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THE HYDROXYLATION OF Δ^5-ANDROSTENES BY *CEPHALOSPORIUM APHIDICOLA*

CAROLINE M. BENSASSON, JAMES R. HANSON* and A. CHRISTY HUNTER

School of Chemistry, Physics and Environmental Science, University of Sussex, Brighton, Sussex, BN1 9QJ, UK

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Key Word Index—*Cephalosporium aphidicola*; microbiological hydroxylation; steroid; dehydroisoandrosterone.

Abstract—Whereas the major hydroxylation product of 3β-hydroxy-5α-androstan-17-one by *Cephalosporium aphidicola* is the 11α-alcohol, the presence of a Δ^5-double bond in the substrate leads to non-stereospecific allylic hydroxylation at C–7. Hydroxylation at C–11 became a minor transformation and there was no detectable hydroxylation at C–14.

INTRODUCTION

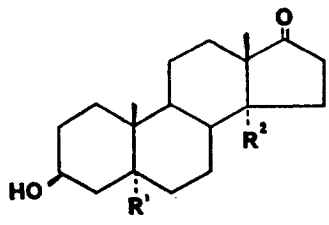
The factors that govern the microbiological hydroxylation of steroids involve a combination of effects based on the site, stereochemistry and nature of the existing functional groups in the substrate. The relative contributions of these factors to hydroxylation by different organisms continues to attract interest [1]. The fungus, *Cephalosporium aphidicola*, has proved to be a useful organism for the microbiological hydroxylation of steroids. We have shown [2] that it will hydroxylate progesterone firstly at C–11α and then at C–6β whilst testosterone is hydroxylated at C–6β with only minor transformations taking place at C–11α and C–14α [3]. The introduction of unsaturation into ring B has been shown to significantly affect the positions of hydroxylation by other organisms. Thus compound **1** was hydroxylated at C–15α by *Fusarium graminearium* whilst the unsaturated analogue **5** was attacked at the allylic position C–7 [4]. The fungus *Cunninghamella elegans* has also been shown [5] to hydroxylate 3β-hydroxy-androst-5-en-17-one (**5**) at C–7α. However, compared to the information available on the hydroxylation of Δ^4-3-keto steroids, less is known about the hydroxylation of the Δ^5-androstenes. We have therefore compared the hydroxylation by *C. aphidicola* of the unsaturated steroids **5** and **10** with that of the saturated analogue **1**.

*Author to whom correspondence should be addressed.

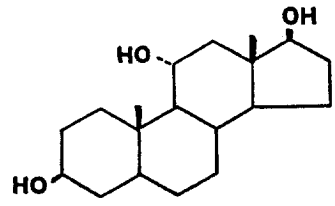
RESULTS AND DISCUSSION

The substrates were incubated with *C. aphidicola* for 8 days. The results are given in Table 1. The sites of hydroxylation were established by the changes in the ^{13}C NMR spectra (see Table 2) [6] whilst the stereochemistry followed from a comparison with the known ^1H NMR patterns [7].

Incubation of 3β-hydroxy-5α-androstan-3-one (**1**) with *C. aphidicola* gave three metabolites which were separated by chromatography on silica. The first metabolite was identified as the known 3β, 14α-dihydroxy-5α-androstan-17-one (**2**) [8] from the downfield shifts of the ^{13}C NMR signals assigned to C–8 ($\Delta\delta_C$ 2.7 ppm), C–13 ($\Delta\delta_C$ 5.0 ppm) and C–15 ($\Delta\delta_C$ 11.3 ppm) and the γ-gauche upfield shifts for the signals assigned to C–7 ($\Delta\delta_C$ 6.2 ppm), C–12 ($\Delta\delta_C$ 6.4 ppm) and C–16 ($\Delta\delta_C$ 6.0 ppm) when compared to the starting material. The second metabolite was 3β, 5α-dihydroxy-5α-androstan-17-one (**3**) [9]. There were downfield shifts for C–4 ($\Delta\delta_C$ 7.2 ppm) and C–6 ($\Delta\delta_C$ 6.5 ppm) and C–10 ($\Delta\delta_C$ 3.8 ppm) and γ-gauche upfield shifts for C–1 ($\Delta\delta_C$ 4.6 ppm), C–7 ($\Delta\delta_C$ 5.3 ppm) and C–9 ($\Delta\delta_C$ 8.6 ppm). The H–3α proton resonance appeared at low field (δ_H 4.75) in accordance with a transannular 1:3-diaxial interaction with a hydroxyl group. The major metabolite was 3β,11α,17β-trihydroxy-5α-androstane (**4**) [10]. The ^1H NMR spectrum contained signals characteristic of H–3α (δ_H 3.91, *t*, J = 11 Hz, of *t*, J = 5 Hz), H–11β (δ_H 4.24, *t*, J = 11.5 Hz, of *d*, J = 5.5 Hz) and H–17α (δ_H 3.94, *t*, J = 9 Hz). The H–11β signal received an n.O.e. enhancement on irradiation



