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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

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ABSTRACT

The fungus *Aspergillus tamarii* metabolizes progesterone to testololactone in high yield through a sequential four step enzymatic pathway which, has demonstrated flexibility in handling a range of steroidal probes. These substrates have revealed that subtle changes in the molecular structure of the steroid lead to significant changes in route of metabolism. It was therefore of interest to determine the metabolism of a range of 5-ene containing steroidal substrates. Remarkably the primary route of 5-ene steroid metabolism involved a 3β-hydroxy-steroid dehydrogenase/Δ^5–Δ^4 isomerase (3β-HSD/isomerase) enzyme(s), generating 3-one-4-ene functionality and identified for the first time in a fungus with the ability to handle both dehydroepiansdrosterone (DHEA) as well as C-17 side-chain containing compounds such as pregnenolone and 3β-hydroxy-16α,17α-epoxypregn-5-en-20-one. Uniquely in all the steroids tested, 3β-HSD/isomerase activity only occurred following lactonization of the steroidal ring-D. Presence of C-7 allylic hydroxylation, in either epimeric form, inhibited 3β-HSD/isomerase activity and of the substrates tested, was only observed with DHEA and its 13α-methyl analogue. In contrast to previous studies of fungi with 3β-HSD/isomerase activity DHEA could also enter a minor hydroxylation pathway. Pregnenolone and 3β-hydroxy-16α,17α-epoxypregn-5-en-20-one were metabolized solely through the putative 3β-HSD/isomerase pathway, indicating that a 17β-methyl ketone functionality inhibits allylic oxidation at C-7. The presence of the 3β-HSD/isomerase in *A. tamarii* and the transformation results obtained in this study highlight an important potential role that fungi may have in the generation of environmental androgens.

1. Introduction

Aspergillus tamarii is unusual amongst fungi in that it converts progesterone **1** to testololactone **5** in high yield [1] as the final product of a flexible four step enzymatic pathway [2]. Initial Baeyer–Villiger oxidation converts the progesterone **1** to testosterone acetate **2**, which is then hydrolyzed generating testosterone **3**, followed by oxidation to afford androst-4-ene-3,17-dione **4** and finally ring-D Baeyer–Villiger oxidation of the C-17 ketone giving testololactone **5** (Fig. 1). These initial studies also demonstrated isolation of the 20(R)-hydroxy analogue **6** of progesterone **1** [3] showing that there is a competitive equilibrium present that exists between the reductase forming the alcohol **6** and the oxidase which regenerates **1**. A minor endogenous hydroxylation pathway is also present within the organism which acts solely on testosterone **3** forming 11β-hydroxytestosterone with no further transformation [1] when starting with progesterone **1** as the molecular probe.

Previous studies involving other steroidal substrates with this fungus have highlighted that a very small change in structure of the steroid can result in markedly different metabolic fate in *A. tamarii*. For example, a comparison of steroids functionalized at C-6 or C-11 demonstrated that a single functional group could control metabolic fate. Substitution at C-6 facilitates access into novel reductive pathway acting exclusively on ring-A and C-11 substitution controls entry into, and degree of metabolism in, the lactonization pathway [4]. Testololactone **5** which retains a 4-ene double bond, and is the end product of the lactonization of progesterone **1** does not undergo further metabolism even with an extra 5 days incubation with the organism. However, transformation of fully saturated steroidal lactones which have an increase in angle of 11° at the A/B ring junction compared to **5** was only handled within the hydroxylation pathway. However, they demonstrated remarkable flexibility within the enzyme site, binding to the hydroxylase in all 4 theoretical binding orientations [5]. Incubation of *quasi* reverse steroids with *A. tamarii* which have a 14-en-16-one functionalized ring-D was exclusively handled in the hydroxylation pathway [6]. It was, therefore, of interest to determine the metabolic fate of 5-ene functionalized steroids to see if they would be metabolized through the endogenous lactonization

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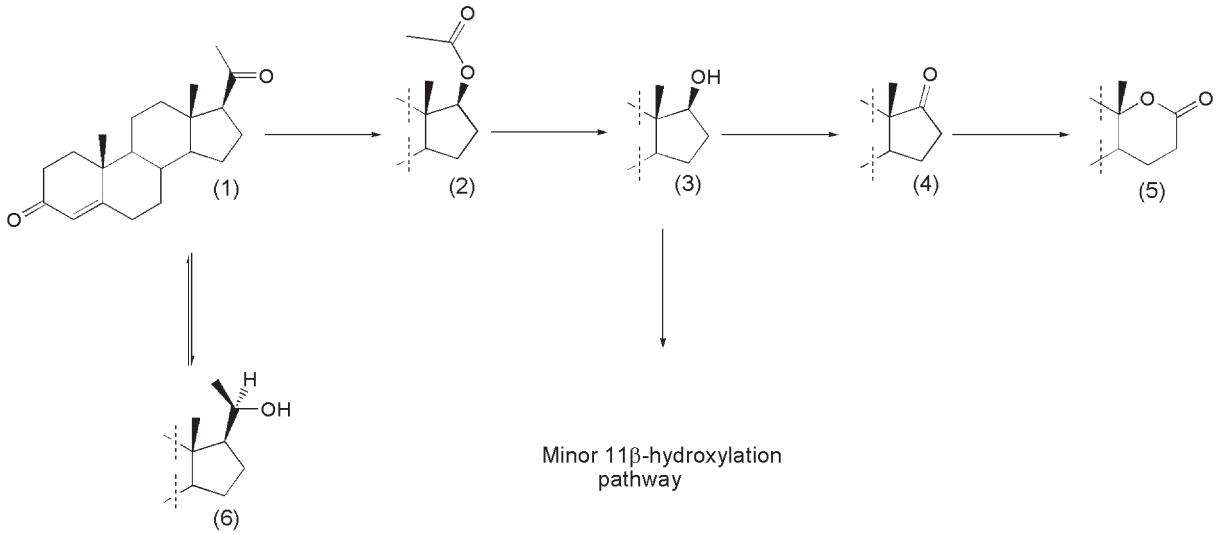

Fig. 1. Endogenous lactonization pathway present in *Aspergillus tamarii KITA*, which converts progesterone **1** to testololactone **5** with preceding C-20 keto-alcohol reducto-oxidase interconverting **1** and **6** and the minor 11β-hydroxylation pathway from testosterone **3**.

or hydroxylation pathway, or would these steroidal probes reveal new metabolic pathways within this fungus and gauge the potential for generation of new biologically active compounds.

