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Flexibility of the endogenous progesterone lactonisation pathway in *Aspergillus tamarii KITA*: transformation of a series of cortical steroid analogues

A. Christy Hunter*, Natasha E. Carragher

Molecular Targeting and Polymer Toxicology Group, School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, East Sussex BN2 4GJ, UK

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Abstract

A range of cortical steroids have been transformed by the fungus *Aspergillus tamarii*, which has the ability to convert progesterone to testololactone in high yield through a four step enzymatic pathway. 16α,17α-Epoxyprogesterone underwent a rare epoxide opening resulting in a unique inversion of stereochemistry to give 16β-hydroxy-17α-oxa-D-homo-androst-4-en-3,17-dione. The metabolism of deoxycorticosterone resulted in relatively efficient transformation to testololactone with no other products isolated. Transformation of 17α-hydroxyprogesterone yielded 17α-oxa-D-homo-androst-1,4-dien-3,17-dione, a lactone not previously isolated from *A. tamarii*. Cortexolone was transformed to the 20(*R*)-alcohol with no further transformation observed. Evidence is also presented for the presence of a highly flexible but stereospecific keto-reductase. All metabolites were isolated by column chromatography and were identified by ^{1}H, ^{13}C NMR, DEPT analysis and other spectroscopic data.

Keywords: Biotransformation; *Aspergillus tamarii*; Baeyer–Villiger oxidation; Lactonisation; Steroid; Cortical steroid; Epoxide opening; Progesterone

1. Introduction

A variety of fungi [1–7] have been identified which can stereospecifically degrade the 17β-acetyl side chain of progesterone in high yield to give the ring D steroidal lactone testololactone (5). Of these fungi *Aspergillus tamarii KITA* is the most efficient, with yields from transformation reported at 70% [1].

The metabolic pathway present in *A. tamarii* has four distinct enzymatic steps that occur in a precise sequence to transform progesterone (1) to testololactone (5) (Fig. 1) [5]. The pathway initiates with a selective Baeyer–Villiger oxidation of the cortical side-chain producing testosterone acetate (2), this is then hydrolysed to form testosterone (3) oxidation of which follows giving androst-4-en-3,17-dione (4) this then undergoes a ring D Baeyer–Villiger oxidation yielding testololactone (5). In a number of reports the (20(*R*)) analogue of progesterone (6) has been isolated [8] and it has been suggested that a competitive equilibrium exists between the reductase forming the alcohol (6) and the oxidase that regenerates (1). Frequently [2] it has not been possible to isolate testosterone acetate (2); this has been explained by the presence of high levels of the esterase enzyme [2] resulting in rapid hydrolysis of the acetate. A minor hydroxylation pathway is also present in this organism, here testosterone is hydroxylated at the 11β-position [1] with no further metabolism observed [9].

In order to test the flexibility of this pathway and determine its ability to generate novel steroidal metabolites we have challenged it with a series of progesterone analogues, namely 17α-hydroxyprogesterone (7), 16α,17α-epoxyprogesterone (8), deoxycorticosterone (9) and cortexolone (10). To date there is a mass of information on the hydroxylation of steroids, however there is relatively little research into the area of steroidal lactonisation pathways with respect to the handling of a variety of cortical steroids. This is important as it offers a potentially new route to novel biologically active steroids. For example if enzymatic reactions can generate carbocation intermediates, such as the Bayer–Villiger

* Corresponding author. Tel.: +44-1273-642088; fax: +44-1273-679333.
E-mail address: c.hunter@bton.ac.uk (A.C. Hunter).

