstane-3, 16-dione (12)	3.1
	3β, 14α-dihydroxy-5α-androstan-16-one (13)	5.6
	7α, 14α-dihydroxy-5α-androstane-3, 16-dione (14)	3.8
	6β-hydroxy-5α-androstane-3, 16-dione (15)	15.4
	3β, 6β-dihydroxy-5α-androstan-16-one (16)	8.9
16α, 17α-Epoxy-5α-androstan-3β-ol (11)		
	starting material	57
	16α, 17α-epoxy-5α-androstane-3β,6β-diol (17)	14
3β, 16β-Dihydroxy-5α-androstane (10)		
	starting material	12
	11α, 16α-dihydroxy-5α-androstan-3-one (18)	4.5
	3β, 11α, 16β-trihydroxy-5α-androstane (19)	19.9

Table 2. ^{13}C NMR data for 5α-androstan-3, 16-dione (9) and its hydroxylation products

	9	**12**	**13**	**14**	**15**	**16**
1	37.95^{+}	39.12	36.80	38.07	39.47	38.19
2	38.87	39.35	32.00	38.19	39.29	31.39
3	211.25	213.01	71.08	211.98	212.34	71.54^{+}
4	44.29	44.11	38.73	43.94	41.94	35.63
5	46.24	47.64^{+}	44.44	38.95^{+}	50.75	47.30
6	28.39	29.51	28.90	37.34	70.34	71.63^{+}
7	31.67	28.00	28.03	69.19	40.06	39.73
8	34.53	37.06	36.97	40.01	29.75	29.93
9	53.42	47.29^{+}	47.11	38.83^{+}	53.66	54.18
10	35.59	38.01	35.84	35.89	35.86	35.20
11	20.71	20.98	19.67	19.82	20.78	20.61
12	37.79^{+}	31.07	30.10	29.89	38.00	39.34
13	38.97	45.58	43.01	43.36	39.21	39.23
14	51.17	82.11	81.27	80.93	51.19	51.41
15	37.80	49.00	47.81	48.00	38.08^{+}	38.14
16	217.97	218.89	217.69	218.46	218.32	218.67
17	55.54	51.90	50.81	50.77	55.77	55.83
18	17.85	23.95	22.87	22.61	18.15	18.18
19	11.22	12.44	12.18	10.49	14.92	15.74

$^{+}$assignments may be interchanged

ever an interesting feature is that whereas the diketone is hydroxylated at C-6β, the diol is hydroxylated at C-11α. This could suggest that a ketone favours one mode of binding whilst an alcohol favours another. The 16, 17-epoxide, a hydrogen bond acceptor, behaves like the 16-ketone.

EXPERIMENTAL

General experimental details have been described previously [10]. Except where stated NMR spectra were determined in $CDCl_3$. Petrol refers to the fraction bp 60–80°. Extracts were dried over Na_2SO_4.

Hydroboration of 3β-hydroxy-5α-androst-16-ene (6).

The steroid (6) (3 g) [11] in dry THF (90 cm^3) was treated with 1 M borane in THF (90 cm^3). After 4 h, H_2O (30 cm^3) was added. The soln. was cooled to O° and 10% aq. NaOH (60 cm^3) and 27.5% H_2O_2 (90 cm^3) were added dropwise. The mixture was left to stir overnight. Na_2SO_3 (6 g) was added followed by AcOH (3 cm^3), H_2O (150 cm^3) and dil. HCl (150 cm^3). The soln. was extracted with EtOAc and the extract was washed with H_2O, brine and dried. The solvent was evaporated to give a residue which was chromatographed on silica. Elution with 30% EtOAc-petrol gave 3β, 17α-dihydroxy-5α-androstane (8) (1.17 g)

Table 3. ^{13}C NMR data for 3β-hydroxy-16α, 17α-epoxy-and 3β, 16β-dihydroxy-5α-androstane and their hydroxylation products

