



The microbiological hydroxylation of 4β -hydroxy- 4α -methyl- 5α -androstanes by *Cephalosporium aphidicola*

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Abstract

Some 4β -hydroxy- 4α -methyl- 5α -androstanes were synthesized and their microbiological hydroxylation by *Cephalosporium aphidicola* was examined in order to explore the possibility of a biosynthetically patterned methylcarbinol: vicinal glycol biotransformation; however the substrates were hydroxylated mainly at C-7. © 1998 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Microbiological hydroxylation; *Cephalosporium aphidicola*; Steroids; 4β -hydroxy- 4α -methyl- 5α -androstanes

1. Introduction

The biosynthesis of the diterpenoid aphidicolin (**1**) by the fungus, *Cephalosporium aphidicola*, involves the efficient hydroxylation of the equatorial 17-methyl of $3\alpha,16\beta,18$ -trihydroxyaphidicolane to afford the $16\beta,17$ -glycol of aphidicolin [1]. The transformation of a methylcarbinol to a vicinal glycol exemplified by this step, is difficult to achieve regiospecifically by chemical means. Consequently we have explored [2] the possibility of using this organism for a biosynthetically-directed biotransformation to achieve this reaction using steroidal methylcarbinols as test substrates. $4\beta,17\beta$ -Dihydroxy- 4α -methyl- 5α -androstane (**2**) and $4\beta,17\beta$ -dihydroxy- $4\alpha,17\alpha$ -dimethyl- 5α -androstane (**3**) were potential substrates since not only is there a formal similarity between these steroids binding in both their normal and reverse modes when compared to aphidicolin but also the methyl groups at C-4 are equatorial substituents.

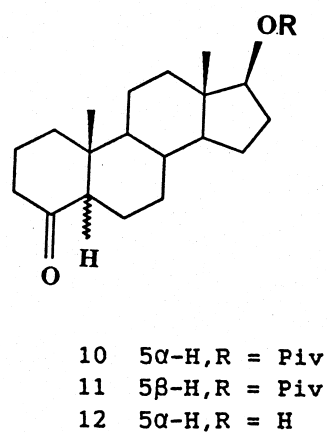
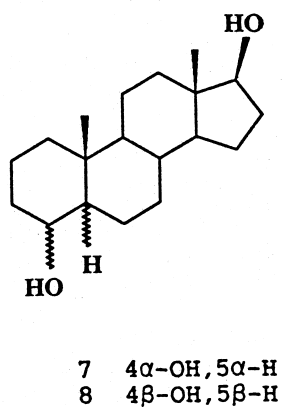
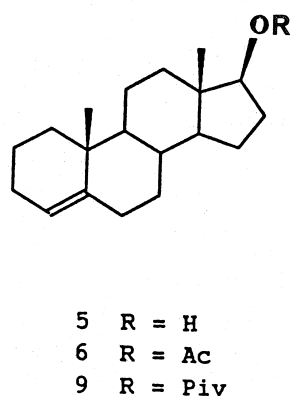
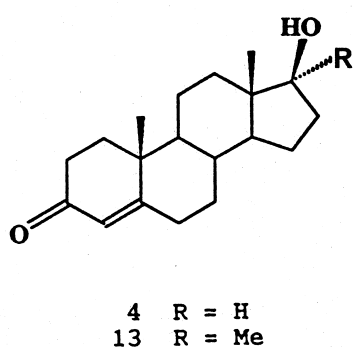
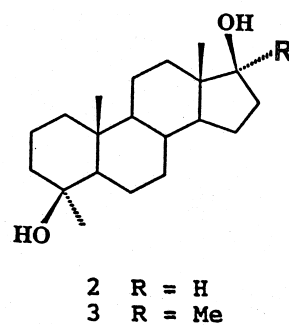
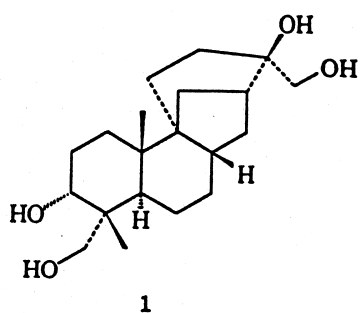
2. Results and discussion

