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Transformation of a series of saturated isomeric steroidal diols by *Aspergillus tamarii KITA* reveals a precise stereochemical requirement for entrance into the lactonization pathway

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ABSTRACT

Four isomers of 5α-androstan-3,17-diol have been transformed by the filamentous fungus *Aspergillus tamarii*, an organism which has the ability to convert progesterone to testololactone in high yield through an endogenous four step enzymatic pathway. The only diol handled within the lactonization pathway was 5α-androstan-3α,17β-diol which, uniquely underwent oxidation of the 17β-alcohol to the 17-ketone prior to its Baeyer–Villiger oxidation and the subsequent production of 3α-hydroxy-17a-oxa-D-homo-5α-androstan-17-one. This demonstrated highly specific stereochemical requirements of the 17β-hydroxysteroid dehydrogenase for oxidation of this specific steroidal diol to occur. In contrast, the other three diols were transformed within the hydroxylation pathway resulting in functionalization at C-11β. Only 5α-androstan-3β,17α-diol could bind to the hydroxylase in multiple binding modes undergoing monohydroxylation in 6β and 7β positions. Evidence from this study has indicated that hydroxylation of saturated steroidal lactones may occur following binding of ring-D in its open form in which an α-alcohol is generated with close spatial parity to the C-17α hydroxyl position. All metabolites were isolated by column chromatography and were identified by ^{1}H, ^{13}C NMR and DEPT analysis and further characterized using infra-red, elemental analysis and accurate mass measurement.

1. Introduction

The fungus *Aspergillus tamarii* contains an endogenous sequential enzymatic pathway (Fig. 1) which converts progesterone (**1**) via Baeyer–Villiger oxidation to testosterone acetate (**2**). This undergoes hydrolysis to form testosterone (**3**) and is then oxidised to androst-4-en-3,17-dione (**4**). A final Baeyer–Villiger oxidation of the C-17 ketone affords testololactone (**5**) in high yield [1]. Divergence from this pathway can occur through testosterone (**3**) which, can be functionalized by a hydroxylase affording 11β-hydroxytestosterone. Progesterone (**1**) can also undergo reversible reduction of the C-20 ketone resulting in (**6**), the equilibrium between the reductase and the oxidase is thought to be dynamic [2]. 20(R)-Reduction products of progesterone analogues have been isolated in a number of studies [2,3].

Steroids differing in side-chain structure [2,3] substituent position [4,5] and ring-D structure [6,7] have been transformed in whole cell cultures of *A. tamarii*. These studies have demonstrated that a wide-range of structurally diverse steroids can be handled by the endogenous pathways within this organism, and have highlighted the powerful effect of single functional groups in directing their metabolic fate (oxidative or reductive) [3,6,7]. To date, no single study has determined the metabolic fate of all four isomers of 5α-androstan-3,17-diol. This is of significance due to the importance of hydrogen bonding in steroid/enzyme interactions [8–16] and in determining if specific stereochemistry is handled within different metabolic pathways. We have also modified a previous [17] synthetic strategy to facilitate an expedient route to 3α-hydroxy containing 17α- and 17β-diols that, as with the other analogues [18–23] have a broad range of interesting biological activity in their own right [24–27].

2. Materials and methods

2.1. Chemicals and reagents

5α-Androstan-3β,17α-diol (**13**) and 5α-androstan-3β,17β-diol (**14**) were synthesized as previously described [17] and were of high purity (99%+ by carbon content) as determined by elemental analysis found: C, 77.39; H, 11.47. $C_{19}H_{32}O_2$ requires C, 78.03; H, 11.03%

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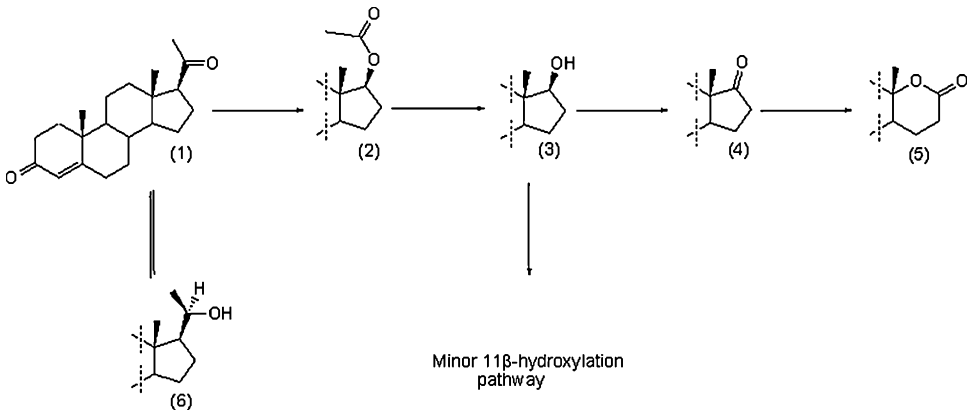

Fig. 1. The endogenous lactonization pathway present in *Aspergillus tamarii KITA*, that converts progesterone (**1**) to testololactone (**5**) with the preceding C-20 keto-alcohol reducto-oxidase interconverting (**1**) and (**6**) and the minor 11β-hydroxylation pathway from testosterone (**3**).

for compound (**13**) and found: C, 77.50; H, 11.54. $C_{19}H_{32}O_2$ requires C, 78.03; H, 11.03% for compound (**14**). 3β-Hydroxy-5α-androstan-17-one was the starting material for the synthetic sequence to compounds (**13**) and (**14**) was purchased from Steraloids Ltd. (UK). From the same supplier 3α-hydroxy-5α-androstan-17-one was obtained and initiated the reaction sequence to 5α-androstan-3α,17α-diol (**11**) and 5α-androstan-3α,17β-diol (**15**) which were also found to be of high purity (99%+ by carbon content) as determined by elemental analysis found: C, 77.75; H, 11.77. $C_{19}H_{32}O_2$ requires C, 78.03; H, 11.03% for compound (**11**) and found: C, 77.76; H, 10.85. $C_{19}H_{32}O_2$ requires C, 78.03; H, 11.03% for (**15**). Solvents were of analytical grade; petroleum ether refers to the fraction with a boiling point of 60–80 °C. Silica for column chromatography was Merck 9385 and TLC was performed with Macherey-Nagel Alugram® SIL G/UV_{254}.