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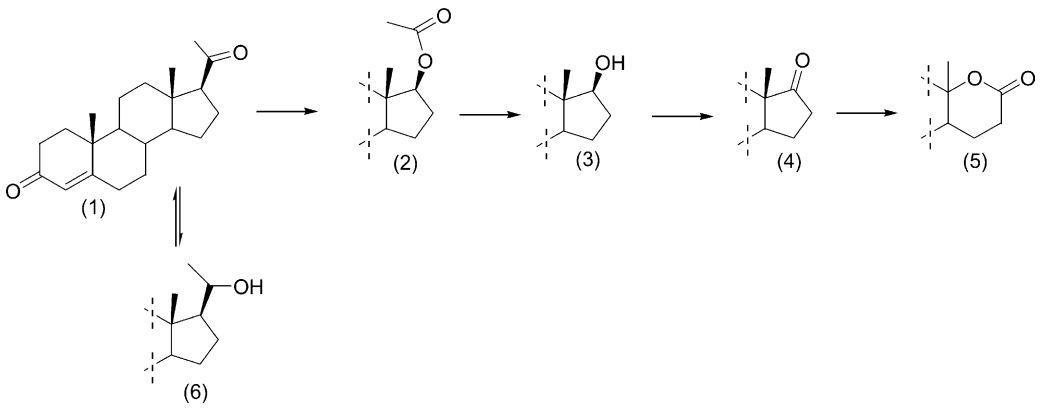

Fig. 1. Metabolic pathway of side-chain cleavage of progesterone (1) in *Aspergillus tamarii*.

rearrangement, there is scope for the generation of new steroidal skeleta.

2. Materials and methods

2.1. Chemicals and reagents

17α-Hydroxyprogesterone (7), epoxyprogesterone (8) and deoxycorticosterone (9) were purchased form Aldrich (UK), cortexolone (10) was supplied by Acros Organics (UK). All compounds were used as supplied. Solvents used were of analytical grade; petroleum ether refers to the fraction bp 60–80 °C. Silica for chromatography was Merck 9385.

2.2. Microorganism

Aspergillus tamarri KITA (QM 1223) was obtained 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

1. Spores were transferred aseptically in a laminar flow hood into 500 ml Erlenmeyer flasks containing 300 ml of sterile media and were incubated for 72 h at 24 °C. The cultures were shaken at 160 rpm on an orbital shaker.
2. 5 ml Aliquots from the seed flask were transferred aseptically to 10 flasks and grown for a further 72 h as above. After this time period 2 g of steroid dissolved in dimethylformamide and made up to 10 ml was evenly distributed between the flasks under sterile conditions and incubated for a further 5 days after which the meatabolites 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 all of the 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 light petroleum ether. Higher polarity metabolites were eluted with 1–5% methanol in ethyl acetate. 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 in ethyl acetate or pure ethyl acetate. A 50:50 sulfuric acid in methanol spray was used to develop the plates.

2.5. Analysis and identification of metabolites

Characteristic shift values [10,14] 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 using tetramethylsilane as the internal standard. Infra-red spectra absorption were recorded directly on a Nicolet avatar 320 FT-IR fitted with a Smart Golden Gate®. All melting points were determined on an electrothermal melting point apparatus and are uncorrected.

3. Results

3.1. Products of metabolism and structural identification

Incubation of 17α-hydroxyprogesterone (7) resulted in three transformation products. 17α,20(*R*)-dihydroxypregna-4-en-3-one (11) was identified by a new quartet signal in the ^{1}H NMR spectrum at δ_H 4.03 ppm (J = 6.1 and 12.2 Hz) consistent with reduction of the C-20 ketone to the (*R*)-alcohol [10,11] as was the upfield shift of 1.1 ppm for the 21-methyl proton signal. Further supporting evidence