1 $R^1 = R^2 = H$
2 $R^1 = H, R^2 = OH$
3 $R^1 = OH, R^2 = H$



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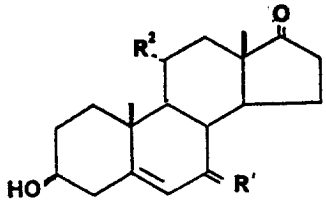
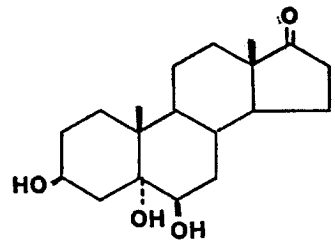


5 $R^1 = H_2, R^2 = H$
6 $R^1 = \alpha\text{-OH}, \beta\text{-H}, R^2 = H$
7 $R^1 = \alpha\text{-H}, \beta\text{-OH}, R^2 = H$
8 $R^1 = H_2, R^2 = OH$



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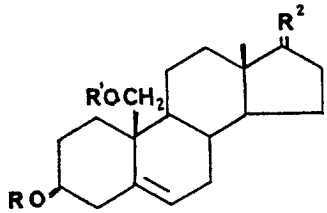
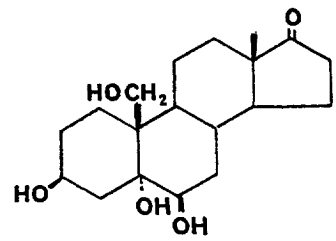


10 $R^1 = H, R^2 = O$
11 $R^1 = Ac, R^2 = O$
12 $R^1 = H, R^2 = \alpha\text{-H}, \beta\text{-OH}$
13 $R^1 = Ac, R^2 = \alpha\text{-H}, \beta\text{-OAc}$



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of both the H–18 (δ_H 1.02) and H–19 (δ_H 1.11) resonances of 3.5% and 4.7%, respectively. Comparison of the ^{13}C NMC spectrum with that of 3β, 17β-dihydroxy-5α-androstane [11] revealed downfield shifts for the resonances assigned to C–9 ($\Delta\delta_C$ 6.1 ppm) and C–12 ($\Delta\delta_C$ 12.3 ppm).

The major metabolites obtained from 3β-hydroxyandrost-5-en-17-one (**5**) were identified as the C–7α and C–7β alcohols **6** and **7**, respectively, by comparison of their ^1H NMR spectral data with literature values [5, 12]. Comparison of their ^{13}C NMR signals with those of the starting material [6] revealed the anticipated downfield shifts for C–6 ($\Delta\delta_C$ 4.3 and 6.6 ppm) and C–8 ($\Delta\delta_C$ 6.5 and 9.1 ppm) and a γ-gauche shielding for C–9 ($\Delta\delta_C$ 7.2 and 1.4 ppm). The fermentation also produced a minor amount of 3β, 11α-dihydroxyandrost-5-en-17-one (**8**). The ^1H NMR spectrum possessed signals at δ_H 3.54, (*t*, J = 10.5 Hz of *t*, J = 5 Hz) and δ_H 3.89 (*t*, J = 11 Hz, of *d*, J = 5 Hz) characteristic of the H–3α and H–11β resonances. The other minor product was 3β, 5α, 6β-trihydroxy-androstan-17-one (**9**) which was identified by comparison with an authentic sample. 3β, 19-Dihydroxy-androst-5-en-17-one (**10**) was relatively poorly metabolized. The 17-ketone was reduced to the 17β-alcohol (**12**) which was purified as its triacetate (**13**). The alkene was also converted to the 5α, 6β-diol