The substrates (**2**) and (**3**) were prepared from testosterone (**4**) and 17α -methyltestosterone (**13**). The ad-

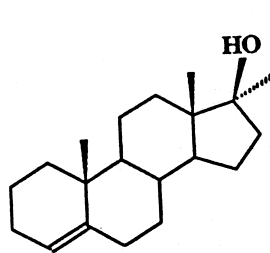
dition of methylmagnesium iodide to steroidal ketones shows a marked preference for the formation of the tertiary alcohol with an equatorial methyl group [3] although this has not been examined previously with steroidal 4-ketones as substrates. The steroidal 4-ketones were prepared via the 4-enes. 17β -Hydroxyandrost-4-ene (**5**) [4], free from double bond isomers, has become readily available from testosterone (**4**) by using a selective reduction with sodium borohydride in a mixture of trifluoroacetic acid, acetic acid, acetonitrile and dichloromethane [5].

Hydroboration of 17β -acetoxyandrost-4-ene (**6**) [6] and oxidation of the borane with alkaline hydrogen peroxide gave a mixture of $4\alpha,17\beta$ -dihydroxy- 5α -androstane (**7**) and $4\beta,17\beta$ -dihydroxy- 5β -androstane (**8**). However the protecting acetoxy group at C-17 had been hydrolysed. Oxidation of the borane with chromium trioxide [7] to avoid the alkaline conditions and form the 4-ketone directly was not successful. Attempts to circumvent the hydrolysis of the 17β -acetate by oxidizing the borane with sodium perborate [8] were also unsuccessful. Hence the 17β -hydroxyl group was protected as its sterically more hindered 17β -pivaloate (**9**). The mixture of 17β -pivaloyloxy- 4α - and 4β -alcohols obtained from the hydroboration, was oxidized with chromium trioxide to a mixture of 17β -pivaloyloxy- 5α - and 5β -androstane-4-ones, (**10**) and (**11**), without further purification. This mixture was then epimerized at C-5 to afford the more

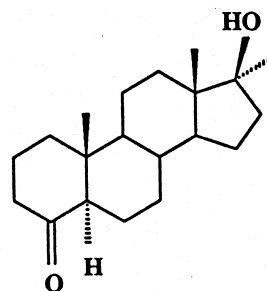
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Table 1. Incubation of steroids with *C. aphidicola*

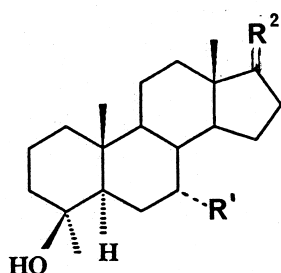
Substrate	Product	% Yield
4 β ,17 β -Dihydroxy-4 α -methyl-5 α -androstane (2)	starting material	20
	4 β -hydroxy-4 α -methyl-5 α -androstane-17-one (16)	37
	4 β ,7 α -dihydroxy-4 α -methyl-5 α -androstane-17-one (17)	10
	4 β ,15 α ,17 β -trihydroxy-4 α -methyl-5 α -androstane (19)	7
4 β ,17 β -Dihydroxy-4 α ,17 α -dimethyl-5 α -androstane (3)	starting material	16
	4 β ,7 α ,17 β -trihydroxy-4 α ,17 α -dimethyl-5 α -androstane (18)	15



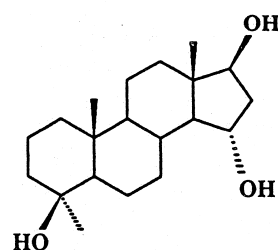
14



15



- 16 $R^1 = H, R^2 = O$
 17 $R^1 = OH, R^2 = O$
 18 $R^1 = OH, R^2 = \alpha\text{-Me}, \beta\text{-OH}$



