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# THE HYDROXYLATION OF $\Delta^5$ -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  $3\beta$ -hydroxy- $5\alpha$ -androstan-17-one by *Cephalosporium aphidicola* is the 11 $\alpha$ -alcohol, the presence of a  $\Delta^5$ -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. © 1998 Elsevier Science Ltd. All rights reserved

## INTRODUCTION

The factors that govern the microbiological hydroxvlation 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-11 $\alpha$  and then at C-6 $\beta$  whilst testosterone is hydroxylated at C-6 $\beta$  with only minor transformations taking place at C-11 $\alpha$  and C-14 $\alpha$  [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-15 $\alpha$  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  $3\beta$ -hydroxy-androst-5-en-17-one (5) at C-7 $\alpha$ . However, compared to the information available on the hydroxylation of  $\Delta^4$ -3-keto steroids, less is known about the hydroxylation of the  $\Delta^5$ -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 <sup>13</sup>C NMR spectra (see Table 2) [6] whilst the stereochemistry followed from a comparison with the known <sup>1</sup>H NMR patterns [7].

Incubation of  $3\beta$ -hydroxy- $5\alpha$ -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  $3\beta$ ,  $14\alpha$ dihydroxy- $5\alpha$ -androstan-17-one (2) [8] from the downfield shifts of the <sup>13</sup>C NMR signals assigned to C-8 ( $\Delta\delta_{\rm C}$  2.7 ppm), C-13 ( $\Delta\delta_{\rm C}$  5.0 ppm) and C-15 ( $\Delta\delta_{\rm C}$  11.3 ppm) and the  $\gamma$ -gauche upfield shifts for the signals assigned to C-7 ( $\Delta \delta_{\rm C}$ ) 6.2 ppm), C-12 ( $\Delta\delta_{\rm C}$  6.4 ppm) and C-16 ( $\Delta\delta_{\rm C}$ 6.0 ppm) when compared to the starting material. The second metabolite was  $3\beta$ ,  $5\alpha$ -dihydroxy- $5\alpha$ androstan-17-one (3) [9]. There were downfield shifts for C–4 ( $\Delta\delta_{\rm C}$  7.2 ppm) and C–6 ( $\Delta\delta_{\rm C}$ 6.5 ppm) and C–10 ( $\Delta\delta_{\rm C}$  3.8 ppm) and  $\gamma$ -gauche upfield shifts for C-1 ( $\Delta\delta_{\rm C}$  4.6 ppm), C-7 ( $\Delta\delta_{\rm C}$ 5.3 ppm) and C–9 ( $\Delta\delta_{\rm C}$  8.6 ppm). The H–3 $\alpha$  proton resonance appeared at low field ( $\delta_{\rm H}$  4.75) in accordance with a transannular 1:3-diaxial interaction with a hydroxyl group. The major metabolite was  $3\beta$ ,11 $\alpha$ ,17 $\beta$ -trihydroxy-5 $\alpha$ -androstane (4) [10]. The <sup>1</sup>H NMR spectrum contained signals characteristic of H-3 $\alpha$  ( $\delta_{\rm H}$  3.91, t, J = 11 Hz, of t, J = 5 Hz), H–11 $\beta$  ( $\delta_{\rm H}$  4.24, t, J = 11.5 Hz, of d, J = 5.5 Hz) and H–17 $\alpha$  ( $\delta_{\rm H}$  3.94, t, J = 9 Hz). The H–11 $\beta$  signal received an n.O.e. enhancement on irradiation



of both the H–18 ( $\delta_{\rm H}$  1.02) and H–19 ( $\delta_{\rm H}$  1.11) resonances of 3.5% and 4.7%, respectively. Comparison of the <sup>13</sup>C NMC spectrum with that of  $3\beta$ , 17 $\beta$ -dihydroxy-5 $\alpha$ -androstane [11] revealed downfield shifts for the resonances assigned to C–9 ( $\Delta\delta_{\rm C}$  6.1 ppm) and C–12 ( $\Delta\delta_{\rm C}$  12.3 ppm).

