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THE HYDROXYLATION OF TESTOSTERONE AND SOME RELATIVES BY *CEPHALOSPORIUM APHIDICOLA*

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

Abstract—The fungus *Cephalosporium aphidicola* has been shown to hydroxylate testosterone, 19-nortestosterone, 1-dehydrotestosterone, 1α-methyltestosterone, androst-4-en-3-one, androst-4-en-3,17-dione and 17α-methyltestosterone predominantly at the C-6β position with a minor hydroxylation occurring at the C-14α position. 19-Nortestosterone was also hydroxylated at the C-10β position. In contrast to the hydroxylation of progesterone by this organism, hydroxylation at C-11α was a minor pathway.

INTRODUCTION

The factors that govern the microbiological transformation of steroids continue to attract interest [1]. We have shown [2] that the fungus *Cephalosporium aphidicola* is capable of hydroxylating progesterone (**1**) sequentially, first at C-11α and then at C-6β with a minor pathway involving attack at C-17α and C-12β. Some of these transformations proceeded in high yield. We have now examined the microbiological transformation of testosterone (**2**) and its relatives by *C. aphidicola* in order to define the directing role of the ring A unsaturated ketone in the transformation of steroids by this organism. We have observed some marked differences from the progesterone series. It has been suggested [3] that the microbial hydroxylation of Δ^4-3-keto steroids at C-6 by *Rhizopus arrhizus* proceeds via binding of the substrate to the hydroxylase as the $\Delta^{3,5}$-dienol.

RESULTS AND DISCUSSION

The following substrates were examined: testosterone (**2**), androst-4-en-3-one (**5**), 19-nortestosterone (**7**), 1-dehydrotestosterone (**11**), 1α-methyltestosterone (**14**), androst-4-en-3,17-dione (**17**), 17α-methyltestosterone (**20**) and ethisterone (**23**). The substrates were incubated with *C. aphidicola* in shake culture for a period of five days. The results are given in Table 1. The structures of the metabolites were established by changes in their ^{1}H and ^{13}C NMR spectra when compared to the starting material and with literature data [4–6] (for ^{13}C NMR data see Table 2).

The major metabolites possessed a C-6β hydroxyl group. The C<u>H</u>(OH) ^{1}H NMR signal had the characteristic position and multiplicity (δ_H 4.3–4.5, narrow triplet, $J = 2.6$ Hz or broad singlet) [7] whilst the H-4 and H-19 resonances showed typical downfield shifts (*ca* 0.08 and 0.20 ppm, respectively). The ^{13}C NMR signal for the C-6 alcohols (see Table 2) appeared at δ 73. The signal assigned to C-7 was shifted downfield whilst that for C-8 showed a *γ-gauche* upfield shift in agreement with the stereochemistry of the hydroxyl group at C-6. The location of a hydroxyl group at the C-14 position in the minor metabolites followed from changes in the position of the ^{13}C NMR signals for the ring-D carbon atoms. There were *γ-gauche* shieldings for C-12 and C-17. The H-17 ^{1}H NMR signal showed a typical downfield shift ($\Delta\delta_H$ 0.67 ppm) arising from a diaxial interaction with the 14α-hydroxyl group. The location of the hydroxyl groups at C-10β and C-11α in the other minor metabolites were established similarly and by comparison with literature data. No metabolites of 17α-ethynyltestosterone (ethisterone) (**23**) were detected and unchanged material was recovered.

A number of points emerge from these results. Whereas this fungus readily hydroxylated progesterone at C-11α and then at C-6β, the testosterone series were hydroxylated mainly at C-6β with only a minor hydroxylation occurring at C-11α. Apart from the major hydroxylation at C-6β, another site of attack was C-14α, a transformation which was not observed in the progesterone series. The poor transformation of 17α-methyltestosterone (**20**) and the lack of transformation of 17α-ethynyltestosterone (**23**) suggest that a 17α-alkyl residue may be an impediment to transformation with this fungus, an observation which has been made previously with *Aspergillus ochraceus* [8].

