SHORT PAPER

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The microbiological hydroxylation of des-ring D androstanes by *Cephalosporium aphidicola*†

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The hydroxylation of ring B of des-ring D androstanes by *Cephalosporium aphidicola* follows the pattern of normal steroids, but that of ring C differs from this, suggesting that the nature of ring D is important in determining the hydroxylation of ring C.

Keywords: Microbiological hydroxylation, des-ring D steroids, phenanthrenes, *Cephalosporium aphidicola*

We have been examining the features which govern the regiospecificity of the hydroxylation of steroids by the fungus *Cephalosporium aphidicola*. Hydroxylation of testosterone takes place at C-6β and then at C-11α, whilst dehydroisoandrosterone is hydroxylated at C-7 and C-14α.[1] On the other hand, progesterone is hydroxylated firstly at C-11α and then at C-6β;[2] the extent of hydroxylation appears to be determined to a certain extent by the nature of ring D. Consequently it was of interest to examine the hydroxylation of steroidal substrates lacking ring D.

Incubation of 3β-hydroxy-des-D-13α-androst-5-en-14-one (the Koster–Logemann ketone, **1**)[3] with *C. aphidicola* for 8 days gave five metabolites. The sites of hydroxylation were established by changes in the ^{13}C NMR spectra (see Table 1).

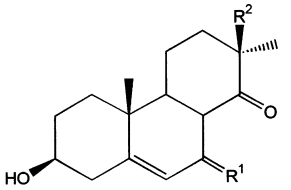
The major metabolite was 3β,13β-dihydroxy-13α-des-D-androst-5-en-14-one (**2**). The ^{1}H NMR spectrum showed that the 13-methyl group resonance (δ_H 1.23) was now a singlet and there was a new tertiary alcohol signal (δ_C 74.2) in the ^{13}C NMR spectrum. The stereochemistry at C-13 was confirmed by n.O.e. experiments. Irradiation at δ_H 1.02 produced an enhancement of 8.6% at δ_H 3.05 (H-8) which had moved downfield, but there was no enhancement of this signal on irradiation of the 13-methyl signal.

The next two metabolites to be isolated from the chromatography column were the 7α- and 7β-alcohols **3** and **4**. They were identified by the changes in the ^{13}C NMR spectra and by the presence of coupling between the alkene C-H resonance and the new CH(OH) resonances. The stereochemistry of the alcohols was established by the magnitude of the H-7:H-8 coupling constants (*J* 5 and 9 Hz respectively).

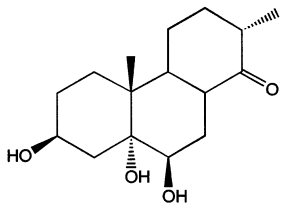
The fourth metabolite was the 3β,7α,13β-triol **5**. The magnitudes of the coupling constants (δ_H 4.78, dd, *J* 2.6 and 5 Hz) of the new CH(OH) resonance were consistent with the C-7α stereochemistry for the alcohol.

The final metabolite was the 3β,5α,6β-triol **6**. The ^{1}H NMR spectrum lacked the alkene C-H resonance, containing in its place a new CH(OH) signal (δ_H 4.16) whilst the angular methyl group resonance (H-19) showed a significant downfield shift to δ_H 1.59.

Oxidation of the alcohol **1** with aluminium isopropoxide gave the unsaturated ketone **7**.[4,5] Incubation of this with *C. aphidicola* gave four metabolites. The first metabolite to be isolated was the epoxide **8** in which an epoxide proton signal (δ_H 3.01) replaced the alkene signal of the starting material. Comparison with the 4α,5α- and 4β,5β-epoxides of testosterone established the 4β,5β-stereochemistry of the epoxide.



1 $R^1 = H_2$; $R^2 = H$
2 $R^1 = H_2$; $R^2 = OH$
3 $R^1 = \alpha$–OH, β–H; $R^2 = H$
4 $R^1 = \alpha$–H, β–OH; $R^2 = H$
5 $R^1 = \alpha$–OH, β–H; $R^2 = OH$



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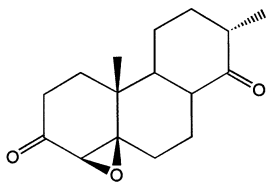

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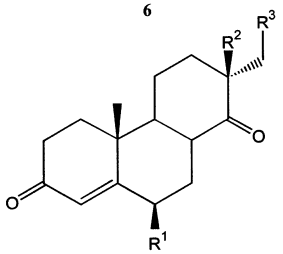

7 $R^1 = R^2 = R^3 = H$
9 $R^1 = R^3 = H$; $R^2 = OH$
10 $R^1 = OH$; $R^2 = R^3 = H$
11 $R^1 = OAc$; $R^2 = R^3 = H$
12 $R^1 = R^2 = H$; $R^3 = OH$
13 $R^1 = R^2 = H$; $R^3 = OAc$

The C-19 signal for the 4α,5α-epoxide is at δ_C 16.53 whilst that of the 4β,5β-epoxide is at δ_C 18.97.[6] In the metabolite it is at δ_C 18.4 (see Table 2).

