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THE HYDROXYLATION OF SOME 13α-METHYLSTEROIDS BY *CEPHALOSPORIUM APHIDICOLA*

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Key Word Index—*Cephalosporium aphidicola*; 13α-methylsteroids; androstanes; microbiological hydroxylation.

Abstract—The fungus, *Cephalosporium aphidicola*, has been shown to hydroxylate 5α,13α-androstan-3,17-dione and the 3β-alcohol at the C-1α and C-7α positions, whereas the corresponding compounds in the normal 13β-methyl series are hydroxylated at the C-11α and C-14α positions. Both series were hydroxylated at the 5α-position. There was some epimerization of the axial 3α-alcohols to the equatorial 3β-epimers. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The microbiological hydroxylation of 13α-methyl steroids by *Cephalosporium aphidicola* has been explored in the context of the factors that govern the relationship between biosynthetically directed and xenobiotic transformations in this organism. The isomerization at C-13 has the effect of substantially distorting the relatively planar steroid geometry of the 5α,13β-androstanes and hence of modifying the 'fit' of the steroid to the Brannon and Jones models [1, 2] for steroid hydroxylation. Secondly, when ring A of the steroid is superimposed on ring A of aphidicolin [3], the characteristic secondary metabolite of *C. aphidicola*, the 13α-methyl group of the steroid lies close to C-17 of aphidicolin. This site undergoes an efficient hydroxylation in the biosynthesis of aphidicolin [4]. Previous studies [5, 6] on the microbiological hydroxylation of steroids by *C. aphidicola* have shown that it will hydroxylate ring D desoxysteroids in the normal series at C-17. In these cases the steroidal C-17 lies nearer to the aphidicolin C-17. It was therefore of interest to see if the fungus would hydroxylate the 13α-methyl group. The unsaturated ketone, progesterone, is hydroxylated by *C. aphidicola* firstly at C-11α and then at C-6β. Recent advances in the study of the microbiological hydroxylation of other steroids have been reviewed [7].

RESULTS AND DISCUSSION

The 13α-methyl steroids were prepared by treatment of the 17-oximes with acetic anhydride in pyridine [8]. This generates the 17-acetylamino-16-enes with the more stable *cis* C–D–ring fusion. Hydrolysis of the enamines affords the 17-ketones. The 3α-alcohols were prepared from the 3β-alcohols using a modified Mitsunobu reaction [9].

The following pairs of compounds were incubated with *Cephalosporium aphidicola*: 3α-hydroxy-5α,13α-androstan-17-one (**1**) and 3α-hydroxy-5α-androstan-17-one (**9**); 5α,13α-androstan-3,17-dione (**2**) and 5α-androstan-3,17-dione (**10**); 3β-hydroxy-5α,13α-androstan-17-one (**3**) and 3β-hydroxy-5α-androstan-17-one (**11**); 3α- and 3β-hydroxy-5α,13α-androstane (**4** and **5**). The results are given in Table 1.

The position of hydroxylation was established by changes in the ^{13}C NMR spectra (see Tables 2 and 3) whilst the stereochemistry of the secondary alcohols followed from the multiplicity of the relevant ^{1}H NMR signal and, where appropriate, nOe studies based on irradiation of the 18-H and 19-H methyl signals. The location of a hydroxyl group at the 1α-position followed from downfield changes to the ^{13}C NMR signals for C-2 and C-10 and from the γ-gauche shieldings exhibited by C-5 and C-9 [10]. Furthermore decoupling experiments in the ^{1}H NMR spectrum showed that both the C<u>H</u>(OH) signals were coupled to the same methylene proton resonances (δ_H 1.65 and 1.97). The new C<u>H</u>(OH) resonance is a broad singlet typical of an equatorial proton. The location of a tertiary hydroxyl group at C-5 followed from the downfield shift in the position of the ^{13}C NMR signals assigned to C-4, C-5, C-6 and C-10 and to the γ-gauche shieldings experienced by C-7 and C-9. Furthermore the 3α-H resonance showed a characteristic downfield shift ($\Delta\delta$ *c* 0.4 ppm) compared to the unsubstituted compound. The location of a group at the C-

