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Transformation of some 3α-substituted steroids by *Aspergillus tamarii* KITA reveals stereochemical restriction of steroid binding orientation in the minor hydroxylation pathway

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ABSTRACT

Aspergillus tamarii contains an endogenous lactonization pathway which can transform progesterone to testololactone in high yield through a sequential four step enzymatic pathway. In this pathway testosterone is formed which primarily undergoes oxidation of the C-17β-alcohol to a C-17 ketone but, can also enter a minor hydroxylation pathway where 11β-hydroxytestosterone is produced. It was recently demonstrated that this hydroxylase could monohydroxylate 3β-hydroxy substituted saturated steroidal lactones in all four possible binding orientations (normal, reverse, inverted normal, inverted reverse) on rings B and C of the steroid nucleus. It was therefore of interest to determine the fate of a series of 3α-substituted steroidal analogues to determine stereochemical effect on transformation. Hydroxylation on the central rings was found to be restricted to the 11β-position (normal binding), indicating that the 3α-stereochemistry removes freedom of binding orientation within the hydroxylase. The only other hydroxylation observed was at the 1β-position. Interestingly the presence of this functional group did not prevent lactonization of the C-17 ketone. In contrast the presence of the 11β-hydroxyl completely inhibited Baeyer–Villiger oxidation, a result which again demonstrates that single functional groups can exert significant control over metabolic handling of steroids in this organism. This may also explain why lactonization of 11β-hydroxytestosterone does not occur. Lactonization of the C-17 ketone was not significantly affected by the 3α-alcohol with significant yields achieved (53%). Interestingly a time course experiment demonstrated that the presence of the 3α-acetate inhibited the Baeyer–Villiger monooxygenase with its activity being observed 24 h later than non-acetate containing analogues. Apart from oxidative transformations observed a minor reductive pathway was revealed with the C-17 ketone being reduced to a C-17β-alcohol for the first time in this organism.

1. Introduction

The fungus *Aspergillus tamarii* KITA has the ability to transform progesterone (**1**) into testololactone (**5**) in high yield through a sequential four step enzymatic pathway [1] (Fig. 1). This initiates with Baeyer–Villiger oxidation of the C-20 ketone generating testosterone acetate (**2**) which is then hydrolysed to testosterone (**3**). This then undergoes oxidation of the 17β-hydroxyl group producing androst-4-en-3,17-dione (**4**). The final Baeyer–Villiger oxidation then takes place at the C-17 ketone generating the end product testololactone (**5**) which does not undergo further metabolism [2]. Within the transformation sequence testosterone (**3**) can also enter into a minor 11β-hydroxylation pathway and progesterone (**1**) can undergo reduction of the C-20 ketone forming the C-20(R)-hydroxy analogue (**6**). A competitive equilibrium exists between the reductase forming the C-20(R)-alcohol and the oxidase that regenerates (**1**).

Further investigation of the metabolic pathways within *A. tamarii* with a range of diverse steroidal probes has revealed a broad spectrum of metabolic fate with this organism [2–6]. For example, a range of cortical steroid analogues with different side-chains were in general readily handled within the lactonization pathway [3]. In contrast a series of *quasi* reverse steroidal substrates, in which ring-A functionality (4-ene-3-one) had been transposed to ring-D (14-ene-16-one) and ring-D C-17 functionality (alcohol, acetate, ketone) to C-3, underwent exclusive metabolism in the 'minor' hydroxylation pathway [4]. Switching of metabolism between ring-A and ring-D has been observed with a range of substrates modified on the central ring-B (C-6) and ring-C (C-11) where the former drives reductive metabolism to ring-A and the later to lactonization of ring-D [5].