2.2. Formation of hydrazone (*8*)

A solution of 3α-hydroxy-5α-androstan-17-one (**7**) (3 g) in ethanol (30 cm^3) was treated with triethylamine (6 cm^3) and hydrazine hydrate (20 cm^3) and the solution was heated under reflux for 3 h. The solution was poured into cold water to give 3α-hydroxy-5α-androstan-17-hydrazone (**8**) (3.1 g) which crystallized from ethanol as cubes m.p. 239–241 °C found: C, 74.79; H, 10.37; N, 9.03. $C_{19}H_{32}N_2O$ requires C, 74.95; H, 10.59; N, 9.20%. For spectroscopic data please refer to Tables 1–3.

2.3. Formation of vinyl iodide (*9*)

3α-Hydroxy-5α-androstan-17-hydrazone (**8**) (3 g) in dry THF (70 cm^3) and triethylamine (30 cm^3) under nitrogen was treated with iodine (4 g in 12.5 cm^3 THF) at room temperature. After nitrogen evolution had ceased the THF was removed *in vacuo* and the mixture was extracted with ethyl acetate. The extract was washed with dilute hydrochloric acid, water, aqueous sodium hydrogen carbonate, saturated sodium chloride and dried over sodium sulfate and isolated as a gum (3 g), found: 423.115 $C_{19}H_{29}INaO$ requires 423.115. For spectroscopic data please refer to Tables 1–3.

Table 2
^{13}C NMR data for synthesis of the 3α-containing diols determined in $CDCl_3$.

Carbon atom	Compounds					
	7	**8**	**9**	**10**	**11**	**12**
1	31.56	31.48	32.01	32.05	32.32	32.22
2	30.85	29.04	31.48	31.97	29.03	29.02
3	66.37	66.49	66.51	66.58	66.55	66.57
4	35.86	35.83	35.81	35.94	35.91	37.24
5	35.04	39.16	39.31	39.36	39.10	39.12
6	28.25	24.35	28.37	28.56	28.55	28.50
7	29.00	28.39	29.00	29.04	32.26	32.13
8	39.11	34.93	34.57	34.12	35.76	35.33
9	51.50	53.75	54.67	55.17	54.13	54.47
10	35.47	36.28	36.30	45.39	36.40	36.23
11	21.75	20.43	20.63	20.75	24.60	20.40
12	32.14	34.28	36.26	37.16	32.32	38.74
13	47.48	44.07	50.04	45.58	45.53	41.89
14	54.43	54.68	54.79	56.17	48.78	52.14
15	20.04	23.28	33.63	32.09	20.28	35.89
16	35.78	32.16	112.93	129.29	31.49	71.87
17	221.10	163.90	137.48	144.00	80.06	52.04
18	11.18	17.06	15.28	17.08	17.11	18.70
19	13.83	11.18	11.14	11.18	11.20	11.19

Table 3
Significant infra-red absorption signals for compounds **7**–**12**.

Compound	–OH	>C=O	>C=C<
3α-Hydroxy-5α-androstan-17-one (**7**)	3379	1724	
3α-Hydroxy-5α-androstan-17-hydrazone (**8**)	3343		
17-Iodo-5α-androst-16-en-3α-ol (**9**)	3343		1628
5α-Androst-16-en-3α-ol (10)	3276		1588
5α-Androstan-3α,17α-diol (11)	3344		
5α-Androstan-3α,16α-diol (12)	3261		

2.4. Microorganism

A. tamarii KITA (QM 1223) was purchased from the collection at CABI Bioscience (UK). Stock cultures were maintained at 4 °C on potato dextrose agar slopes. Steroid transformation studies were carried out in 3% malt extract medium (Oxoid, UK).

Table 1
^1H NMR data for synthesis of the 3α-containing diols determined in $CDCl_3$.

Compound	3β-H	18-H_3	19-H_3	Other significant signals
3α-Hydroxy-5α-androstan-17-one (**7**)	4.05 (t, J = 2.6 Hz)	0.86	0.80	
3α-Hydroxy-5α-androstan-17-hydrazone (**8**)	4.04 (t, J = 2.6 Hz)	0.84	0.80	4.73 (2H, brs, $N\underline{H}_2$)
17-Iodo-5α-androst-16-en-3α-ol (**9**)	4.05 (t, J = 2.6 Hz)	0.72	0.80	6.11 (1H, dd, J = 2 Hz, J = 3.5 Hz, 16-H)
5α-Androst-16-en-3α-ol (10)	4.04 (t, J = 2.6 Hz)	0.75	0.81	5.69 (1H, brs, 17-H) 5.83 (1H, dd, J = 1.4 Hz, J = 4.3 Hz, 16-H)
5α-Androstan-3α,17α-diol (11)	4.04 (t, J = 2.6 Hz)	0.65	0.79	3.71 (1H, d, J = 6 Hz, 17β-H)
5α-Androstan-3α,16α-diol (12)	4.04 (t, J = 2.6 Hz)	0.70	0.77	4.44 (1H, m, ω/2 = 10 Hz, J = 6.5 Hz, 16β-H)

Table 4
^{1}H NMR data for steroidal starting material (except compound **11**, see Table 1) and transformation products determined in $CDCl_3$.