in the ^{13}C NMR spectrum was loss of the quaternary resonance signal at δ_C 211 ppm in the starting material replaced by a C*H*OH signal at 71 ppm. Also the 21-methyl signal underwent an upfield shift of 8.4 ppm consistent with the presence of the less electronegative alcohol. This metabolite crystallised from acetone as needles mp 204 °C which was consistent with reported literature values lit., 204–205.5 °C [12]; lit., 203–204 °C [13] (found: 332.236 $C_{21}H_{32}O_3$ requires 332.235). The ^1H NMR spectrum of the next metabolite isolated was devoid of the 21-methyl signal (δ_H 2.28 ppm) present in the starting material suggesting loss of the cortical side chain. This was fully supported by loss of signals at δ_C 211.6 and 27.8 ppm in the ^{13}C NMR spectrum of the starting material assigned to C-20 and C-21 and a downfield shift of the C-13 resonance signal of δ_C 35.4 ppm consistent with insertion of oxygen adjacent to this position on ring D. This coupled with the upfield shift in the 18-methyl resonance signal of δ_H 0.61 ppm and a new quaternary resonance signal at δ_C 171.9 ppm were consistent with lactone formation [14] all other spectral data were consistent with the structure of testololactone (5) as was the melting point 208 °C following crystallisation from ethyl acetate in light petroleum as needles lit., 208 °C [1,9] (found: 302.187 $C_{19}H_{26}O_3$ requires 302.188). The final transformation product was identified as 17α-oxa-D-homo-androst-1,4-diene-3,17-dione (12). Resonance signals in both the ^1H and ^{13}C NMR spectra were consistent with the formation of a ring D lactone as in (5). Two new downfield signals in the ^1H NMR spectrum at δ_H 6.8 (d *J* 8.5 Hz) and 7.12 (d *J* 8.5 Hz) ppm indicated dehydrogenation on ring A. This was supported by two methylene resonance signals in (6) being replaced by two methyne resonances at δ_C 155.2 (C-1) and 130.5 (C-2) ppm in the product. A melting point of 219–220 °C obtained following crystallisation from ethyl acetate in light petroleum, which was consistent with the literature value, 218–220 °C [15] (found: 300.172 $C_{19}H_{24}O_3$ requires 300.172).

Incubation of 16α,17α-epoxyprogesterone (8) yielded two transformation products, the first 16α,17α-epoxy-20(*R*)-hydroxy-pregna-4-en-3-one (13) was identified by a new signal in the ^1H NMR spectrum at δ_H 4.22 ppm (broad singlet) and an upfield shift of δ_H 0.7 ppm for the 21-methyl proton signal. This suggested a loss of electronegativity at C-21 consistent with reduction of the of the C-20 ketone to the (*R*)-alcohol [10,11]. Further supporting evidence in the ^{13}C NMR spectrum was loss of the quaternary resonance signal at δ_C 204.5 ppm in the starting material replaced by a C*H*OH signal at 63.6 ppm. Also the 21-methyl signal underwent an upfield shift of 4 ppm consistent with the presence of the less electronegative alcohol. Spectroscopic data were consistent with that previously reported for the (*R*)-hydroxy keto reduction [11] (found: 330.218 $C_{21}H_{30}O_3$ requires 330.219).

16β-Hydroxy-17α-oxa-D-homo-androst-4-en-3,17-dione (14) contained resonance signals in both the ^1H and ^{13}C NMR spectra that were consistent with the formation of a ring D-lactone [14]. Loss of the resonance signal at δ_H 3.71 ppm suggested epoxide opening. This was supported by a new resonance signal in the ^1H NMR spectrum at δ_H 4.47 ppm (dd) indicating the presence of a hydroxyl group, as did the new C*H*OH resonance signal at δ_C 65.2 ppm. The multiplicity of the signal in the ^1H NMR spectrum indicated equatorial stereochemistry for the hydroxyl group. The quaternary resonance at C-17 underwent a downfield shift of δ_C 28 ppm indicating that the hydroxyl is *beta* to this position. Hence a 16-hydroxyl group was assigned on the basis of these observations. This metabolite crystallised from ethyl acetate in petroleum ether as needles mp 268 °C (expected: C 71.67; H 8.22 $C_{19}H_{26}O_3$; found C 71.18; H 8.30).

Incubation of deoxycorticosterone (9) resulted in the production of one major metabolite, testololactone (5) that was identified by direct comparison with an authentic sample.

Incubation of cortexolone (10) resulted in the isolation of one transformation product, namely 17α,20(*R*),21-trihydroxy-pregna-4-en-3-one (15). A 3H multiplet at δ_H 3.8 ppm replaced the 2H resonance signal for the protons at C-21 in the starting material (δ_H 4.6 and 4.3 ppm). This indicated a reduction in the electronegativity of the environment beta to these protons indicated reduction of the C-20 ketone to an alcohol which was consistent with 20(*R*) stereochemistry [10,16]. Reduction of the ketone was supported further by the loss of a quaternary resonance signal at δ_C 211 ppm present in the starting material, replaced with a C*H*OH signal at δ_C 73.3 ppm. This triol crystallised readily from ethyl acetate mp 192 °C (expected: C 72.38; H 9.25 $C_{21}H_{32}O_3$; found: C 71.90; H 9.34) lit., 188–189 °C [17]. This structural assignment was fully supported by X-ray crystallography (Fig. 4).