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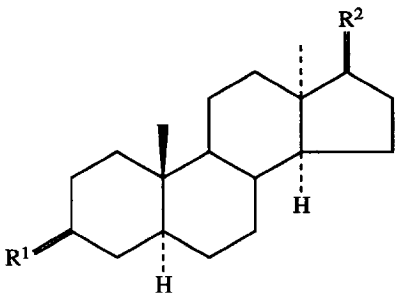

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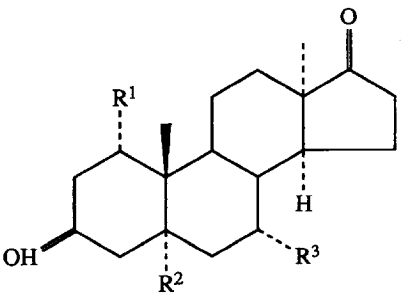

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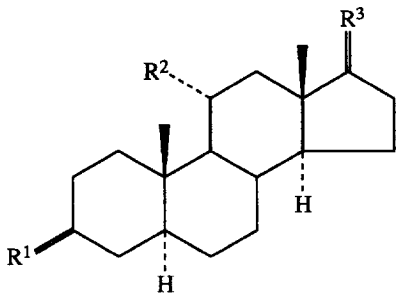

9 R^1 = α–OH, β–H; R^2 = H, R^3 = O

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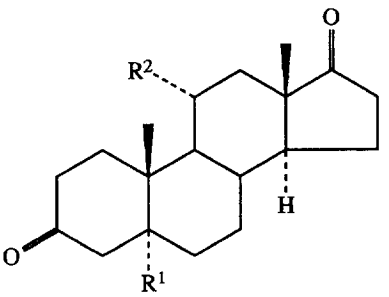
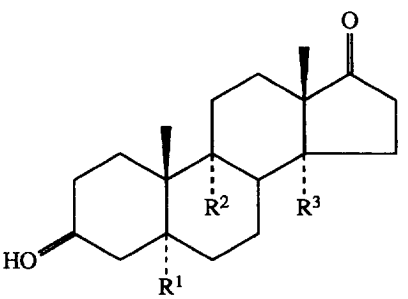


14 R^1 = OH; R^2 = H

15 R^1 = H; R^2 = OH



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17 R^1 = R^3 = H; R^2 = OH

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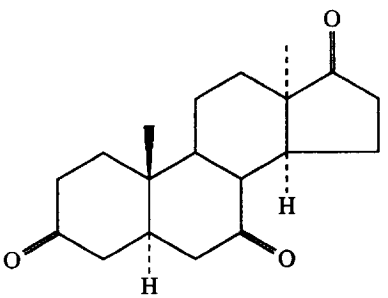

19

Table 1. Biotransformation of steroids by *Cephalosporium aphidicola*

Substrate	Product	% Yield
13α-Methyl Series		
3α-Hydroxy-5α,13α-androstan-17-one (**1**) S.M.		51
	3β-hydroxy-5α,13α-androstan-17-one (**3**)	37
5α,13α-Androstan-3,17-dione (**2**) S.M		28
	3β-hydroxy-5α,13α-androstan-17-one (**3**)	19.5
	1α,3β-dihydroxy-5α,13α-androstan-17-one (**6**)	13
	3β,5α-dihydroxy-13α-androstan-17-one (**7**)	0.5
	3β,7α-dihydroxy-5α,13α-androstan-17-one (**8**)	2.5
3β-Hydroxy-5α,13α-androstan-17-one (**3**) S.M.		22
	1α,3β-dihydroxy-5α,13α-androstan-17-one (**6**)	7.8
	3β,5α-dihydroxy-5α,13α-androstan-17-one (**7**)	17
	3β,7α-dihydroxy-5α,13α-androstan-17-one (**8**)	4.4
3α-Hydroxy-5α,13α-androstane (**4**) S.M.		96
	3β-hydroxy-5α,13α-androstane (**5**)	3
3β-Hydroxy-5α,13α-androstane (**5**) no metabolism		
13β-Methyl Series		
3α-Hydroxy-5α-androstan-17-one (**9**) S.M.		79
	3β,9α-dihydroxy-5α-androstan-17-one (**17**)	10.2
	5α-hydroxyandrostan-3,17-dione (**14**)	5.3
5α-Androstan-3,17-dione (**10**) S.M.		17
	3β-hydroxy-5α-androstan-17-one (**11**)	9
	3β,17β-dihydroxy-5α-androstane (**12**)	5.5
	11α-hydroxy-5α-androstan-3,17-dione (**15**)	3.5
	3β,5α-dihydroxy-5α-androstan-17-one (**16**)	1
3β-Hydroxy-5α-androstan-17-one (**11**) S.M.		14
	3β,5α-dihydroxy-5α-androstan-17-one (**16**)	4
	3β,14α-dihydroxy-5α-androstan-17-one (**18**)	2.5
	3β,11α,17β-trihydroxy-5α-androstane (**13**)	12.2

S.M. = Starting material recovered.