2. Materials and methods

2.1. Chemicals and reagents

Dehydroepiandrosterone (DHEA) **7**, 3β,17β-dihydroxyandrost-5-ene **9**, pregnenolone **10** and 3β-hydroxy-16α,17α-epoxypregn-5-en-20-one **11** were all purchased from Steraloids UK. 3β-Hydroxy-13α-androstan-5-en-17-one **8** was synthesized as described previously [7], and had identical spectral characteristics to an authentic sample [8] which was found to be in excess of 99% purity following elemental analysis (found: C, 78.81; H, 9.85. $C_{19}H_{28}O_2$ calc. for C, 79.12; H, 9.78%). All other chemicals and reagents used were supplied by the Aldrich Chemical Company (UK). Solvents were of analytical grade; light petroleum ether refers to the fraction with a boiling point 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

Aspergillus tamarri KITA (QM 1223) was obtained from the collection at CABI Bioscience (UK). Stock cultures were grown on potato dextrose agar (Oxoid, UK) slopes (3 days) and maintained at 4 °C until use. 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 each containing 300 mL of sterile media and were incubated for 72 h at 24 °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, 2 g of steroid, dissolved in dimethylformamide (DMF) and made up to 10 mL, 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 separation 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 anhydrous sodium sulfate and the solvent was 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 light 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 light petroleum ether in ethyl acetate or pure ethyl acetate. A 50:50 sulfuric acid in methanol spray was used to develop the TLC plates.

2.5. Analysis and identification of metabolites

Characteristic shift values [9,10] in the ^{1}H and ^{13}C NMR spectra from the starting compounds were used to determine metabolite structure in combination with DEPT analysis to identify the nature of the carbon. Spectra were recorded on a Bruker WM 360 spectrometer, all samples were analysed in deuteriochloroform or deuteriopyridine using tetramethylsilane as the internal standard. Infra-red spectra were recorded directly on a Nicolet avatar 320 FT-IR fitted with a Smart Golden Gate®.

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 media) for each of the substrates **7**, **8**, **9**, **10**, **11**. 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 presence and steroidal nature of the extracts.

2.7. Crystallographic structure determination of 17a-oxa-D-homo-androst-5-en-3β,7α-diol **14**

$C_{19}H_{28}O_4$, Mr 320.41: orthorhombic space group $P2_12_12_1$ (No. 19), a=8.5466(4), b=10.4454(6), c=18.30.40(11) Å, $\alpha=\beta=\gamma=90°$,

V=1634.05(16) Å^3, Z=4, $D_{calc.}$=1.30 Mg/m^3, u=0.09 mm^{-1}, F(000) 696. Data were collected using a crystal of dimension 0.10×0.10×0.05 mm^3 on a KappaCCD diffractometer. A total of 6693 reflections were collected for $3.80<\theta<26.03°$ and $-10\le h\le 8$, $-12\le k\le 9$, $-20\le 1\le 22$. There were a total of 3150 independent reflections and 2762 reflections with $I>2\sigma(I)$ used in the refinement. No absorption correction was applied. The structure was solved by direct methods and refined using SHELXL-97. The final R indices were [$I>2\sigma(I)$ R_1=0.057, wR_2=0.151 and R indices (all data) R_1=0.067, wR_2=0.156. The goodness-of-fit on F^2 was 1.144 and the largest difference peak and hole was 0.20 and −0.21e Å^{-3}.

3. Results

3.1. Products isolated following transformation

3.1.1. Transformation of dehydroepiandrosterone 7

Starting material DHEA **7** (197 mg, 9.8%) was recovered from the chromatography column with 20% ethyl acetate in light petroleum ether. Comparison of the ^{1}H and ^{13}C NMR spectra to an authentic sample [11] confirmed structural identity. Elution with 50% ethyl acetate in light petroleum ether afforded 17a-oxa-D-homo-androst-5-en-3β-ol **13** (541 mg, 27%) which crystallized from ethyl acetate as needles, m.p. 185 °C (found: C, 72.37; H, 8.80 $C_{19}H_{28}O_3.0.5H_2O$ requires C, 72.81; H, 9.32%); IR$_{\upsilon max}$ cm^{-1}=3442, 1722, 1651; ^{1}H NMR ($CDCl_3$) 0.98 (3H, s, 19-H), 1.34 (3H, s, 18-H) 3.54 (1H, tt, J=5 Hz, J=12 Hz, 3α-H), 5.35 (1H, d, J=5 Hz, 6-H).

