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

Caroline S. Bensasson, James R. Hanson*, Yvan Le Huerou

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

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Abstract

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

Keywords: Microbiological hydroxylation; *Cephalosporium aphidicola*; Steroids; 3α,5-cycloandrostanes

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 3α,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 3α,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 3α,5-cycloandrostanes **1**, **3** and **6**.

Prior studies (Holland, Chernishenko, Conn, Munoz, Manoharan & Zawadski, 1990) have shown that 17β-hydroxy-3α,5-cycloandrostane was hydroxylated at C-2α and C-7β by *Rhizopus arrhizus*. 6β-Hydroxy-3α,5-cycloandrostan-17-one **1** was hydroxylated (Prochazka, Budesinsky & Prekajski, 1974) at C-2α, C-11α and C-7β by *R. nigricans* whilst the 6β-methoxy analogue was hydroxylated (Thoa, Prochazka, Budesinsky & Kocovsky, 1978) at C-1β. The fungus, *Calonectria decora*, has been shown (Chambers et al., 1975) to hydroxylate 6β-hydroxy-3α,5-cycloandrostan-17-one **1** at C-11α and the corresponding diketone **3** at C-2α, C-11α, C-15α and C-19.

2. Results and discussion

Incubation of 6β-hydroxy-3α,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 14α-hydroxy-3α,5-cycloandrostane-6,17-dione **4**. The ^{13}C-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 6β-alcohol had been oxidized to a ketone. The location of the new tertiary alcohol at C-14α 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

* Corresponding author.

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

Substrate	Product	% Yield
6β-Hydroxy-3α,5-cycloandrostan-17-one	**1**	
14α-Hydroxy-3α,5-cycloandrostane-6,17-dione	**4**	2
3β,14α-Dihydroxyandrost-5-en-17-one	**9**	6
6β,14α-Dihydroxy-3α,5-cycloandrostan-17-one	**2**	10
3β,7α-Dihydroxyandrost-5-en-17-one	**10**	15
3β,7β-Dihydroxyandrost-5-en-17-one	**11**	13
3β,5α,6β-Trihydroxyandrostan-17-one	**12**	9
3α,5-Cycloandrostane-6,17-dione	**3**	
14α-Hydroxy-3α,5-cycloandrostane-6,17-dione	**4**	9
2α-Hydroxy-3α,5-cycloandrostane-6,17-dione	**5**	6
3α,5-Cycloandrost-6-en-17-one	**6**	
6β,7α-Dihydroxy-3α,5-cycloandrostan-17-one	**7**	15
3β,7α-Dihydroxyandrost-5-en-17-one	**10**	7

ppm, respectively). The second metabolite, 3β,14α-dihydroxyandrost-5-en-17-one **9**, lacked the ^{1}H- and ^{13}C-NMR signals associated with the cyclopropane ring possessing instead signals characteristic of a substituted 3β-hydroxyandrost-5-en-17-one. These included ^{1}H-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-14α followed from the downfield shifts of the resonances assigned to C-8, C-13 and C-15 (Δδ 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 (Δδ 4.7, 5.6 and 9.9 ppm, respectively) when compared to 3β-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 6β,14α-dihydroxy-3α,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-14α 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 3β,7α- and 3β,7β-dihydroxyandrost-5-en-17-one **10** and **11** (Dodson, Nicholson & Muir, 1959; Crabb, Dawson & Williams, 1980; Bensasson, Hanson & Hunter, 1998) from their ^{1}H- and ^{13}C-NMR spectra. The final metabolite was the known 3β,5α,6β-trihydroxyandrostan-17-one **12** (Bensasson, Hanson & Hunter, 1998).

Incubation of 3α,5-cycloandrostane-6,17-dione **3** gave 14α-hydroxy-3α,5-cycloandrostane-6,17-dione **4** and 2α-hydroxy-3α,5-cycloandrostane-6,17-dione **5**. The ^{1}H-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 (Δδ 8.4 and 2.7 ppm, respectively) and a γ-gauche shielding for the signal assigned to C-4 (Δδ 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 3α,5-cycloandrost-6-en-17-one **6** with *C. aphidicola* gave 6β,7α-dihydroxy-3α,5-cycloandrostan-17-one **7**. In the ^{1}H-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 3α,5-cycloandrostan-17-one.

Products involving the cleavage of the cyclopropane ring were detected in the fermentations involving 6β-hydroxy-3α,5-cycloandrostan-17-one **1** and in the fermentation in which the 6β,7α-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. 6β-Hydroxy-3α,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 ^{1}H-NMR. In both of the latter, the cyclo-steroid was converted to 3β-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 3β-hydro-

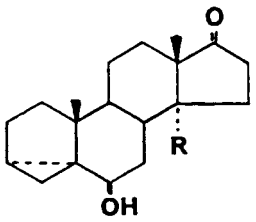
xyandrost-5-ene moiety arose via an acid-catalysed rather than from a microbial cleavage of the cyclopropane ring. The biotransformations with *C. aphidicola* follow the pattern of other fungal hydroxylations of 3α,5-cyclo-steroids in which the cyclopropane ring is not readily cleaved by the micro-organism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

3. Experimental

^{1}H-NMR Spectra were recorded in deuteriochloroform at 300 MHz and ^{13}C-NMR spectra were determined 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).

