SHORT PAPER

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The effect of a 4-formyl and hydroxymethyl substituent on steroid biotransformations by *Mucor plumbeus*†

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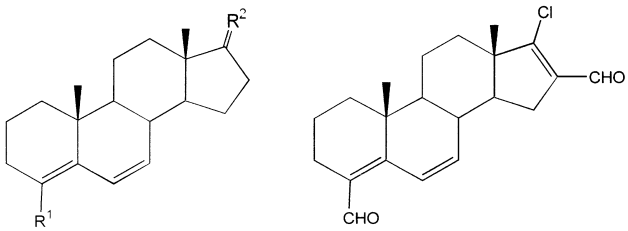
Although steroids possessing a 4-formyl substituent undergo reduction on incubation with *Mucor plumbeus*, they do not appear to be substrates for efficient microbiological hydroxylation by this organism indicating that the structural requirements for these two enzyme systems are different.

Keywords: steroids, microbiological reduction, *Mucor plumbeus*, formylation

Micro-organisms can carry out a variety of transformations including oxidations, reductions and hydroxylations utilising different enzyme systems. The models that have been proposed for rationalising the microbiological hydroxylation of steroids are based on triangular relationships between two binding sites and the site of hydroxylation.[1–3] In these models the C-3 and C-17 carbonyl and hydroxyl groups can act as binding groups orienting the steroid within the hydroxylase so that microbial hydroxylation may occur at a specific centre, *e.g.* C-11. In an effort to define the structural requirements of these models in terms of the efficient microbiological hydroxylation of steroids, we have examined the effect of moving one binding site away from the steroid skeleton whilst maintaining the distance between the binding groups. In this paper we report on the biotransformation by the fungus *Mucor plumbeus* of 4-formylandrosta-4,6-dien-17-one **1**, 4-formyl-17β-hydroxyandrosta-4,6-diene **2** and 17β-hydroxy-4-hydroxymethylandrosta-4,6-diene **3**, in which the C-4 substituent mimics the C-3 functional group. In particular, the distance between a C-4 formyl oxygen atom and C-17 is the same as that between a C-3 carbonyl oxygen and C-17.

The C-4 formyl substituent was introduced by the Vilsmeier-Haack formylation[4] of 17β-acetoxyandrosta-4, 6-diene **4**[5] with N-formylmorpholine and phosphorus oxychloride.[6] The presence of alkene doublet resonances [δ_H 5.96 (H-6) and 7.08 (H-7); $J_{6:7}$ 10.2; $J_{7:8}$ 2.1 Hz] in the ^{1}H NMR spectrum of the product **5** showed that the formyl group was at C-4 rather than at C-7. Formylation had taken place on the more highly substituted double bond. Hydrolysis of 5 with aqueous methanolic potassium carbonate gave the 17β-alcohol **2**. In order to avoid further oxidation of the formyl group, the 17β-alcohol was oxidised using the Swern conditions (oxalyl chloride and dimethylsulfoxide)[7] to give 4-formylandrosta-4, 6-dien-17-one **1**. Reduction of the 4-formyl group of **2** with sodium borohydride gave 17β-hydroxy-4-hydroxymethylandrosta-4,6-diene **3**. An alternative attempt to synthesise 4-formylandrosta-4,6-dien-17-one **1** involved a Vilsmeier- Haack formylation of androsta-4,6-dien-17-one **6**. This was obtained by the Swern oxidation of 17β-hydroxyandrosta-4, 6-diene. However, the product was 17-chloro-4,16-diformylandrosta-4,6,16-triene **7** [δ_H 5.47 (H-6), 7.13(H-7), 9.99 (16-CHO), 10.34(4-CHO)]. Nevertheless, this compound gave crystals which were suitable for X-ray crystallography (see Fig. 1). The distance between the 4-formyl oxygen and C-17 was 9.91Å compared to a typical distance of 10.05Å between a C-3 carbonyl oxygen and C-17.