The second metabolite was the tertiary alcohol **9**. The H-18 ^{1}H NMR signal was now a singlet (δ_H 1.32) whilst the H-8 resonance had shifted downfield to δ_H 3.17.

The third metabolite was the 6β-alcohol **10** which was purified as its acetate **11**. The ^{1}H NMR spectrum possessed a new signal at δ_H 5.50 (triplet, *J* 3 Hz) characteristic of a 6α-H.

The final metabolite to be isolated was the primary alcohol **12**, which was also purified as its acetate **13**. The ^{1}H NMR spectrum showed that the doublet methyl group signal of the starting material had been replaced by two new signals (δ_H 4.03 and 4.40, double-doublets, *J* 11.5 and 6 Hz).

In conclusion, the biotransformations of ring B typical of dehydroisoandrosterone and testosterone still occur, but the hydroxylations of ring C are quite different and in particular there was no hydroxylation at C-11. The hydroxylation of the 13α-methyl group is quite interesting as this position can be superimposed on the C-17 position of testosterone.

Experimental

Silica for chromatography was Merck 9385. Light petroleum refers to the fraction b.p. 60 – 80 °C. ^{1}H and ^{13}C NMR spectra were determined at 300 and 75 MHz respectively in deuteriochloroform unless

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† This is a Short Paper, there is therefore no corresponding material in *J Chem. Research (M).*

Table 1 ^{13}C NMR data of the metabolites of **1**

Carbon atom	Compound **1**(Ac)[a]	**2**[a]	**3**[b]	**4**[b]	**5**[b]
1	37.1	37.2	36.5	36.7	37.2
2	28.0	30.6	31.1	31.3	32.3
3	73.9	70.3	70.8	71.3	70.9
4	38.2	41.8	41.8	41.5	43.1
5	138.5	139.6	145.7	141.9	145.5
6	122.4	120.0	122.1	124.4	124.0
7	25.4	24.6	62.2	67.1	63.0
8	52.5	51.7	51.3	53.5	48.3
9	45.9	40.8	45.7	49.9	44.7
10	38.1	37.4	38.4	38.0	38.1
11	25.5	19.4	24.4	24.7	19.1
12	35.0	39.5	33.6	33.9	38.8
13	45.5	73.4	45.3	45.4	73.4
14	214.0	214.0	216.2	214.9	213.9
18	14.9	23.8	14.2	14.2	24.6
19	19.3	18.8	18.1	18.9	17.1
AcO	21.9 170.8				

[a] in $CDCl_3$; [b] in pyridine-d_5.

Table 2 ^{13}C NMR data of the metabolites of **7**[a]

Carbon atom	Compound **7**	**8**	**9**	**11**	**13**
1	35.6	34.9	35.4	37.0	36.0
2	33.9	28.4	33.5	34.1	34.3
3	198.9	205.6	198.9	199.3	198.3
4	124.4	62.4	124.2	129.2	125.0
5	168.9	69.1	168.8	160.3	168.8
6	31.6	26.1	31.5	73.5	31.9
7	24.9	24.4	25.6	30.8	24.8
8	54.5	48.7	54.0	54.2	54.7
9	49.2	47.7	44.3	45.0	49.4
10	39.0	37.6	39.0	38.5	39.4
11	25.9	25.9	19.6	24.9	25.9
12	34.8	32.4	39.5	34.7	30.1
13	44.8	44.8	74.6	44.6	49.9
14	213.7	212.7	212.7	212.3	205.2
18	14.4	14.4	14.4	14.4	63.4
19	17.3	18.4	18.4	19.0	17.7
AcO		21.4 170.7		21.4 170.8	21.4 170.7

[a]In $CDCl_3$.

otherwise stated. IR spectra were determined as nujol mulls. Extracts were dried over sodium sulfate.