	11	**17**	**10**	**18**†	**19**†
1	36.77	38.35	36.96	36.24	37.94
2	31.26^{+}	31.42	31.49	33.48	33.00^{+}
3	70.95	71.54	71.31	209.28	70.51
4	37.96	35.23	38.17	44.03	39.74
5	44.81	47.48	44.83	46.33	45.84
6	28.48	71.82	28.62	28.21	29.89
7	31.74^{+}	39.28	32.33	30.83	33.08^{+}
8	33.47	28.51	35.27	37.26	35.27
9	54.63	54.65	54.33	58.83	61.01
10	35.56	35.61	35.59	36.34	38.00
11	20.78	20.68	20.91	66.68	68.26
12	27.26	27.38	37.22	49.96	51.78
13	40.52	40.69	40.21	39.33	40.98
14	43.94	43.83	54.44	51.93	53.68
15	32.37	32.43	39.01	39.33	40.08
16	53.72	53.80	72.12	69.43	71.05
17	62.16	62.25	51.38	50.14	51.80
18	15.13	15.60	19.11	18.97	20.56
19	12.17	15.60	12.35	10.28	13.12

$^{+}$ assignments may be interchanged† determined in pyridine-d_5

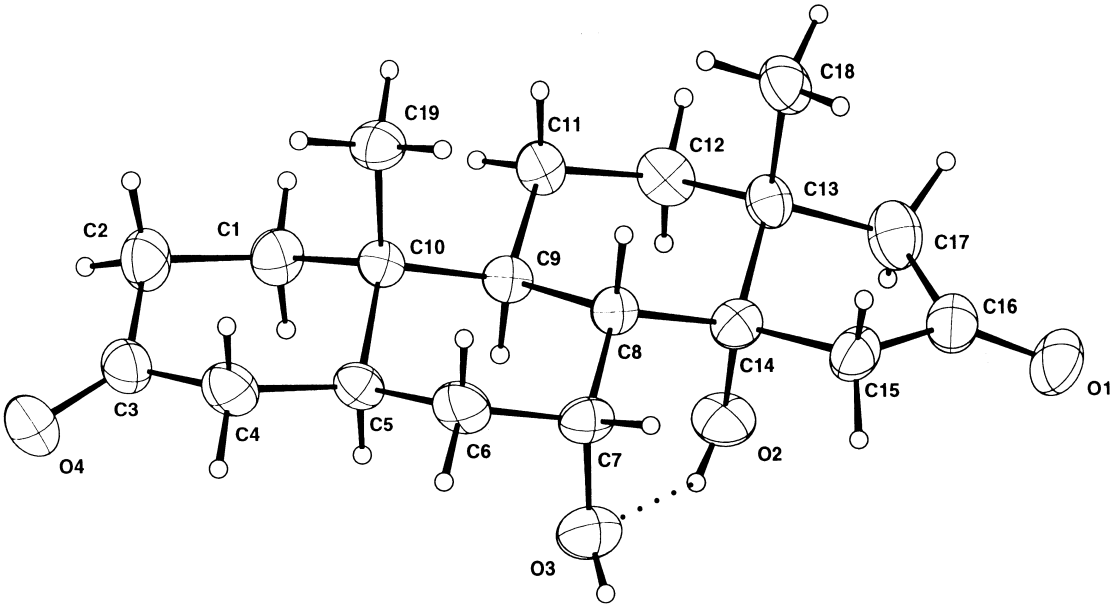
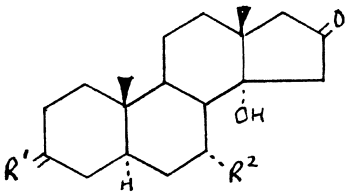


Fig. 1.

which crystallized from EtOAc-petrol as needles, mp 212° (lit.[12], 214°); IR $\upsilon_{max}.cm^{-1}$ 3252; ^1H NMR δ_H 0.65 (3H,*s*, H-18), 0.82 (3H,*s*, H-19), 3.60 (1H,*tt*, *J* = 5 and 11 Hz, H-3), 3.73 (1 H, *br.d.*, J = 5 Hz, H-17). Further elution gave 3β, 16α-dihydroxy-5α-androstane (7) (1.58 g) which crystallized from EtOAc-petrol as needles, mp 190° (Found: C, 73.7; H, 11.4. $C_{19}H_{32}O_2$. H_2O requires C, 73.5; H, 11.0%); IR $\upsilon_{max}\,cm^{-1}$ 3262; ^1H NMR δ_H 0.70 (3 H,*s*, H-18), 0.80 (3H,*s*, H-19), 3.60 (1H,*tt*, J = 5 and 11 Hz, H-3), 4.45 (1H,m, H-16).