1 R^1 = CHO, R^2 = =O
2 R^1 = CHO, R^2 = α-H, β-OH
3 R^1 = CH_2OH, R^2 = α-H, β-OH
4 R^1 = H, R^2 = α-H, β-OAc
5 R^1 = CHO, R^2 = α-H, β-OAc
6 R^1 = H, R^2 = =O

7

Fig. 1 Caption needed

Incubation of 4-formylandrosta-4,6-dien-17-one **1** with *Mucor plumbeus* for 7 days on shake culture gave a mixture of the alcohol **2** (13% yield) and the diol 3 (33% yield) whilst 4-formylandrosta-4,6-diene-17β-ol **2** gave a poor yield (17.5%) of the diol **3**. The latter did not undergo any further biotransformation by *M.plumbeus.*

These results show that these steroids still behave as substrates for the redox enzymes within the organism. However there were no microbiological hydroxylations. Although the correct distance between the binding groups has been maintained, their geometrical relationship on the carbon skeleton does not place a carbon atom at a suitable site for hydroxylation. This suggests that the structural conditions for ring A fitting the steroid hydroxylase in this organism may differ from those of the redox enzymes.

Experimental

Silica for chromatography was Merck 9385. Petrol refers to the fraction, b-p. 60–80°C. Extracts were dried over sodium sulfate. ^{1}H and ^{13}C NMR spectra were determined at 300 and 75 MHz respectively for solutions in deuteriochloroform. The ^{13}C NMR data are given in Table 1. IR spectra were determined as nujol mulls.

Preparation of 17β-acetoxy-4-formylandrosta-4,6-diene **5**: The Vilsmeier reagent was prepared by the slow addition of N-formylmorpholine (4 cm^3) to a solution of phosphorus oxychloride (3 cm^3) in 1,2-dichloroethane (6 cm^3) below 5°C. After 10 min a solution of 17β-acetoxyandrosta-4,6-diene[5] (700 mg) in dry dichloromethane was added to the Vilsmeier reagent at 0°C and the mixture was then heated at 50–55°C for 3 days. The mixture was neutralised with aqueous sodium hydrogen carbonate. The organic phase was separated, washed with water and dried. The solvent was evaporated and the

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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 determined in deuteriochloroform

Carbon atom	Compound 1	2	3	5	6	7
1	35.7	36.5	30.2	34.1	34.4	32.8
2	21.3	22.8	20.7	22.8	22.9	22.8
3	22.8	22.9	22.9	23.0	25.2	28.0
4	131.4	130.9	129.1	131.1	125.6	131.4
5	157.3	158.1	137.6	158.1	142.1	157.0
6	121.5	120.7	122.7	120.9	124.6	121.6
7	134.7	136.6	131.4	136.3	129.8	136.2
8	36.8	37.4	34.8	37.1	36.4	36.8
9	48.9	48.4	48.9	48.2	49.5	51.2
10	34.1	34.1	36.8	36.5	35.7	33.8
11	17.1	17.2	18.0	17.2	18.2	17.1
12	36.6	36.6	34.6	36.7	34.7	35.8
13	48.1	43.6	43.5	43.3	48.4	51.1
14	51.1	51.2	51.4	51.1	51.5	51.4
15	20.4	20.7	18.2	20.6	20.2	20.6
16	31.4	30.4	28.2	27.5	30.6	134.6
17	219.8	81.3	80.8	82.2	220.7	161.9
18	13.8	11.1	11.1	12.0	13.6	15.2
19	18.2	18.2	15.1	18.2	18.3	18.1
4-c	190.9	191.0	61.4	191.0		190.7
16-C						187.8
OAC				171.2		
				21.2		

residue chromatographed on silica. Elution with 5% ethyl acetate:light petroleum gave 17β-acetoxy-4-formylandrosta-4,6-diene 5 (450 mg) as a foam which resisted crystallisation, (Found: C,69.4; H, 8.2 $C_{22}H_{30}O_3$ requires C, 69.7; H, 8.2%),υ_{max}/cm^{-1} 1736,1665,1629,1586;δ_H 0.89(3H,s, H-18), l.02(3H,s,H-19), 0.90–2.30 (17H, overlapping multiplets), 2.06 (3H,s,OAc), 4.60 (lH,t,*J* 8.4 Hz,H-17), 5.96(1H,d, *J* 10.2 Hz, H-6), 7.08(lH,dd, *J* 10.2 and 2.1 Hz, H-7), 10.34 (lH,s,CHO). Under similar conditions androsta-4,6-dien-17-one **6** (1 g) gave 17-chloro-4,16-diformylandrosta-4,6,16-triene **7** (700 mg) which crystallised from acetone: light petroleum as needles, m.p. 157–162°C, (Found: C,70.8; H,7.2. M^+ 344.155 $C_{21}H_{25}C10_2$ requires C,71.3: H, 7.4%, M^+ 344.154), υ_{max}/cm^{-1} 1673, 1614, 1582, 1570; δ_H 1.06 and 1.07(each 3H,s,H-18 and H-19), 0.85–2.60 (15H overlapping multiplets), 5.97 (lH,d, *J* 10.7 Hz, H-6), 7.13(1H,dd, *J* 10.2 and 2.6 Hz, H-7), 9.99 (lH,s,16-CHO) 10.34 (4-CHO).

