The microbiological hydroxylation of some methoxysteroids by *Cephalosporium aphidicola*

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The effect of replacing a hydroxyl group by a methoxyl group on the microbiological hydroxylation of some steroids by the fungus, *Cephalosporium aphidicola*, has been examined.

Keywords: *Cephalosporium aphidicola*, microbiological hydroxylation, steroids, methoxyl groups

The microbiological hydroxylation of steroids has been rationalised in terms of a triangular relationship between two binding sites on the steroid framework and the site of hydroxylation.[1-3] The dimensions of this triangle vary with the organism but typically the binding sites might be C-3 and C-17 with hydroxylation taking place at C-6β or C-11α. The binding groups are typically hydroxyl or carbonyl groups. This pattern of hydroxylation has been observed[4] with the fungus, *Cephalosporium aphidicola*. In the androstane series, apart from hydroxylations at C-6β and C–11α, hydroxylation occurred to a minor extent at positions C-5α and C-14α whilst the presence of a Δ^5-double bond in the substrate led to non-stereospecific hydroxylation at C-7.[5] In the present study, we have examined the effect on the microbiological hydroxylation of replacing a hydroxyl group, a hydrogen bond donor, by a methoxyl group which can only act as an acceptor. The substrates, 17β-methoxy-5α-androstan-3-one **1**,[6] 17β-methoxyestra-4-en-3-one **4** and 3β-methoxyandrost-5-en-17-one **6**[7] were prepared by methylating the corresponding alcohols with methyl iodide in the presence of freshly prepared silver oxide.

The steroids were incubated with *C.aphidicola* in shake culture for 5–7 days and the metabolites were then isolated and separated by chromatography. 17β-Methoxy-5α-androstan-3-one gave two products, **2** and **3**. These were identified by their ^{1}H and ^{13}C NMR spectra. The presence of a 3β-hydroxyl group in 17β-methoxy-5α-androstan-3β-ol **2** was revealed by its ^{1}H NMR spectrum in which the H-3α resonance (δ_H 3.57) appeared as a triplet (*J*=10.5 Hz) of triplets (*J*=5 Hz) whilst there was a ^{13}C NMR signal (see Table 1) at the characteristic position (δ_C 71.3). 6β,11α-Dihydroxy-17β-methoxy-5α-androstan-3-one **3** possessed two new C**H**(OH) resonances in the ^{1}H NMR spectrum. The signal at δ_H 3.75 was a broad singlet indicative of hydroxylation at an axial position. A significant downfield shift (Δδ 0.3 ppm) in the H-19 resonance suggested that a hydroxyl group might be at C-6β. This was confirmed by the ^{13}C NMR spectrum (see Table 1) which showed that the resonance assigned to C-6 had moved downfield whilst there was a characteristic γ-gauche shielding of C-8 (Δδ 7.1 ppm). The location of the second hydroxyl group at C-11α followed from the multiplicity of the C**H**(OH) resonance at δ_H 4.00 (triplet of doublets, ***J*** 10 and 5 Hz) typical of an equatorial hydroxyl group at C-11. The disappearance of the C-11 methylene ^{13}C resonance and the appearance of a new methine signal at δ_C 68.8 was consistent with this assignment.

Incubation of 17β-methoxyestra-4-en-3-one **4** with *C. aphidicola* gave one major metabolite **5**. The location of the new hydroxyl grop at C-6β followed from the multiplicity of the new C**H**(0H) resonance (δ_H 4.35) which was a narrow triplet (*J*=2.4 Hz) and from the ^{13}C NMR spectrum (see Table 1) including changes in the position of C-6 (δC=72.1) and the characteristic shielding of C-8 (Δδ=6.8 ppm).

Table 1 ^{13}C NMR Data for compounds 1–6 and 8 determined in $CDC1_3$ at 75 MHz

Carbon atom	Compound 1	2	3	4	5	6	8
1	38.1	38.2	38.4	36.9	36.7	36.2	36.0
2	38.6	31.6	39.1	38.1	38.0	28.3	27.9
3	212.0	71.3	212.7	200.4	201.2	80.5	79.2
4	44.7	38.1	43.5	124.9	125.6	37.5	39.1
5	46.7	44.9	49.5	167.1	165.9	141.5	166.6
6	28.8	28.6	70.6	31.1	72.1	121.2	126.3
7	31.2	31.5	42.6	35.9	38.1	31.2	201.5
8	35.2	35.3	28.1	40.6	33.8	31.9	44.7
9	53.9	54.5	59.8	50.0	49.8	50.7	46.1
10	35.7	35.5	37.3	42.9	38.5	37.4	37.5
11	21.1	20.9	68.8	27.0	26.5	20.7	20.9
12	37.9	37.0	49.9	28.0	28.0	39.0	36.6
13	42.9	43.0	41.3	43.3	43.5	47.9	48.2
14	51.1	51.3	49.8	50.3	50.3	52.2	50.5
15	23.3	23.3	23.3	23.5	23.4	22.3	24.6
16	27.7	27.7	27.8	26.6	26.4	31.8	31.1
17	90.7	90.8	90.0	90.9	90.9	220.1	220.5
18	11.6	12.3	12.7	11.9	12.0	13.9	14.1
19	11.5	11.6	15.1	–	–	19.8	17.8
OMe	57.6	57.8	57.9	58.2	58.2	56.0	56.4