Table 1. Microbiological hydroxylation of testosterone and related compounds

Substrate	Metabolites	Yield (%)
Testosterone (**2**)		
	6β,17β-Dihydroxyandrost-4-en-3-one (**3**)	47
	14α,17β-Dihydroxyandrost-4-en-3-one (**4**)	3
Androst-4-en-3-one (**5**)		
	6β,17β-Dihydroxyandrost-4-en-3-one (**3**)	18
	6β,11α-Dihydroxyandrost-4-en-3-one (**6**)	2
19-Nortestosterone		
	6β,17β-Dihydroxy-19-norandrost-4-en-3-one (**8**)	47
	10β,17β-Dihydroxy-19-norandrost-4-en-3-one (**9**)	4
	19-Norandrost-4-ene-3,17-dione (**10**)	1
1-Dehydrotestosterone (**11**)		
	6β,17β-Dihydroxyandrosta-1,4-dien-3-one (**12**)	48
	14α,17β-Dihydroxyandrosta-1,4-dien-3-one (**13**)	2
1α-Methyltestosterone (**14**)		
	6β,17β-Dihydroxy-1α-methylandrost-4-en-3-one (**15**)	51
	14α,17β-Dihydroxy-1α-methylandrost-4-en-3-one (**16**)	1.5
Androst-4-en-3,17-dione (**17**)		
	6β-Hydroxyandrost-4-ene-3,17-dione (**18**)	25
	14α-Hydroxyandrost-4-ene-3,17-dione (**19**)	2
17α-Methyltestosterone (**20**)		
	6β,17β-Dihydroxy-17α-methylandrost-4-en-3-one (**21**)	17
	6β,11α,17β-Trihydroxy-17α-methylandrost-4-en-3-one (**22**)	4

EXPERIMENTAL

General experimental details have been described previously [9].

(*a*) Incubation of steroids with *C. aphidicola*. The fungus was grown on shake culture (100 ml medium) in 250 ml conical flasks as described previously [9]. Two days after inoculation, **2** (2 g) in EtOH (25 ml) was evenly distributed between 50 flasks. After a further 5 days, the mycelium was filtered and the broth extracted with EtOAc. The extract was dried over Na_2SO_4 and the solvent evapd to give a gum which was chromatographed on silica gel. Elution with EtOAc–petrol (1:1) gave 6β,17β-dihydroxyandrost-4-en-3-one (**3**) (1 g), mp 215–220° (lit. [10] 216–222°); IR ν_{max}/cm^{-1}: 3500, 1662, 1620. ^1H NMR ($CDCl_3$): δ 0.83 (3H, *s*, H-18), 1.39 (3H, *s*, H-19), 3.66 (1H, *t*, $J = 8.5$ Hz, H-17), 4.35 (1H, *t*, $J = 2.9$ Hz, H-6), 5.81 (1H, *s*, H-4). Further elution gave 14α,17β-dihydroxyandrost-4-en-3-one (**4**) (50 mg), mp 181–185° (lit., [11] 183.5–186°).

Table 2. ^{13}C NMR signals of testosterone and its derivatives determined in $CDCl_3$ at 125 MHz

	Compound						
Carbon	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	36.1	36.4	35.8	35.7	37.9	27.0	31.3
2	34.1	34.2	34.0	33.9	34.2	36.6	36.6
3	198.0	200.4	199.5	199.7	202.4	198.6	199.7
4	124.2	126.4	124.0	123.7	126.2	124.8	124.6
5	170.4	168.3	170.6	171.7	170.5	166.2	167.4
6	32.8	73.0	32.5	32.9	72.5	35.6	71.4
7	32.2	37.1	28.6	32.3	38.7	31.3	39.1
8	36.1	30.0	38.9	35.9	28.7	41.0	34.3
9	54.6	53.7	46.8	54.0	59.1	49.2	50.0
10	39.0	38.0	38.9	38.7	39.3	42.2	38.6
11	21.2	20.6	19.7	21.0	68.7	26.6	26.5
12	37.1	38.0	32.8	38.4	49.9	37.1	37.2
13	43.2	42.9	47.0	40.6	41.3	43.4	43.9
14	51.1	50.5	83.4	54.0	53.3	48.9	50.2
15	23.8	23.3	26.1	25.4	25.1	23.6	23.6
16	30.7	30.5	29.6	20.4	20.4	30.7	31.0
17	81.3	81.6	78.6	40.6	40.0	81.4	81.3
18	11.3	11.1	14.9	17.3	18.3	11.4	11.8
19	17.3	19.5	17.3	17.4	19.8		