Hydrolysis of 17β-acetoxy-4-formylandrosta-4,6-diene **5**: The steroid 5 (1 g) in methanol (60 cm^3) was treated with aqueous potassium carbonate (2.5 g) in water (20 cm^3) at room temperature for 3 h. Acetic acid (3 cm^3) was added and the solution was concentrated *in vacuo*. The product was extracted with chloroform. The extract was washed with water, brine and dried. The solvent was evaporated and the residue chromatographed on silica. Elution with 10% ethyl acetate:light petroleum gave 4-formyl-17β-hydroxy-4-androsta-4,6-diene **2** (850 mg) as needles, m.p. 165–167°C (Found: C, 79.7; H, 9.5. $C_{20}H_{28}O_2$ requires C, 80.0; H,9.4%), υ_{max}/cm^{-1} 3484,1691,1644; δ_H 0.84(3H,s, H-18), 1.05(3H,s,H-19), 0.80-2.50 (18H, overlapping multiplets), 3.65 (lH,t, *J* 8.2 Hz, H-17), 5.96(1H,d, *J* 10.2 Hz, H-6), 7.06 (lH,dd, *J* 10.2 and 2.6 Hz, H-7), 10.33 (lH,s, CHO).

Preparation of 17β-hydroxy-4-hydroxymethylandrosta-4,6-diene **3**: 4-Formyl-17β-hydroxyandrosta-4,6-diene 2 (750 mg) in tetrahydrofuran (100 cm^3) and methanol (2 cm^3) was treated with sodium borohydride (750 mg) for 3 h at room temperature. Acetic acid (2 cm^3) and water (5 cm^3) were added and the solvents were evaporated *in vacuo*. The products were recovered in ethyl acetate and chromatographed on silica. Elution with 10% ethyl acetate:light petroleum gave 17β-hydroxy-4-hydroxymethylandrosta-4,6-diene 3 (680 mg) which crystallised as needles, m.p. 190–193°C (Found: C, 78.8; H, 10.1. $C_{20}H_{30}O_2$ requires C,79.4: H,10.0%), υ_{max}/cm^{-1} 3270, 1665; δ_H 0.82 (3H,s,H-18), 0.95(3H,s, H-19), 0.80–2.10 (19H, overlapping multiplets), 3.63(1H,t, *J*, 8.2 Hz, H-17), 4.07 and 4.24 (each lH,d, *J* 11.2 Hz, 4-CH_2OH), 5.59 (lH,d, *J* 10.1 Hz, H-6), 6.38 (lH,dd, *J* 10.1 and 2.4 Hz, H-7).