Incubation of 3β-methoxyandrost-5-en-17-one **6** gave an inseparable mixture of C-7α and C-7β-alcohols **7** (δ_H=3.90 and 4.05). Oxidation of this mixture with chromium trioxide gave the Δ^5-7-ketone **8.** This had IR absorption at 1658 and 1625 cm^{-1} and NMR signals at δ_H=5.65 (H-6) and δ_C=166.6 (C-5), 126.3(C-6) and 201.5(C-7) typical of an unsaturated ketone.

In conclusion, these results show that the microbiological hydroxylation at C-6β and at C-11α is not affected by the conversion of a hydroxyl group at C-17 to a methoxyl group. However, there was no detectable hydroxylation at C-14α. The non-stereospecific allylic hydroxylation at C-7 was unaffected by the conversion of the 3β-hydroxyl group into a methoxyl group. However, we were unable to detect any hydroxylation at C-11α or the consequences of epoxidation of the Δ^5-double bond which were observed in the presence of a 3β-hydroxyl group. These results suggests that the presence of a hydroxyl group rather than a methoxyl group, may be required for some hydroxylations.

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 on a Bruker DPX 300 spectrometer. IR spectra were determined as nujol mulls. Mass spectra were obtained by electrospray ionisation on a Bruker Daltonics Apex mass spectrometer. Extracts were dried over anhydrous sodium sulfate.

Preparation of the substrates: (a) 17β-Hydroxy-5α-androstan-3-one (3 g) in dry dimethylformamide (60 cm^3) was treated with freshly

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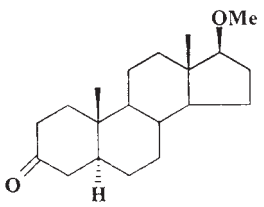
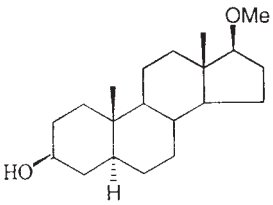


1



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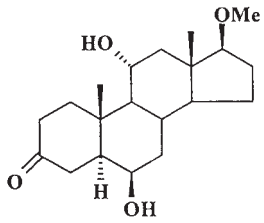

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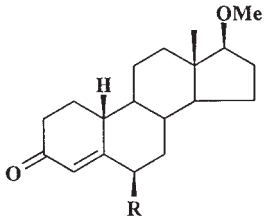
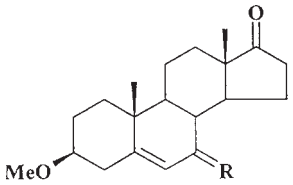


4 R = H
5 R = OH



6 R = H_2
7 R = OH
8 R = O

prepared silver oxide (4.8 g) and iodomethane (12 cm^3) at room temperature for 5 days. The reaction mixture was filtered through a Celite pad, concentrated and the product recovered in ethyl acetate. The extract was washed with aqueous sodium hydrogen carbonate, brine and dried. The solvent was evaporated and the residue chromatographed on silica. Elution with 10% ethyl acetate:light petroleum gave 17β-methoxy-5α-androstan-3-one **1** (558 mg) as needles, m.p.108–110°C (lit.,[6] 110-112°C), ν_{max}/cm^{-1} 1718: δ_H 0.77 (3H,s, H-18). 1.02 (3H,s, H-19), 0.8–2.4 (22H, overlapping multiplets), 3.23 (1H, t, *J*=8.3 Hz, H-17), 3.35 (3H,s, OMe).

(b) Under similar conditions 17β-hydroxyestra-4-en-3-one (2 g) gave 17β-methoxyestra-4-en-3-one **4** (1 g) as an oil, [Found M^+ 599.410 $(C_{19}H_{28}0_2)_2Na^+$ requires 599.407], ν_{max}/cm^{-1} 1680, 1625; δ_H=0.75 (3H, s, H-18), 0.8–2.3 (20H, overlapping multiplets), 3.17 (1H, t, *J*=8.5Hz, H-17), 3.28 (3H, s, OMe), 5.81 (lH, s, H-4).