Examination of a range of 3β-hydroxy-5-ene containing steroids revealed the fungus has the enzymatic capacity to isomerize the double bond and oxidize the 3β-alcohol forming 3-one-4-ene on

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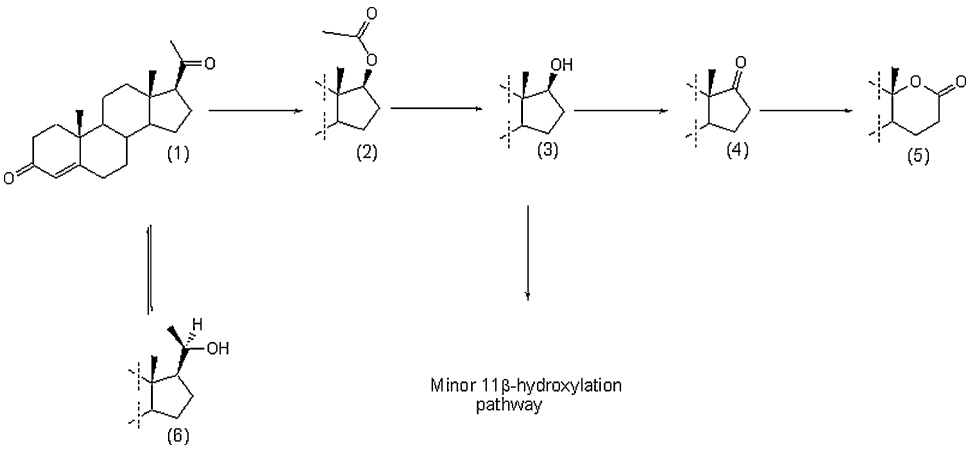

Fig. 1. The endogenous lactonization pathway present in *Aspergillus tamarii* KITA which converts progesterone (**1**) to testololactone (**5**) with preceding C-20 keto-alcohol isomerise (**1**) and (**6**) and the minor 11β-hydroxylation pathway from testosterone.

ring-A thus generating androgens from some of the substrates tested [6].

Remarkably a range of fully saturated ring-D lactones were incubated with *A. tamarii* this resulted in monohydroxylation (11β, 6β, 7β, 11α) which geometrically related to the four previously hypothesized [7–10,14] possible binding positions (normal, reverse, inverted normal and inverted reverse) for a terminally (C-3β alcohol and a C-17 ketone) substituted steroid [2]. In order to further determine the effect of stereochemistry at C-3 on hydroxylation and lactonization we have investigated a series of analogues containing ring-A 3α-alcohols and acetates and ring-D ketones and lactones.

2. Materials and methods

2.1. Chemicals and reagents

3α-Hydroxy-5α-androstan-17-one (**7**) and 3α-acetoxy-5α-androstan-17-one (**8**) were purchased from Steraloids Ltd (UK) and were used as supplied. Steroidal lactones (**9**) and (**10**) were synthesized using previously described methodology [11] and were found to be in excess of 99.6% purity following elemental analysis, found: C, 74.14; H, 10.04. $C_{19}H_{30}O_3$ requires C, 74.47; H 9.87% for compound (**9**) and for compound (**10**) found: C, 72.35; H, 9.36. $C_{21}H_{32}O_4$ requires C, 72.38; H, 9.26%. 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. 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).

2.3. 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 40 °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.

2.4. 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

Table 1
^{1}H NMR data for steroidal starting material and transformation products determined in $CDCl_3$.

	3β-H	18-H_3	19-H_3	Other significant signals
(*a*) *Reference data*				
3α-Hydroxy-5α-androstan-17-one (**7**)	4.06, t, *J* = 2.5 Hz	0.86	0.80	
3α-Acetoxy-5α-androstan-17-one (**8**)	5.01 brs	0.86	0.82	2.05 (3H, s, 21-H_3)
3α-Hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**9**)	4.06, t, *J* = 2.5 Hz	1.30	0.75	
3α-Acetoxy-17a-oxa-D-homo-5α-androstan-17-one (**10**)	5.01, t, *J* = 2.5 Hz	1.30	0.76	2.06 (3H, s, 21-H_3)
(*b*) *Transformation products*				
3α,17β-Dihydroxy-5α-androstane (**11**)	4.04, t, *J* = 2.6 Hz	0.79	0.73	3.63 (1H, t, *J* = 8 Hz, 17α-H)
1β,3α-Dihydroxy-5α-androstan-17-one (**12**)	4.09, t, *J* = 2.5 Hz	0.85	0.83	3.82 (1H, dd, *J* = 4.5 Hz, *J* = 11.2 Hz, 1α-H)
3α,11β-Dihydroxy-5α-androstan-17-one (**13**)	4.05, t, *J* = 2.4 Hz	1.10	1.05	4.43 (1H, brs, 11α-H)
1β,3α-Dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**14**)	4.11, t, *J* = 2.5 Hz	1.25	0.84	3.82 (1H, dd, *J* = 4.8 Hz, *J* = 11.5 Hz, 1α-H)
3α,11β-Dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**15**)	4.21, brs	1.50	0.98	4.47, brs, 11α-H)

Table 2
^{13}C NMR data for steroidal starting material and transformation products determined in $CDCl_3$.