3.2. Crystallographic structure determination of (15)

$C_{21}H_{32}O_4$, M_r: 348.47, orthorhombic space group $P2_12_12_1$ (No. 19), $a = 7.2314(10)$, $b = 13.1794(2)$ $c = 19.0484(4)$ Å, $\alpha = \beta = \gamma = 90°$, $V = 1815.42(5)$ Å^3, $Z = 4$, $D_{calc'} = 1.28$ mg/m^3, $u = 0.09$ mm^{-1}, $F(0\,0\,0)$ 760. Data were collected using a crystal of size 0.20 mm × 0.10 mm × 0.10 mm on a KappaCCD diffractometer. A total of 3197 reflections were collected for $3.76 < \theta < 25.04°$ and $-8 \le h \le 8$, $-14 \le k \le 15$, $-22 \le l \le 22$. There were 3197 independent reflections and 2859 reflections with $I > 2\sigma(I)$ were used in the refinement. No absorption correction was applied. The structure was solved by direct methods and refined using SHELXL-97. The diagrams used ORTEP-3 for windows. The final *R* indices were [$I > 2\sigma$(I)] $R_1 = 0.032$, $WR_2 = 0.078$ and *R* indices (all data) $R_1 = 0.039$, $WR_2 = 0.083$. The goodness-of-fit on F^2 was 0.911 and the largest difference peak and hole was 0.13 and −0.18 eÅ^{-3}. The crystallographic data have been deposited with the Cambridge Crystallographic Data Centre.

Yield values and the spectroscopic details for ^1H and ^{13}C NMR and infra-red absorption signals for the metabolites isolated are given in Tables 1–4.

Table 1
^{1}H NMR data for *Aspergillus tamarii* starting compounds and transformation products

Compound	4-H	20-H	18-H_3	19-H_3	21-H_3	Other significant signals
(a) Reference data						
17α-Hydroxyprogesterone (7)	5.73	–	0.75	1.19	2.28	3.0 brs (OH)
16α,17α-Epoxyprogesterone (8)	5.72	–	1.08	1.19	2.03	3.71 s (16β-H)
Deoxycorticosterone (9)	5.74	–	0.69	1.19	–	4.2 dd *J* 4.8 Hz (21-H)
Cortexolone (10)	5.73	–	0.69	1.19	–	4.3 and 4.6 dd *J* 20 Hz, J 20 Hz (21-H)
(b) Transformation products						
17α,20(*R*)-Dihydroxypregna-4-en-3-one (11)	5.73	4.03 q *J* 6.1 Hz *J* 12.2 Hz	0.85	1.19	1.17	
Testololactone (5)	5.76	–	1.36	1.18	–	
17α-Oxa-D-homo-androst-1,4-dien-3,17-dione (12)	5.77	–	1.36	1.17	–	6.80 d *J* 8.5 Hz (1-H)
16α,17α-Epoxy-20(*R*)-hydroxy-pregna-4-en-3-one (13)	5.73	4.38 q *J* 6.4 Hz	0.92	1.19	1.10 d *J* 6.4 Hz	7.12 d *J* 8.5 Hz (2-H) 3.34 s (16β-H)
16β-Hydroxy-17α-oxa-D-homo-androst-4-en-3,17-dione (14)	5.76	–	1.39	1.17	–	4.47 dd *J* 5 Hz *J* 10 Hz (16α-H)
17α,20(*R*),21-Trihydroxy-pregna-4-en-3-one (15)	5.73	–	0.85	1.19	–	3.8 m W_h 20 Hz (20-H and 21-H)

Table 2
^{13}C NMR of starting compounds and their metabolites (7–15) in $CDCl_3$