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Table 2. ^{13}C NMR spectra of 4β -hydroxy- 4α -methylandrostanes (determined in CDCl_3 at 75 MHz)

Carbon	Compound					
	2	3	16	17	18	19
1	38.74	38.81	38.69	38.47	38.45	39.37
2	18.11	18.16	18.07	18.03	17.93	18.56
3	40.91	40.97	40.97	41.20	40.82	41.43
4	72.17	72.20	72.12	72.13	72.03	71.32
5	53.84	53.93	53.79	45.94	45.81	54.27
6	20.05	20.15	20.33	29.23	28.75	20.31
7	31.94	32.13	31.22	66.90	67.14	33.18
8	35.08	35.96	34.62	38.73	39.88	35.34
9	55.84	55.82	55.80	47.53	47.21	56.16
10	36.67	36.72	36.77	36.95	36.70	37.07
11	20.42	20.51	19.76	19.55	19.75	21.03
12	36.71	39.05	36.71	35.76	38.45	37.58
13	42.82	45.42	47.68	47.46	45.20	44.63
14	51.23	50.93	51.65	45.94	44.55	59.30
15	23.31	23.20	21.71	21.31	22.45	72.14
16	30.53	31.66	35.85	31.13	31.06	43.14
17	81.94	81.72	221.45	221.10	81.47	78.67
18	11.11	14.10	13.80	13.48	13.53	13.16
19	14.05	13.98	14.02	12.95	12.78	14.41
4-Me	30.83	30.86	30.87	30.78	30.24	31.05
17-Me		25.78			25.43	

stable 5α -androstan-4-one (**12**) by refluxing with methanolic sodium hydroxide. The C-5 epimers may be readily distinguished by the position of the H-19 resonance in the ^1H NMR spectrum [9]. The pivaloyloxy group was hydrolysed at the same time. Treatment of the ketone with methylmagnesium iodide in ether gave $4\beta,17\beta$ -dihydroxy- 4α -methyl- 5α -androstan-4-one (**2**). We were unable to detect any of the 4β -methyl isomer.

A similar sequence was applied to 17α -methyltestosterone (**13**) except that there was no need to protect the tertiary 17β -hydroxyl group. Thus the ring A unsaturated ketone was reduced to the 4-ene (**14**) with sodium borohydride in a mixture of trifluoroacetic acid, acetic acid, acetonitrile and dichloromethane and thence via hydroboration, oxidation and isomerization, converted to the 4-ketone (**15**). Reaction with methylmagnesium iodide gave $4\beta,17\beta$ -dihydroxy- $4\alpha,17\alpha$ -dimethyl- 5α -androstan-4-one (**3**).

The stereochemistry at C-4 followed from an examination of the ^1H NMR spectra of the 4β -hydroxy- 4α -methyl- 5α -androstanes. Whilst there was no n.O.e. enhancement of the C-4 methyl group signal on irradiation of the C-10 methyl group signal, the trans-

annular 1:3-diaxial interaction with a 4 β -hydroxyl group produced a significant downfield shift ($\Delta\delta$ 0.24 ppm) in the position of the C-10 methyl group signal when compared to 17 β -hydroxy-5 α -androstane [9].

The substrates were incubated with *C. aphidicola* for 8 days and the metabolites were then isolated and separated by chromatography. The results are tabulated (see Table 1). The metabolites were identified from their ^1H and ^{13}C NMR spectra (see Table 2).