17a-Oxa-D-homo-androst-5-en-3β,7α-diol **14** (416 mg, 20.8%) was eluted at 60% ethyl acetate in light petroleum ether and crystallized from ethanol as cubes, m.p. 213–215 °C (found C, 71.55; H, 9.11, $C_{19}H_{28}O_4$ requires C, 71.22; H, 8.81%); IR$_{\upsilon max}$ cm^{-1}=3310 br, 1737, 1675; ^{1}H NMR ($CDCl_3$) 0.91 (3H, s, 19-H), 1.24 (3H, s, 18-H), 3.57 (1H, tt, J=5 Hz, J=10 Hz, 3α-H), 3.95 (1H, s, 7β-H), 5.72 (1H, d, J=5 Hz, 6-H). Continued elution with 60% ethyl acetate in light petroleum ether afforded 17a-oxa-D-homo-androst-4-ene-3,17-dione (testololactone) **5** (114 mg, 5.7%) the structure of which was confirmed by comparison of ^{1}H and ^{13}C NMR spectra with that of an authentic sample [2]. The final metabolite eluted from pure ethyl acetate and was identified as 3β,7β-dihydroxyandrost-5-en-17-one **12** (240 mg, 12%) and was isolated as a gum (HRMS ESI calc. for M+Na$^+$ 327.193 $C_{19}H_{28}O_3Na$ requires 327.193); IR$_{\upsilon max}$ cm^{-1}=3236 br, 1739, 1666; ^{1}H NMR ($CDCl_3$) 0.89 (3H, s, 18-H), 1.07 (3H, s, 19-H), 3.56 (1H, tt, J=5 Hz, J=11 Hz, 3α-H), 3.96 (1H, m, $\omega/2$=3.5 Hz, 7α-H), 5.31 (1H, s, 6-H).

3.1.2. Transformation of 3β-hydroxy-13α-androst-5-en-17-one 8

Both products of transformation were eluted at a concentration of 50% ethyl acetate in light petroleum ether initially affording 3β,7β-dihydroxy-13α-androst-5-en-17-one **15** (140 mg, 7%) as a gum (HRMS ESI: calc. for M+Na$^+$ 327.190, $C_{19}H_{28}O_3Na$ requires 327.193); IR$_{\upsilon max}$ cm^{-1}= 3226 br, 1726, 1698; ^{1}H NMR ($CDCl_3$) 0.91 (3H, s, 18-H), 1.00 (3H, s, 19-H), 3.55 (1H, tt, J=5 Hz, J=10 Hz, 3α-H), 3.98 (1H, d, J=8 Hz, 7α-H), 5.28 (1H, s, 6-H). 3β,7α-Dihydroxy-13α-androsta-1,5-dien-17-one **16** (31 mg, 1.6%) was also isolated as a gum (HRMS ESI: calc. for M+Na$^+$ 325.175, $C_{19}H_{26}O_3Na$ requires 325.177); IR$_{\upsilon max}$ cm^{-1}=3453 br, 1719, 1700, 1696; ^{1}H NMR ($CDCl_3$) 0.83 (3H, s, 18-H), 1.03 (3H, s, 19-H) 3.56 (1H, m, $\omega/2$=15.6 Hz, 3α-H), 4.00 (1H, s, 7β-H), 5.66 (1H, d, J=4.0 Hz, 6-H), 6.80 (1H, d, J=8 Hz, 1-H), 7.14 (1H, d, J=8 Hz, 2-H).

3.1.3. Transformation of 3β,17β-dihydroxyandrost-5-ene 9

3β,17β-Dihydroxyandrost-5-ene **9** (546 mg, 27.3%) eluted from the chromatography column at a concentration of 50% ethyl acetate in light petroleum ether, comparison of the ^{1}H and ^{13}C NMR spectra to that of an authentic sample confirmed molecular structure. 17a-Oxa-D-homo-androst-5-en-3β-ol **13** (392 mg, 19.6%) eluted at 55% ethyl acetate in light petroleum ether and was identified by comparison of ^{1}H and ^{13}C NMR spectra (see Section 3.1.1). Elution with 60% ethyl acetate in light petroleum ether afforded 17a-oxa-D-homo-androsta-1,5-dien-3β-ol **17** (300 mg, 15%) as a gum (HRMS ESI: calc. for M+Na$^+$, 325.177, $C_{19}H_{26}O_3Na$ requires 325.177); IR$_{\upsilon max}$ cm^{-1}=3398, 1700, 1696, 1684; ^{1}H NMR ($CDCl_3$) 0.97 (3H, s, 19-H), 1.32 (3H, s, 18-H), 3.56 (1H, br s, 3α-H), 5.35 (1H, d, J=5 Hz, 6-H), 6.78 (1H, d, J=8.5 Hz, 1-H), 7.14 (1H, d, J=8.5 Hz, 2-H).

3.1.4. Transformation of pregnenolone 10

Pregnenolone **10** (880 mg, 44%) was recovered from the chromatography column with 30% ethyl acetate in light petroleum ether. Comparison of the ^{1}H and ^{13}C NMR spectra to an authentic sample [11] confirmed structural identity. Elution with 60% ethyl acetate in light petroleum ether afforded 17a-oxa-D-homo-androst-5-en-3β-ol **13** (420 mg, 21%) which was identified by comparison of the ^{1}H and ^{13}C NMR spectra to that of an authentic sample. Testololactone **5** (513 mg, 25.6%) eluted from the chromatography column in 70% ethyl acetate in light petroleum ether and was identified by comparison of ^{1}H and ^{13}C NMR spectra to that of an authentic sample [2].

3.1.5. Transformation of 3β-hydroxy-16α,17α-epoxy pregn-5-en-20-one 11

16β-Hydroxy-17a-oxa-D-homo-androsta-4-ene-3,17-dione **18** (392 mg, 19.6%) was isolated following elution with 60% ethyl acetate in light petroleum ether, from which it crystallized as cubes, m.p. 267–269 °C, Lit., 268 °C [2]; IR$_{\upsilon max}$ cm^{-1}=3355, 1722, 1649, 1609; ^{1}H NMR ($CDCl_3$) 1.17 (3H, s, 19-H), 1.39 (3H, s, 18-H), 4.47 (1H, dd, J=5 Hz, J=10 Hz, 16α-H), 5.76 (1H, s, 4-H). These spectra and ^{13}C NMR were compared to that of an authentic sample [2] and found to be consistent.