Carbon atom	Compounds									
	(7)	(8)	(9)	(10)	(11)	(5)	(12)	(13)	(14)	(15)
1	35.6	35.5	36.4	35.2	36.4	36.2	155.2	36.3	36.2	35.6
2	33.4	33.7	34.6	33.4	34.5	34.5	130.5	34.6	34.5	33.9
3	199.6	199.2	200.1	197.4	200.6	199.8	199.5	200.1	199.7	199.9
4	123.8	123.9	124.7	123.0	124.5	124.8	124.0	124.8	125.0	123.7
5	171.2	170.4	171.4	170.4	172.5	170.0	169.7	171.4	169.6	171.8
6	32.8	31.3	33.4	31.9	32.8	33.0	32.3	32.2	33.0	32.9
7	31.9	32.5	32.6	32.1	32.7	31.1	30.3	33.4	31.4	32.0
8	35.4	33.1	36.2	35.2	36.5	38.7	37.9	34.4	39.4	35.7
9	53.2	53.7	54.3	53.0	54.1	53.2	52.4	54.5	52.9	53.4
10	38.5	38.5	39.2	38.1	39.3	39.2	40.0	39.3	39.1	38.6
11	20.4	20.2	21.6	20.3	21.4	22.5	21.8	21.3	22.5	20.7
12	30.0	31.0	39.1	30.1	33.6	39.7	38.4	33.5	39.8	31.7
13	48.0	41.4	45.4	47.0	47.9	83.4	82.8	41.9	85.3	47.4
14	49.9	44.7	56.8	49.9	50.4	46.4	45.6	46.3	46.5	49.0
15	23.8	27.2	25.2	23.2	24.5	20.6	19.8	27.4	30.9	23.8
16	33.8	60.2	23.6	33.4	34.6	29.2	28.5	59.5	65.2	33.3
17	89.8	70.5	59.7	88.4	85.8	171.9	171.4	74.0	199.7	85.2
18	15.3	15.0	14.2	14.5	15.8	20.8	20.0	16.4	18.1	15.1
19	17.4	17.0	18.1	17.0	18.1	18.1	17.3	17.9	20.0	17.3
20	211.6	204.5	210.8	211.3	71.0	–	–	63.6	–	73.3
21	27.8	25.8	70.1	65.9	19.4	–	–	21.8	–	64.6

Compounds are numbered as follows: 17α-hydroxyprogesterone (7), 16α,17α-epoxyprogesterone (8), deoxycorticosterone (9), cortexolone (10), 17α,20(*R*)-dihydroxypregna-4-ene-3-one (11), testololactone (5), 17α-oxa-D-homo-androst-1,4-dien-3,17-dione (12), 16α,17α-epoxy-20(*R*)-hydroxy-pregna-4-en-3-one (13), 16β-hydroxy-17α-oxa-D-homo-androst-4-en-3,17-dione (14), 17α,20(*R*),21-trihydroxypregna-4-en-3-one (15).

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

Compound	>C=O	>C=CH–	–OH	Lactone	Epoxide
17α,20(*R*)-Dihydroxypregna-4-en-3-one (11)	1698	1635	3456	–	–
Testololactone (5)	1664	1614	–	1714	–
17α-Oxa-D-homo-androst-1,4-dien-3,17-dione (12)	1653	1618 br	–	1716	–
16α,17α-Epoxy-20(*R*)-hydroxy-pregna-4-en-3-one (13)	1657	1625	3416	–	3100
16β-Hydroxy-17α-oxa-D-homo-androst-4-en-3,17-dione (14)	1649	1609	3355	1722	–
17α,20(*R*),21-Trihydroxy-pregna-4-en-3-one (15)	1649	1612	3425 br	–	–

Table 4
Product yields following chromatography

Starting material	Metabolites	Yield (%)
17α-Hydroxy progesterone (7)		1.1
	17α,20(*R*)-Dihydroxypregna-4-en-3-one (11)	2.3
	Testolactone (5)	31
	17α-Oxa-D-homo-androst-1,4-dien-3,17-dione (12)	1
16α,17α-Epoxyprogesterone (8)		2.5
	16α,17α-Epoxy-20(*R*)-hydroxy-pregna-4-en-3-one (13)	9.3
	16β-Hydroxy-17α-oxa-D-homo-androst-4-en-3,17-dione (14)	2
Deoxycorticosterone (9)		1.3
	Testolactone (5)	47
Cortexolone (10)		26
	17α,20(*R*),21-Trihydroxy-pregna-4-en-3-one (15)	28

