



The microbiological hydroxylation of 3 α ,5-cycloandrostanes by *Cephalosporium aphidicola*

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Abstract

The microbiological hydroxylation of some 3 α ,5-cycloandrostanes by the fungus, *Cephalosporium aphidicola* has been shown to take place at C-2 α and C-14 α and a 6 β -alcohol was oxidized to the 6-ketone. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The microbiological hydroxylation of steroids provides a useful mild synthetic method for obtaining access to rare steroids (Mahato & Majumdar, 1993). The cyclopropane ring of the 3 α ,5-cyclosteroids is sensitive to a variety of chemical reagents restricting many chemical transformations. The fission of a cyclopropane ring in the presence of an adjacent radical has provided a probe for enzyme mechanism (Suckling, 1988; Nonhebel, 1993). Chemical studies have shown that the cyclopropane ring of a 3 α ,5-cyclocholestane undergoes fission induced by an adjacent C-6 radical (Cristol & Barbour, 1968). In continuation of our studies (Bensasson, Chevolot, Hanson & Quinton, 1999) on the application of *Cephalosporium aphidicola* to the microbiological hydroxylation of steroids, we have examined the transformation of the 3 α ,5-cycloandrostanes **1**, **3** and **6**.

Prior studies (Holland, Chernishenko, Conn, Munoz, Manoharan & Zawadski, 1990) have shown that 17 β -hydroxy-3 α ,5-cycloandrostan-17-one was hydroxylated at C-2 α and C-7 β by *Rhizopus arrhizus*. 6 β -Hydroxy-3 α ,5-cycloandrostan-17-one **1** was hydroxyl-

ated (Prochazka, Budesinsky & Prekajski, 1974) at C-2 α , C-11 α and C-7 β by *R. nigricans* whilst the 6 β -methoxy analogue was hydroxylated (Thoa, Prochazka, Budesinsky & Kocovsky, 1978) at C-1 β . The fungus, *Calonectria decora*, has been shown (Chambers et al., 1975) to hydroxylate 6 β -hydroxy-3 α ,5-cycloandrostan-17-one **1** at C-11 α and the corresponding diketone **3** at C-2 α , C-11 α , C-15 α and C-19.

2. Results and discussion

Incubation of 6 β -hydroxy-3 α ,5-cycloandrostan-17-one **1** with *C. aphidicola* for 8 days gave six metabolites (see Table 1) which were separated by chromatography. The first metabolite to be isolated was 14 α -hydroxy-3 α ,5-cycloandrostan-6,17-dione **4**. The ¹³C-NMR spectrum (see Table 2) possessed a carbonyl signal at δ_C 209.9 in place the secondary alcohol at δ_C 73.3 and a tertiary alcohol at δ_C 81.0 in place of a methine signal in the starting material. Hence the 6 β -alcohol had been oxidized to a ketone. The location of the new tertiary alcohol at C-14 α followed from the downfield shift of the signals assigned to C-8, C-13 and C-15 ($\Delta\delta$ 7.6, 5.4 and 11.8 ppm, respectively) together with γ -gauche shieldings for the signals assigned to C-7, C-12 and C-16 ($\Delta\delta$ 3.0, 6.6 and 5.7

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Table 1
Hydroxylation of 3 α ,5-cycloandrostanes by *C. aphidicola*

| Substrate | Product | % Yield |
|---|-----------|---------|
| 6 β -Hydroxy-3 α ,5-cycloandrostan-17-one | 1 | |
| 14 α -Hydroxy-3 α ,5-cycloandrostan-6,17-dione | 4 | 2 |
| 3 β ,14 α -Dihydroxyandrost-5-en-17-one | 9 | 6 |
| 6 β ,14 α -Dihydroxy-3 α ,5-cycloandrostan-17-one | 2 | 10 |
| 3 β ,7 α -Dihydroxyandrost-5-en-17-one | 10 | 15 |
| 3 β ,7 β -Dihydroxyandrost-5-en-17-one | 11 | 13 |
| 3 β ,5 α ,6 β -Trihydroxyandrost-17-one | 12 | 9 |
| 3 α ,5-Cycloandrostan-6,17-dione | 3 | |
| 14 α -Hydroxy-3 α ,5-cycloandrostan-6,17-dione | 4 | 9 |
| 2 α -Hydroxy-3 α ,5-cycloandrostan-6,17-dione | 5 | 6 |
| 3 α ,5-Cycloandro-6-en-17-one | 6 | |
| 6 β ,7 α -Dihydroxy-3 α ,5-cycloandrostan-17-one | 7 | 15 |
| 3 β ,7 α -Dihydroxyandrost-5-en-17-one | 10 | 7 |

