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Biotransformation of 3-keto-androstanes by *Gongronella butleri* VKM F-1033

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Abstract

The activity of *Gongronella butleri* VKM F-1033 towards androst-4-ene-3,17-dione (AD), androsta-1,4-diene-3,17-dione (ADD) and 9α-hydroxyandrost-4-ene-3,17-dione (9α-OH-AD) was studied. Bioconversion products were purified by column chromatography and preparative TLC, and identified by HPLC, mass-spectrometry and ^{1}H NMR spectroscopy.

The 7α-hydroxy-AD and 14α-hydroxy-AD were revealed as major products from AD, while 7β-, 6α-, 6β-hydroxylated AD derivatives were observed in small amounts. The presence of 1(2)-double bond in ADD molecule resulted in the accumulation of 14α-, 6β- and 7β-hydroxylated ADD derivatives, whereas no 7α-hydroxy steroid was formed from ADD. Along with hydroxylation, 17-ketone reduction was observed during AD and ADD bioconversion. Unlike other fungal biocatalysts, the strain carried out neither 1(2)-dehydrogenation of AD, nor 1(2)-hydrogenation of ADD. During incubation with 9α-OH-AD, two products, 9α, 14α-dihydroxy-AD and 6β, 9α-dihydroxy-AD were revealed. The results demonstrate the dependence of hydroxylation position on the structure of steroid nucleus.

Keywords: 3-Keto-steroid; Androst-4-ene-3,17-dione; Androsta-1,4-diene-3,17-dione; 9α-Hydroxy-androst-4-ene-3,17-dione; Biotransformation; Hydroxylation; *Gongronella butleri*

1. Introduction

Hydroxylation is one of key reactions of steroid metabolism in microorganisms and currently used for steroid biotransformations [1]. At its realization the dominant role is traditionally featured to mycelial fungi which perform the process more effectively as compared with other microorganisms [2].

Since inventing in the 50th of progesterone 11α-hydroxylation by *Rhizopus orchidis*, the possibility of one or more hydroxyl groups introduction at different sites of steroid nucleus was described for fungi of various taxonomic position. Reactions leading to the formation of physiologically active or chemically valuable steroids with hydroxyl function in positions 11 (α and β), 7 (α and β), 6 (α and β), 9α, 14 (α and β), 15 (α and β) are of maximal interest [3,4].

By now some insight was formed that oxidation of steroid molecule is catalyzed by non-specific steroid monooxygenases and the position of hydroxylation is determined in many respects by the structure of steroid substrate. For example, the fungus of *Curvularia lunata* was known to introduce hydroxyl group in 11β-position of Reichstein compound "S". The reaction found a wide application for the industrial production of hydrocortisone [5,6]. However, the same culture converted androst-4-ene-3,17-dione (AD) to 14α-OH-AD [7], 11α-OH-AD, 15α-OH-AD and formed also 1(2)-dehydro-derivatives [8]. The presence of additional double bond or substitute (e.g. methyl group) in steroid substrate can notably influence on the ratio of hydroxylated products. For instance, mycelium of *Absidia coerulea* hydroxylated AD, testosterone (T) and progesterone at position 14α, while transformed 1(2)-dehydro-17α-methyl progesterone mainly to 7α- and 11β-hydroxy derivatives with accumulation of 14α,15α-epoxy-steroid in small amounts [9].

The position of 3-keto-steroid hydroxylation can also depend on the structure of substitutes. For example, transformation of

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4-methyl-T by *Fusarium culmorum* AM 282 was accompanied by predominant hydroxylation at 6β-position, whereas bioconversion of 4-Cl-T resulted in formation of 15α-OH-derivative and transition of 3-keto-4-ene to 3β-OH-5-ene moiety [10].

Significant criterion also influencing position of hydroxylation is the presence of side-chain at C-17 in steroid substrate. The fungus of *Phycomyces blakesleeanus* converted progesterone (C_{21}-steroid) to the corresponding 7α-, 6β-, 14α- and 15β-hydroxyderivatives. While, at T (C_{19}-steroid) transformation by the same culture, the reaction was directed to 1(2)-dehydrogenation and 17β-reduction with accumulation of ADD and 1(2)-dehydro-T as major products, and 6β-OH- and 7α-OH-derivatives of T were fixed in traces [11]. The strain of *Botryosphaerica obtusa* actively hydroxylated progesterone at 7β-position, whilst expressed very low 7β-hydroxylase activity towards AD and T. These C_{19}-steroids were mainly hydroxylated by the strain at the positions 7α and 6β [12].

In some cases the position of hydroxyl group introduction was the same for both pregnane and androstane steroids. The culture of *Mucor piriformis* hydroxylated AD forming a number of products with two of them determined as 14α-OH-T and 14α-OH-1(2)-dehydro-T [13]. Microsomes of this strain also performed 14α-hydroxylation of progesterone in the presence of NADPH and O_2 [14].

The fact that configuration of the steroid nucleus – 3-keto-4-ene or 3β-OH-5-ene – did affect the site of microbial hydroxylation was revealed. Culture of *Beauveria bassiana* hydroxylated 3-keto-4-ene-steroids (T and the derivatives) in 11α-position, but introduced hydroxyl group additionally in 7α-position of 3β-OH-5-ene-steroid (DHEA) [15].