Fermentation conditions:- The fungus *Cephalosporium aphidicola* was grown on shake culture in conical flasks (250 cm^3) containing medium (100 cm^3) comprising (per litre): glucose (80 g), ammonium nitrate (4.8 g), potassium dihydrogen phosphate (5 g), magnesium sulfate (1 g) and a solution (2 cm^3) of trace elements. The last contained (per 100 cm^3): cobalt nitrate (0.01 g), iron(II) sulfate (0.1 g), copper sulfate (0.015 g), zinc sulfate (0.161 g), manganese sulfate (0.01 g) and ammonium molybdate (0.01 g). The substrates were added after 3 days growth and the fermentation was continued for a further 8 days. The mycelium was filtered off and the broth extracted with ethyl acetate. The extract was dried and the solvent evaporated to give a residue which was chromatographed on silica.

Biotransformation of 3β-hydroxy-des-D-13α-androst-5-en-14-one (**1**):- The ketone[3] [δ_H 1.02 (3H, d, *J* 6.5 Hz, 18-H), 1.07 (3H, s, 19-H), 3.52 (1H, tt, *J* 4 and 11 Hz, 3-H), 5.38 (1H, t, *J* 2.5 Hz,6-H)] (1.85 g) in dimethylsulfoxide (25 cm^3) and ethanol (5 cm^3) was evenly distributed between 48 flasks of *C. aphidicola*. After 8 days incubation the metabolites were isolated and chromatographed on silica. Elution with 20% ethyl acetate : light petroleum gave *3β,13β-dihydroxy-des-D-13α-androst-5-en-14-one* (**2**) (498 mg) which crystallised from ethyl acetate : light petroleum as prisms, m.p. 184-186°C (Found: C, 70.2; H, 9.0. $C_{16}H_{24}O_3 \cdot 0.5H_2O$ requires C, 70.3; H, 9.2 %); ν_{max}/cm^{-1} 3398, 3304, 1709; δ_H(pyridine) 1.02 (3H, s, 19-H), 1.23 (3H, s, 18-H), 3.05 (1H, td, *J* 11.7 and 5.5 Hz, H-8), 3.47 (1H, tt, *J* 11.0 and 5.0 Hz, 3-H), 5.27 (1H, t, *J* 2.5 Hz, 6-H).

Elution with 40% ethyl acetate : light petroleum gave a mixture of two compounds which were separated by a series of fractional recrystallisations from diethyl ether. *3β,7α-Dihydroxy-des-D-13α-androst-5-en-14-one* (**3**) (306 mg) which crystallized first, had m.p. 123–127 °C. (Found: C, 72.7; H, 9.3. $C_{16}H_{24}O_3$ requires C, 72.7; H, 9.15%), ν_{max}/cm^{-1} 3278 and 1706; δ_H 1.05 (3H, d, J 6.5 Hz, 18-H), 1.06 (3H, s, 19-H), 3.66 (1H, tt, J 11.0 and 4.7 Hz, 3-H), 4.47 (1H, dd, J 5 and 2.5 Hz, 7-H), 5.59 (1H, d, 2.5 Hz, 6-H). *3β,7β-Dihydroxy-des-D-13α-androst-5-en-14-one* (**4**) (204 mg) had m.p.141–143°C. (Found: C, 72.7; H, 9.3 $C_{16}H_{24}O_3$ requires C,72.7; H, 9.15%); ν_{max}/cm^{-1} 3280, 1708; δ_H 1.06 (3H, d, *J* 6.5 Hz, 18-H), 1.12 (3H, s, 19-H), 3.57 (1H, tt, *J* 11 and 4.5 Hz, 3-H), 4.50 (1H, dd, *J* 9 and 2 Hz, H-7), 5.59 (1H, d,*J* 2 Hz, 6-H).

Elution with 60% ethyl acetate:light petroleum gave *3β,7α,13β-trihydroxy-des-D-13α-androst-5-en-14-one* (**5**) (47 mg) which crystallised as needles from chloroform, m.p.157–159°C. (Found: C, 67.9; H, 8.8. $C_{16}H_{24}O_4$ requires C, 68.5; H, 8.6%) ν_{max}/cm^{-1} 3450,1710; δ_H (pyridine) 1.09 (3H, s, 19-H), 1.54 (3H, s, 18-H), 3.19 (1H, td, *J* 11 and 2 Hz, 8-H), 3,83 (1H, tt, *J* 11 and 5 Hz, 3-H), 4.78 (1H, m, 7-H), 5.78 (1H, d, *J* 5 Hz 6-H).