3.2. Identification of metabolites

Incubation of DHEA **7** resulted in the isolation of 4 products of metabolism. 17a-Oxa-D-homo-androst-5-en-3β-ol **13** was recovered following elution with 50% ethyl acetate in petroleum ether. In comparison to the ^{1}H NMR spectrum of DHEA **7** no new signals were present for **13**; however, the 18-methyl resonance signal had undergone a significant downfield shift (0.42 ppm) suggesting that heteroatom insertion had occurred. Loss of the 5 ring-D ketone resonance in DHEA **7** at 221.32 ppm, replaced by a nonprotonated resonance in the product ^{13}C NMR spectrum at 171.45 ppm, was consistent with ring-D expansion. The position of oxygen insertion in the 17a position was confirmed by a significant downfield shift in the product C-13 signal (Δ 35.60 ppm), and a displacement of the 18-methyl resonance signal from 13.53 ppm in DHEA **7** to 20.05 ppm in the product ^{13}C NMR spectrum.

Elution with 60% ethyl acetate in petroleum ether afforded 17a-oxa-D-homo-androst-5-en-3β,7α-diol **14**. Comparison of the product ^{1}H NMR spectrum to that of **13** revealed a new signal at 3.95 ppm (1H, s), indicating monohydroxylation and this was fully supported by elemental analysis. Comparison of the ^{13}C NMR spectrum demonstrated downfield β-carbon shifts for C-6 (Δ 4.42 ppm), C-8 (Δ 8.99 ppm) and upfield γ-carbon shifts for C-9 (Δ 7.79 ppm), all of which fully support hydroxylation at C-7α. As did X-ray crystallography confirming ring-D lactonization and hydroxylation with axial stereochemistry (Fig. 2).

Further elution with 60% ethyl acetate in petroleum ether afforded 17a-oxa-D-homo-androst-4-ene-3,17-dione **5**. In comparison to the spectra of the starting material the ^{1}H NMR of **5** demonstrated a significant downfield shift for the 18-methyl protons (Δ 0.48 ppm) indicating oxygen insertion into ring-D. The product ^{13}C NMR spectrum supported this notion with downfield shifts observed for C-13 (Δ 35.93 ppm), C-18 (Δ 7.33 ppm) and an upfield shift for C-17 (Δ 49.40 ppm), all of which are consistent with lactonization of ring-D. Absence of the signatory 3α-H resonance (tt) from the ^{1}H NMR spectrum of the product combined with significant

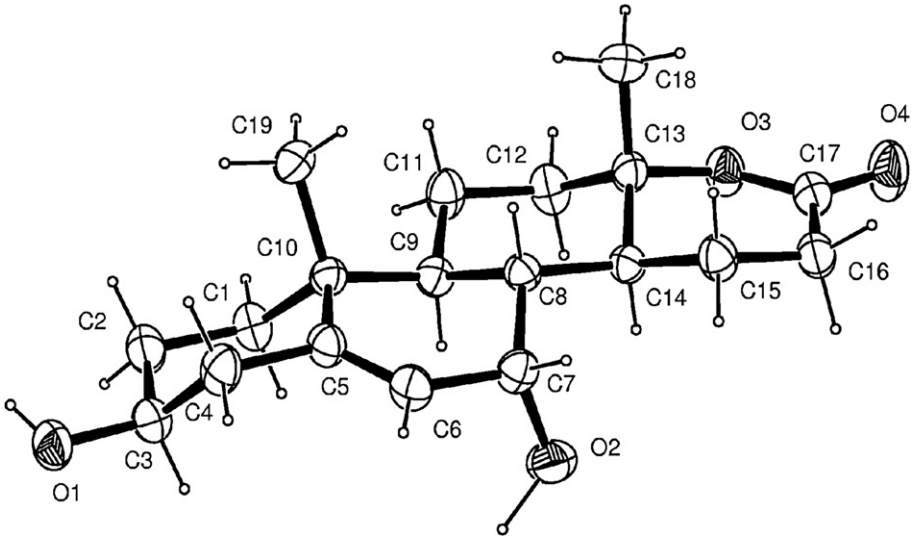

Fig. 2. X-ray crystal structure of 17a-oxa-D-homo-androst-5-en-3β,7α-diol **14**, a metabolite isolated following metabolism of DHEA **7**.

increases in the 19-methyl (Δ 0.14 ppm) and C-6 (Δ 0.38 ppm) resonance signals indicated isomerization of the double bond with its formation between C-4 and C-5 and oxidation of the C-3 alcohol to a ketone. Double bond migration was fully supported in the product ^{13}C NMR spectrum with downfield shifts observed for C-4 (Δ 81.94 ppm), C-5 (Δ 28.22 ppm), C-10 (Δ 2.58 ppm). Oxidation of the alcohol was fully supported with loss of a methine signal in the starting material ^{13}C NMR spectrum at 71.49 ppm being replaced by a new non-protonated resonance signal in the product ^{13}C NMR spectrum at 199.09 ppm.

3β,7β-Dihydroxyandrost-5-en-17-one **12** eluted from the chromatography column in pure ethyl acetate. A new signal in the ^{1}H NMR spectrum of the product at 3.96 ppm (m, $\omega/2$=3.5 Hz) compared to DHEA **7** indicated monohydroxylation which was confirmed by accurate mass measurement. Downfield shifts in the product ^{13}C NMR resonance signals for C-6 (Δ 4.59 ppm), C-8 (Δ 8.94 ppm) and an upfield shift for C-9 (Δ 3.49 ppm) supported hydroxylation at C-7.