Reference data	3-H	18-H_3	19-H_3	Other significant signals
5α-Androstan-3β,17α-diol (13)	3.60 (1H, tt, *J* = 5 Hz, *J* = 10 Hz, 3α-H)	0.65	0.82	3.73 (1H, d, *J* = 5 Hz, 17β-H)
5α-Androstan-3β,17β-diol (14)		0.73	0.81	3.60 (2H, m, ω/2 = 16 Hz, 3α-H, 17α-H)
5α-Androstan-3α,17β-diol (15)	4.04 (1H, t, *J* = 2.6 Hz, 3β-H)	0.73	0.79	3.63 (1H, t, *J* = 8 Hz, 17α-H)
Transformation products				
5α-Androstan-3α,11β,17α-triol (16)	4.05 (1H, t, *J* = 2.3 Hz, 3β-H)	0.90	1.00	3.70 (1H, d, *J* = 6 Hz, 17β-H) 4.61 (1H, s, 11α-H)
5α-Androstan-3β,6β,17α-triol (17)	3.60 (1H, tt, *J* = 5 Hz, *J* = 10 Hz, 3α-H)	0.89	1.26	3.70 (1H, d, *J* = 6 Hz, 17β-H) 4.60 (1H, s, 6α-H)
5α-Androstan-3β,7β,17α-triol (18)	3.60 (1H, tt, *J* = 5 Hz, *J* = 10 Hz, 3α-H)	0.68	0.85	3.40 (1H, td, *J* = 5 Hz, *J* = 10 Hz, 7α-H) 3.70 (1H, d, *J* = 6 Hz, 17β-H)
5α-Androstan-3β,11β,17α-triol (19)	3.60 (1H, tt, *J* = 5 Hz, *J* = 10 Hz, 3α-H)	0.89	1.00	3.70 (1H, d, *J* = 6 Hz, 17β-H) 4.61 (1H, s, 11α-H)
5α-Androstan-3β,11β,17β-triol (20)		0.98	1.05	3.59 (2H, m, ω/2 = 16 Hz, 3α-H, 17α-H) 4.33 (1H, s, 11α-H)
3α-Hydroxy-17a-oxa-D-homo-5α-androstan-17-one (21)	4.06 (1H, t, *J* = 2.6 Hz, 3β-H)	0.75	1.30	

2.5. Conditions of cultivation and transformation

Spores were transferred aseptically in a category 2 biological safety cabinet into 500 ml Erlenmeyer flasks containing 300 ml of sterile media and were incubated for 72 h at 30 °C. The cultures were shaken at 180 rpm on an orbital shaker. Aliquots (5 ml) from the seed flask were transferred aseptically to 10 flasks and grown for a further 72 h as above, at the end of which the fungus is in log phase growth. After this time period steroid dissolved in dimethylformamide (DMF) was evenly distributed between the flasks (1 mg/ml) under sterile conditions and incubated for a further 5 days after which, the metabolites were extracted from the broth.

2.6. Extraction and identification of metabolites

The fungal mycelium was separated from the broth by filtration under vacuum. Following completion the mycelium was rinsed with ethyl acetate (0.5 L) to ensure the entire available steroid was removed. The mycelial broth was then extracted thrice with ethyl acetate (1.5 L). The organic extract was dried over sodium sulfate and the solvent evaporated *in vacuo* to give a gum. This gum was adsorbed onto silica and chromatographed on a column of silica; the steroidal metabolites were eluted with increasing concentrations of ethyl acetate in petroleum ether. The solvent was collected in aliquots (10 ml) and analysed by thin layer chromatography (TLC) to identify the separated metabolite fractions. The solvent systems used for running the TLC plates were 50:50 petroleum ether in ethyl acetate or pure ethyl acetate. A 50:50 sulfuric acid in methanol spray was used to develop the TLC plates.

Table 6
Significant metabolite infra-red absorption signals.

Compound	–OH	Lactone
5α-Androstan-3β,17α-diol (13)	3253 br	
5α-Androstan-3β,17β-diol (14)	3456, 3196	
5α-Androstan-3α,17β-diol (15)	3322 br	
5α-Androstan-3α,11β,17α-triol (16)	3366 br	
5α-Androstan-3β,6β,17α-triol (17)	3492, 3320	
5α-Androstan-3β,7β,17α-triol (18)	3358 br	
5α-Androstan-3β,11β,17α-triol (19)	3358 br	
5α-Androstan-3β,11β,17β-triol (20)	3381 br	
3α-Hydroxy-17a-oxa-D-homo-5α-androstan-17-one (21)	3534 br	1712

2.7. Analysis and identification of metabolites

Characteristic splitting patterns [28] and shift values [29] in the ^{1}H and ^{13}C NMR spectra in comparison to the starting compounds were used to determine metabolite structure in combination with DEPT analysis to identify the nature of the carbon (Tables 1, 2, 4 and 5). Spectra were recorded on a Bruker WM 360 Spectrometer, all samples were analysed in deuteriochloroform using tetramethylsilane as the internal standard. High resolution mass measurement (HRMS) was determined in electrospray ionization (ESI) mode using a Bruker Daltonics Microtof spectrometer. Infra-red absorption spectra (Tables 3 and 6) were recorded directly

Table 5
^{13}C NMR data for starting materials (13–15) and biotransformation products (16–21) determined in $CDCl_3$.