Continued

Table 2. *Continued*

Carbon	Compound						
	9	**10**	**11**	**12**	**13***	**14**	**15**
1	33.5	26.6	155.9	157.5	156.4	36.7	40.9
2	33.5	36.4	127.4	126.9	127.5	42.5	42.6
3	200.3	199.6	186.3	186.5	185.9	198.9	199.9
4	124.2	124.8	123.8	125.2	123.0	123.3	126.2
5	165.9	165.7	169.2	168.3	161.4	167.6	165.3
6	25.7	35.2	32.7	73.3	29.6	30.6	72.8
7	31.3	31.3	33.1	35.2	32.7	32.8	37.5
8	35.2	39.8	33.0	30.7	39.6	35.5	29.4
9	52.8	49.5	52.4	52.7	46.6	46.7	46.6
10	69.9	42.4	43.0	43.7	43.8	41.5	38.0
11	20.0	25.7	22.4	22.6	22.3	19.8	19.7
12	36.1	31.3	37.1	40.7	32.7	36.3	36.4
13	42.7	47.7	44.1	44.1	48.1	42.8	43.0
14	50.1	50.1	50.1	50.5	82.3	50.6	50.6
15	23.3	21.9	23.5	23.9	28.6	23.3	23.3
16	30.1	35.2	30.2	30.9	32.8	30.4	30.5
17	81.3	220.3	81.3	81.1	78.2	81.6	81.7
18	10.8	13.7	11.1	11.8	15.7	11.1	11.1
19			18.7	20.8	18.5	19.8	22.5
Me						15.2	15.4

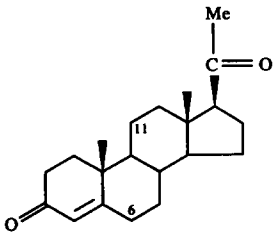
Carbon	Compound						
	16	**17**	**18**	**19**	**20**	**21***	**22***
1	37.0	35.5	37.2	37.8	36.0	37.6	38.8
2	42.5	33.8	34.1	33.5	34.4	34.7	35.1
3	199.0	199.1	200.4	200.5	198.3	199.5	200.4
4	123.4	124.0	126.3	123.4	124.1	125.9	126.4
5	167.1	170.2	168.2	171.6	170.7	169.8	170.8
6	28.5	32.4	72.6	32.9	32.9	72.5	72.9
7	32.7	31.2	35.7	32.3	32.1	39.5	39.8
8	38.8	35.0	29.4	37.8	36.6	31.2	30.2
9	39.7	53.7	53.6	46.5	54.0	54.2	60.0
10	41.6	38.5	38.0	38.5	38.8	38.6	40.2
11	18.9	20.2	20.2	18.9	21.0	21.1	68.9
12	32.9	35.6	36.9	35.3	32.1	32.1	44.6
13	47.0	47.4	47.6	52.6	46.0	46.1	50.0
14	83.4	50.7	50.8	79.9	50.6	50.7	50.3
15	25.2	21.6	21.6	24.2	23.7	23.8	23.8
16	29.6	31.7	31.2	29.5	39.4	39.4	39.5
17	78.6	220.2	220.6	220.1	80.5	80.6	80.2
18	14.9	13.6	13.7	17.0	14.7	14.7	16.1
19	20.0	17.6	19.5	17.5	17.2	19.6	20.6
Me	15.3				26.7	26.7	26.7

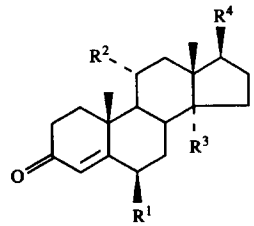
*In pyridine-d_5.

IR ν_{max} cm^{-1}: 3450, 1662, 1640; ^1H NMR ($CDCl_3$): δ 0.92 (3H, *s*, H-8), 1.22, (3H, *s*, H-19), 4.32 (1H, *dd*, $J = 6.9$, 8.8 Hz, H-17), 5.73 (1H, *s*, H-4).