Carbon atom	Compounds								
	(**7**)	(**8**)	(**9**)	(**10**)	(**11**)	(**12**)	(**13**)	(**14**)	(**15**)
1	32.13	32.83	31.94	32.62	32.22	74.02	31.92	74.03	31.20
2	28.95	26.06	28.92	25.95	29.02	40.02	31.26	40.06	27.61
3	66.42	69.93	66.29	69.77	66.56	66.75	66.35	66.56	66.21
4	35.74	32.83	35.55	32.58	36.76	35.72	35.94	35.72	31.88
5	39.09	40.05	38.47	39.40	39.20	38.25	39.94	38.26	39.18
6	28.23	28.06	28.12	27.92	28.43	27.89	28.65	27.89	28.51
7	30.84	30.77	30.52	30.42	31.57	30.78	31.26	30.78	28.70
8	35.03	35.02	37.89	37.85	35.55	35.56	30.79	35.57	32.85
9	54.40	54.32	53.07	52.93	54.47	54.78	58.45	54.81	56.35
10	36.23	35.98	36.08	35.06	36.20	42.05	40.77	42.56	36.12
11	20.03	20.06	19.75	19.73	23.36	23.73	67.77	23.75	66.53
12	31.55	31.57	28.68	28.64	35.88	31.79	40.40	28.71	34.96
13	47.83	47.82	83.49	82.66	42.99	47.33	47.18	82.75	82.84
14	51.48	51.52	46.33	46.34	51.09	51.38	53.02	48.04	48.05
15	21.75	21.74	21.59	21.58	20.36	21.95	21.65	21.96	19.48
16	35.86	35.83	39.32	39.30	30.52	35.72	35.94	35.75	47.20
17	221.65	222.00	171.67	170.84	81.99	221.50	220.17	174.30	171.84
18	13.82	13.82	20.16	20.11	11.21	13.75	16.20	22.44	23.25
19	11.19	11.34	11.05	11.19	11.15	5.61	14.20	5.59	14.05
20	–	170.66	–	169.88	–	–	–	–	–
21	–	21.53	–	21.51	–	–	–	–	

Table 3
Significant metabolite infra-red absorption signals cm^{-1}.

Compound	–OH	)C=O	Lactone
3α,17β-Dihydroxy-5α-androstane (**11**)	3322 br	–	–
1β,3α-Dihydroxy-5α-androstan-17-one (**12**)	3153 br	1724	–
3α,11β-Dihydroxy-5α-androstan-17-one (**13**)	3439	1723	–
1β,3α-Dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**14**)	3100 br	–	1722
3α,11β-Dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**15**)	3369	–	1715

sulfuric acid in methanol spray was used to develop the TLC plates

2.5. Analysis and identification of metabolites

Characteristic splitting patterns [12] and shift values [13] 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 and 2). 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 (Table 3) were recorded directly on a Nicolet avatar 320 FT-IR fitted with a Smart Golden Gate®. All yields obtained from the transformation experiments are listed in Table 4.

2.6. Time course experiment

Experimental conditions were identical to those in Section 2.3 except that steroid (1.2 g) dissolved in dimethylformamide (6 ml) was evenly distributed between 6 flasks (each containing 200 ml of media) for each of the substrates (**7**, **8**, **9**, **10**). One flask was harvested after 8 h, then one every 24 h and extracted as in Section 2.4. Following 6 h under high vacuum, the product ^{1}H NMR spectra were determined in $CDCl_3$ to confirm the presence and

Table 4
Product yields following chromatography.