(c) Under similar conditions 3β-hydroxyandrosta-5-en-17-one (2 g) gave 3β-methoxyandrosta-5-en-17-one **6** (1.2 g), m.p. 135–136°C (lit.,[7] 142°C) (Found M^+ 325.214 $C_{20}H_{30}O_2Na^+$ requires 325.214), ν_{max}/cm^{-1} 1735, 1667; δ_H 0.82 (3H, s, H-18), 0.95 (3H, s, H-19), 0.8–2.4 (19H, overlapping multiplets), 3.09 (1H, tt, *J*=5 and 11Hz, H-3), 3.25 (3H, s, OMe), 5.30 (1H, brs, H-5).

Incubation experiments: The fungus, *Cephalosporium aphidicola*, was grown on shake culture in 10 conical flasks (250cm^3) containing a medium (100cm^3) comprising (per litre), glucose (80g), ammonium nitrate (4.8g), potassium dihydrogen phosphate (5g), magnesium sulfate (1g) and a solution (2cm^3) of trace elements. This contained (per 100cm^3) cobalt nitrate (0.01g), iron(II) sulfate (0.lg), copper sulfate (0.015g), zinc sulfate (0.161g), manganese sulfate (0.01g) and ammonium molybdate (0.01g). The substrates (500mg) were added after two days growth and the fermentations were continued for a further 5–7 days. The mycelium was filtered off and the broth was acidified with dil. hydrochloric acid and extracted with ethyl acetate.

The extract was dried and the solvent evaporated to give a residue which was chromatographed on silica with increasing concentrations of ethyl acetate in light petroleum.

(a) Incubation of 17β-methoxy-5α-androstan-3-one **1** (500mg) and chromatography of the products gave 17β-methoxy-5α-androstan-3β-ol **2** (37mg) which crystallised from methanol as needles, m.p. 146°C (lit.,[8] 150–152°C) ν_{max}/cm^{-1} 3354; δ_H 0.77 (3H, s, H-18), 0.84 (3H, s, H-19), 0.8–2.2 (22H, overlapping multiplets), 3.21 (1H, t, *J*=8 Hz, H-17), 3.34 (3H, s, OMe), 3.57 (1H, tt, *J*=10.5 and 5 Hz, H-3), Further elution gave 6β, 11α-dihydroxy-17β-methoxy-5α-androstan-3-one **3** (79 mg) as a gum, (Found: M^+ 336.230 $C_{20}H_{32}O_4$ requires M^+ 336.231), ν_{max}/cm^{-1} 3491, 3435; δ_H 0.83 (3H, s, H-18), 1.34 (3H, s, H-19), 0.8–2.4 (20H, overlapping multiplets), 3.28 (1H, t, *J*=8.4 Hz, H-17), 3.35(3H, s, OMe), 3.75 (1H, br. s, H-6), 4.00 (1H, td, *J*=10.5 and 5 Hz, H-11).

(b) Incubation of 17β-methoxyestra-4-en-3-one **4** (500mg) gave the starting material (250mg) followed by 6β-hydroxy-17β-methoxyestra-4-en-3-one **5** (80mg) as a gum, (Found: M^+ 327.193 $C_{19}H_{28}O_3Na^+$ requires M^+ 327.193), ν_{max}/cm^{-1} 3400, 1680; δ_H=0.75 (3H.s. H-18). 0.9–2.3 (16H, overlapping multiplets), 3.18 (1H, t, *J*=8.4 Hz, H-17), 3.30 (3H, s, OMe), 4.35 (1H, t *J*=2.4Hz, H-6), 5.81 (1H, s, H-4).

(c) Incubation of 3β-methoxyandrost-5-en-17-one **6** (500mg) gave a mixture of 7α- and 7β-hydroxy-3β-methoxyandrost-5-en-17-one 7 (70mg) (δ_H=3.95 and 4.05) (approx. 0.5H each) which could not be obtained pure. The mixture was dissolved in acetone (2cm^3) and treated with the Jones' reagent (chromium trioxide in sulfuric acid) (0.5cm^3) for 15 min.

The excess reagent was destroyed with methanol, the solvent was evaporated and the residue was chromatographed on silica to give 3β-methoxyandrost-5-ene-7, 17-dione **8** (45mg) which crystallised as needles, m.p. 172-175°C, (Found: M^+ 339.193 $C_{20}H_{28}O_3Na^+$ requires M^+ 339.193), ν_{max}/cm^{-1} 1738, 1658, 1625; δ_H=0.83 (3H, s, H-18), 1.15 (3H, s, H-19). 0.9–2.3 (17H, overlapping multiplets), 3.17 (1H, tt, *J*=5 and 11 Hz, H-3), 3.31 (3H, s, OMe), 5.65 (1H, s, H-6).

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