Oxidation of 3β, 16α-*dihydroxy*-5α-*androstane* (7).

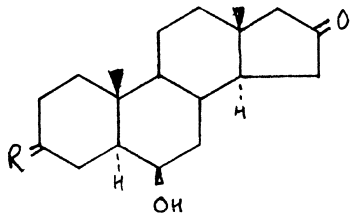
The diol (7) (1.2 g) in Me_2CO (120 cm^3) was treated with 8 N CrO_3 reagent at O° until the orange colour persisted. The soln. was allowed to warm to room



12 $R' = O$, $R^2 = H$

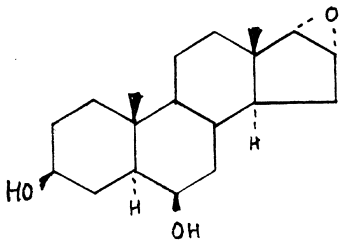
13 $R' = \alpha\text{-}H, \beta\text{-}OH$, $R^2 = H$

14 $R' = O$, $R^2 = \alpha\text{-}OH, \beta\text{-}H$

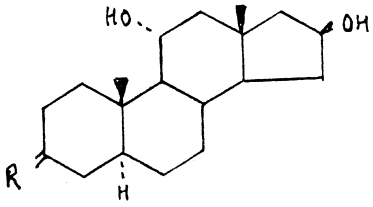


15 $R = O$

16 $R = \alpha\text{-}H, \beta\text{-}OH$



17



18 $R = O$

19 $R = \alpha\text{-}H, \beta\text{-}OH$.

temp. over 30 min. MeOH was added and the soln. was concentrated, diluted with H_2O, and the product recovered in EtOAc. The extract was washed with aq. $NaHCO_3$, H_2O, dried and the solvent evaporated. The residue was crystallized from EtOAc to give 5α-androstane-3, 16-dione (9) (1 g) as cubes, mp 162–163° (lit.[6], 162–163°); IR υ_{max} cm^{-1} 1738, 1703; ^1H NMR δH 0.83 (3 H,*s*, H-18), 0.99 (3H,*s*, H-19).

Reduction of 5α-*androstane*-3, 16-*dione* (9).

The diketone (9) (0.9 g) in MeOH (30 cm^3) was treated with $NaBH_4$ (1 g) at O° for 40 min. AcOH (3 cm^3) was added and the solvent was evaporated. H_2O was added and the product was recovered in EtOAc. The extract was washed with aq.$NaHCO_3$, H_2O and dried. The solvent was evaporated to give 3β, 16β-dihydroxy-5α-androstane (10) (0.85 g) which crystallized from EtOAc-petrol as needles, mp 178° (Found: C, 78.2; H, 11.1. $C_{19}H_{32}O_2$ requires C, 78.0; H, 11.0%), IR υ_{max} cm^{-1} 3343; ^1H NMR δH 0.82 (3H,*s*, H-18), 0.94 (3H,*s*, H-19), 3.60 (1H,*tt*, J = 4.8 and 11.0 Hz, H-3), 4.37 (1 H,*tdd*, J = 7. 5,5.6 and 1.8 Hz, H-16).

Epoxidation of 3β-*hydroxy*-5α-*androst*-16-*ene* (6).