Carbon atom	Compounds								
	13	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
1	32.40	36.75	32.22	32.71	37.64	32.68	37.00	36.89	37.49
2	31.48	30.54	29.02	28.65	31.14	31.35	31.20	30.35	31.95
3	71.36	71.31	66.56	68.56	68.87	71.06	71.09	72.23	66.30
4	38.11	38.18	36.76	35.31	32.73	36.96	37.70	37.69	35.57
5	44.80	44.91	39.20	39.89	50.32	42.07	45.74	45.80	39.33
6	28.68	28.57	28.43	27.99	71.05	37.69	28.16	28.05	28.13
7	32.28	31.63	31.57	32.48	40.41	75.44	32.73	31.96	30.53
8	35.75	35.55	35.55	31.30	31.33	43.59	31.35	31.33	36.09
9	54.48	54.47	54.47	57.98	58.03	52.26	58.04	58.18	53.70
10	37.73	36.81	36.20	30.97	35.38	35.08	35.71	35.70	35.57
11	20.75	20.84	20.36	66.43	20.58	20.86	68.91	71.13	28.68
12	37.08	37.03	35.88	40.42	36.98	38.17	40.43	45.84	30.49
13	43.02	42.49	42.99	44.00	42.74	46.18	43.34	42.05	83.44
14	48.74	51.01	51.09	50.38	45.80	47.84	50.33	52.51	46.35
15	24.61	23.40	23.36	24.54	24.55	27.22	24.56	23.45	19.76
16	31.62	31.51	30.52	37.00	32.56	31.42	32.54	31.17	28.68
17	80.08	81.99	81.99	79.51	79.44	78.88	79.49	82.42	172.50
18	17.09	11.15	11.15	19.43	17.16	17.18	19.41	13.61	20.14
19	12.35	12.36	11.21	14.35	15.49	12.47	15.52	15.46	11.07

Table 7
Product yields following chromatography.

Starting material	Metabolites	Yield (%)
5α-Androstan-3α,17α-diol (**11**)		6
	5α-Androstan-3α,11β,17α-triol (**16**)	12
5α-Androstan-3β,17α-diol (**13**)		8
	5α-Androstan-3β,6β,17α-triol (**17**)	3
	5α-Androstan-3β,7β,17α-triol (**18**)	55
	5α-Androstan-3β,11β,17α-triol (**19**)	8
5α-Androstan-3β,17β-diol (**14**)		1
	5α-Androstan-3β,11β,17β-triol (**20**)	1
5α-Androstan-3α,17β-diol (**15**)		3
	3α-Hydroxy-5α-androstan-17-one (**7**)	23
	3α-Hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**21**)	29

on a Nicolet avatar 320 FT-IR fitted with a Smart Golden Gate®. All yields obtained from the transformation experiments are listed in Table 7.

3. Results

3.1. Confirmation of structure for the novel synthetically derived compounds (*8*) and (*9*)

Comparison of the ^{1}H NMR spectrum of (**8**) with the starting material (**7**) demonstrated the presence of a new signal at 4.73 ppm (2H, brs) which was consistent with the amino protons (–N$\underline{H}_2$). Examination of the ^{13}C NMR spectrum revealed a range of signal shifts in the product consistent with replacement of the more electronegative C-17 carbonyl by the hydrazone. These included upfield shifts in C-16 (12.5 ppm), C-17 (57.2 ppm), C-13 (3.41) and a downfield shift for C-18 (5.88 ppm). The structure was fully supported by elemental analysis (Section 2.2) and accurate mass measurement (HRMS ESI: calc. for M+Na 327.240 $C_{19}H_{32}N_2NaO$ obsd. 327.240).

Comparison of the ^{1}H and ^{13}C NMR spectra of the vinyl iodide (**9**) with that of the hydrozone (**8**) enabled confirmation of structure. The product spectrum (**9**) was devoid of the resonance signal at 4.73 (2H, brs) consistent with the loss of the –N$\underline{H}_2$ and contained a new signal at 6.11 ppm (1H, dd) synonymous with the presence of a ring-D double bond. This was further confirmed in the ^{13}C NMR spectrum with the presence of resonance signals for C-17 (137.48 ppm) and C-16 (112.93 ppm) consistent with a double bond and a concomitant downfield shift for C-15 to 33.63 ppm (Δ10.35 ppm). The presence of the iodine was fully supported by accurate mass measurement (Section 2.2).

3.2. Products of metabolism and structural identification

Transformation of 5α-androstan-3α,17α-diol (**11**) resulted in the isolation of one product. The structure of 5α-androstan-3α,11β,17α-triol (**16**) was determined by comparison of its' spectra with that of (**11**). The ^{1}H NMR revealed significant downfield shifts for both 18-H and 19-H methyl protons respectively of 0.25 ppm and 0.21 ppm and taken into account with a new signal at 4.60 ppm (1H, s) this strongly suggest substitution at the 11β-position. This was confirmed by the product ^{13}C NMR spectra which demonstrated α-carbon downfield shift for C-11 (41.83 ppm), β-carbon downfield shifts for C-9 (3.85 ppm) and C-12 (8.19 ppm) and γ-carbon upfield shifts for C-8 (4.43 ppm), C-10 (5.43 ppm) and C-13 (1.53 ppm). Comparatively both methyl groups were also shifted downfield C-18 (2.32 ppm) and C-19 (3.15 ppm) which is consistent with the presence of a C-11β hydroxyl group. The monohydroxylation was fully supported by accurate mass measurement (HRMS ESI: calc. for M+Na 331.224 $C_{19}H_{32}NaO_3$ obsd. 331.223) (Fig. 2).

Transformation of 5α-androstan-3β,17α-diol (**13**) afforded three products. In the ^{1}H NMR spectrum for 5α-androstan-3β,6β,17α-triol (**17**) the 19-methyl protons had undergone a significant downfield shift (0.44 ppm) in comparison to the starting material (**13**). The product spectrum also contained a new resonance signal at 4.61 ppm (1H, s) indicating hydroxylation at an axial proton, possibly 6β-H. This notion was confirmed by comparison of the ^{13}C NMR spectrum of (**17**) with that of (**13**). The beta carbons (C-5 and C-7) had undergone downfield shifts (5.52 ppm and 8.13 ppm respectively) as had C-19 (3.14 ppm) and carbons gamma to C-6 had undergone upfield shifts C-4 (5.38 ppm), C-8 (4.42 ppm) and C-10 (2.35 ppm). Monohydroxylation was confirmed by accurate mass measurement (HRMS ESI: calc. for M+Na 331.224 $C_{19}H_{32}NaO_3$ obsd. 331.223).