ppm, respectively). The second metabolite, 3 β ,14 α -dihydroxyandrost-5-en-17-one **9**, lacked the ^1H - and ^{13}C -NMR signals associated with the cyclopropane ring possessing instead signals characteristic of a substituted 3 β -hydroxyandrost-5-en-17-one. These included ^1H -NMR signals at δ_{H} 3.54 (*tt*, $J = 4.5$ and 11 Hz) and 5.42 (*d*, $J = 2.2$ Hz) assigned to H-3 and H-6 and ^{13}C -NMR signals at δ_{C} 73.0, 142.1 and 122.2 assigned to C-3, C-5 and C-6. The location of the second hydroxyl group at C-14 α followed from the downfield shifts of the resonances assigned to C-8, C-13 and C-15 ($\Delta\delta$ 5.7, 6.1 and 12.9 ppm, respectively) and the γ -gauche shieldings of the signals assigned to C-7, C-12 and C-16 ($\Delta\delta$ 4.7, 5.6 and 9.9 ppm, respectively) when compared to 3 β -hydroxyandrost-5-en-17-one **8**.

Table 2
 ^{13}C -NMR Spectra of steroids 1–9

| Carbon atom | Steroid | | | | | | | | |
|-------------|---------|-------|-------|-------|-------|-------|-------|-------|-------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 1 | 33.6 | 31.2 | 33.4 | 33.8 | 41.8 | 31.4 | 33.1 | 37.2 | 37.2 |
| 2 | 25.4 | 25.4 | 25.8 | 26.2 | 71.9 | 25.0 | 24.9 | 31.5 | 31.6 |
| 3 | 24.7 | 24.7 | 35.5 | 36.1 | 38.2 | 25.7 | 24.5 | 71.4 | 71.5 |
| 4 | 11.6 | 12.2 | 11.8 | 12.5 | 9.5 | 14.7 | 9.4 | 42.2 | 42.2 |
| 5 | 38.9 | 39.3 | 46.3 | 46.8 | 46.4 | 42.6 | 35.5 | 141.3 | 141.6 |
| 6 | 73.3 | 73.9 | 208.5 | 209.9 | 207.4 | 132.9 | 77.1 | 120.8 | 120.7 |
| 7 | 35.9 | 33.7 | 43.4 | 38.9 | 43.9 | 124.8 | 70.0 | 31.5 | 31.2 |
| 8 | 29.9 | 32.7 | 34.3 | 37.5 | 34.4 | 35.9 | 33.9 | 31.5 | 34.2 |
| 9 | 47.8 | 41.3 | 46.2 | 40.0 | 48.5 | 46.3 | 39.7 | 50.3 | 44.0 |
| 10 | 42.9 | 43.6 | 46.7 | 46.7 | 45.8 | 36.7 | 42.9 | 36.7 | 36.8 |
| 11 | 21.9 | 21.0 | 22.1 | 21.2 | 22.5 | 21.5 | 21.8 | 20.4 | 20.4 |
| 12 | 31.7 | 25.4 | 31.4 | 25.1 | 31.7 | 31.8 | 31.4 | 30.8 | 25.1 |
| 13 | 47.9 | 53.3 | 47.8 | 53.3 | 48.2 | 48.4 | 47.7 | 47.5 | 54.5 |
| 14 | 51.4 | 82.0 | 51.9 | 81.0 | 52.2 | 50.1 | 45.8 | 51.8 | 82.2 |
| 15 | 21.7 | 33.7 | 21.6 | 33.5 | 22.0 | 21.8 | 21.2 | 21.8 | 33.6 |
| 16 | 35.8 | 30.5 | 35.7 | 30.2 | 36.1 | 35.9 | 35.8 | 35.8 | 30.5 |
| 17 | 221.3 | 219.3 | 220.0 | 218.7 | 221.2 | 220.7 | 221.2 | 221.3 | 218.7 |
| 18 | 13.9 | 18.5 | 13.7 | 18.2 | 14.2 | 13.9 | 13.6 | 13.5 | 17.6 |
| 19 | 20.2 | 20.6 | 19.7 | 20.0 | 20.0 | 17.8 | 20.2 | 19.4 | 19.4 |