Table 2. ^{13}C NMR data for 13α-methyl steroids (determined in $CDCl_3$)

	Compound								
Carbon	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**19**
1	32.2	37.9	36.8	32.0	36.8	72.5	30.8	36.7	36.6
2	28.9	38.2	31.4	28.9	31.4	37.6	30.5	31.6	37.2
3	66.4	211.8	71.2	66.5	71.4	66.5	67.3	71.1	209.2
4	35.7	44.4	37.9	35.8	38.1	37.8	43.9	37.4	43.7
5	38.6	46.1	44.3	38.7	44.4	37.6	74.9	36.1	41.8
6	28.4	28.7	28.5	28.7	28.8	28.3	34.4	31.2	45.2
7	32.9	32.6	32.2	32.9	33.0	31.8	26.6	66.2	208.5
8	37.8	37.7	37.8	37.6	37.6	36.9	37.0	44.5	46.5
9	50.8*	50.5	50.8	52.5	52.6	43.8	43.4	43.5	52.2
10	36.2	35.7	35.6	36.2	35.6	39.7	38.8	35.6	35.7
11	21.2	21.3	21.3	21.0	21.2	21.3	21.2	20.6	23.3
12	31.9	32.0	33.0	33.4	33.4	32.6	32.1	36.5	31.0
13	50.1	50.1	50.1	41.7	41.7	50.2	50.2	50.2	49.3
14	51.6*	51.1	51.6	53.3	53.2	50.7	50.7	41.6	50.8
15	22.2	22.9	22.7	28.1	28.1	21.9	22.9	22.5	23.9
16	33.9	33.8	33.8	20.4	20.8	33.8	33.7	33.8	33.3
17	222.6	222.1	222.4	35.8	35.7	222.5	222.3	222.5	221.6
18	25.3	25.2	25.3	30.2	30.2	25.3	25.2	25.2	24.6
19	10.9	11.2	12.0	11.1	12.2	12.4	16.0	11.0	10.7

* These assignments may be interchanged.

7α-position followed from the downfield shift of the ^{13}C NMR signals assigned to C-6, C-7 and C-8 and the γ-gauche shielding of C-9 and C-14. Oxidation gave a 3,7,17-triketone (**19**) which was identical to a sample prepared from 3β-hydroxy-13α-androst-5-en-17-one.

Table 3. ^{13}C NMR data for 13β-methyl steroids (determined in $CDCl_3$)

Carbon	Compound 13[a]	14	16*	17	18
1	40.1	32.6	32.3	29.9	36.9
2	33.0	37.8	31.9	31.2	31.1
3	68.5	210.6	66.6	70.7	70.8
4	39.7	51.9	45.2	38.2	37.7
5	45.9	77.4	74.2	36.3	44.4
6	29.8	34.2	34.9	28.2	28.0
7	32.2	25.0	25.3	26.9	24.7
8	35.4	34.4	34.3	37.3	37.7
9	61.0	45.9	45.3	75.6	47.2
10	37.9	39.3	39.4	40.3	35.6
11	70.5	20.7	21.0	24.5	19.1
12	49.8	31.6	31.5	27.3	25.2
13	44.1	47.7	48.1	47.6	52.7
14	50.8	50.9	51.2	44.2	81.0
15	23.9	21.7	22.0	21.6	33.1
16	31.0	35.8	36.1	35.8	29.8
17	81.1	220.8	220.5	220.7	219.7
18	13.0	13.8	14.1	14.3	17.9
19	13.1	15.7	16.2	12.7	12.1

* Determined in pyridine-d_5.