Starting material	Metabolites	Yield (%)
3α-Hydroxy-5α-androstan-17-one (**7**)		18
	3α-Hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**9**)	53
	3α,17β-Dihydroxy-5α-androstane (**11**)	3
	1β,3α-Dihydroxy-5α-androstan-17-one (**12**)	4
	3α,11β-Dihydroxy-5α-androstan-17-one (**13**)	7
	1β,3α-Dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**14**)	2
	3α,11β-Dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**15**)	11
3α-Acetoxy-5α-androstan-17-one (**8**)		10
	3α-Hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**9**)	13
	3α-Acetoxy-17a-oxa-D-homo-5α-androstan-17-one (**10**)	5
3α-Hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**9**)		
	3α,11β-Dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**15**)	24
3α-Acetoxy-17a-oxa-D-homo-5α-androstan-17-one (**10**)		0
	3α-Hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**9**)	49
	3α,11β-Dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**15**)	27

Table 5
Time course experiment.

Time points (h)	24	48	72	96	120
Metabolites identified following metabolism of 3α-hydroxy-5α-androstane-17-one (**7**)	**9**, **11**	**12**, **13**, **14**, **15**			
Metabolites identified following metabolism of 3α-acetoxy-5α-androstane-17-one (**8**)		**9**, **10**			
Metabolites identified following metabolism of 3α-hydroxy-17a-oxa-D-homo-5α-androstane-17-one (**9**)		**15**			
Metabolites identified following metabolism of 3α-acetoxy-17a-oxa-D-homo-5α-androstane-17-one (**10**)	**9**	**15**			
Metabolites identified following metabolism of 1β,3α-dihydroxy-5α-androstane-17-one (**12**)	**14**				
Metabolites identified following metabolism of 3α,11β-dihydroxy-5α-androstane-17-one (**13**)					No change observed to **13**

steroidal nature of the extracts (Table 5). For metabolites **12** and **13** total isolated yield was evenly distributed between 6 flasks (each containing 100 ml of media) and handled as above.

3. Results

3.1. Products of metabolism and structural identification

Transformation of 3α-hydroxy-5α-androstan-17-one (**7**) generated six products of metabolism. 3α-Hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**9**) was identified by comparison of its spectra to (**7**) which demonstrated a significant downfield shift for the 18-methyl group (Δ 0.44 ppm) which is consistent with hetero atom insertion following biocatalytic Baeyer–Villiger oxidation of the C-17 ketone [3,5,6]. This was further supported by the product ^{13}C NMR spectrum in which downfield shifts were observed for C-13 (35.66 ppm), C-18 (6.34 ppm) with an upfield shift for C-14 (5.15 ppm) that was consistent with reduced ring junction strain due to six membered ring formation, as was the accurate mass measurement (HRMS ESI: calc. for M+Na$^+$ 329.207 $C_{19}H_{30}NaO_3$ obsd. 329.208).

The structure of 3α,17β-dihydroxy-5α-androstane (**11**) was identified by comparison of its' NMR spectra with that of (**7**). The ^{1}H NMR spectrum of (**11**) contained a new resonance signal at 3.63 ppm (1H, t) which indicated that the C-17 ketone had been reduced to a C-17β-alcohol. Examination of the product ^{13}C NMR spectrum confirmed this notion with loss of the non-protonated resonance signal at 221.65 ppm, being replaced with a C*H*OH resonance signal at 85.06 ppm. Further evidence of the reduction was provided by upfield shifts in the product ^{13}C NMR spectrum for C-13 (4.84 ppm) and C-11 (2.61 ppm) as did the infra-red spectrum which was devoid of carbonyl absorption signals but did contain a broad alcohol absorption signal at 3322 cm^{-1}. Accurate mass measurement fully supported the proposed structure (HRMS ESI: Calc for M+Na$^+$ 315.229 $C_{19}H_{32}NaO_2$ obsd. 315.225).

1β,3α-Dihydroxy-5α-androstan-17-one (**12**) was identified by comparison of its' ^{1}H and ^{13}C NMR spectra with that of the starting material (**7**). The ^{1}H NMR spectrum of the product contained a new signal (1H) at 3.82 ppm, the coupling constants of which (J = 4.5 Hz, J = 11.2 Hz) indicated substitution at an equatorial proton. Monohydroxylation was fully supported by accurate mass measurement (HRMS ESI: Calc for M+Na$^+$ 329.208 $C_{19}H_{30}NaO_3$ obsd. 329.208). The ^{13}C NMR spectrum of the product indicated a signatory shift for hydroxylation at C-1β with the C-19 methyl resonance undergoing an upfield shift of 5.58 ppm. This position of substitution was also supported by downfield shifts in C-2 (11.02 ppm) and C-11 (3.33 ppm) [13].