Incubation of 3β-hydroxy-13α-androst-5-en-17-one **8** generated two products of metabolism, both of which eluted from the chromatography column at a concentration of 50% ethyl acetate in light petroleum ether. The ^{1}H NMR spectrum of 3β,7β-dihydroxy-13α-androst-5-en-17-one **15** in comparison to that of the starting material demonstrated no significant shifts for the methyl groups, but did contain a new resonance signal at 3.98 ppm. This suggested hydroxylation distant from the methyl groups at an equatorial proton such as C-7β. Carbons beta to C-7, C-6 (Δ 4.88 ppm), and C-8 (Δ 7.44 ppm) had undergone significant downfield shifts, and C-14 (Δ 1.00 ppm) had undergone an upfield shift, all of which supported hydroxylation at C-7β.

The second product isolated was identified as 3β,7α-dihydroxy-13α-androsta-1,5-dien-17-one **16**.

Comparison of the product ^{1}H NMR spectrum revealed no significant shifts for either methyl group. This, coupled with a new C*H*OH signal at 4.00 ppm (s) and a downfield shift for the 6-H, strongly suggested allylic oxidation at carbon 7 with alpha stereochemistry. This was supported in the product ^{13}C NMR spectrum in which β-carbon shifts were observed for C-6 (Δ9.59 ppm) and C-8 (Δ 5.32 ppm) and γ-carbon upfield shifts for C-9 (Δ 9.60 ppm) and C-14 (Δ 5.99 ppm). Two new one proton signals at 6.80 ppm and 7.14 ppm indicated the insertion of a new double bond into ring-A. This was confirmed by a DEPT experiment which demonstrated the loss of two methylene signals at 36.87 ppm and 31.62 ppm, replaced by two methine resonances at 115.56 and 123.58 ppm. This was consistent with previously observed metabolic behaviour by *A. tamarii* which resulted in double bond insertion between C1 and C2. The resonance values for the obtained 1,5-diene-3β-ol structure were consistent with those previously reported [12].

Incubation of 3β,17β-dihydroxyandrost-5-ene **9** resulted in the isolation of two metabolites. 17a-oxa-D-homo-androst-5-en-3β-ol **13** was recovered following elution with 55% ethyl acetate in petroleum ether. The 17α-H resonance signal in the product ^{1}H NMR spectrum (3.58 ppm, t) was not present in the ^{1}H NMR spectrum of **13**. The 18-methyl protons in **13** underwent a significant downfield shift (0.54 ppm), indicative of heteroatom insertion in ring-D. The lactonization of this ring was supported by the ^{13}C NMR spectrum of the product where the nonprotonated resonance for C-13 had undergone a significant downfield shift (39.36 ppm) and a new nonprotonated resonance for C-17 appeared at 171.45 ppm. Comparison to an authentic sample fully confirmed this metabolites' structure.

The second metabolite, contained new resonance signals in the product ^{1}H NMR spectrum at 6.78 ppm (1H) and 7.14 ppm (1H), and this suggested the presence of a double bond. This was supported by the ^{13}C NMR spectrum in which two methylene signals in **13** at 36.91 and 31.46 ppm were replaced by two methine signals at 115.57 and 130.55 ppm. The resonance values were consistent with a C1–C2 double bond generating the diene structure in **17** [12].

Transformation of pregnenolone **10** resulted in the isolation of two metabolites. Both metabolite spectra were devoid of the side-chain methyl-ketone resonance signals in the ^{1}H NMR spectrum at 2.07 ppm (21-H_3) and ^{13}C NMR spectra at 31.57 ppm (C-21) and 209.72 ppm (C-20) and this fully supported the fact that removal of the side-chain had occurred. A significant downfield shift was observed for the 18-methyl resonance signals for both products metabolite spectra from the 18-methyl resonance in pregnenolone (0.63 ppm) [11] to 1.30 ppm for **13** and 1.36 ppm for **5** due to heteroatom insertion into ring-D via Baeyer–Villiger oxidation. The structure of **13** was readily identified by comparison with authentic spectra *vide supra*, as was the isomerization product testololactone **5** [2].

Transformation of 3β-hydroxy-16α,17α-epoxypregn-5-en-20-one **11** resulted in the isolation of one product of biotransformation **18** which was identified spectroscopically. Comparison of the ^{1}H NMR spectrum of **11** and that of **18** revealed loss of the 21-methyl signal, suggesting removal of the methyl ketone side-chain. This was supported by the ^{13}C NMR spectrum of **18** which contained only 19

carbon resonance signals and was devoid of signals present in **11** for C-20 (205.10 ppm) and C-21 (26.00 ppm). A significant downfield shift was observed in the ^{1}H NMR spectrum of **18** for the 18-methyl group (Δ 0.76 ppm), which is consistent with heteroatom insertion into ring-D. This was supported in the ^{13}C NMR in which C-13 had undergone a downfield shift of 43.15 ppm. The heteroatom insertion suggested lactonization of ring-D which would undergo concomitant ring expansion and retain a non-protonated carbon resonance, present in the spectrum of **18** at 174.91 ppm (C-17) [13]. The epoxide 16β-proton (3.68 ppm) was absent from the spectrum of **18** indicating epoxide opening. This was supported by a new signal in the ^{1}H NMR spectrum of **18** at 4.47 ppm (dd), which demonstrated the presence of a hydroxyl group as did the new *CH*OH ^{13}C NMR resonance signal at 64.45 ppm. The multiplicity of the signal in the ^{1}H NMR spectrum confirmed equatorial stereochemistry which is consistent with a 16β-hydroxyl group.