Preparation of 4-formylandrosta-4,6-dien-17-one **1**: Dimethylsulfoxide (0.38 cm^3) in dichloromethane (1 cm^3) was added to a stirred solution of oxalyl chloride (0.23 cm^3) in dichloromethane (10 cm^3) at between –50 and –60°C. The mixture was stirred for 5 min and then 17β-hydroxy-4-formylandrosta-4,6-diene 2 (600 mg) in dichloromethane (40 cm^3) was added over 10 min. The mixture was stirred for a further 30 min. The solution was allowed to warm to room temperature and water was added. The organic layer was separated, washed with water and dried. The solvent was evaporated and the residue was chromatographed on silica. Elution with 10% ethyl acetate:light petroleum gave 4-formylandrosta-4,6-dien-17-one **1** (370 mg) as needles, m.p. 133–134°C, (Found: C,80.3: H,8.8 $C_{20}H_{26}O_2$ requires C,80.5; H, 8.8%), υ_{max}/cm^{-1} 1732, 1655, 1615, 1576: δ_H 0.98(3H,s,H-18), 1.08(3H,s,H-19), 0.80-2.50 (17H, overlapping multiplets), 6.05(1H,d, *J* 10.2 Hz, H-6), 7.14 (lH,dd, *J* 10.2 and 2.6 Hz, H-7) and 10.35.(1H,s, CHO). Under similar conditions 17β-hydroxyandrosta-4,6-diene (800 mg) gave androsta-4,6-dien-17-one **6** (720 mg) as needles, m.p. 92–95°C, (Found: C,84.0; H, 9.7. $C_{l9}H_{26}O$ requires C,84.4; H,9.7%,υ_{max}/cm^{-1} 1746, 1645, 1620; δ_H 0.93 and 0.95 (each 3H,s, H-18 and H-19), 0.80–2.50 (17H overlapping multiplets), 5.43 (lH,t, *J* 3.8 Hz, H-4), 5.51(1H,d, *J* 9.8 Hz, H-6), 5.94 (lH,dd, *J* 9.8 and 2.6 Hz).

Biotransformations with Mucor plumbeus: The fungus was grown on shake culture in 250 cm^3 conical flasks (100 cm^3 medium per flask) on a medium comprising (per litre) glucose (15 g), potasssium dihydrogen phosphate (1 g), magnesium sulfate (1 g), ammonium tartrate (1 g), yeast extract (0.5 g), calcium chloride (0.25 g), sodium chloride 0.5 g), ferrous ammonium sulfate (0.1 g) and a trace elements solution (1 cm^3). The trace elements solution comprised (per litre), zinc sulfate (1.6 g), ferrous sulfate (1.0 g), cobalt nitrate (1.0 g), ammonium molybdate (1.0 g) copper sulfate (0.1 g) and manganese sulfate (0.1 g). The substrates dissolved in ethanol (12 cm^3), were added to 25 flasks after 2 days and the fermentations were harvested after a further 7 days. The metabolites were extracted with ethyl acetate, the extracts were dried and the solvent evaporated to give a residue which was chromatographed. The metabolites were identified by their ^1H NMR spectra. 4-Formylandrosta-4,6-dien-17-one **1** (300 mg) gave the starting material (60 mg), 4-formyl-17β-hydroxyandrosta-4,6-diene **2** (40 mg) and 17β-hydroxy-4-hydroxymethylandrosta-4,6-diene **3** (100 mg).

4-Formyl-17β-hydroxyandrosta-4,6-diene 2 (400 mg) gave the starting material (200 mg) and 17β-hydroxy-4-hydroxymethylandrosta-4,6-diene **3** (70 mg).

17β-Hydroxy-4-hydroxymethylandrosta-4,6-diene **3** (400 mg) gave only the starting material (300 mg).

Crystal data and structure determination: Compound **7** $C_{21}H_{25}C10_2$, M_r 344.86, monoclinic, space group $P2_1$ (No. 4), a = 7.2757(2), b = 17.4590(4), c = 14.0143(3)Å, $\alpha = \gamma = 90°$, β = 100.868(1)°, V = 1748.26(7)Å^3, Z = 4, D_C = 1.31g cm^3, μ = 0.23mm^{-1}, F(000) = 736, λ = 0.71073Å. Data were collected on a crystal of size 0.3 × 0.3 × 0.2 mm on a KappaCCD diffractometer. A total of 11074 reflections were collected for $3.70 < \theta < 25.66°$ and $-6 <= h <= 8$, $-21 <= k <= 19$, $-16 <= l <= 14$. There were 5512 independent reflections and 5226 reflections with $I > 2\sigma(I)$ that were used in the refinement. No absorption correction was applied. The structure was solved using direct methods and refined using SHELXL-97 with full matriz least-squares on F^2. The final R indices were $[I>2\sigma(I)]$ R_1 = 0.043, wR_2 = 0.111, and (all data) R_1 = 0.047, wR_2 = 0.114. The goodness-of-fit on F^2 was 1.073 and the largest difference peak and hole was 0.16 and –0.19 e$Å^{-3}$. The data will be deposited at the Cambridge Crystallographic Data Centre.

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