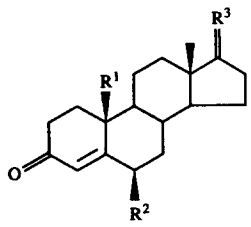
(*b*) Under similar fermentation conditions, **7** (2 g) gave, on chromatography on silica gel in EtOAc–petrol (2:3), 19-norandrost-4-en -3,17-dione (**10**) (15 mg), mp 160–165° (lit. [12] 170–171°). IR ν_{max} cm^{-1}: 1743, 1664; ^1H NMR ($CDCl_3$): δ 0.93 (3H, *s*, H-18), 5.85 (1H, *s*, H-4). Further elution with EtOAc–petrol (1:1) gave 10β,17β-dihydroxy-19-norandrost-4-en-3-one (**9**) (80 mg), mp 207–209° (lit. [13] 205–210°). IR ν_{max} cm^{-1}: 3305, 1660. ^1H NMR ($CDCl_3$): δ 0.83 (3H, *s*, H-18), 3.67 (1H, *t*, $J = 8.5$ Hz, H-17), 5.78 (1H, *s*, H-4). Elution with EtOAc–petrol (7:3) gave 6β,17β-dihydroxy-19-norandrost-4-en-3-one (**8**) (1 g) mp 212–215° (lit. [14] 209–213°). IR ν_{max} cm^{-1}: 3372, 1660. ^1H NMR (pyridine-d_5): δ 1.07 (3H, *s*, H-18), 3.92 (1H, *t*, $J = 8.5$ Hz, H-17), 4.60 (1H, *t*, $J = 2.8$ Hz, H-6), 6.14 (1H, *s*, H-4). ^1H NMR ($CDCl_3$): δ 0.79, (3H, *s*, H-18), 3.88 (1H, *t*, $J = 8.5$ Hz, H-17), 4.35 (1H, *t*, $J = 2.8$ Hz, H-6), 5.88 (1H, *s*, H-4).

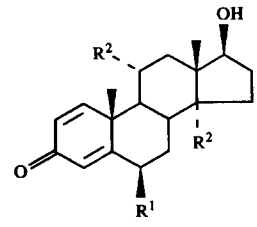
(*c*) Under similar fermentation conditions, **11** (2 g) gave, on chromatography on silica gel in EtOAc–petrol (1:1) 6β,17β-dihydroxyandrosta-1,4-dien-3-one (**12**)





	R^1	R^2	R^3	R^4
2	H	H	H	OH
3	OH	H	H	OH
4	H	H	OH	OH
5	H	H	H	H
6	OH	OH	OH	OH

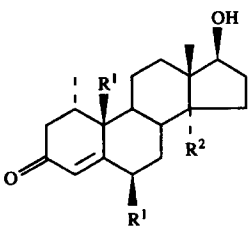


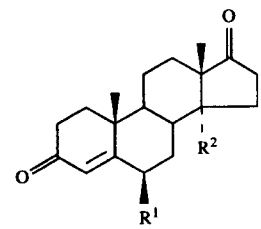


	R^1	R^2	R^3
7	H	H	α-H, β-OH
8	H	OH	α-H, β-OH
9	OH	H	α-H, β-OH
10	H	H	O

	R^1	R^2
11	H	H
12	OH	H
13	H	OH

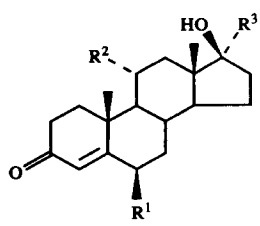
(1 g), mp 189–194° (lit. [15] 194–195°). IR ν_{max} cm^{-1}: 3500, 1651, 1600. ^1H NMR ($CDCl_3$): δ 0.83 (3H, *s*, H-18), 1.45 (3H, *s*, H-19), 3.66 (1H, *t*, $J = 8.5$ Hz, H-17), 4.55 (1H, *s*, H-6), 6.16 (1H, *s*, H-4), 6.22 (1H, *d*, $J = 10$ Hz, H-2), 7.06 (1H, *d*, $J = 10$ Hz, H-1). Elution with MeOH–EtOAc (1:19) gave 14α,17β-dihydroxyandrosta-1,4-dien-3-one (**13**) (34 mg), mp 230–235° (lit. [16] 224–226°). IR ν_{max} cm^{-1}: 3500, 1651, 1600. ^1H MMR (pyridine-d_5): δ 1.17 (3H, *s*, H-18), 1.20 (3H, *s*, H-19), 4.91 (1H, *t*, $J = 8.5$ Hz, H-17), 6.22 (1H, *s*, H-4), 6.39 (1H, *d*, $J = 10$ Hz, H-2), 7.03 (1H, *d*, $J = 10$ Hz, H-1).