The major metabolite from the incubation of 4 β ,17 β -dihydroxy-4 α -methyl-5 α -androstane (**2**) was the 17-ketone (**16**). The H-17 α signal at δ_{H} 3.61 had disappeared from the ^1H NMR spectrum whilst the ^{13}C NMR spectrum (see Table 2) showed a new carbonyl resonance at δ_{C} 221.45 ppm. The second metabolite to be isolated from the column, was assigned the structure of 4 β ,7 α -dihydroxy-4 α -methyl-5 α -androstane-17-one (**17**). The H-17 α signal had disappeared and a carbonyl signal had appeared at δ_{C} 221.1 ppm. The ^{13}C NMR spectrum also contained a CH(OH) resonance at δ_{C} 66.70 ppm in place of a methylene carbon. Compared to the starting material, the signals assigned to C-6 and C-8 had moved downfield ($\Delta\delta$ 8.81 and 3.65 ppm respectively) whilst there were γ -gauche upfield shifts for the signals assigned to C-5, C-9 and C-14 ($\Delta\delta$ 7.90, 8.31 and 5.29 ppm respectively). Consequently the new alcohol was located at C-7. The ^1H NMR spectrum showed a CH(OH) signal at δ_{H} 4.09 (doublet, J 2.5 Hz) resembling that of a 7 α -alcohol [10]. The third metabolite to be isolated was assigned the structure of 4 β ,15 α ,17 β -trihydroxy-4 α -methyl-5 α -androstane (**19**). Compared to the starting material, the ^{13}C NMR spectrum revealed downfield shifts for the signals assigned to C-14 and C-16 ($\Delta\delta$ 8.07 and 12.61 ppm, respectively) whilst there was a γ -gauche upfield shift for the signal assigned to C-17 ($\Delta\delta$ 3.27 ppm). The ^1H NMR spectrum showed a new signal at δ_{H} 4.27 ppm as a triplet (J , 9 Hz) of doublets (J , 3 Hz) consistent with the presence of a 15 α -alcohol.

Incubation of 4 β ,17 β -dihydroxy-4 α ,17 α -dimethyl-5 α -androstane (**3**) gave the corresponding 7 α -alcohol (**18**). The presence of the 7 α -alcohol was established by the downfield shifts of the ^{13}C NMR resonances of C-6 and C-8 ($\Delta\delta$ 8.6 and 3.9 ppm, respectively) and the γ -gauche shieldings for C-5, C-9 and C-14 ($\Delta\delta$ 8.12, 8.61 and 6.38 ppm, respectively) when compared to the starting material. The H-7 β proton resonance (δ_{H} 3.95 ppm) showed a comparable multiplicity to other 7 α -alcohols [10].

In conclusion the transformations which we have observed with these substrates have followed the typical pattern of steroidal hydroxylations by this organism [11,12] rather than a biosynthetically patterned pathway. It may be that the systems responsible for steroidal transformations are more readily accessible to the exogenous substrates and are present throughout

the fermentation whilst those involved in aphidicolin biosynthesis may only be present for a limited period, for example during the idiophase, of the fermentation.

3. Experimental

^1H NMR spectra were recorded in deuteriochloroform at 300 or 500 MHz. ^{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 sodium sulfate. Jones reagent refers to a solution of chromium trioxide (26.72 g) in conc. sulfuric acid (23 cm³) diluted to 100 cm³ with water. *Cephalosporium aphidicola* was cultured as described previously [13].

3.1. 17 β -Pivaloyloxyandrost-4-en-3-one

Testosterone (**3**) (5 g) in dry pyridine (50 cm³) was treated with pivaloyl chloride (5 g) at room temp. overnight. The mixture was poured into dil. HCl (200 cm³) and the steroid was recovered in EtOAc. The extract was washed with dil. HCl, aq. NaHCO₃, H₂O and dried. The solvent was evap. to give 17 β -pivaloyloxy-androst-4-en-3-one (4.53 g) which crystallized from petrol as prisms, m.p. 155–157° (found: C, 77.6; H, 10.1; C₂₄H₃₆O₃ requires C, 77.4; H, 9.7%), IR, ν_{max} 1722, 1679, 1617 cm⁻¹; ^1H NMR δ_{H} 0.85 (3H, s, H-18), 1.20 (9H, s OPiv), 1.25 (3H, s, H-19), 4.57 (1H, t, J 8.5 Hz, H-17), 5.73 (1H, s, H-4).