The double bond signal in the ^{1}H NMR spectrum of the product had undergone a significant downfield shift (Δ 0.44 ppm), as had the ^{13}C NMR resonance for C-5 (Δ 27.84 ppm). This, coupled with the generation of a new nonprotonated carbon at C-3, indicated isomerization of the double bond had taken place with oxidation of the 3β-alcohol to a ketone generating the 3-one-4-ene system in **18**. Comparison of the spectra to an authentic sample [2] fully supported the proposed structure.

3.3. Determination of the metabolic pathway

^{1}H NMR was used to follow the steroid transformations and thus enable the determination of time of formation. Lactonization of DHEA **7** ring-D ketone was identified following 24 h incubation, with the 18-methyl resonance signal in shifting from 0.88 ppm in **7** to 1.3 ppm in **13** following heteroatom insertion. Isomerization of **13** had occurred following 48 h incubation with the emergence of the 4-H signal at 5.8 ppm in **5**. The allylic hydroxylation occurred following 48 h incubation with *CH*OH signal for hydroxylation at C-7α and 7β at 3.9 ppm. The transformation of **8** also resulted in allylic oxidation resulting in C-7β and C-7α hydroxylation in compounds **15** and **16** respectively. Insertion of the ring-A double bond into **8** forming **16** was observed at the 48 h time point by two resonance signals (1H) at 6.8 ppm and 7.1 ppm. Comparable double bond resonance signals were also identified following transformation of **9** to **17** following 48 h.

As with DHEA **7**, lactone formation was observed at the 24 h time point for each of the compounds **9**, **10**, **11**, identified by the

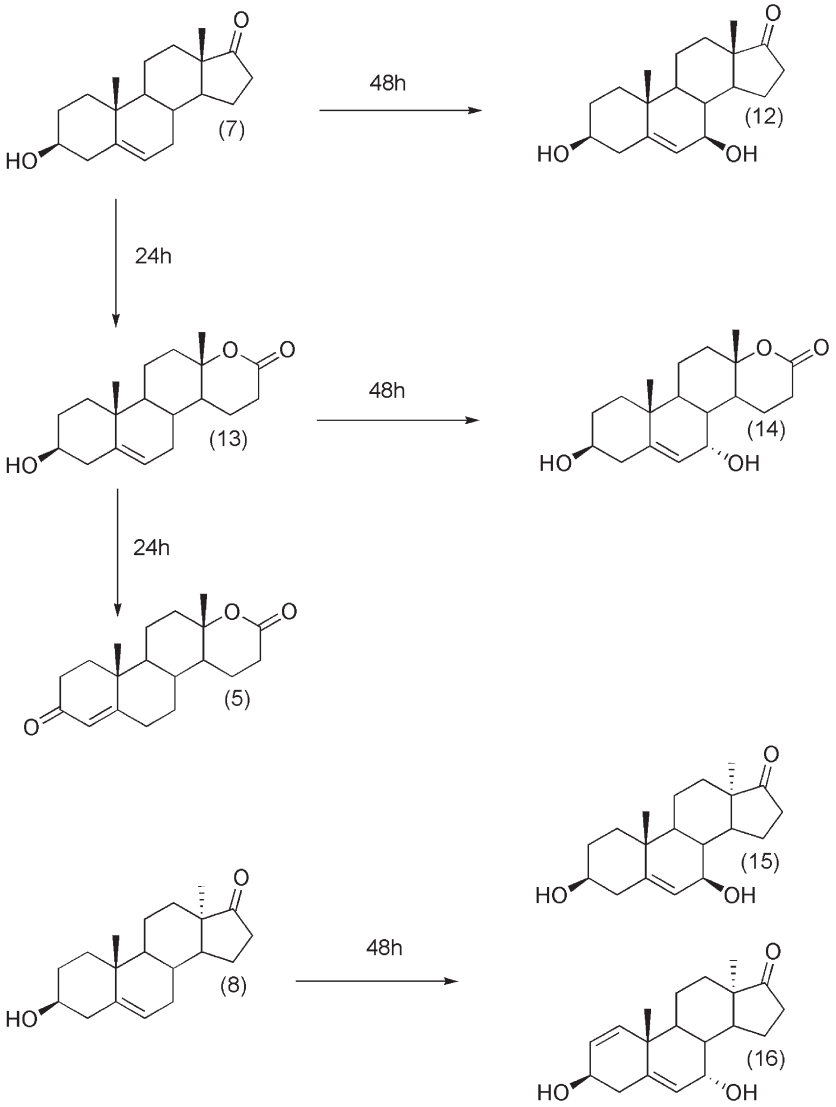

Fig. 3. Metabolism of DHEA **7** and its 13α-methyl analogue **8**. DHEA 7 undergoes ring-D lactonization, allylic oxidation and isomerization of the 5-ene double bond and oxidation of the 3β-hydroxyl generating the 4-en-3-one ring-A system. Comparatively **8** undergoes allylic oxidation and the case of **16** C1–C2 double bond formation.