The structure of 3α,11β-dihydroxy-5α-androstan-17-one (**13**) was determined following comparison of its' spectroscopic data with that of the starting material (**7**). A new signal was observed in the product ^{1}H NMR spectrum at 4.43 ppm (brs) which suggested monohydroxylation. This notion was supported by accurate mass measurement of the product (HRMS ESI: Calc for M+Na$^+$ 329.208 $C_{19}H_{30}NaO_3$ obsd. 329.208). The product ^{1}H NMR spectrum also showed that both methyl groups had undergone significant downfield shifts [18-H_3 (0.24 ppm), 19-H_3 (0.25 ppm)] which, coupled with the new signal in the ^{1}H NMR spectrum (4.43 ppm, brs) was signatory for hydroxylation at C-11β. This assignment was further supported by the product ^{13}C NMR spectrum in which a gamma carbon upfield shift was observed for C-8 (4.24 ppm) and downfield shifts for C-9 (4.05 ppm), C-10 (4.54 ppm), C-12 (8.8 ppm), C-14 (1.54 ppm) [13], C-18 (2.38 ppm) and C-19 (3.01 ppm).

1β,3α-Dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**14**) was identified following comparison with the spectra of compound (**9**). A new signal in the product ^{1}H NMR spectrum indicated monohydroxylation at a methylene, confirmed by accurate mass measurement (HRMS ESI: Calc for M+Na$^+$ 345.203 $C_{19}H_{30}NaO_4$ obsd. 345.202). Examination of the product ^{13}C NMR spectrum revealed that the 19-methyl signal had undergone an upfield shift (5.46 ppm) resulting in a resonance signal at 5.59 ppm which is signatory for C-1β hydroxylation. This assignment was fully supported by downfield shifts for C-2 (11.14 ppm), C-10 (6.84 ppm) and C-11 (4.00 ppm).

In comparison to the ^{1}H NMR spectrum of (**9**) 3α,11β-dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**15**) a new signal at 4.47 ppm (1H, brs) and significant downfield shifts for the 18-methyl (0.2 ppm) and the 19-methyl (0.23 ppm) protons, confirmed 11β-hydroxylation. This was fully supported in the ^{13}C NMR spectrum of the product which demonstrated an upfield shift for C-8 (5.04 ppm) and downfield shifts for C-9 (3.28 ppm), C-12 (6.28 ppm) C-14 (1.72 ppm) [13], C-18 (3.09 ppm) and C-19 (3.00 ppm).

Transformation of 3α-acetoxy-5α-androstan-17-one (**8**) generated two products of transformation, namely 3α-acetoxy-17a-oxa-D-homo-5α-androstan-17-one (**10**) and 3α-hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**9**) the structures of which were confirmed by comparison of their spectra to authentic samples (see above) which, were found to be identical.

Transformation of 3α-hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**9**) resulted in the isolation of one product, 3α,11β-dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**15**) which was readily identified by comparison of its' spectra with that of an authentic sample determined above.

Transformation of 3α-acetoxy-17a-oxa-D-homo-5α-androstan-17-one (**10**) yielded two products, 3α-hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**9**) and 3α,11β-dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**15**). The structure of these compounds was confirmed by comparison to the authenticated samples above.