3.2. 17 β -Pivaloyloxyandrost-4-ene (**9**)

NaBH₄ (1.3 g) was gradually added to a stirred mixture of CF₃CO₂H (8 cm³), HOAc (8 cm³) and CH₃CN (8 cm³) which was cooled in an ice-bath. 17 β -Pivaloyloxyandrost-4-en-3-one (2.4 g) in CH₂Cl₂ (40 cm³) was added to the mixture which was then stirred for 6 h (TLC control). Sat. aq. NaHCO₃ was added to neutralize the mixture. The steroid was extracted with CH₂Cl₂. The extract was thoroughly washed with H₂O and dried. The solvent was evap. to give 17 β -pivaloyloxyandrost-4-ene (**9**)-(1.84 g) which crystallized from MeOH as needles, m.p. 114–116° (found: C, 80.4; H, 11.1; C₂₄H₃₈O₂ requires C, 80.4; H, 10.7%), IR, ν_{max} 1733 cm⁻¹; ^1H NMR δ_{H} 0.82 (3H, s, H-18), 1.02 (3H, s, H-19), 1.19 (9H, s, OPiv), 4.57 (1H, t, J 8.5 Hz, H-17), 5.27 (1H, br.s. H-4).

3.3. Preparation of 17 β -hydroxy-5 α -androstane-4-one (**12**)

17 β -Pivaloyloxyandrost-4-ene (**3** g) in THF (100 cm³) was treated with 1 M borane in THF

(20 cm³) at 0° for 4 h under N₂. H₂O (10 cm³) was added dropwise at 0° followed by 10% aq. NaOH (30 cm³) and 30% aq. H₂O₂ (30 cm³). The mixture was stirred for 1 h. Na₂SO₃ (6 g) was then added followed by HOAc (3 cm³), H₂O (150 cm³), dil. HCl (150 cm³) and EtOAc (150 cm³). The mixture was stirred for a further 15 min and then the organic layer was separated, washed with H₂O and dried. The solvent was evap. to give a residue which was taken up in Me₂CO (75 cm³) and treated with the Jones reagent (3 cm³) dropwise. The mixture was stirred for 45 min and then MeOH was added until the soln. remained a green colour. After 30 min the solvents were evap. and the residue was suspended in H₂O. The steroids were extracted with EtOAc. The extract was washed with aq. NaHCO₃ and H₂O. The solvent was evap. to give a gum (1.5 g) which was dissolved in MeOH (50 cm³). This soln. was treated with NaOH (8 g) in H₂O (20 cm³) under N₂ and heated under reflux for 1 h. The soln. was cooled and neutralized with dil. HCl. The MeOH was evap. and the steroids were recovered in EtOAc. The extract was washed with aq. NaHCO₃, H₂O and dried. The solvent was evap. to give 17 β -hydroxy-5 α -androstan-4-one (1 g) which was recrystallized from petrol as needles, m.p. 121° (lit. [14], 125°), IR $\nu_{\max}$ 3526, 1712 cm⁻¹; ¹H NMR δ_{H} 0.74 and 0.75 (each 3H, s, H-18 and H-19), 3.61 (1H, t, J 8.5 Hz, H-17).

3.4. 4 β ,17 β -Dihydroxy-4 α -methyl-5 α -androstan-4-one (2)

17 β -Hydroxy-5 α -androstan-4-one (1 g) in dry THF (30 cm³) was treated with 3 M methylmagnesium iodide in Et₂O (8 cm³) under N₂ at room temp. overnight. 10% Aq. NH₄Cl (100 cm³) was added and the mixture was left to stand for 1 h. The steroid was extracted with EtOAc and the extract was washed with brine and dried. The solvent was evap. to give 4 β ,17 β -dihydroxy-4 α -methyl-5 α -androstan-4-one (2) (0.7 g) which was recrystallized from MeOH as needles, m.p. 186–189° (found: C, 78.4; H, 11.4. C₂₀H₃₄O₂ requires C, 78.4; H, 11.7%); IR, $\nu_{\max}$ 3383 cm⁻¹. ¹H NMR δ_{H} 0.73 (3H, s, H-18), 1.03 (3H, s, H-19), 1.15 (3H, s, 4-Me), 3.61 (1H, t, J 8.5 Hz, H-17).