The third metabolite was 6 β ,14 α -dihydroxy-3 α ,5-cycloandrostan-17-one **2**. The ^{13}C -NMR spectrum showed that one C–H resonance of the starting material had been replaced by a tertiary alcohol (δ_{C} 82.1). This was located at C-14 α from the pattern of downfield shifts and γ -gauche shieldings of the resonances assigned to C-8, C-13 and C-15 on the one hand and C-7, C-12 and C-16 on the other. The fourth and fifth metabolites were identified as 3 β ,7 α - and 3 β ,7 β -dihydroxyandrost-5-en-17-one **10** and **11** (Dodson, Nicholson & Muir, 1959; Crabb, Dawson & Williams, 1980; Bensasson, Hanson & Hunter, 1998) from their ^1H - and ^{13}C -NMR spectra. The final metabolite was the known 3 β ,5 α ,6 β -trihydroxyandrost-17-one **12** (Bensasson, Hanson & Hunter, 1998).

Incubation of 3 α ,5-cycloandrostan-6,17-dione **3** gave 14 α -hydroxy-3 α ,5-cycloandrostan-6,17-dione **4** and 2 α -hydroxy-3 α ,5-cycloandrostan-6,17-dione **5**. The ^1H -NMR spectrum of the latter possessed a new CH(OH) signal at δ_{H} 4.63 as a doublet ($J = 12.5$ Hz) of triplets ($J = 4$ Hz). The location of this hydroxyl group at C-2 followed from the downfield shifts of the signals assigned to C-1 and C-3 ($\Delta\delta$ 8.4 and 2.7 ppm, respectively) and a γ -gauche shielding for the signal assigned to C-4 ($\Delta\delta$ 2.3 ppm) when compared to the starting material **3**. The stereochemistry of the hydroxyl group followed from the multiplicity of the CH(OH) resonance (one diaxial and two axial: equatorial couplings) and from the NOE enhancement (ca. 1%) of the signal on irradiation of the H-19 resonance (δ_{H} 1.04).

Incubation of 3 α ,5-cycloandro-6-en-17-one **6** with *C. aphidicola* gave 6 β ,7 α -dihydroxy-3 α ,5-cycloandrostan-17-one **7**. In the ^1H -NMR spectrum of this biotransformation product, the alkene C–H signals had been replaced by two CH(OH) signals at δ_{H} 3.86 and 3.22. This metabolite was then identified by comparison with an authentic sample of the diol (Cambie, Thomas & Hanson, 1975). No hydroxylation products were obtained from the incubation of 3 α ,5-cycloandrostan-17-one.

Products involving the cleavage of the cyclopropane ring were detected in the fermentations involving 6 β -hydroxy-3 α ,5-cycloandrostan-17-one **1** and in the fermentation in which the 6 β ,7 α -diol **7** was formed. The possibility was explored that these were artefacts arising from an acid-catalysed reversal of the *i*-steroid reaction in the acidic medium. 6 β -Hydroxy-3 α ,5-cycloandrostan-17-one **1** was shaken for 8 days with sterile medium at the natural pH (4.5) and in media in which the pH had been adjusted to pH 1.5 and 3.

The crude extract was then examined by ^1H -NMR. In both of the latter, the cyclo-steroid was converted to 3 β -hydroxyandrost-5-en-17-one **8** whilst at the natural pH there was a 20% conversion. It therefore seems probable that the metabolites containing the 3 β -hydro-

xyandrost-5-ene moiety arose via an acid-catalysed rather than from a microbial cleavage of the cyclopropane ring. The biotransformations with *C. aphidicola* follow the pattern of other fungal hydroxylations of 3 α ,5-cyclo-steroids in which the cyclopropane ring is not readily cleaved by the micro-organism even though microbial reaction occurs at an adjacent centre. This particular biotransformation provides access to C-2 α and C-14 α . Chemical hydroxylation of C-14 α normally involves oxidation with chromium trioxide (Saint-Andre et al., 1952) and would be precluded by the presence of the cyclopropane ring. The formation of the 6 β ,7 α -diol from the 6,7-ene may involve an acid-catalysed cleavage of the 6 α ,7 α -epoxide.