The major metabolites obtained from  $3\beta$ -hydroxyandrost-5-en-17-one (5) were identified as the C-7 $\alpha$  and C-7 $\beta$  alcohols 6 and 7, respectively, by comparison of their <sup>1</sup>H NMR spectral data with literature values [5, 12]. Comparison of their <sup>13</sup>C NMR signals with those of the starting material [6] revealed the anticipated downfield shifts for C-6 ( $\Delta\delta_{\rm C}$  4.3 and 6.6 ppm) and C-8 ( $\Delta\delta_{\rm C}$  6.5 and

9.1 ppm) and a  $\gamma$ -gauche shielding for C-9 ( $\Delta\delta_{\rm C}$  7.2 and 1.4 ppm). The fermentation also produced a minor amount of  $3\beta$ , 11 $\alpha$ -dihydroxyandrost-5-en-17-one (8). The <sup>1</sup>H NMR spectrum possessed signals at  $\delta_{\rm H}$  3.54, (t, J = 10.5 Hz of t, J = 5 Hz) and  $\delta_{\rm H}$  3.89 (t, J = 11 Hz, of d, J = 5 Hz) characteristic of the H-3 $\alpha$  and H-11 $\beta$  resonances. The other minor product was  $3\beta$ ,  $5\alpha$ ,  $6\beta$ -trihydroxy-androstan-17-one (9) which was identified by comparison with an authentic sample.  $3\beta$ , 19-Dihydroxyandrost-5-en-17-one (10) was relatively poorly metabolized. The 17-ketone was reduced to the 17 $\beta$ alcohol (12) which was purified as its triacetate (13). The alkene was also converted to the  $5\alpha$ ,  $6\beta$ -diol

Table 1. Incubation of Steroids with C. aphidicola

Substrate	Product	% Yield
3β-Hydrox	$xy-5\alpha$ -androstan-17-one (1)	
, ,	$3\beta$ , $11\alpha$ , $17\beta$ -trihydroxy- $5\alpha$ -androstane (4)	12
	$3\beta$ , $14\alpha$ -dihydroxy- $5\alpha$ -androstan-17-one (2)	2
	$3\beta$ , $5\alpha$ -dihydroxy- $5\alpha$ -androstan-17-one (3)	4
3β-Hydrox	xyandrost-5-en-17-one (5)	
	$3\beta$ , $7\alpha$ -dihydroxyandrost-5-en-17-one (6)	25
	$3\beta$ , $7\beta$ -dihydroxyandrost-5-en-17-one (7)	31
	$3\beta$ , $11\alpha$ -dihydroxyandrost-5-en-17-one (8)	6
	$3\beta$ , $5\alpha$ , $6\beta$ -trihydroxyandrostan-17-one (9)	6
$3\beta$ , 19-Dih	ydroxyandrost-5-en-17-one (10)	
	$3\beta$ , $17\beta$ , 19-trihydroxyandrost-5-ene (12) <sup>+</sup>	6
	$3\beta$ , $5\alpha$ , $6\beta$ , 19-tetrahydroxyandrostan-17-one (14)	8

<sup>+</sup> 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 ( $\Delta^5$ -double bond significantly alters the biotransformation of  $3\beta$ -hydroxy- $5\alpha$ -androstan-17one by *C. aphidicola* leading to non-stereospecific allylic hydroxylation at C-7 in place of attack at C-14 $\alpha$  and reducing the extent of hydroxylation at C-11 $\alpha$ . However, this allylic hydroxylation did not appear to take place to any significant extent in the presence of a 19-hydroxyl group. The  $5\alpha$ ,  $6\beta$ -diols were probably formed via the  $5\alpha$ ,  $6\alpha$ -epoxide. In this case hydroxylation at C- $5\alpha$  in the saturated steroid has been replaced by epoxidation of the  $\Delta^5$ 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)  $3\beta$ -Hydroxy- $5\alpha$ -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 EtOAcpetrol (1:3) gave the starting material (137 mg). Elution with EtOAc-petrol (1:1) gave  $3\beta$ ,  $11\alpha$ ,  $17\beta$ trihydroxy- $5\alpha$ -androstane (4) (130 mg) which crystallized from  $Me_2CO$  as needles, mp 245–247° (lit. [10], 247–249°), IR  $v_{max}$  cm<sup>-1</sup>: 3316. <sup>1</sup>H NMR (pyridine- $d_5$ , 500 MHz after <sup>2</sup>H<sub>2</sub>O wash):  $\delta$  1.02 (3H, s, H<sub>3</sub>-18), 1.11 (3H, s, H<sub>3</sub>-19), 3.91 (1H, tt, J = 5 and 11 Hz, H-3 $\alpha$ ), 3.94 (1H, t, J = 9 Hz, H-17 $\alpha$ ), 4.24 (1H, td, J = 11.5 and 5.5 Hz, H-11 $\beta$ ). Further elution gave  $3\beta$ ,  $14\alpha$ -dihydroxy- $5\alpha$ -androstan-17-one (2) (23 mg) which crystallized from EtOAc-petrol as needles, mp 217-220° (lit. [8], 218–220°), IR v<sub>max</sub> cm<sup>-1</sup>: 3342. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 0.85 (3H, s, H<sub>3</sub>-18), 1.00 (3H, s H<sub>3</sub>-19), 3.60 (1H, tt, J = 4.8 and 10.5 Hz, H-3 $\alpha$ ). Further elution gave  $3\beta$ ,  $5\alpha$ -dihydroxy- $5\alpha$ -androstan-17-one (3) (43 mg) which crystallized from EtOAc as needles, mp 279-280° (lit. [9], 281-282°), IR v<sub>max</sub> cm<sup>-1</sup>: 3393, 1724. <sup>1</sup>H NMR (pyridine- $d_5$ ):  $\delta$  0.80 (3H, s, H<sub>3</sub>-18), 1.03 (3H, s, H<sub>3</sub>-19), 4.75 (1H, tt, J = 5.3 and 10.3 Hz, H-3 $\alpha$ ).