3.5. 17 β -Hydroxy-17 α -methylandrost-4-ene (14)

NaBH₄ (1.3 g) was gradually added to a stirred mixture of CF₃CO₂H (8 cm³), HOAc (8 cm³) and CH₃CN (8 cm³) which was cooled in an ice-bath. 17 β -Hydroxy-17 α -methylandrost-4-en-3-one (2.4 g) in CH₂Cl₂ (40 cm³) was added to the mixture which was then stirred for 4 h. (TLC control). Sat. aq. NaHCO₃ was added to neutralize the mixture. The steroid was extracted with CH₂Cl₂. The extract was thoroughly washed with H₂O and dried. The solvent was evap. to

give 17 β -hydroxy-17 α -methylandrost-4-ene (14) (2 g) which crystallized from petrol as needles, m.p. 98–100° (found: C, 82.8; H, 11.4; C₂₀H₃₂O requires C, 83.3; H, 11.2%), IR, $\nu_{\max}$ 3361 cm⁻¹; ¹H NMR δ_{H} 0.88 (3H, s, H-18), 1.03 (3H, s, H-19), 1.20 (3H, s, H-20), 5.27 (1H, br s, H-4).

3.6. 17 β -Hydroxy-17 α -methyl-5 α -androstan-4-one (15)

17 β -Hydroxy-17 α -methylandrost-4-ene (3 g) in dry THF (100 cm³) was treated with 1 M borane in THF (20 cm³) at 0° for 6 h under N₂. H₂O (10 cm³) was added dropwise at 0° followed by 10% aq. NaOH (30 cm³) and 30% aq. H₂O₂ (30 cm³). The mixture was stirred for 1 h. Na₂SO₃ (6 g) was then added followed by HOAc (3 cm³), H₂O (150 cm³), dil. HCl (150 cm³) and EtOAc (150 cm³). The mixture was stirred for a further 15 min and then the organic layer was separated, washed with H₂O and dried. The solvent was evap. to give a residue which was taken up in Me₂CO (75 cm³) and treated with the Jones reagent (3 cm³) dropwise. The mixture was stirred for 45 min and then MeOH was added until the soln. remained a green colour. After 30 min the solvents were evap. and the residue was suspended in H₂O. The steroids were extracted with EtOAc. The extract was washed with aq. NaHCO₃ and H₂O. The solvent was evap. to give a gum (1.5 g) which was dissolved in MeOH (50 cm³). This soln. was treated with NaOH (8 g) in H₂O (20 cm³) under N₂ and heated under reflux for 1 h. The soln. was cooled and neutralized with dil. HCl. The MeOH was evap. and the steroids were recovered in EtOAc. The extract was washed with aq. NaHCO₃, H₂O and dried. The solvent was evap. to give 17 β -hydroxy-17 α -methyl-5 α -androstan-4-one (1.36 g) which was recrystallized from EtOAc: petrol as needles, m.p. 149–151° (found: C, 77.2; H, 10.5. C₂₀H₃₂O₂ requires C, 76.7; H, 10.5%) IR $\nu_{\max}$ 3344, 1711 cm⁻¹; ¹H NMR δ_{H} 0.76 (3H, s, H-19), 0.85 (3H, s, H-18), 1.22 (3H, s, H-20).