Comparison of the ^{1}H NMR of 5α-androstan-3β,7β,17α-triol (**18**) with that of (**13**) revealed a new signal at 3.40 ppm (1H, td) indicating hydroxylation at an equatorial proton with no concomitant shift in either of the methyl signals suggesting substitution at C-7β. This was supported by comparison of the ^{13}C NMR spectrum of this product and that of (**13**). Carbons beta to C-7 underwent downfield shifts C-6 (9.01 ppm) and C-8 (7.84 ppm) with those γ-carbon signals shifting C-5 (2.73 ppm), C-9 (2.22 ppm) and C-14 (0.9 ppm). Accurate mass measurement confirmed that this compound was monohydroxylated (HRMS ESI: calc. for M+Na 331.224 $C_{19}H_{32}NaO_3$ obsd. 331.224).

The ^{1}H NMR spectrum of 5α-androstan-3β,11β,17α-triol (**19**) contained a new signal at 4.61 ppm (1H, s) indicating axial substitu-

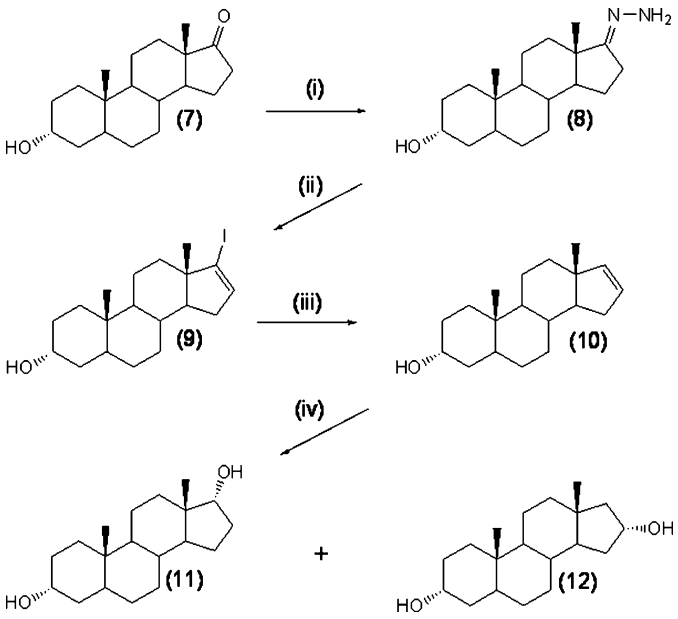

Fig. 2. Synthetic reaction pathway to 5α-androstan-3α,17α-diol (**11**): (i) $H_2NNH_2/(CH_3CH_2)_3N$/EtOH; (ii) I/$(CH_3CH_2)_3N$/THF; (iii) Na/EtOH; (iv) (a) B_2H_6/THF and (b) H_2O_2/NaOH.

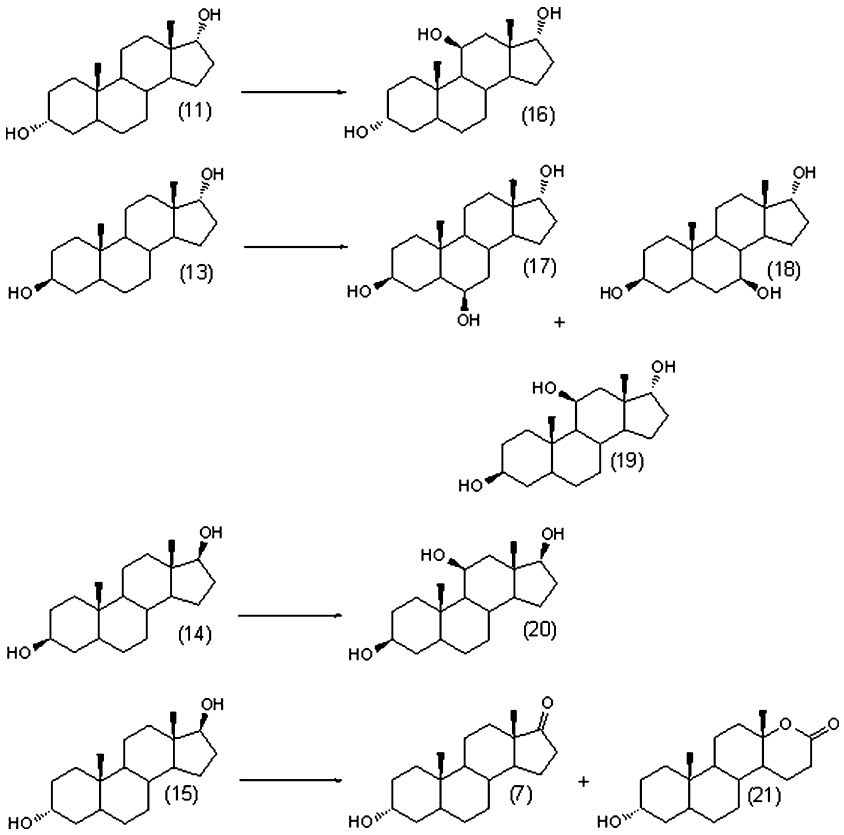

Fig. 3. Transformation of steroidal diols following 5 days incubation with *Aspergillus tamarii KITA*.

tion. In comparison to the spectrum of (**13**) both methyl groups had undergone significant downfield shifts 18-H (0.24 ppm) and 19-H (0.18 ppm) strongly suggesting hydroxylation at the 11β-position. This was confirmed following comparison of the product ^{13}C NMR spectrum to that of (**13**) which demonstrated β-carbon downfield shifts for C-9 (3.56 ppm) and C-12 (3.35 ppm) and γ-carbon upfield shifts for C-8 (4.40 ppm) and C-10 (2.02 ppm). Monohydroxylation was confirmed by accurate mass measurement (HRMS ESI: calc. for M+Na 331.224 $C_{19}H_{32}NaO_3$ obsd. 331.223).