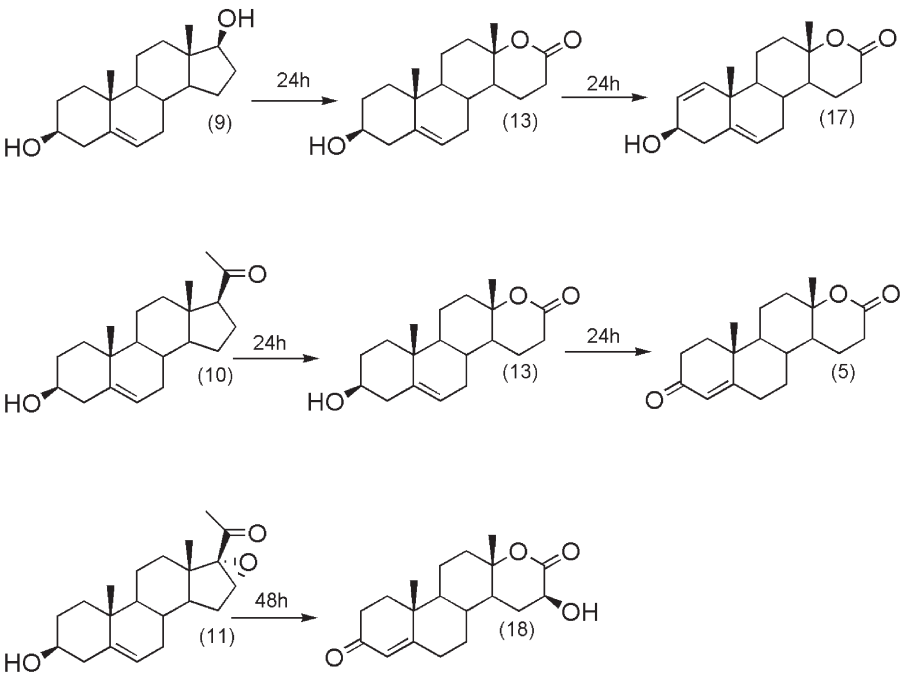

Fig. 4. Metabolism of 5-ene steroids **9**, **10**, **11** all of which undergo ring-D lactionization and isomerization of the 5-ene double bond and oxidation of the 3β-hydroxyl generating the 4-en-3-one ring-A system for **10**, **11**. Comparatively metabolism of **9** results in C1–C2 double bond formation.

characteristic downfield shift for the 18-methyl protons. Isomerization of the 5-ene double bond was observed at the 48 h post incubation of compounds **10** and **11**, with the appearance of the 4-H signal in the ^{1}H NMR spectrum.

4. Discussion

The predominant metabolism of the incubated 5-ene steroids by *A. tamarii* occurs through a newly identified fungal 3β-hydroxy-steroid dehydrogenase/Δ^5–Δ^4 isomerase (3β-HSD/isomerase) putative pathway, resulting in the generation of a 3-one-4-ene system on ring-A with the capability of handling DHEA **7** as well as C-17 side chain containing steroids such as pregnenolone **10** and 3β-hydroxy-16α,17α-epoxypregn-5-en-20-one **11**. This is in contrast to previous studies in which the present 3β-HSD/isomerase would only be active in the presence of a C-17-ketone as in DHEA or a C-17 side chain such as pregnenolone but not both [14,15]. For all of the steroids being handled by the 3β-HSD/isomerase enzyme system a number of common structural steroidal features were present. All 3β-HSD/isomerase activity occurred following ring-D lactonization, as

Table 1
^{13}C NMR data for starting materials determined in $CDCl_3$ or d_5-pyr[§]

Carbon atom	Compounds				
	7	8	9[§]	10	11
1	37.17	36.87	37.86	37.23	37.08
2	31.51	31.62	32.82	31.53	31.48
3	71.49	71.64	71.74	71.60	71.58
4	42.16	42.01	44.04	42.18	42.13
5	141.06	140.32	141.52	140.78	141.12
6	120.85	120.92	121.65	121.23	120.96
7	31.41	33.05	32.40	31.74	31.40
8	31.47	34.10	32.82	31.81	29.68
9	51.72	51.07	51.26	49.92	50.23
10	36.62	36.73	37.52	36.49	36.64
11	20.34	22.05	21.63	21.06	20.41
12	30.76	31.45	33.12	38.80	31.32
13	47.54	50.06	43.78	44.00	41.47
14	50.19	47.96	52.19	56.87	45.49
15	21.87	22.97	24.36	22.76	27.49
16	35.84	34.17	31.44	24.47	60.55
17	221.32	222.35	81.79	63.67	71.01
18	13.53	25.15	12.21	13.22	15.17
19	19.42	19.14	20.17	19.38	19.32
20				209.72	205.10
21				31.57	26.00

Compounds are numbered as follows: dehydroepiandrosterone **7**, 3β-hydroxy-13α-androst-5-en-17-one **8**, 3β,17β-dihydroxyandrost-5-ene **9**, pregnenolone **10**, 3β-hydroxy-16α,17α-epoxypregn-5-en-20-one **11**.

Table 2
^{13}C NMR data for transformation products determined in $CDCl_3$

Carbon atom	Compounds							
	5	12	13	14	15	16	17	18
1	36.27	36.87	36.91	36.59	36.59	115.56	115.57	35.47
2	34.51	31.40	31.46	29.13	31.34	123.58	130.55	33.78
3	199.09	72.88	71.46	71.02	71.45	71.35	71.51	199.05
4	124.10	41.56	41.88	41.34	41.35	41.66	41.79	124.26
5	169.28	143.72	140.67	143.93	143.45	145.84	140.61	168.96
6	33.03	125.44	120.55	124.97	125.80	130.51	120.60	32.27
7	31.19	71.29	31.07	63.36	76.30	63.89	28.78	30.67
8	38.73	40.41	34.45	43.44	41.54	39.42	34.43	38.68
9	53.27	48.23	48.99	41.20	49.99	41.47	48.94	52.12
10	39.20	36.66	36.59	36.40	37.08	37.62	36.58	38.34
11	22.59	20.39	21.95	21.44	22.66	21.65	21.95	21.81
12	39.79	31.21	38.92	39.01	34.63	36.68	38.88	39.05
13	83.47	47.88	83.14	83.29	50.41	49.90	83.31	84.62
14	46.40	51.17	46.73	43.34	47.25	41.97	46.68	46.54
15	20.61	24.19	19.90	21.95	23.40	22.47	19.85	30.21
16	29.22	36.00	28.80	31.41	31.89	31.13	36.88	64.45
17	171.92	221.46	171.45	171.60	223.28	222.98	175.81	174.91
18	20.86	13.59	20.05	20.26	25.32	25.15	20.07	17.37
19	18.19	19.18	19.28	18.95	18.75	18.15	19.27	19.27
20								
21								