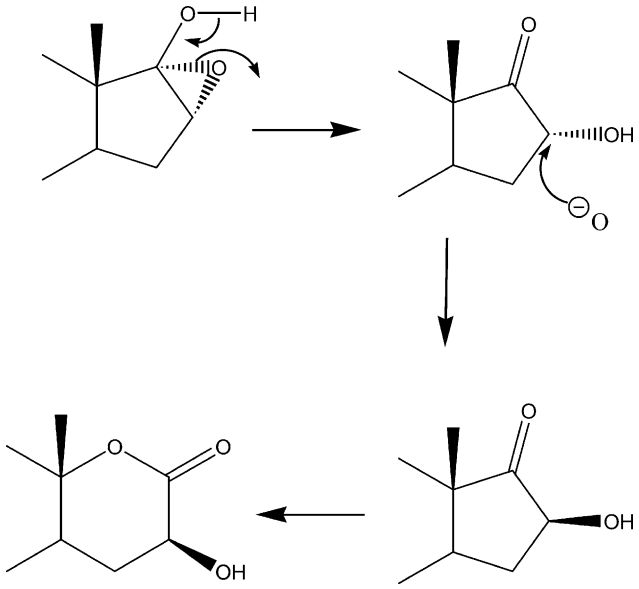

Fig. 2. Postulated reaction sequence to give 16β-hydroxy-17α-oxa-D-homo-androst-4-en-3,17-dione (14).

4. Discussion

In contrast to microbiological epoxidation of steroids reports of opening steroidal epoxides are rare. Of those, which have reported in yeast [19] and fungi [7,20], they have resulted in retention of *alpha* stereochemical configuration. For example 16α,17α-epoxyprogesterone was incubated with the fungus *Cylindrocarpon radicicola* [7] resulting in 16α-hydoxy-17α-oxa-D-homo-androst-1,4-dien-17-one. Although epoxide opening with inversion of stereochemistry can occur, for example by an activated oxygen species such as a per acid, it is unlikely that this is occurring in our transformation as it would result in a diol being formed [18,21] on ring D. A more likely scenario can be centred around the mechanism where the epoxide opens to form a 16α-hydroxy metabolite reported with the fungus *C. radicicola* [7] (Fig. 2). This was based on the initial formation of the 17-keto-16α-hydroxy functionalised ring D intermediate following oxidation of the 17β-hydroxyl group. Here oxidase activity at the 17β-hydroxyl promotes epoxide opening resulting in the formation of a 16α-alcohol. However, in the case of *A. tamarii* it undergoes a further nucleophilic attack (S_{n2}) by an activated oxygen species that is likely to occur in the enzyme site, thus achieving the observed inversion of stereochemistry to afford the 16β-hydroxyl group. If this is the case then the nucleophilic reaction following epoxide opening may be rapid as no 16α-hydroxy metabolites were isolated. However, absolute proof of the mechanism for epoxide opening will await the isolation of the oxidase responsible from *A. tamarii*. Finally, to complete this series of transformations Baeyer–Villiger oxidation of the 17-ketone would follow resulting in the metabolite isolated (15).

The reduction of the C-20 ketone of 16α,17α-epoxyprogesterone (8) is also of interest. In the case of this steroid and other epoxy analogues X-ray crystallography has demonstrated that the C-20 ketone eclipses (i.e. is parallel to the C-13–C-17 bond), which, is contrast to the majority of cortical steroids where the C-20 ketone is positioned over ring D [22–25]. The retention of this configuration in solution for 16α,17α-epoxyprogesterone (8) is supported by the signal in the ^{1}H NMR spectrum for the 18-methyl protons which has shifted significantly downfield (δ_H 1.08 ppm) compared to 17α-hydroxyprogesterone (7) (δ_H 0.75 ppm) and the other compounds (9,10) fed (δ_H 0.69 ppm). This would suggest that the reductase present in this fungus responsible for C-20 reduction can reduce the presented ketone over an angle of approximately 180°. Hence this enzyme has a high degree of flexibility but posses the ability to lock the presented C-20 ketone into a strict specific stereochemical orientation irrespective of the intramolecular forces present, which retains the epoxy steroid side-chain in its usual position to give a 20(*R*)-alcohol as is common to fungi.