Table 1. Incubation of Steroids with *C. aphidicola*

Substrate	Product	% Yield
3β-Hydroxy-5α-androstan-17-one (**1**)		
	3β, 11α, 17β-trihydroxy-5α-androstane (**4**)	12
	3β, 14α-dihydroxy-5α-androstan-17-one (**2**)	2
	3β, 5α-dihydroxy-5α-androstan-17-one (**3**)	4
3β-Hydroxyandrost-5-en-17-one (**5**)		
	3β, 7α-dihydroxyandrost-5-en-17-one (**6**)	25
	3β, 7β-dihydroxyandrost-5-en-17-one (**7**)	31
	3β, 11α-dihydroxyandrost-5-en-17-one (**8**)	6
	3β, 5α, 6β-trihydroxyandrostan-17-one (**9**)	6
3β, 19-Dihydroxyandrost-5-en-17-one (**10**)		
	3β, 17β, 19-trihydroxyandrost-5-ene (**12**)[+]	6
	3β, 5α, 6β, 19-tetrahydroxyandrostan-17-one (**14**)	8

[+]isolated as the triacetate (**13**)

(**14**). We were unable to isolate any products arising from hydroxylation at C–7 or C–11.

In conclusion we have shown that the introduction of a (Δ^5-double bond significantly alters the biotransformation of 3β-hydroxy-5α-androstan-17-one by *C. aphidicola* leading to non-stereospecific allylic hydroxylation at C–7 in place of attack at C–14α and reducing the extent of hydroxylation at C–11α. However, this allylic hydroxylation did not appear to take place to any significant extent in the presence of a 19-hydroxyl group. The 5α, 6β-diols were probably formed via the 5α, 6α-epoxide. In this case hydroxylation at C–5α in the saturated steroid has been replaced by epoxidation of the Δ^5-double bond.

EXPERIMENTAL

General experimental and fermentation details have been described previously [13]. The fungus, *C. aphidicola* was grown on shake culture.

Incubation of steroids

(a) 3β-Hydroxy-5α-androstan-17-one (**1**) (1 g) in EtOH (50 ml) was evenly distributed between 50 flasks of a 3 day old culture. After a further 7 days the mycelium was filtered and the broth was extracted with EtOAc. The extract was dried and the solvent evaporated to give a residue which was chromatographed on silica. Elution with EtOAc–petrol (1:3) gave the starting material (137 mg). Elution with EtOAc–petrol (1:1) gave 3β, 11α,17β-trihydroxy-5α-androstane (**4**) (130 mg) which crystallized from Me_2CO as needles, mp 245–247° (lit. [10], 247–249°), IR ν_{max} cm^{-1}: 3316. ^1H NMR (pyridine-d_5, 500 MHz after ^2H_2O wash): δ 1.02 (3H, *s*, H_3–18), 1.11 (3H, *s*, H_3–19), 3.91 (1H, *tt*, J = 5 and 11 Hz, H–3α), 3.94 (1H, *t*, J = 9 Hz, H–17α), 4.24 (1H, *td*, J = 11.5 and 5.5 Hz, H–11β). Further elution gave 3β, 14α-dihydroxy-5α-androstan-17-one (**2**) (23 mg) which crystallized from EtOAc–petrol as needles, mp 217–220° (lit. [8], 218–220°), IR ν_{max} cm^{-1}: 3342. ^1H NMR ($CDCl_3$): δ 0.85 (3H, *s*, H_3–18), 1.00 (3H, *s* H_3–19), 3.60 (1H, *tt*, J = 4.8 and 10.5 Hz, H–3α). Further elution gave 3β,5α-dihydroxy-5α-androstan-17-one (**3**) (43 mg) which crystallized from EtOAc as needles, mp 279–280° (lit. [9], 281–282°), IR ν_{max} cm^{-1}: 3393, 1724. ^1H NMR (pyridine-d_5): δ 0.80 (3H, *s*, H_3–18), 1.03 (3H, *s*, H_3–19), 4.75 (1H, *tt*, J = 5.3 and 10.3 Hz, H–3α).