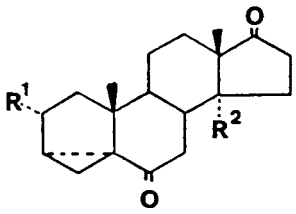
3.1. Biotransformation of 6β-hydroxy-3α,5-cycloandrostan-17-one *1*

The substrate **1** (2g) in DMSO (25 cm^3) and EtOH (5 cm^3) 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 14α-hydroxy-3α,5-cycloan-



1 R = H

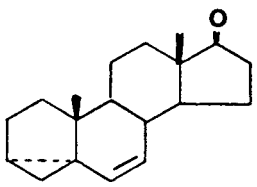
2 R = OH



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

4 $R^1 = H$, $R^2 = OH$

5 $R^1 = OH$, $R^2 = H$



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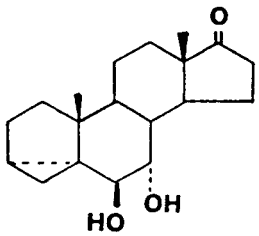

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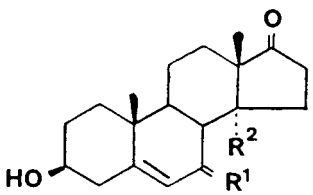
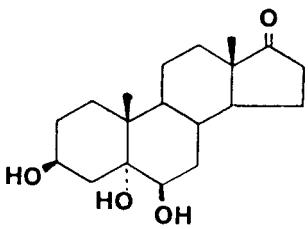


8 $R^1 = H_2$, $R^2 = H$

9 $R^1 = H_2$, $R^2 = OH$

10 $R^1 = \alpha$-OH,β-H, $R^2 = H$

11 $R^1 = \alpha$-H,β-OH, $R^2 = H$



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 3β,14α-dihydroxyandrost-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 6β,14α-dihydroxy-3α,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 3β,7β-dihydroxyandrost-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 3β,7α-dihydroxyandrost-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 3β,5α,6β-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 3α,5-cycloandrostane-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 14α-hydroxy-3α,5-cycloandrostane-6,17-dione **4** (136 mg) identical to the material described above. Elution with 75% EtOAc: petrol gave 2α-hydroxy-3α,5-cycloandrostane-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 3α,5-cycloandrost-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 6β,7α-dihydroxy-3α,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 3β,7α-dihydroxyandrost-5-en-17-one **10** (170 mg) identical to the material described above.

References

Bensasson, C. S., Hanson, J. R., & Hunter, A. C. (1998). *Phytochemistry*, *49*, 2355.

Bensasson, C. S., Chevolot, Y., Hanson, J. R., & Quinton, J. (1999). *Phytochemistry*, *50*, 25.

Cambie, R. C., Thomas, P. W., & Hanson, J. R. (1975). *Journal of the Chemical Society Perkin Trans*, *1*, 323.

Chambers, V. E. M., Denny, W. A., Jones, Sir Ewart R. H., Meakins, G. D., Miners, J. O., Pinhey, J. T., & Wilkins, A. L. (1975). *Journal of the Chemical Society Perkin Trans*, *1*, 1359.

Crabb, T. A., Dawson, P. J., & Williams, R. O. (1980). *Journal of the Chemical Society Perkin Trans*, *1*, 2535.

Cristol, S. J., & Barbour, R. V. (1968). *Journal of the American Chemical Society*, *90*, 2832.

Dodson, R. M., Nicholson, R. T., & Muir, R. D. (1959). *Journal of the American Chemical Society*, *81*, 6295.

Hanson, J. R., & Nasir, H. (1993). *Phytochemistry*, *33*, 831.

Holland, H. J., & Diakow, M. A. (1979). *Canadian Journal of Chemistry*, *57*, 436.

Holland, H. J., Chernishenko, M. J., Conn, M., Munoz, A., Manoharan, T. S., & Zawadski, M. A. (1990). *Canadian Journal of Chemistry*, *68*, 696.

Mahato, S. B., & Majumdar, I. (1993). *Phytochemistry*, *34*, 883.

Nonhebel, D. C. (1993). *Chemical Society Reviews*, *22*, 347.

Prochazka, Z., Budesinsky, M., & Prekajski, P. (1974). *Collection of Czechoslovak Chemical Communications*, *39*, 984.

Saint-Andre, A. F., MacPhillamy, H. B., Nelson, J. A., Shabica, A. C., & Scholz, C. R. (1952). *Journal of the American Chemical Society*, *74*, 5506.

Suckling, C. J. (1988). *Angewandte Chemie (International Edition in English)*, *27*, 537.

Thoa, H. K., Prochazka, Z., Budesinsky, M., & Kocovsky, P. (1978). *Collection of Czechoslovak Chemical Communications*, *43*, 2305.