A number of general features were recurrent in the handling of the cortical steroid analogues by the metabolic pathway present in *A. tamarii* (Fig. 3). Firstly all of the analogues tested were accepted by the pathway and all but one [cortexolone (10)] underwent the Baeyer–Villiger oxidation to form a ring D lactone. In all cases, except deoxycorticosterone (9), the 20(*R*)-alcohol was isolated from the fermentation; this stereochemical reduction is common

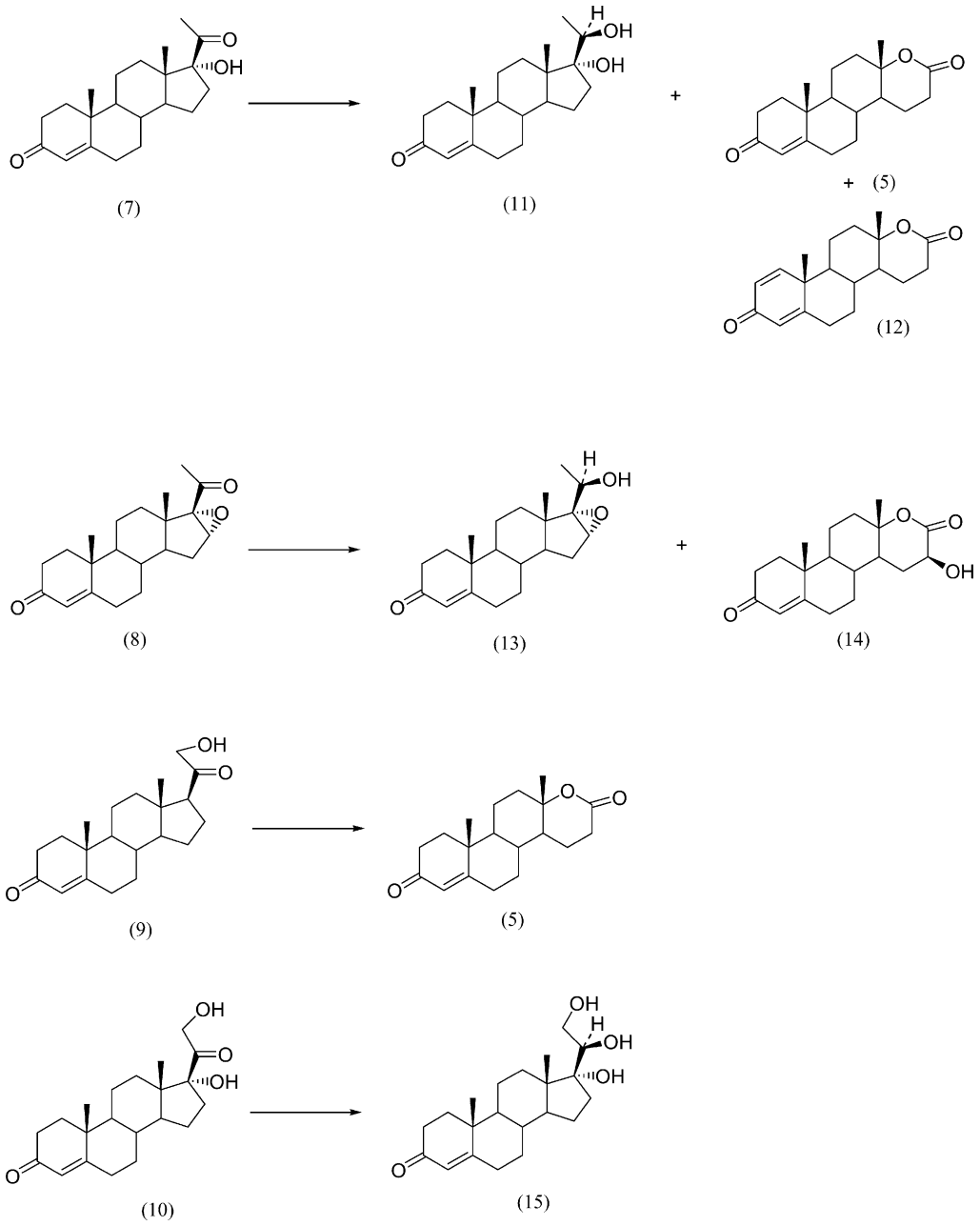

Fig. 3. Cortical steroid transformation.

to a wide range of fungi [18]. It may therefore be speculated that the oxidase required to oxidise the alcohol to progesterone is affected by ring D side chain functionality e.g. 17α,20(*R*),21-trihydroxy-pregna-4-en-3-one (15) which did not undergo further transformation. Interestingly this 20(*R*) reduction product was not observed following incubation of progesterone by the researchers who first reported the pathway [1] and we have not isolated it in our own comparable experiments (unpublished results). This may indicate that progesterone is not accessible to the reductase or it does not activate it.