(b) 3β-Hydroxyandrost-5-en-17-one (**5**) (1.5 g) in DMSO–EtOH (5:1, 30 ml) was evenly distributed between 50 flasks of a 3 day old culture. After a further 8 days, the mycelium was filtered and the broth was extracted with EtOAc. The extract was dried and the solvent evaporated to give a residue which was chromatographed on silica. Elution with

Table 2. ^{13}C NMR signals of androstanes determined in $CDCl_3$ at 75 MHz

Carbon	Compound											
	1	**2**	**3**[+]	**4**[+]	**5**	**6**	**7**	**9**	**10**	**11**[a]	**13**[b]	**14**[+]
1	36.9	36.9	32.3	40.0	37.2	37.5	37.6	32.3	33.9	33.9	33.9	33.9
2	31.4	31.1	31.9	32.1	31.5	32.0	32.0	33.2	31.9	28.2	28.2	32.5
3	70.9	70.8	66.6	68.5	71.4	70.9	71.0	67.3	70.8	73.5	73.6	67.4
4	38.0	37.7	45.2	39.7	42.2	43.4	43.0	42.6	42.7	38.5	38.5	42.7
5	44.8	44.4	74.2	45.9	141.3	145.2	142.7	75.8	137.5	135.2	135.0	75.6
6	28.4	28.0	34.9	29.8	120.8	125.0	127.8	75.9	126.8	126.4	124.6	76.2
7	30.9	24.7	25.3	31.0	31.5	63.7	72.4	34.4	32.1	32.0	31.3	33.0
8	35.0	37.7	34.8	35.4	31.5	38.0	40.6	30.8	32.8	32.9	33.1	32.1
9	54.5	47.2	45.8	61.0	50.3	43.1	48.9	46.0	50.8	50.6	50.4	46.6
10	35.6	35.6	39.4	37.9	36.7	37.8	37.0	39.2	41.7	40.3	40.1	43.9
11	20.5	17.1	21.0	70.5	20.4	20.5	20.8	20.9	21.0	21.3	21.5	21.7
12	31.6	25.2	31.5	49.8	30.8	32.4	32.6	32.4	30.4	30.6	37.3	29.7
13	47.7	52.7	48.1	44.1	47.5	47.3	47.9	48.0	47.8	48.0	42.9	48.6
14	51.4	81.0	51.5	50.8	51.8	45.6	51.9	51.4	52.5	52.5	51.9	52.4
15	21.8	33.1	22.0	23.9	21.8	22.3	25.0	22.0	21.8	22.2	23.9	22.1
16	35.8	29.8	36.1	33.0	35.8	36.0	36.2	35.9	35.8	36.2	27.9	36.1
17	220.8	219.7	220.5	81.1	221.3	220.1	220.3	220.1	221.2	221.2	83.0	220.4
18	13.8	17.9	14.1	13.0	13.5	13.4	13.6	13.8	13.9	14.1	12.5	14.4
19	12.3	12.1	16.2	13.1	19.4	18.4	19.1	17.0	62.6	64.8	64.9	64.7

[+]determined in pyridine-d_5
[a]acetates 21.5, 21.8 and 171.0 (×2)
[b]acetates 21.6, 21.8 (×2), 171.0, 171.2 and 171.7