	R^1	R^2
14	H	H
15	OH	H
16	H	OH

	R^1	R^2
17	H	H
18	OH	H
19	H	OH



	R^1	R^2	R^3
20	H	H	Me
21	OH	H	Me
22	OH	OH	Me
23	H	H	-C≡CH

(*d*) Under similar fermentation conditions, (**14**) (2 g) gave, on chromatography on silica gel in EtOAc–petrol (1:1) 6β,17β-dihydroxy-1α-methylandrost-4-en-3-one (**15**) (1.05 g), mp 203–207°, (Found: C, 75.2; H, 9.5. $C_{20}H_{30}O_3$ requires C, 75.5; H, 9.5%). IR ν_{max} cm^{-1}: 3382, 1673. ^1H NMR ($CDCl_3$): δ 0.82 (3H, *s*, H-18), 0.91 (3H, *d*, $J = 7$ Hz, Me-1), 1.47 (3H, *s*, H-19), 3.67 (1H, *t*, $J = 8.5$ Hz, H-17), 4.34 (1H, *t*, $J = 3$ Hz, H-6), 5.82 (1H, *s*, H-4). Further elution with EtOAc–petrol (3:2) gave 14α,17β-dihydroxy-1α-methylandrost-4-en-3-one (**16**) (30 mg), mp 179–180° (Found: C, 75.4; H, 9.4. $C_{20}H_{30}O_3$ requires C, 75.5; H, 9.5%). IR ν_{max} cm^{-1}: 3436, 1646. ^1H NMR ($CDCl_3$): δ 0.81 (3H, *s*, H-18), 0.92 (3H, *d*, $J = 7$ Hz, Me-1), 1.30 (3H, *s*, H-19), 4.31 (1H, *t*, $J = 8.5$ Hz, H-17), 5.70 (1H, *s*, H-4).

(*e*) Under similar fermentation conditions, **5** (1.5 g) gave, on chromatography on silica gel and elution with EtOAc–petrol (2:3) 6β,11α-dihydroxyandrost-4-en-3-one (**6**) (70 mg), mp 250–254° (lit. [17] 252–255°). IR ν_{max} cm^{-1}: 3436, 1646. ^1H NMR ($CDCl_3$): δ 0.83 (3H, *s*, H-18), 1.52 (3H, *s*, H-19), 4.10 (1H, *t*, $J = 10.5$ of *d* 4.6 Hz, H-11) 4.35 (1H, *s*, H-6), 5.83 (1H, *s*, H-4). Further elution with the same solvent gave **3** (300 mg), mp 214–215°, identical to the material described above.

(*f*) Under similar fermentation conditions, **17** (2 g) gave, on chromatography on silica gel and elution with EtOAc–petrol (1:4), 14α-hydroxyandrost-4-en-3,17-dione (**19**) (40 mg), mp 255–260° (lit. [11] 261–262°) IR ν_{max} cm^{-1}: 3421, 1744, 1656. ^1H NMR ($CDCl_3$): δ 1.06 (3H, *s*, H-18), 1.23 (3H, *s*, H-19), 5.76 (1H, *s*, H-4). Further elution with EtOAc–petrol (2:3) gave 6β-hydroxyandrost-4-en-3,17-dione (**18**) (500 mg), mp 190–193° (lit. [10] 191–194°). IR ν_{max} cm^{-1}: 3418, 1736, 1661. ^1H NMR ($CDCl_3$): δ 0.93 (3H, *s*, H-18), 1.39 (3H, *s*, H-19) 4.34 (1H, *br s*, H-6), 5.80 (1H, *s*, H-4).

(*g*) Under similar fermentation conditions, **20** (1.1 g) gave, on chromatography on silica gel and elution with EtOAc–petrol (3:2), 6β,17β-dihydroxy-17α-methylandrost-4-en-3-one (**21**) (176 mg), mp 248–250° (lit. [10] 252–253°), IR ν_{max} cm^{-1}: 3450, 1675. ^1H NMR (pyridine-d_5): δ 1.15 (3H, *s*, H-18), 1.43 (3H, *s*, Me-17), 1.56 (3H, *s*, H-19), 4.55 (1H, *s*, H-6), 6.05 (1H, *s*, H-4). Further elution with EtOAc gave 6β,11α,17β-trihydroxy-17α-methylandrost-4-en-3-one (**22**) (39 mg), mp 266–268°. (Found: C, 69.2; H, 8.9. $C_{20}H_{30}O_4$. 0.5 H_2O requires C, 69.9; H, 8.7%). IR ν_{max} cm^{-1}: 3450, 1680. ^1H NMR (pyridine-d_5): δ 1.24 (3H, *s*, H-18), 1.46 (3H, *s*, Me-17), 1.86 (3H, *s*, H-19), 4.48 (1H, *dt*, $J = 4$, 10 Hz, H-11), 4.58 (1H, *br s*, H-6), 6.09 (1H, *s*, H-4).

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