Transformation of 5α-androstan-3β,17β-diol (**14**) afforded one product, 5α-androstan-3β,11β,17β-triol (**20**). This was identified by inspection of its' spectra with the ^{1}H NMR, compared to (**14**), which, contained a new signal at 4.33 ppm (1H, s) suggestive of axial substitution. This information combined with the downfield shifts observed for 18-H (0.25 ppm) and 19-H (0.26 ppm) was supportive of C-11β hydroxylation. The ^{13}C NMR spectrum of (**20**) was consistent with beta carbon downfield shift for C-9 (3.71 ppm) and C-12 (37.03 ppm) and γ-carbon upfield shifts for C-8 (4.22 ppm) and C-10 (1.11 ppm). Monohydroxylation was confirmed by accurate mass determination (HRMS ESI: calc. for M+Na 331.224 $C_{19}H_{32}NaO_3$ obsd. 331.224).

Transformation of 5α-androstan-3α,17β-diol (**15**) resulted in two products. 3α-Hydroxy-5α-androstan-17-one (**7**) was devoid of the triplet signal (4.05 ppm) generated by the 17α-H in the starting material indicating that oxidation to a ketone may have taken place. This was confirmed by inspection of the product ^{13}C NMR spectrum that contained a resonance signal at 221.10 ppm and is fully consistent with a 5-ring ketone. Comparison of the ^{1}H and ^{13}C NMR spectra of (**7**) to that of an authentic sample [6] fully supported the proposed structure (Fig. 3).

3α-Hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**21**) was identified by comparison of its' spectra to that of (**7**) this demonstrated a significant downfield shift for the 18-methyl protons of 0.44–1.30 ppm which is fully indicative of hetero-atom insertion [2,3,6] in ring-D. This was supported in the ^{13}C NMR spectrum with downfield shifts for C-13 (35.96 ppm) and C-18 (8.96 ppm), directly associated with insertion of the hetero-atom, and upfield shifts consistent with the reduced 6 membered ring strain for C-14 (8.08 ppm) and C-17 (48.60 ppm). Comparison of the product spectrum to that of an authentic sample [6] fully supported the proposed structure.

4. Discussion

Transformation of this series of the four isomers of androstan-3,17-diol revealed exclusive handling of 5α-androstan-3α,17β-diol (**15**) within the lactonization pathway, ultimately resulting in the generation of 3α-hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**21**). Before the lactone (**21**) is formed the oxidation of the C-17β-alcohol to a C-17 ketone must occur via a 17β hydroxysteroid dehydrogenase (17β-HSD). As this only occurs with one of the four isomers it demonstrates that there is a very precise stereochemical requirement for the oxidation of the C-17β hydroxyl group. This is interesting in that some 17β-HSD enzymes show a degree of flexibility in structure with respect to handling steroidal substrates e.g. in bacteria [30] and plant [31], or an ability to accept steroids in alternative binding conformations in human cells [32,33]. The 17β-HSD was unable to oxidise the three other steroidal diol isomers (**11**,**13**,**14**) all of which underwent 11β-hydroxylation. It is remarkable that the oxidation of 5α-androstan-3β,17β-diol (**14**) did not occur, as it is only structurally different at C-3 where the alcohol has beta stereochemistry. This is another example of the importance of single functional groups [3,6] and their stereochemistry in guiding metabolic fate within this organism. Interestingly, 5α-androstan-3β,17α-diol (**13**) demonstrated the greatest range of monohydroxylated products and if

these occurred solely within the 11β-hydroxylase, it would be consistent with reverse (6β), inverted normal (7β) and normal (11β) binding. Identical positions of hydroxylation have been observed following metabolism of the 6-membered ring-D containing lactone 3β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one [7]. This may indicate that during the hydroxylation process the lactone ring is open, thus generating an alcohol with 17α-stereochemistry in remarkably spatial proximity to the 17α-alcohol of (**13**). The metabolite 5α-androstan-3β,7β,17α-triol was isolated in significant yield (55%) presumably demonstrating that optimal binding, if in the 11β-hydroxylase, is in the inverted normal binding position. The recovered total yields of steroid from the transformation of compounds (**11**) and (**14**) were low compared to those from (**13**) and (**15**) (Table 7). Interestingly the steroids with the low recovery retained both hydroxyl groups on the same side of the molecule (3α,17α and 3β,17β).

It could be speculated that the unique metabolic handling resulting in compound (**21**) may either be directly or indirectly due to compound (**15**) having inhibitory activity against the 11β-hydroxylase. This notion may be supported from previous work in which the ring-D lactone in a range of steroidal analogues does not appear to inhibit 11β-hydroxylase activity [1,6]. No oxidation of the 3α-alcohols to 3-ketones was observed, this is consistent with previous observations [6] with this organism. Previous studies with *A. tamarii* have also demonstrated that 3α-alcohols and acetates [6] do not block Baeyer–Villiger oxidation of the C-17 ketone, this also holds true in the presence of 11α-hydroxyl groups [3].