3.7. 4 β ,17 β -Dihydroxy-4 α ,17 α -dimethyl-5 α -androstan-4-one (3)

17 β -Hydroxy-17 α -methyl-5 α -androstan-4-one (0.8 g) in dry THF (30 cm³) was treated with 3 M methylmagnesium iodide in Et₂O (8 cm³) under N₂ at room temp. overnight. 10% Aq. NH₄Cl (100 cm³) was added and the mixture was left to stand for 1 h. The steroid was extracted with EtOAc and the extract was washed with brine and dried. The solvent was evap. to give 4 β ,17 β -dihydroxy-4 α ,17 α -dimethyl-5 α -androstan-4-one (3) (0.3 g) which was recrystallized from MeOH as needles, m.p. 155–157° (found: C, 78.6; H, 11.5. C₂₁H₃₆O₂ requires C, 78.8; H, 11.3%); IR, $\nu_{\max}$

3400 cm^{-1} ; ^1H NMR δ_{H} 0.84 (3H, s, H-18), 1.03 (3H, s, H-19), 1.15 (3H, s, 4-Me), 1.20 (3H, s, H-20).

3.8. Biotransformation of 4 β ,17 β -dihydroxy-4 α -methyl-5 α -androstane (**2**)

The substrate (**2**) (970 mg) 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 10% EtOAc:petrol gave 4 β -hydroxy-4 α -methyl-5 α -androstane-17-one (**16**) (360 mg) which crystallized from EtOAc:petrol as prisms, m.p. 196–198° (found: C, 78.9; H, 10.2. $\text{C}_{20}\text{H}_{32}\text{O}_2$ requires C, 78.9; H, 10.6%); ^1H NMR δ_{H} 0.86 (3H, s, H-18), 1.05 (3H, s, H-19), 1.17 (3H, s, Me-4). Elution with 13% EtOAc:petrol gave the starting material (190 mg). Elution with 45% EtOAc:petrol gave 4 β ,7 α -dihydroxy-4 α -methyl-5 α -androstane-17-one (**17**) (93 mg) which crystallized from EtOAc:petrol as prisms, m.p. 203–205° (found: C, 74.9; H, 10.1. $\text{C}_{20}\text{H}_{32}\text{O}_3$ requires C, 75.0; H, 10.1%); ^1H NMR δ_{H} 0.87 (3H, s, H-18), 1.07 (3H, s, H-19), 1.21 (3H, s, Me-4), 4.09 (1H, d, J 2.5 Hz, H-7). Further elution gave 4 β ,15 α ,17 β -trihydroxy-4 α -methyl-5 α -androstane (**19**) (72 mg) which crystallized from EtOAc:petrol as prisms, m.p. 263–265° (found: C, 74.2; H, 10.9. $\text{C}_{20}\text{H}_{34}\text{O}_3$ requires C, 74.5; H, 10.6%); IR, ν_{max} 3422 cm^{-1} ; ^1H NMR δ_{H} ($\text{C}_5\text{D}_5\text{N}$) 0.98 (3H, s, H-19), 1.30 (3H, s, H-18), 1.31 (3H, s, Me-4), 4.10 (1H, t, J 9 Hz, H-17 α), 4.27 (1H, t, d J 9 and 3 Hz, H-15 β).

3.9. Biotransformation of 4 β ,17 β -dihydroxy-4 α ,17 α -dimethyl-5 α -androstane (**3**)

The substrate (**3**) (740 mg) 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*. The fermenta-

tion was continued for a further 8 days. The mycelium was filtered and the broth was extracted with EtOAc. The extract was dried and the solvent evaporated to give a residue which was chromatographed on silica. Elution with 12% EtOAc:petrol gave the starting material (120 mg). Elution with 25% EtOAc: petrol gave 4 β ,7 α ,17 β -trihydroxy-4 α ,17 α -dimethyl-5 α -androstane (**18**) (110 mg) which crystallized from CHCl_3 as prisms, m.p. 168–170° (found: C, 70.8; H, 10.5. $\text{C}_{21}\text{H}_{36}\text{O}_3 \cdot \text{H}_2\text{O}$ requires C, 71.1; H, 10.8%), IR, ν_{max} 3495 cm^{-1} ; δ_{H} 0.84 (3H, s, H-18), 1.04 (3H, s, H-19), 1.15 (3H, s, Me-4), 1.22 (3H, s, H-20), 3.95 (1H, d, J 2 Hz, H-7 β).

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