A common feature observed by previous researchers [1,9] in which the metabolism of both progesterone and a series of androgens by *A. tamarii* demonstrated an active but minor 11β-hydroxylation pathway. In the case of all these cortical steroids not one was hydroxylated let alone in the 11β position, indicating that functionality at C-17, e.g. epoxide or alcohol or alcohol at C-21 were not handled by this pathway. This may be due to the polarity of the molecule and/or stereochemical factors which precludes the molecule from binding to the hydroxylase, i.e. these analogues all contain a diketo-alcohol functionality compared to progesterone which has a less polar diketo structure.

Deoxycorticosterone (9) underwent the most efficient metabolism to form testololactone (5) in 47% yield, followed by 17α-hydroxyprogesterone (7) (31%) both of

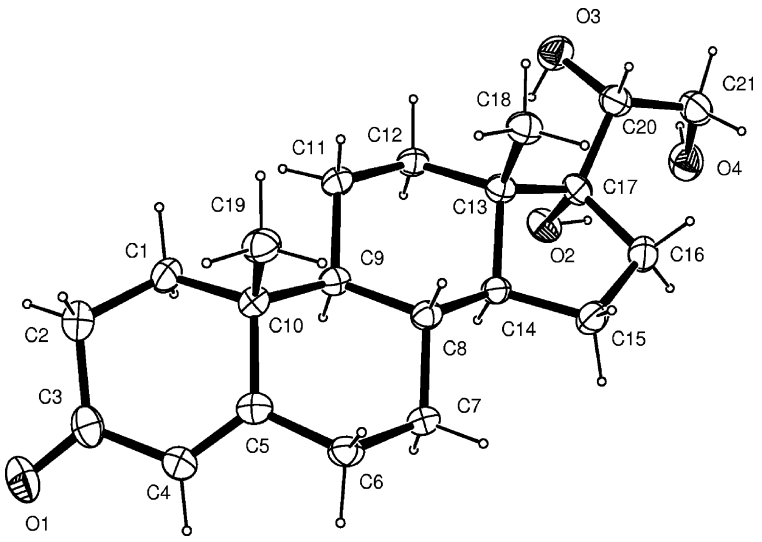

Fig. 4. X-ray crystal structure of 17α,20(*R*),21-trihydroxypregna-4-en-3-one (15).

which are below the yield (60%) routinely found with the transformation of progesterone (1) in our laboratory. This indicates that although the pathway has a degree of flexibility there is clear structural preference on metabolism of these analogues, further examples include a low yield of the 16β-hydroxy lactone (14) and no transformation following reduction of the C-20 ketone of cortexolone (10) to the (*R*)-alcohol (16). In this case the oxidase cannot be accessed, this may be due to tri-hydroxy ring D functionality rendering the molecule too polar to partition to the oxidase or that intramolecular hydrogen bonding may be inhibiting substrate binding. The latter notion has been refuted by X-ray crystallographic evidence (Fig. 4), which demonstrates only weak hydrogen bonding present between the 17α-hydroxyl group and the 21-hydroxy group (1.993 Å) and C-20 hydroxyl to the 17α-hydroxyl group (2.141 Å).

In conclusion this work has demonstrated a high degree of flexibility in the lactonisation pathway of *A. tamarii* with respect to the cortical steroids incubated and has given insight not only into a unique epoxide opening but also the rigor in which the fungal reductase deals with the C-20 ketone with variation of side chain orientation. Clearly this approach to the 'high jack' of steroidal lactonisation pathways with various side-chain analogues of progesterone has been fruitful and hopefully will prompt further investigation of other microorganisms with similar pathways to gain further illumination into mechanism and importance of this approach to the generation of new steroidal entities.

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