(b)  $3\beta$ -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. <sup>13</sup>C NMR signals of androstanes determined in CDCl<sub>3</sub> at 75 MHz

Carbon	Compound													
	1	2	<b>3</b> <sup>+</sup>	<b>4</b> <sup>+</sup>	5	6	7	9	10	11 <sup>a</sup>	13 <sup>b</sup>	<b>14</b> <sup>+</sup>		
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		

<sup>+</sup> determined in pyridine-*d*<sub>5</sub>

<sup>a</sup>acetates 21.5, 21.8 and 171.0 (×2)

<sup>b</sup>acetates 21.6, 21.8 (×2), 171.0, 171.2 and 171.7

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

(c)  $3\beta$ , 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<sub>2</sub>O-pyridene overnight and chromatography. Elution with EtOAc-petrol (1:4) gave  $3\beta$ ,  $17\beta$ , 19triacetoxy-androst-5-ene (13) (102 mg) which crystalized from EtOAc-petrol as plates, mp 90° (lit. [15], 88–89°), <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  0.82 (3H, s, H<sub>3</sub>-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–17 $\alpha$ ), 4.63 (1H, tt, J = 5 and 11 Hz, H $-3\alpha$ ), 5.66 (1H, br.s, H-6). Elution of the original column with MeOH–EtOAc (1:9) gave  $3\beta$ ,  $5\alpha$ ,  $6\beta$ , 19tetrahydroxyandrostan-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  $v_{max}$  cm<sup>-1</sup>: 3200 (*br*), 1730 <sup>1</sup>H NMR (pyridine- $d_5$ ):  $\delta$  0.78 (3H, *s*, H<sub>3</sub>–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–3 $\alpha$ ).

#### REFERENCES

- Mahato, S. B. and Majumdar, I., *Phytochemistry*, 1993, 34, 883.
- Farooq, A., Hanson, J. R. and Iqbal, Z., *Phytochemistry*, 1994, **37**, 723.
- Hanson, J. R., Nasir, H. and Parvez, A., *Phytochemistry*, 1996, 42, 411.
- DeFaye, G., Luche, M. H. and Chambaz, E. M., J. Steroid Biochem., 1978, 9, 331.
- Crabb, T. A., Dawson, P. J. and Williams, R. O., J. Chem. Soc., Perkin Trans. I, 1980, 2535.
- Holland, H. L., Diakow, P. R. P. and Taylor, G. J., *Canad. J. Chem.*, 1978, 56, 3121.
- Bridgeman, J. E., Cherry, P. C., Clegg, A. S., Evans, J. M., Jones, Sir E. R. H., Kumar, V., Meakins, G. D., Morisawa, Y. and Richards, E. E., J. Chem. Soc. (C), 1970, 250.
- Chambers, V. E. M., Jones, Sir E. R. H., Meakins, G. D., Miners, J. O. and Wilkins, J., J. Chem. Soc., Perkin Trans. I, 1975, 55.
- Williams, K. I. H., Rosenfield, R. S., Smulowitz, M. and Fukushima, *Steroids*, 1963, 1, 377.
- Bell, A. M., Browne, J. W., Denny, W. A., Denny, W. A., Jones, Sir E. R. H. and Meakins, G. D., *J. Chem. Soc, Perkin Trans. I*, 1972, 2930.
- 11. Blunt, J. W. and Stothers, J. B., Organic Magnetic Resonance, 1977, 9, 439.
- Dodson, R. M., Nicholson, R. T. and Muir, R. D., J. Am. Chem. Soc., 1959, 81, 6295.
- 13. Hanson, J. R. and Nasir, H., *Phytochemistry*, 1993, **33**, 831.
- Holland, H. L. and Diakow, P. R. P., Canad. J. Chem., 1979, 57, 436.
- Hill, R. A., Kirk, D. N., Makin, H. L. J. and Murphy, G. M., eds., *Dictionary of Steroids*. Chapman and Hall, London, 1991, p. 60.