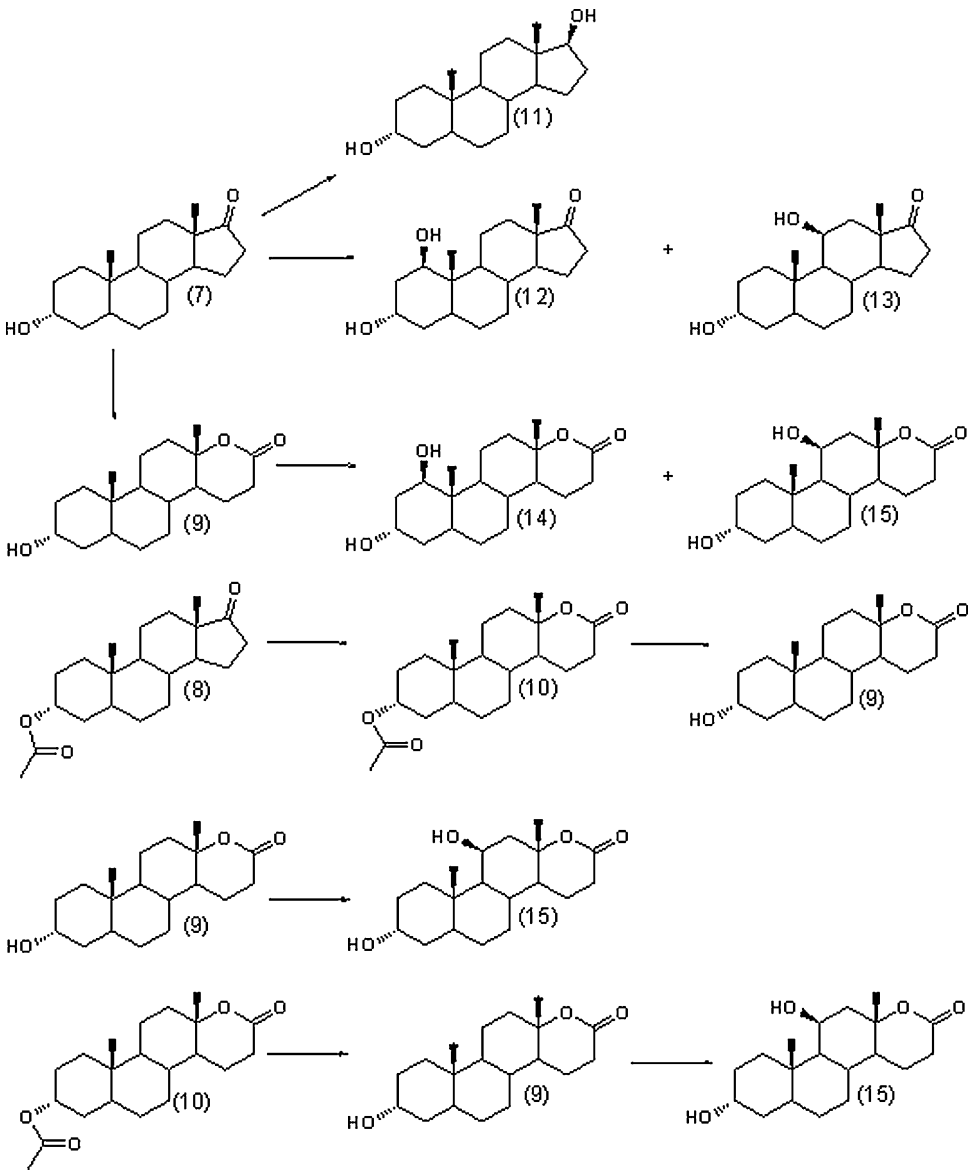

Fig. 2. Transformation of steroidal substrates following 5 days incubation with *Aspergillus tamarii* KITA.

*3.2. Further transformation of 1β,3α-dihydroxy-5α-androstan-17-one (**12**) and 3α,11β-dihydroxy-5α-androstan-17-one (**13**)*

Transformation of 1β,3α-dihydroxy-5α-androstan-17-one (**12**) resulted in lactonization of the C-17 ring-D ketone in the first 24 h time period affording 1β,3α-dihydroxy-17a-oxa-D-homo-androstan-17-one (**14**). This was readily identified by comparison of the NMR data with that of an authentic sample.

Transformation 3α,11β-dihydroxy-5α-androstan-17-one (**13**) with the fungus for 5 days resulted in no observable transformation with full recovery of the starting material.

4. Discussion

The metabolism of the saturated lactone 3β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one by *A. tamarii* has been previously investigated [2]. This study uniquely demonstrated that this steroidal substrate could undergo hydroxylation on rings B and C at positions which correspond to the four possible sites of attack (C-11β, C-6β, C-7β, 11α) following binding of a C-3 and C-17 terminally functionalized molecule [7–10] within the 11β-hydroxylase. In contrast, the incubation of the 3α-analogue (**9**) resulted in exclusive hydroxylation at the 11β-position. This strongly suggests that the 3α-alcohol, with its axial stereochemistry, is responsible for locking these 3α-substituted steroidal probes in the "normal" binding position within the hydroxylase, thus inhibiting the complete molecular freedom of orientation observed in the 3β-series. It is of note that to achieve this, the polar binding portion of the hydroxylase which attaches the C-3 alcohols must be relatively broad, with an angle of interaction within the enzyme of at least 109.5° to this steroidal carbon.