The steroid (6) (1.8 g) [11] in $CHCl_3$ (100 cm^3) was treated with m-chloroperbenzoic acid (3 g) at O° in portions over 10 min. The mixture was allowed to warm to room temp. After 2 h, the soln was diluted with $CHCl_3$ and washed thoroughly with aq. Na_2SO_3, aq. $NaHCO_3$, H_2O and brine. The solvent was evaporated to give 16α, 17α-epoxy-5α-androstane-3β-ol (11) (1.1 g) which crystallized from petrol as needles, mp 149–151°, (Found: C,78.3; H,10.4. $C_{19}H_{30}O_2$ requires C,78.6; H, 10.4%); IR υ_{max} cm^{-1} 3479; ^1H NMR δH 0.72 (3H,*s*, H-18), 0.81 (3H,*s*, H-19), 3.09 (1H,*d*, J = 3 Hz, H-17), 3.33 (1H,*m*, H-16), 3.57 (1H,*tt*, J = 5.5 and 11 Hz, H-3).

Incubation of steroids with C. aphidicola.

(a) The fungus was grown on shake culture (100 cm^3 medium) in 250 cm^3 conical flasks as described previously [10]. Three days after inoculation, 5α-androstane-3, 16-dione (9) (1.1 g) in EtOH (50 cm^3) was evenly distributed between 50 flasks. After a further 7 days, the mycelium was filtered and the broth extracted with EtOAc. The extract was dried and the solvent evaporated to give a residue which was chromatographed on silica. Elution with 20% EtOAc-petrol gave the starting material (121 mg). Elution with 30% EtOAc-petrol gave 14α-hydroxy-5α-androstane-3, 16-dione (12) (36 mg) which crystallized from EtOAc-petrol as needles, mp 198° (decomp.), (Found: C, 75.0; H, 9.2. $C_{19}H_{28}O_3$ requires C, 75.0; H, 9.3%);

IR υ_{max} cm^{-1} 3475, 1725, 1712; ^1H NMR δH 1.07 and 1.08 (each 3H,*s*, H-18 and H-19). Elution with 45% EtOAc-petrol gave 3β, 14α-dihydroxy-5α-androstan-16-one (13) (65 mg) which crystallized from EtOAc-petrol as needles, mp 236° (decomp.) (Found: C, 74.5; H,9.7.$C_{19}H_{30}O_3$ requires C,74.5; H,9.9%); IR υ_{max} cm^{-1} 3421, 1737; ^1H NMR δH 0.87 (3H,*s*, H-18), 1.05 (3H,*s*, H-19), 3.61 (1H,*tt*, J = 5 and 11 Hz, H-3). Elution with 50% EtOAc-petrol gave 7α, 14α-dihydroxy-5α-androstane-3, 16-dione (14) (46 mg) which crystallized from EtOAc-petrol as needles, mp 238–239° (Found: C, 71.4; H, 8.9. $C_{19}H_{28}O_4$ requires C,71.2; H,8.8%); IR υ_{max} cm^{-1} 3411, 3340, 1732, 1712; ^1H NMR δH 1.05 and 1.06 (each 3H,*s*, H-18 and H-19), 4.15 (^1H,*br.s*., H-7), 4.69 (^1H,*s*, OH). Further elution with 50% EtOAc-petrol gave 6β-hydroxy-5α-androstane-3, 16-dione (15) (179 mg) which crystallized from EtOAc-petrol as needles, mp 219° (Found: C,70.9; H,9.2. $C_{19}H_{28}O_4$ requires C,71.2; H,8.8%); IR υ_{max} cm^{-1} 3451, 1724, 1707; ^1H NMR δH 0.95 (3H,*s*, H-18), 1.25 (3H,*s*, H-19), 3.80 (1H,*br.s*, H-6).

Further elution with 60% EtOAc-petrol gave 3β, 6β-dihydroxy-5α-androstan-16-one (16) (109 mg) which crystallized from EtOAc-petrol as needles, mp 184–185°, (Found: C, 74.2; H, 9.8. $C_{19}H_{30}O_3$ requires C,74.5; H,9.9%); IR υ_{max} cm^{-1} 3414, 1738; ^1H NMR δH 0.91 (3H,*s*, H-18), 1.17 (3H,*s*, H-19), 3.68 (1H,*tt*, J = 5.2 and 10.7 Hz, H-3), 3.84 (1H,*d*, J = 2 Hz, H-6).