EtOAc–petrol (1:1) gave 3β, 7β-hydroxyandrost-5-en-17-one (**7**) (482 mg) which crystallized from EtOAc–petrol as needles, mp 207° (lit. [11], 215°), IR ν_{max} cm^{-1}; 3440, 1726. ^1H NMR (pyridine-d_5): δ 0.84 (3H, *s*, H_3–18), 0.99 (3H, *s*, H_3–19), 3.85 (1H, *tt*, *J* = 5 and 10.5 Hz, H–3α), 4.15 (1H, *d*, *J* = 7.5 Hz, H–7α), 5.71 (1H, *s*, H–6). Elution with EtOAc–petrol (3:2) gave 3β, 7α-dihydroxyandrost-5-en-17-one (**6**) (389 mg) which crystallized from EtOAc–petrol as needles, mp 177° (lit. [11], 182–183°), IR ν_{max} cm^{-1}: 3400, 1723. ^1H NMR (pyridine-d_5): δ 0.86 (3H, *s*, H_3–18), 1.02 (3H, *s* H_3–19), 3.75 (1H, *tt*, *J* = 4.5 and 11 Hz, H–3α), 4.15 (1H, *br.s.*, H–7β), 5.89 (1H, *br.s*, H–6). Elution with EtOAc–petrol (7:3) gave 3β, 11α-dihydroxyandrost-5-en-17-one (**8**) (95 mg) which crystallized from EtOA–petrol as prisms, mp 210° (lit. [10], 211–213°), IR ν_{max} cm^{-1}: 3595, 1740. ^1H NMR ($CDCl_3$): δ 1.15(3H, *s*, H_3–18), 1.33 (3H, *s*, H_3–19), 3.54 (1H, *tt*, *J* = 5 and 10.5 Hz, H–3α), 3.89 (1H, *t*, *J* = 11 Hz of *d*, *J* = 5 Hz, H–11β), 5.41 (1H, *d*, *J* = 1.5 Hz, H–6). Elution with EtOAc–petrol (9:1) gave 3β, 5α, 6β-trihydroxyandrostan-17-one (**9**) (89 mg) which crystallized from EtOAc as prisms, mp 298–301° (lit. [14], 292–297°) IR ν_{max} cm^{-1} 3551, 3444, 3348, 1726. ^1H NMR (pyridine-d_5): δ 0.82 (3H, *s*, H_3–18), 1.59 (3H, *s*, H_3–19), 4.16 (1H, *s*, H–6), 4.82 (1H, *tt*, *J* = 5.5 and 10.5 Hz, H–3α).

(c) 3β, 19-Dihydroxyandrost-5-en-17-one (**10**) (1.6 g) in DMSO–EtOH (5:1, 30 ml) was evenly distributed between 50 flasks of a 3 day old culture. After a further 8 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 EtOAc–petrol (7:3) gave the starting material (635 mg). Elution with EtOAc–petrol (4:1) gave a mixture of the starting material and a second compound which was further purified by acetylation with Ac_2O-pyridene overnight and chromatography. Elution with EtOAc–petrol (1:4) gave 3β,17β,19-triacetoxy-androst-5-ene (**13**) (102 mg) which crystalized from EtOAc–petrol as plates, mp 90° (lit. [15], 88–89°), ^1H NMR ($CDCl_3$): δ 0.82 (3H, *s*, H_3–18), 2.03, 2.04 and 2.05 (each 3H, *s*, OAc), 3.96 and 4.49 (1H, *d*, *J* = 12 Hz, H–19), 4.60 (1H, *t*, *J* = 8.5 Hz, H–17α), 4.63 (1H, *tt*, *J* = 5 and 11 Hz, H–3α), 5.66 (1H, *br.s*, H–6). Elution of the original column with MeOH–EtOAc (1:9) gave 3β,5α,6β,19-tetrahydroxyandrostan-17-one (**14**) (146 mg) which crystallized from EtOAc as prisms, mp 264–266° (Found: C, 67.0; H, 8.9. $C_{19}H_{30}O_5$ requires C, 67.4; H, 8.9%). IR ν_{max} cm^{-1}: 3200 (*br*), 1730 ^1H NMR (pyridine-d_5): δ 0.78 (3H, *s*, H_3–18), 3.94 (1H, *d*, *J* = 2 Hz, H–6), 4.03 and 4.66 (1H, *d*, *J* = 12 Hz, H–19), 4.70 (1H, *tt*, *J* = 5 and 10.5 Hz, H–3α).

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