Comparison of these results reveals some interesting trends. The ability of this organism to hydroxylate in the 1:3-position to a functional group is observed in the hydroxylation at C-1α, C-5α and C-14α. This finds a parallel in the biosynthetic transformation of 16,18-dihydroxy-aphidicolane to 3α,16,18-trihydroxyaphidicolane [4]. Secondly the 7α-position of the 13α-methyl steroids corresponds to the 11α-position of the 13β-methyl series when the former is bound in the 'reversed-capsized' conformation [1, 2]. Finally, there was no evidence for hydroxylation of the C-13α methyl group despite its congruence with C-17 of the aphidicolane series.

EXPERIMENTAL

General experimental methods have been described previously [5]. 3β-Hydroxy-5α,13α-androstan-17-one (**3**) [11]: mp 125–128° (lit. [11] 129–130°). ^1H NMR ($CDCl_3$): δ 0.65 (3H, *s*, 18-H), 0.97 (3H, *s*, 19-H), 3.60 (1H, *tt*, $J = 5$ and 11 Hz, 3-H). 5α,13α-Androstan-3,17-dione (**2**) [11]: mp 165–166° (lit. [11] 165–167°), ^1H NMR ($CDCl_3$): δ 0.86 (3H, *s*, 18-H), 0.99 (3H, *s*, 19-H). 3β-Hydroxy-5α,13α-androstane (**5**) [11]: mp 94–95° (lit. [11] 104°, dimorphic). ^1H NMR ($CDCl_3$): δ 0.73 (3H, *s*, 18-H), 0.86 (3H, *s*, 19-H), 3.59 (1H, *tt*, $J = 5.4$ and 12.1 Hz, 3-H).

Preparation of 3α-hydroxy-5α,13α-androstan-17-one (**1**). Triphenylphosphine (3 g) and dry chloroacetic acid (1.2 g) were added to a sol of 3β-hydroxy-5α,13α-androstan-17-one (2 g) in dry toluene (60 ml). Diethyl azodicarboxylate (1 ml) was added dropwise and the resultant yellow soln was stirred at room temp. overnight. The solvent was evapd and the residue was chromatographed on silica gel in EtOAc–petrol (60–80°) (1:9) to give 3α-chloroacetoxy-5α,13α-androstan-17-one (2 g) which crystallized from EtOAc–petrol as needles, mp 85–87° (found: C, 68.3; H, 8.5. $C_{21}H_{31}O_3Cl$ requires C, 68.7; H, 8.5%). IR ν_{max} cm^{-1}: 1745, 1728. ^1H NMR ($CDCl_3$): δ 0.57 (3H, *s*, 18-H), 0.91 (3H, *s*, 19-H), 4.00 (2H, *s*, $O.CO.CH_2Cl$), 5.05 (1H, *br s* 3β-H). The chloroacetate in MeOH (60 ml) was then treated with soln of K_2CO_3 (4 g) in E_2O (20 ml) at room temp. for 1 hr. HOAc (3 ml) was added, the MeOH was evapd *in vacuo* and the product recovered with EtOAc. The extract was washed with aq. $NaHCO_3$, H_2O, satd aq. NaCl and dried over Na_2SO_4. The solvent was evapd and the residue chromatographed on silica gel. Elution with 7% EtOAc–petrol gave 3α-hydroxy-5α,13α-androstan-17-one (**1**) (1.25 g) which crystallized from EtOAc–petrol as plates, mp 145–146° (lit. [12] 145–146°) (Found: C, 78.5; H, 10.4. Calc. for $C_{19}H_{30}O_2$: C, 78.6; H, 10.4%). IR ν_{max} cm^{-1}: 3324, 1726. ^1H NMR ($CDCl_3$): δ 0.62 (3H, *s*, 18-H), 0.97 (3H, *s*, 19-H), 4.05 (1H, *br s*, 3β-H).

Under similar conditions 3β-hydroxy-5α,13α-androstane (**5**) (2 g) gave 3α-chloroacetoxy-5α,13α-androstane (1.7 g) which crystallized from MeOH as needles, mp 67–68° (Found: C, 71.2; H, 9.4. $C_{21}H_{33}O_2Cl$ requires C, 71.5; H, 9.4%). IR ν_{max} cm^{-1}: 1742. ^1H NMR ($CDCl_3$): δ 0.72 (3H, *s*, 18-H), 0.87 (3H, *s*, 19-H), 4.07 (2H, *s*, $OCOCH_2Cl$), 5.12 (1H, *br s* 3β-H). Hydrolysis as above, gave 3α-hydroxy-5α,13α-androstane (**4**) (1.27 g) which crystallized from EtOAc–petrol as needles, mp 115–117° (Found: C, 79.9; H, 11.7. $C_{19}H_{32}O$. 0.5 H_2O requires C, 79.9; H, 11.6%). IR ν_{max} cm^{-1}: 3306. ^1H NMR ($CDCl_3$): δ 0.70 (3H, *s*, 18-H), 0.87 (3H, *s*, 19-H), 4.04 (1H, *br s* 3β-H).