Compounds are numbered as follows: testololactone **5**, 3β,7β-dihydroxy-androst-5-en-17-one **12**, 17a-oxa-D-homo-androst-5-en-3β-ol **13**, 17a-oxa-D-homo-androst-5-en-3β,7α-diol **14**, 3β,7β-dihydroxy-13α-androst-5-en-17-one **15**, 3β,7α-dihydroxy-13α-androsta-1,5-dien-17-one **16**, 17a-oxa-D-homo-androsta-1,5-dien-3β-ol **17**, 16β-hydroxy-17a-oxa-D-homo-androst-4-ene-3,17-dione **18**.

confirmed by timed experiments (Figs. 3 and 4), and it is an interesting notion that the aforementioned enzyme is activated. Allylic oxidation at C-7 either in the presence, or absence, of a lactone on ring-D inhibits 3β-HSD/isomerase activity. This may be due to individual effects and/or a combination of the increased polarity of the monohydroxylated derivatives, or the allylic hydroxyl group at C-7 inhibits access into a hydrophobic pocket in the enzyme, or affects orientation of the molecule to the active site, inhibiting oxidation of the 3β-alcohol to the 3-ketone which is the first mechanistic step carried out by the dehydrogenase [16].

Interestingly, DHEA **7** also underwent metabolism within the two commonly observed xenobiotic metabolic routes in *A. tamarii*, the lactonization pathway, generating D-homo derivatives and the minor hydroxylation pathway (C-7α, C-7β). This is distinct in comparison to previously observed patterns of steroid metabolism with this organism, where one predominant route of metabolism has been observed, either through the lactonization pathway [2,4] or the hydroxylation pathway [5,6]. So clearly the 5-ene double bond coupled with a 5-carbon ring-D retaining a 17-ketone but not a C-17 methyl ketone side-chain, facilitates access into all three observed metabolic pathways. In contrast the metabolism of the 13α-methyl analogue resulted only in entrance into the hydroxylation pathway. In part this must be due to the out-of-plane ring-D inhibiting access of the Baeyer–Villiger oxidase to the 17-ketone. This is further supported as metabolism of the C-17 substituents [alcohol (**9**), ketone (**7**), methyl ketone (**10**), epoxymethyl ketone (**11**)] in the presence of a normal series 13β-methyl did not affect access into the lactonization pathway. This strongly supports the notion that the out-of-plane ring system is structurally fundamental in inhibiting access to the Baeyer–Villiger oxidase. Furthermore, the presence of a 5-ene double bond has no significant effect on inhibiting access of Baeyer-Villager oxidases to compounds **7**, **10**, **11**, or alcohol oxidase to compound **9**. Interestingly no allylic oxidation was observed following metabolism of **9**, **10**, **11**, suggesting that 17β-stereochemical orientation of the 17β-alcohol **9** and 17β-methyl ketones **10** and **11** inhibit activity of the C-7 allylic hydroxylase compared to the planar C-17 ketone of DHEA **7**. It is intriguing that the 13α-methyl analogue **8** of DHEA **7** also undergoes allylic oxidation at C-7, clearly demonstrating that the inverted 13α-methyl group or the out-of plane ring-D does not inhibit the allylic oxidase. This suggests a pivotal role for the C-17 ketone in the case of *A. tamarii*, for this allylic oxidation to occur compared to other fungi which hydroxylate at the C-7 allylic carbon irrespective of the C-17 functionality [11,17].

As has been observed in previous studies with *A. tamarii* with structurally diverse steroids [1,2], a C1–C2 double bond has been generated on ring-A of compound **16** (and also compound **17**). This has been attributed to result from the dehydration of a 1β-alcohol as opposed to a dehydrogenation reaction [5]. Transformation of **11** demonstrates a highly unusual epoxide opening which has been observed previously following transformation of epoxyprogesterone [2]. The inversion of stereochemistry following opening of the epoxide is most likely generated through either epimerization via an epimerase enzyme, or the 16α-alcohol is oxidised to a 16-ketone which is then reduced to the 16β-alcohol. Alternately, the 16α,17α-epoxide **11** is hydrolysed to the 16β,17α-diol, which involves nucleophilic attack of water at the less hindered C16. Scission at C17–C20 gives the 16β-hydroxy-17-ketone. The latter is then converted to the hydroxylactone **18** via Baeyer–Villiger chemistry (Tables 1 and 2).

3β-HSD/isomerase enzymes are pivotal in conversion of 5-ene-3β-hydroxy steroids into 4-ene-3-keto steroids, generating a wide range of classes of steroids such as androgens, estrogens, glucocorticoids, progestogens and mineralocorticoids [18], and have been identified in a range of different species including amphibians, birds and mammals [19–25]. The presence of the 3β-HSD/isomerase enzyme system in *A. tamarii* and other fungi [26–28] raises an intriguing question, do these organisms have a role of androgen generation in nature and if so, what degree of impact does it have on the environment? Considering the high biomass of fungi globally, with only 74000 of the estimated 1.5 million fungi described [29], either terrestrial or aquatic, the effects could potentially be a significant, but yet unexplored link to environmental androgens. For example, there could be a role in the observed induction of masculinization in mammals or sex change in fish [30–32], or initiate other observed forms of endocrine disruption [32,33] including behaviour modification [34,35] by exogenously generated androgens.

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