This study has not only revealed unique effects of stereochemistry and polar binding on steroidal diol fate in *A. tamarii* but it has also generated a range of novel triols, one in significant yield, which may be of biological interest, as steroids containing this type of functionality have distinct activity and/or play important intermediate roles in metabolism [34–37].

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References

[1] D.R. Brannon, J. Martin, A.C. Oehlschlager, N.N. Durham, L.H. Zalkow, Transformation of progesterone and related steroids by *Aspergillus tamarii*, J. Org. Chem. 30 (1965) 760–762.
[2] A.C. Hunter, N.E. Carragher, Flexibility of the endogenous progesterone lactonisation pathway in *Aspergillus tamarii KITA*: transformation of a series of cortical steroid analogues, J. Steroid Biochem. Mol. Biol. 87 (2003) 301–308.
[3] A.C. Hunter, J. Elsom, L. Ross, R. Barrett, Ring-B functionalized androst-4-en-3-ones and ring-C substituted pregn-4-en-3-ones undergo differential transformation in *Aspergillus tamarii KITA*: ring-A transformation with all C-6 substituted steroids and ring-D transformation with C-11 substituents, Biochim. Biophys. Acta 1761 (2006) 360–366.
[4] A.C. Hunter, S. Kennedy, S.-J. Clabby, J. Elsom, Fate of novel *Quasi* reverse steroidal substrates by *Aspergillus tamarii KITA*: bypass of lactonisation and an exclusive role for the minor hydroxylation pathway, Biochim. Biophys. Acta 1734 (2005) 190–197.
[5] A.C. Hunter, E. Coyle, F. Morse, C. Dedi, H.T. Dodd, S.-J. Koussoroplis, Transformation of 5-ene steroids by the fungus *Aspergillus tamarii KITA*: mixed molecular fate in lactonization and hydroxylation pathways with identification of a putative 3β-hydroxy-steroid dehydrogenase/Δ^5-Δ^4 isomerase pathway, Biochim. Biophys. Acta 1791 (2009) 110–117.
[6] A.C. Hunter, H. Khuenl-Brady, P. Barrett, H.T. Dodd, C. Dedi, Transformation of some 3α-substituted steroids by *Aspergillus tamarii* KITA reveals stereochemical restriction of steroid binding orientation in the minor hydroxylation pathway, J. Steroid Biochem. Mol. Biol. 118 (2010) 171–176.
[7] A.C. Hunter, H. Bergin-Simpson, Distinct metabolic handling of 3β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one by the filamentous fungus *Aspergillus tamarii KITA*: evidence in support of the steroid/hydroxylase binding hypothesis, Biochim. Biophys. Acta 1771 (2007) 1254–1261.
[8] D.A. Kraut, P.A. Sigala, T.D. Fenn, D. Herschlag, Dissecting the paradoxical effects of hydrogen bond mutations in the ketosteroid isomerase oxyanion hole, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 1960–1965.
[9] D.K. Chakravorty, A.V. Soudackov, S. Hammes-Schiffer, Hybrid quantum/classical molecular dynamics simulations of the proton transfer reactions catalysed by ketosteroid isomerase: analysis of hydrogen bonding, conformational motions and electrostatics, Biochemistry 48 (2009) 10608–10619.
[10] U. Dhagat, S. Endo, R. Sumii, A. Hara, O. El-Kabbani, Selectivity determinants of inhibitor binding to human 20 alpha-hydroxysteroid dehydrogenase: crystal structure of the enzyme internary complex with coenzyme and potent inhibitor 3,5-dichlorosalicylic acid, J. Med. Chem. 51 (2008) 4844–4848.
[11] N. Mast, S.E. Graham, U. Andersson, I. Bjorkhem, C. Hill, J. Peterson, I.A. Pikuleva, Cholesterol binding to cytochrome P450 7A1, a key enzyme in bile acid biosynthesis, Biochemistry 44 (2005) 3259–3271.
[12] R Shi, S.X. Lin, Cofactor hydrogen bonding onto the protein main chain is conserved in the short chain dehydrogenase/reductase family and contributes to nicotinamide orientation, J. Biol. Chem. 279 (2004) 16778–16785.
[13] D. Mazumder, K. Khan, T.C. Bruice, Computational study of ketosteroid isomerase: insights from molecular dynamics simulation of enzyme bound substrate and intermediate, J. Am. Chem. Soc. 125 (2003) 7553–7561.
[14] F. Henot, R.M. Pollack, Catalytic activity of the D38A mutant of 3-oxo-Δ^5-steroid isomerase: recruitment of aspartate-99 as the base, Biochemistry 39 (2000) 3351–3359.
[15] M.A. Massiah, C. Abeygunawardana, A.G. Gittis, A.S. Mildvan, Solution structure of Δ^5-3-ketosteroid isomerase complexed with the steroid 19-nortestosterone hemisuccinate, Biochemistry 37 (1998) 14701–14712.
[16] M. Numazawa, M. Tachibana, Y. Tateda, 4-Oxygenated androst-5-en-17-ones and their 7-oxo derivatives as aromatase inhibitors, J. Steroid Biochem. Mol. Biol. 58 (1996) 431–438.
[17] J.R. Hanson, P.B. Hitchcock, A.C. Hunter, The microbiological hydroxylation of 3,16-disubstituted androstanes by *Cephalosporium aphidicola*, Phytochemistry 49 (1998) 1287–1293.
[18] A.G. Oliveira, P.H. Coelho, F.D. Guedes, G.A.B. Mahecha, R.A. Hess, C.A. Oliveira, 5α-Andrsotane-3β,17β-diol (3β-diol) an estrogenic metabolite of 5α-dihydrotestosterone, is a potent modulator of estrogen receptor ER beta expression in the ventral prostate of adult rats, Steroids 72 (2007) 914–922.
[19] H. Pettersson, L. Holmberg, M. Axelson, M. Norlin, CYP7B1-mediated metabolism of dehydroepiansdrosterone and 5α-androstane-3β,17β-diol—potential role(s) for estrogen signalling, FEBS J. 275 (2008) 1778–1789.
[20] N. Sugiyama, S. Andersson, R. Lathe, X. Fan, P. Alonso-Magdalena, T. Schwend, I. Nalvarte, M. Warner, J.A. Gustafsson, Spatiotemporal dynamics of the expression of estrogen receptors in the postnatal mouse brain, Mol. Psychiatry 14 (2009) 223–232.
[21] M.J. Sikora, K.E. Cordero, J.M. Larious, M.D. Johnson, M.E. Lippman, J.M. Rae, The androgen metabolite 5α-androstane-3β,17β-diol induces breast cancer growth via estrogen receptor: implications for aromatase inhibitor resistance, Breast Cancer Res. Treat. 115 (2009) 289–296.
[22] Y. Omoto, R. Lathe, M. Warner, J.A. Gustafsson, Early onset of puberty and early ovarian failure in CYP7B1 knockout mice, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 2814–2819.
[23] S.R. Aspinall, S. Stamp, A. Davison, B.K. Shenton, T.W.J. Lennard, The proliferative effects of 5-androstene-3β,17β-diol and 5α-dihydrotestosterone on cell cycle analysis and cell proliferation in MCF7, T47D and MDAMB231 breast cancer cell lines, J. Steroid Biochem. Mol. Biol. 88 (2004) 37–51.
[24] E.H. Nunlist, I. Dozmorov, Y. Tang, R. Cowan, M. Centola, H.-K. Lin, Partitioning of 5α-androstane-3α,17β-diol activated pathways for stimulating human prostate cancer LNCaP cell proliferation, J. Steroid Biochem. Mol. Biol. 91 (2004) 157–170.
[25] H. Pattersson, J. Lundqvist, E. Oliw, M. Norlin, CYP7B1-mediated metabolism of 5α-androstane-3α,17β-diol (3α-Adiol): a novel pathway for potential regulation of the cellular levels of androgens and neurosteroids, Biochim. Biophys. Acta. Mol. Cell Biol. Lipid 1791 (2009) 1206–1215.
[26] T. Higashi, Y. Nagura, K. Shimada, T. Toyo'oka, Studies on neurosteroids XXV1. Fluoxetine-evoked changes in rat brain and serum levels of neuroactive androgen, 5α-androstane-3α,17β-diol, Biol. Pharm. Bull. 32 (2009) 1636–1638.
[27] C.A. Frye, A. Ryan, M. Rhodes, Antiseizure effects of 3α-androstanediol and/or 17β-estradiol may involve actions at the estrogen receptor β, Epilepsy Behav. 16 (2009) 418–422.
[28] D.N. Kirk, H.C. Toms, C. Douglas, A. White, A survey of the high-field ^{1}H NMR spectra of steroid hormones, their hydroxylated derivatives and related compounds, J. Chem. Soc. Perkin Trans. 2 (1990) 1567–1594.
[29] J.W. Blunt, J.B. Stothers, ^{13}C NMR of steroids—a survey and commentary, Org. Magn. Reson. 9 (1977) 439–463.
[30] J. Benach, C. Filling, U.C.T. Oppermann, P. Roversi, G. Bricogne, K.D. Berndt, H. Jörnvall, R. Ladenstein, Stucture of bacterial 3β/17β-hydroxysteroid dehydrogenase at 1.2 Å resolution: a model for multiple steroid recognition, Biochemistry 41 (2002) 14659–14668.
[31] V. Heri, J. Frankenstein, N. Meitinger, F. Muller-Uri, W. Kreis, A(5)-3β-hydroxysteroid dehydrogenase (3β-HSD) from *Digitalis lanata*. Heterologous expression and characterisation of the recombinant enzyme, Planta Med. 73 (2007) 704–710.
[32] A. Gangloff, R. Shi, V. Nahoum, S.-X. Lin, Pseudo-symmetry of C-19-steroids, alternative binding orientations and multispecificity in human estrogenic 17β-hydroxysteroid dehydrogenase, FASEB J. 16 (2002), doi:10.1096/fj.02-0026fje.
[33] W. Qui, M. Zhou, F. Labrie, S-X. Lin, Crystal structures of the multispecific 17β-hydroxysteroid dehydrogenase type 5: critical androgen regulation in human peripheral tissues, Mol. Endocrinol. 18 (2004) 1798–1807.