Biotransformation experiments. Cephalosporium aphidicola was grown in shake culture (100 ml) in 250 ml conical flasks as described previously [5]. The steroid (1 g) in EtOH–DMSO (25 ml) was added to 50 flasks 3 days after inoculation. After a further 10 days, the mycelium was filtered and the broth was extracted with EtOAc. The extract was dried (Na_2SO_4) and the solvent evapd to give a gum which was chromatographed on silica. The column was eluted with a gradient of increasing concs of EtOAc in petrol to give the following results:

(*a*) *3α-Hydroxy-5α,13α-androstan-17-one* (**1**). (1 g) gave the starting material (507 mg) and on elution with EtOAc–petrol (1:4), 3β-hydroxy-5α,13α-androstan-17-one (**3**) (374 mg), mp 110–112°C, identified by its ^1H NMR spectrum.

(*b*) *5α, 13α-Androstan-3,17-dione* (**2**). (1 g) gave the starting material (275 mg) followed in EtOAc–petrol (1:3) by 3β-hydroxy-5α,13α-androstan-17-one (200 mg) identified by its ^1H NMR spectrum. Further elution with EtOAc–petrol (1:1) gave 1α,3β-dihydroxy-5α,13α-androstan-17-one (**6**) (135 mg) as a gum (Found: M^+ 306.218, $C_{19}H_{30}O_3$ requires 306.219), IR ν_{max} cm^{-1}: 3383, 1725. ^1H NMR ($CDCl_3$): δ 0.65 (3H, *s*, 18-H), 0.98 (3H, *s*, 19-H), 3.84 (1H, *br s* 1β-H), 4.01

(1H, *tt*, $J = 5.2$ and 10.6 Hz, 3α-H). Further elution gave 3β,7α-dihydroxy-5α,13α-androstan-17-one (**8**) (27 mg) which crystallized from EtOAc–petrol as needles, mp 203–204°, (Found: M^+ 306.219, $C_{19}H_{30}O_3$ requires 306.219). IR ν_{max} cm^{-1}: 3406, 1732; ^1H NMR ($CDCl_3$): δ 0.66 (3H, *s*, 18-H), 0.98 (3H, *s* 19-H), 3.63 (1H, *tt*, $J = 4.7$ and 11.0 Hz, 3α-H), 4.06 (1H, *br s* 7β-H). The triketone was identical to authentic material prepared from 3β-hydroxy-13α-androst-5-en-17-one. Further elution gave 3β,5α-dihydroxy-13α-androstan-17-one (**7**) (7 mg) as a gum (Found: M^+ 306.220, $C_{19}H_{30}O_3$ requires 306.219). IR ν_{max} cm^{-1}: 3389, 1732. ^1H NMR ($CDCl_3$): δ 0.83 (3H, *s*, 18-H), 0.98 (3H, *s*, 19-H), 4.08 (1H, *tt*, $J = 5.9$ and 10.0 Hz, 3α-H).

(*c*) *3β-Hydroxy-5α,13α-androstan-17-one* (**3**). (500 mg) gave the starting material (110 mg). Elution with EtOAc–petrol (1:1) gave successively 1α,3β-dihydroxy-5α,13α-androstan-17-one (**6**) (41 mg), 3β,7α-dihydroxy-5α,13α-androstan-17-one (**8**) (23 mg) and 3β,5α-dihydroxy-13α-androstan-17-one (**7**) (90 mg) each identified by their ^1H NMR spectra.

(*d*) *3α-Hydroxy-5α,13α-androstane* (**4**). (1 g) gave the starting material (960 mg) and on elution with EtOAc–petrol (3:22) 3β-hydroxy-5α,13α-androstane (**5**) (31 mg), mp 92–94°, identified by its ^1H NMR spectrum.

