Journal of Steroid Biochemistry & Molecular Biology 118 (2010) 171–176

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

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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ARTICLE INFO

Article history:
Received 30 October 2009
Received in revised form
10 December 2009
Accepted 11 December 2009

Keywords:
Aspergillus tamarii
3α-Steroid transformation
Hindered hydroxylase binding
Baeyer–Villiger oxidation
Enzyme induction
Biocatalyst

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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doi:10.1016/j.jsbmb.2009.12.003

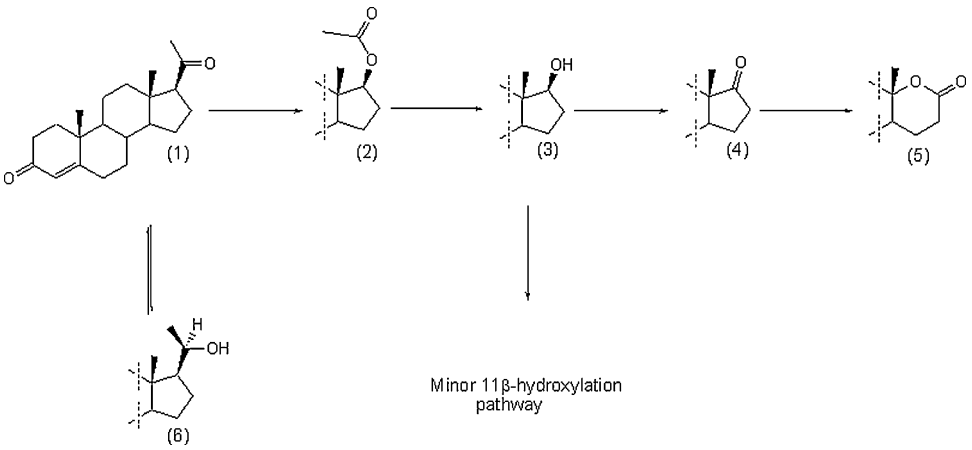

Fig. 1. The endogenous lactonization pathway present in *Aspergillus tamarii* KITA which converts progesterone (**1**) to testololactone (**5**) with preceding C-20 keto-alcohol isomerise (**1**) and (**6**) and the minor 11β-hydroxylation pathway from testosterone.

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)