The occurrence of hydroxylation in the presence of the 3α-alcohol, following 48 h incubation with the fungus, indicates that this functionality is responsible for induction of the 11β-hydroxylase. Thus it would appear that both fully saturated stereoisomers of the C-3 alcohols can induce this hydroxylase [2,4]. This is in contrast to previous studies with *A. tamarii* that have investigated the transformation of none saturated (4-ene-3-one) side-chain containing steroids where activity by the enzyme at C-11 is not observed [3,5,6].

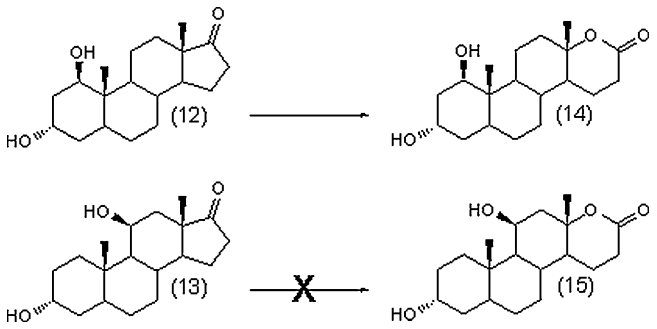

Fig. 3. Transformation of C-1 and C-11 substituted metabolites with *Aspergillus tamarii* KITA.

Incubation of the C-17 keto containing compound (**7**) revealed both 1β- and 11β-hydroxylase activity. In order to determine if these could act as precursor metabolites to the lactones (**14**) and (**15**) they were isolated and subsequently incubated with *A. tamarii*. Interestingly the presence of a 1β-hydroyl group (**12**) did not inhibit the generation of the ring-D lactone as opposed to the 11β containing keto-diol (**13**) which did not undergo further metabolism. This indicates that the presence of the 11β-hydroxy group can inhibit the activity of the terminal Baeyer–Villiger monooxygenase. In support of this the minor hydroxylation pathway following metabolism of progesterone (1) (Fig. 1) generates 11β-hydroxytestosterone which does not undergo further metabolism demonstrating that the 17β-hydroxy oxidase is also inhibited [1]. This is another example in this fungus where a single functional group on a steroid can have a profound effect on route of metabolism [5]. Interestingly, it has been previously demonstrated that the presence of an 11α-alcohol on progesterone [5] has no effect on the activity of either the 17β-hydroxy oxidase or the Baeyer–Villiger oxidase in this organism which, underlines the subtlety of single functional group interaction on metabolic fate of these steroids.

1β-Hydroxylase activity has been previously observed in the transformation of the 3β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one [2], an analogue of compound (**9**), which, suggests that the presence of a 3α-hydroxyl group does not inhibit the activity of this enzyme. Furthermore in both recorded cases the activity of this enzyme was observed post 48 h which suggests that the 1β-hydroxylase responsible is induced. This also appears to be a highly unusual position of hydroxylation especially when compared to the wide range of fungi studied to date which can perform steroidal Baeyer–Villiger oxidation [15–20] including those of which can hydroxylate at C-11 [21–23] (Figs. 2 and 3).

Generation of the lactone (**9**) was achieved in significant yield (53%) following incubation of the 17-keto-3α-ol (**7**) with the Baeyer–Villiger monooxygenase activity observed in the first 24 h. In comparison to a recently reported recombinant cyclohexanone monooxygenase [24] on identical steroidal substrates (**7**, **8**) our whole cell system achieves a 6 times higher yield starting with an identical substrate. Interestingly the 3α-acetate analogue (**8**) appeared to inhibit the activity of the monooxygenase activity where Baeyer–Villiger oxidation of the C-17 ketone was first observed at 48 h. Overall lower yields of lactones (**9**, **10**) were obtained following transformation of the 3α-acetate in comparison to the transformation of the 3α-alcohol (**7**), a pattern also paralleled with the recombinant technology [24].

Aside from oxidation, a minor reduction pathway was observed with transformation of the C-17 ketone of compound (**7**) to a C-17β-alcohol. Although this reduction is common in a wide range of fungi [25] this is the first time that diol generation has been observed with *A. tamarii*.

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