(*e*) *3β-Hydroxy-5α,13α-androstane* (**5**). (500 mg) gave the starting material (493 mg) and no other transformation product was detected.

(*f*) *3α-Hydroxy-5α-androstan-17-one* (**9**). (1 g) gave the starting material (787 mg). Elution with EtOAc–petrol (1:1) gave 3β,9α-dihydroxy-5α-androstan-17-one (**17**) (108 mg) which crystallized from EtOAc–petrol as cubes, mp 192–193° (lit. [13] 192–194°) IR ν_{max} cm^{-1}: 3354, 1739. ^1H NMR ($CDCl_3$): δ 0.87 (3H, *s*, 18-H), 0.96 (3H, *s*, 19-H), 3.60 (1H, *tt*, $J = 4.5$ and 11.5 Hz, 3α-H). Further elution gave 5α-hydroxy-5α-androstan-3,17-dione (**14**) (56 mg) which crystallized from EtOAc–petrol as needles, mp 212–213° (lit. [14] 213–214.5°). IR ν_{max} cm^{-1}: 3352, 1724, 1692. ^1H NMR ($CDCl_3$): δ 0.89 (3H, *s*, 18-H), 1.20 (3H, *s*, 19-H).

(*g*) *5α-Androstan-3,17-dione* (**10**). (1 g) gave, after incubation for 7 days, in the fr. eluted with EtOAc–petrol (3:7), 3β-hydroxy-5α-androstan-17-one (**11**) (93 mg), which crystallized from EtOAc–petrol as needles, mp 175–177° (lit. [13] 176–177.5°), identified by its ^1H NMR spectrum. Further elution gave 3β,17β-dihydroxy-5α-androstane (**12**) (57 mg) which crystallized from EtOAc–petrol as needles, mp 167–168° (lit. [15] 168°) identified by its ^1H NMR spectrum Elution with EtOAc–petrol (1:1) gave 11α-hydroxy-5α-androstan-3,17-dione (**15**) (37 mg) which crystallized from petrol as needles, mp 191–193°, (lit., [13] 192–194°) IR ν_{max} cm^{-1}: 3334, 1732, 1717; ^1H NMR ($CDCl_3$): δ 0.91 (3H, *s*, 18-H), 1.16 (3H, *s*, 19-H), 4.03 (1H, *dt*, $J = 5.2$ and 11.0 Hz, 11β-H). Elution with EtOAc–petrol (3:2) gave 3β,5α-dihydroxyandrostan-17-one (**16**) (13 mg) which crystallized from EtOAc–petrol as needles, mp 280° (lit. [14] 281–282°). IR ν_{max} cm^{-1}: 3393, 1724, ^1H NMR (pyridine-d_5): δ 0.80 (3H, *s*, 18-H), 1.03 (3H, *s*, 19-H), 4.75 (1H, *tt*, $J = 5.3$ and 10.3 Hz, 3α-H).

(*h*) *3β-Hydroxy-5α-androstan-17-one* (**11**). (1 g) gave the starting material (137 mg). Elution with EtOAc–petrol (1:1) gave 3β,14α-dihydroxy-5α-androstan-17-one (**18**) (27 mg) which crystallized from EtOAc–petrol as needles, mp 217–220° (lit. [16] 281–220°). IR ν_{max} cm^{-1}: 3342, 1743. ^1H NMR ($CDCl_3$): δ 0.85 (3H, *s*, 18-H), 1.00 (3H, *s*, 19-H), 3.60 (1H, *tt*, $J = 4.8$ and 10.5 Hz, 3α-H). Continued elution gave 3β,5α-dihydroxy-5α-androstan-17-one (**16**) (43 mg), mp 279–280°, identified by its ^1H NMR spectrum. Further elution then gave 3β,11α,17β-trihydroxy-5α-androstane (**13**) (130 mg), which crystallized from Me_2CO as needles, mp 245–247°, (lit. [13] 247–249°). IR ν_{max} cm^{-1}: 3316; ^1H NMR (pyridine-d_5-^2H_2O wash): δ 1.02 (3H, *s*, 18-H), 1.11 (3H, *s*, 19-H), 3.91 (1H, *tt*, $J = 5$ and 11 Hz, 3α-H), 3.94 (1H, *t*, $J = 9$ Hz, 17α-H), 4.24 (1H, *dt*, $J = 5.5$ and 11.5 Hz, 11β-H).

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