(b) Under similar fermentation conditions 16α, 17α-epoxy-5α-androstane-3β-ol (11) (800 mg) gave, on chromatography on silica in 20% EtOAc-petrol the starting material (470 mg). Further elution with 60% EtOAc-petrol gave 16α, 17α-epoxy-5α-androstane-3β, 6β-diol (17) (125 mg) as a gum, (Found: M^+ 306.218 $C_{19}H_{30}O_3$ requires 306.219); IR υ_{max} cm^{-1} 3479; ^1H NMR δH 0.76 (3H,*s*, H-18), 1.14(3H,*s*, 19-H), 3.11 (1H,*d*, J = 3 Hz, H-17), 3.36 (1H,*m*,H-16), 3.65 (1H,*tt*, J = 5 and 11 Hz, H-3), 3.80 (1H,*d*, J = 2 Hz, H-6).

(c) Under similar fermentation conditions 3β, 16β-dihydroxy-5α-androstane (10) (1 g) gave, on chromatography on silica in 40% EtOAc-petrol, the starting material (12 mg). Elution with 50% EtOAc-petrol gave 11α, 16β-dihydroxy-5α-androstane-3-one (18) (47 mg) which crystallized from EtOAc-petrol as cubes, mp 204° (lit.[1], 206–207°); IR υ_{max} cm^{-1} 3348, 1715; ^1H NMR (pyridine-d_5) δH 1.16 (3H,*s*, H-18), 1.21 (3H,*s*, H-19), 4.20 (1H,*dt*, J = 4.8 and 10 Hz, H-11), 4.61 (1H,*dd*, J = 5.6 and 8 Hz, H-16). Elution with 60% EtOAc-petrol gave 3β, 11α, 16β-trihydroxy-5α-androstane (19) (210 mg) which crystallized from EtOAc-petrol as cubes, mp 245–249° (lit.[1], 250–251°); IR υ_{max} cm^{-1} 3384, 3371; ^1H NMR (pyridine-d_5) δH 1.09, (3H,*s*, H-18), 1.20 (3H,*s*, H-19), 3.90 (1H,*tt*, J = 5 and 10 Hz, H-3), 4.23 (1H,*dt*, J = 4.8 and 10 Hz, H-11), 4.57 (1H,*dd*, J = 5.5 and 8 Hz, H-16).

Crystallographic Structure Determination of (14).

$C_{19}H_{28}O_4$, M-320.4, orthorhombic, space group $P2_12_12_1$ (No.19), a = 6.2790(10), b = 11.802(2), c = 22.629(3) A, $\alpha = \beta = \gamma = 90°$ V = 1676.9 (4) A^3 Z = 4, $D_{calc.}$ = 1.27 $g.cm^{-3}$, F(000) 696, monochromated Mo-K_α radiation γ = 0.71073 A, u = 0.09 mm^{-1}

Data were collected using a crystal ca.0.30 × 0.15 × 0.15 mm on an Enraf–Nonius CAD 4 diffractometer. A total of 3367 reflections were collected with $2 < \theta < 25°$ for $0 < h < 7$, $0 < k < 14$, $-26 < 26$ and 2199 reflections with $1 > 20(I)$ were used in the refinement. There was no crystal decay.

The structure was solved by direct methods using SHELXS-86 and non-hydrogen atoms were refined anisotropically by full matrix least squares on all F^2 using SHELXS-93. The hydrogen atoms were included in riding mode with U_{iso}(H) equal to $1.2U_{eq}$(C) or $1.5U_{eq}$(C) for methyl groups. Hydroxyl groups were fixed at idealised geometry but with the torsion angle defining the H atom positions refined and U_{iso} (H) equal to $1.5U_{eq}$(O). The final R indices [$I > 20(I)$] were $R_1 = 0.062$, $WR_2 = 0.155$ and R indices (all data) $R_1 = 0.086$, $WR_2 = 0.173$. The crystallographic data have been deposited with the Cambridge Crystallographic Data Centre.

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