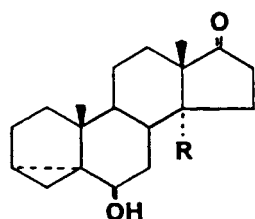
3. Experimental

¹H-NMR Spectra were recorded in deuteriochloroform at 300 MHz and ¹³C-NMR spectra were deter-

mined at 75 MHz. IR Spectra were recorded as nujol mulls. Chromatography was carried out on silica, Merck 9385. Light petroleum refers to the fraction b.p. 60–80°. Extracts were dried over anhydrous sodium sulfate. *Cephalosporium aphidicola* was cultured as described previously (Hanson & Nasir, 1993).

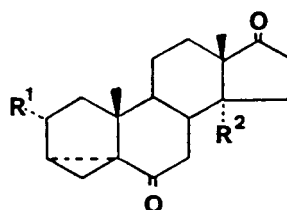
3.1. Biotransformation of 6 β -hydroxy-3 α ,5-cycloandrostan-17-one **1**

The substrate **1** (2g) in DMSO (25 cm³) and EtOH (5 cm³) was evenly distributed between 50 flasks of a 3 day old culture of *C. aphidicola*. After a further 8 days, the mycelium was filtered and the broth was extracted with EtOAc. The extract was dried and the solvent was evap. to give a residue which was chromatographed on silica. Elution with 30% EtOAc: petrol gave the starting material (181 mg). Elution with 35% EtOAc: petrol gave 14 α -hydroxy-3 α ,5-cycloan-