[34] A-K.Ö. Farrants, I. Björkhem, J.I. Pedersen, Identification of 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid as an intermediate in the peroxisomal conversion of 3α,7α,12α-trihydroxy-5β-cholestenoic acid to cholic acid, Biochim. Biophys. Acta. Lipids Lipid Metab. 1002 (1989) 198–202.
[35] L.-H. Shan, H.-M. Liu, K.-X. Huang, G.-F. Dai, C. Cao, R.-J. Dong, Synthesis of 3β,7α,11α-trihydroxy-pregn-21-benzylidene-5-en-20-one derivatives and their cytotoxic activities, Bioorg. Med. Chem. Lett. 19 (2009) 6637–6639.
[36] A.Y. Reeder, G.E. Joannou, 15β-Hydroxysteroids (Part IV). Steroids of the human perinatal period: the synthesis of 3α,15β,17α-trihydroxy-5α-pregnan-20-one and it's A/B-ring configurational isomers, Steroids 60 (1995) 796–801.
[37] S. Christakoudi, D.A. Cowan, N.F. Taylor, A new marker for early diagnosis of 21-hydroxylase deficiency: 3β,16α,17α-trihydroxy-5α-pregnane-7,20-dione, J. Steroid Biochem. Mol. Biol. 121 (2010) 574–581.