Elution with 80% ethyl acetate : light petroleum gave *3β,5α,6β-trihydroxy-des-D-13α-androstan-14-one* (**6**) (213 mg) which crystallised from ethyl acetate:light petroleum as prisms, m.p.216–218°C. (Found: C, 68.3; H, 9.5. $C_{16}H_{26}O_4$ requires C, 68.1; H, 9.3%); ν_{max}/cm^{-1} 3389, 1683; δ_H (pyridine) 1.05 (3H, d, *J* 7 Hz, 18-H), 1.59 (3H, s, 19-H), 4.16 (1H, br.s, H-6), 4.79 (1H, tt, *J* 11 and 5 Hz,3-H).

Biotransformation of des-D-13α-androst-4-ene-3,14-dione (**7**):- The unsaturated ketone **7** had m.p.138–140°C (lit.,[6] 140-141°C), ν_{max}/cm^{-1} 1706, 1669, 1614; δ_H 1.03 (3H, d, *J* 6.5 Hz, 18-H), 1.26 (3H, s, 19-H), 5.76 (1H, s, 4-H). The substrate **7** (2.15 g) in dimethylsulfoxide (25 cm^3) and ethanol (5 cm^3) was evenly distributed between 50 flasks of *C. aphidicola* and the fermentation was continued for 8 days. The metabolites were isolated and separated by chromatography on silica. Elution with 25% ethyl acetate: light petroleum gave *4β,5β-epoxy-des-D-13α-androstan-3,14-dione* (**8**) which crystallised from light petroleum as plates, m.p.144–146°C. (Found: C,73.2; H,8.6. $C_{16}H_{22}O_3$ requires C, 73.25; H, 8.45%); ν_{max}/cm^{-1} 1701; δ_H 1.02 (3H, d, *J* 6.5 Hz,18-H), 1.22 (3H, s, 19-H), 3.01 (1H, s, 4-H).

Elution with 35% ethyl acetate:light petroleum gave *13β-hydroxy-des-D-13α-androst-4-ene-3,14-dione* (**9**) (92 mg) which crystallised from ethyl acetate:light petroleum as prisms, m.p.156–158°C. (Found: C, 73.1; H, 8.5. $C_{16}H_{22}O_3$ requires C, 73.25; H, 8.45%); ν_{max}/cm^{-1} 3333, 1718, 1675; δ_H 1.29 (3H, s, 19-H), 1.32 (3H, s, 18-H), 3.17 (1H, td, *J* 12 and 3,5 Hz, 8-H), 5.76 (1H, s, 4-H).

Further elution with the same solvent gave a mixture of *6β-hydroxy-des-D-13α-androst-4-ene-3,14-dione* (**10**) and *18-hydroxy-des-D-13α-androst-4-ene-3,14-dione* (**12**) which was acetylated with acetic anhydride (5 cm^3) in pyridine (10 cm^3) overnight. The mixture was poured into dil. hydrochloric acid and the steroids were extracted with ethyl acetate and chromatographed on silica.

Elution with 30% ethyl acetate:light petroleum gave *6β-acetoxy-des-D-13α-androst-4-ene-3,14-dione* (**11**) (112 mg) which crystallised from ethyl acetate:light petroleum as plates, m.p. 126–127°C. (Found: C, 70.8; H, 7.5 $C_{18}H_{24}O_4$ requires C, 71.0; H, 7.9%) ν_{max}/cm^{-1} 1730, 1675; δ_H 1.05 (3H, d, *J* 6.5 Hz, 18-H), 1.36 (3H, s, 19-H), 2.02 (3H, s, OAc),5.50 (1H, d, *J* 3 Hz, H-6), 5.98 (1H, s, 4-H). Further elution with 40% ethyl acetate : light petroleum gave *18-acetoxy-des-D-13α-androst-4-ene-3,14-dione* (**13**) (96 mg) which crystallised from ethyl acetate:light petroleum as needles, m.p. 156–160°C. (Found: C, 70.8; H, 8.1. $C_{18}H_{24}O_4$ requires C, 71.0; H, 7.9%); ν_{max}/cm^{-1} 1730, 1680; δ_H 1.26 (3H, s, 19-H),2.05 (3H, s, OAc), 4.03 (1H, dd, *J* 11.5 and 6.0 Hz) and 4.40 (1H, dd, *J* 11.5 and 6.0 Hz) (each 18-H), 5.77 (1H, s, 4-H).

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