1 R = H

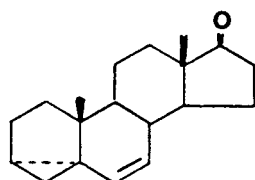
2 R = OH



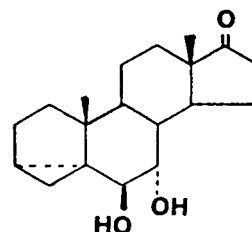
3 R¹ = R² = H

4 R¹ = H, R² = OH

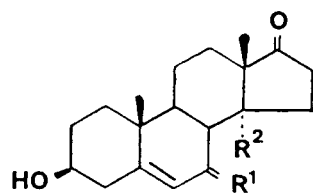
5 R¹ = OH, R² = H



6



7

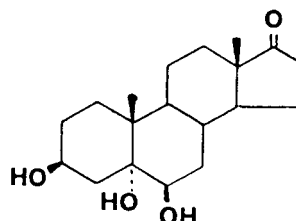


8 R¹ = H₂, R² = H

9 R¹ = H₂, R² = OH

10 R¹ = α -OH, β -H, R² = H

11 R¹ = α -H, β -OH, R² = H



12

drostane-6,17-dione **4** (48 mg) which crystallized from EtOAc: petrol as needles, m.p. 265–270° (found: C, 75.5; H, 8.8. $C_{19}H_{26}O_3$ requires C 75.5; H, 8.7%), ν_{\max} 3440, 1739, 1696 cm^{-1} ; δ_H 0.78 (1H, *t*, *J* = 4.5 Hz, H-3), 1.05 and 1.06 (each 3H, *s* H-18 and H-19). Elution with 37% EtOAc: petrol gave 3 β ,14 α -dihydroxyandrostan-5-en-17-one **9** (114 mg) which crystallized from EtOAc: petrol as needles, m.p. 205–209° (Saint-Andre, MacPhillamy, Nelson, Shabica & Scholz, 1952, 215°), ν_{\max} 3386, 3200, 1729 cm^{-1} ; δ_H 1.03 (3H, *s*, H-18), 1.05 (3H, *s*, H-19), 3.54 (1H, *tt*, *J* = 4.5 and 11 Hz, H-3), 5.42 (1H, *d*, *J* = 2.2 Hz, H-6). Elution with 40% EtOAc: petrol gave 6 β ,14 α -dihydroxy-3 α ,5-cycloandrostan-17-one **2** (194 mg) which crystallized from EtOAc: petrol as needles, m.p. 118–120° (found: C, 74.3; H, 9.7. $C_{19}H_{28}O_3$ requires C, 74.9; H, 9.3%), ν_{\max} 3413, 1733 cm^{-1} ; δ_H 1.06 (3H, *s*, H-18), 1.11 (3H, *s*, H-19), 3.39 (1H, *t*, *J* = 3 Hz, H-6). Elution with 45% EtOAc: petrol gave 3 β ,7 β -dihydroxyandrostan-5-en-17-one **11** (269 mg) which crystallized from EtOAc: petrol as needles, m.p. 207° (Dodson et al., 1959, 215–216°), identified by comparison (IR and NMR) with an authentic sample. Elution with 60% EtOAc: petrol gave 3 β ,7 α -dihydroxyandrostan-5-en-17-one **10** (292 mg) which crystallized from chloroform as needles, m.p. 177° (Dodson et al., 1959, 182°), identified by comparison (IR and NMR) with an authentic sample. Elution with 20% MeOH: EtOAc gave 3 β ,5 α ,6 β -trihydroxyandrostan-17-one **12** (180 mg) which crystallized from MeOH: EtOAc as prisms, m.p. 298–301° (Holland & Diakow, 1979), identified by comparison (IR and NMR) with an authentic sample.

3.2. Biotransformation of 3 α ,5-cycloandrostan-6,17-dione **3**

The substrate **3** (1.5 g) in DMSO (25 cm^3) and EtOH (5 cm^3) was evenly distributed between 50 flasks of *C. aphidicola* 3 days after inoculation. After a further 8 days the mycelium was filtered and the broth was extracted with EtOAc. The extract was dried and evap. and the residue chromatographed on silica. Elution with 30% EtOAc: petrol gave the starting material **3** (125 mg). Elution with 45% EtOAc: petrol gave 14 α -hydroxy-3 α ,5-cycloandrostan-6,17-dione **4** (136 mg) identical to the material described above. Elution with 75% EtOAc: petrol gave 2 α -hydroxy-3 α ,5-cycloandrostan-6,17-dione **5** (32 mg) which crystallized from EtOAc: petrol as needles, m.p. 241–243° (found: C, 75.5; H, 8.8. $C_{19}H_{26}O_3$ requires C, 75.5; H, 8.7%); ν_{\max} 3401, 1734, 1697 cm^{-1} ; δ_H 0.92 (3H, *s*, H-18), 1.04 (3H, *s*, H-19), 4.63 (1H, *dt*, *J* = 12.5 and 4 Hz, H-2).

3.3. Biotransformation of 3 α ,5-cycloandrostan-6-en-17-one **6**

The substrate **6** (1.2 g) in DMSO (30 cm^3) and EtOH (10 cm^3) was evenly distributed between 50 flasks of *C. aphidicola* 3 days after inoculation. After a further 8 days the mycelium was filtered and the broth was extracted with EtOAc. The extract was dried and the solvent evap. to give a residue which was chromatographed on silica. Elution with 10% EtOAc: petrol gave the starting material **6** (360 mg). Elution with 80% EtOAc: petrol gave 6 β ,7 α -dihydroxy-3 α ,5-cycloandrostan-17-one **7** (332 mg) which crystallized from EtOAc: petrol as needles, m.p. 170° (Cambie et al., 1975, 172°); ν_{\max} 3450, 1730 cm^{-1} ; δ_H 0.27 (1H, *dd* *J* = 5 and 8 Hz, H-4), 0.52 (1H, *t*, *J* = 5 Hz, H-4), 0.93 (3H, *s*, H-18), 1.07 (3H, *s*, H-19), 3.22 (1H, *d*, *J* = 3 Hz, H-6), 3.86 (1H, *m*, H-7). Elution with EtOAc gave 3 β ,7 α -dihydroxyandrostan-5-en-17-one **10** (170 mg) identical to the material described above.

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