The knowledge of biotransformation peculiarities of steroids with different structures by one or another culture-hydroxylator conditioned remarkably production of various hydroxylated derivatives which are known to be valuable intermediates in the combined synthesis of steroidal drugs.

Recently, in our laboratory the screening of more than 450 mycelial fungi was carried out, and the strains capable of introducing hydroxyl function in 7α- or 7β-positions of 3β-OH-5-ene-steroid (DHEA) were selected. Along with many others, the strain of *Gongronella butleri* VKM F-1033 demonstrated sufficient level of 7α-hydroxylase activity towards DHEA [4].

In the present work, we examined whether the selected strains would express hydroxylase activity towards 3-keto-4-ene-androstanes, and whether the presence of 1(2)-double bond, or 9α-hydroxyl group would influence the position of hydroxyl group introduction. The strain of *G. butleri* VKM F-1033 was used as a model organism in this research.

2. Experimental

2.1. Materials

Steroids: androst-4-ene-3,17-dione (AD), androsta-1,4-diene-3,17-dione (ADD) were purchased from Sigma, USA; 9α-hydroxy-androst-4-ene-3,17-dione (9α-OH-AD) (of 98% purity, mp 217–220 °C, [E] (0.01% in ethanol) —14,780 m^{-1} cm^{-1}) was obtained from Laboratory MTOC (IBPM RAS). Other reagents were of analytical grade and purchased from domestic companies (Russia).

2.2. Microorganisms and cultivation

The strain of *G. butleri* VKM F-1033 was obtained from All-Russian Collection of Microorganisms (VKM IBPM RAS). The strain was cultivated in two stages. Firstly, mycelium was grown on malt extract for 24–28 h on a rotary shaker (220 rpm) at 29 °C. At the second stage, 10% (v/v) of the first mycelium obtained were added to the growing medium containing (g/l): sucrose, 50; lyophilized corn extract, 10; KH_2PO_4, 5; $MgSO_4$, 5 (pH 6.0) and incubated for 32–36 h at the same conditions. The mycelium grown was separated by filtration, washed with 0.05 M K-phosphate buffer (pH 6.0) and used for steroid transformation.

2.3. Steroid transformation

Steroid transformations were carried out in 750 ml Erlenmeyer flasks contained 100 ml of 0.1 M NaOH–K_2HPO_4 buffer (pH 6.0). The washed pressed biomass was re-suspended in the buffer, and then steroid substrate (1 g/l) was added as a methanol solution (final methanol concentration in the conversion medium was 2%). Incubation was carried on a rotary shaker (220 rpm) at 29 °C.

2.4. Isolation of steroids

At the end of incubation period, steroids were isolated by means of preparative thin layer chromatography (TLC) or column chromatography. The former was carried out as described earlier [3]. For the latter, the column (16 mm × 450 mm) with Silica gel 60 (Merck, 0.040–0.063 mm) as a sorbent was applied using hexane/ethyl acetate mixtures of various percentage. The composition of fractions was followed by TLC and high pressure liquid chromatography (HPLC). The separate fractions showing the presence of metabolites with the same R_F and R_T values were concentrated.

2.5. Analyses

Samples (1 ml) of conversion medium were taken every 24 h. For TLC, steroids were extracted with ethyl acetate (1:5, v/v). The extracts were applied to Kieselgel 60 F_{254} (Merck, Germany) plates, developed in benzene/acetone (3:1, v/v), and visualized under UV-light (254 nm) on CN-15MC chemiscope (Vilber Lourmat, France).

For HPLC, a reverse-phase column Symmetry C_{18} (250 mm × 4.6 mm, Waters, USA) and guard column Symmetry C_{18} (4.6 mm × 10 mm, Waters, USA) were used with acetonitrile:H_2O (60:40, v/v) as a mobile phase (50 °C), at a flow rate of 1 ml/min with UV-detection at 240 nm.

For mass-spectrometry (MS), a Finnigan SSQ-710 (USA) was used with direct inlet of a sample into an ionization chamber

Table 1
Identification of metabolites of AD, ADD and 9α-OH-AD conversion by *G. butleri* F-1033

Compound	R_F	R_T	Characteristics of major fragments, m/z ($I_{\%}$)	Putative structure
AD (**I**), standard	0.93	6.05	M^+286 (100), 244 (20), 148 (27), 145 (16), 131 (16), 124 (39), 119 (18), 117 (16), 109 (19), 107 (29), 105 (32)	AD
9α-OH-AD (**II**), standard	0.63	3.62	M^+302 (100), 137 (28), 136 (44), 124 (34), 123 (15), 121 (17), 110 (18), 109 (37), 108 (16), 107 (11), 95 (10)	9α-OH-AD
ADD (**III**), standard	0.87	4.9	M^+284 (41), 159 (21), 150 (8), 134 (8), 123 (12), 122 (100), 121 (28), 108 (8), 107 (12), 105 (10)	ADD
IV	0.66	3.57	M^+302 (100), 284 (32), 211 (29), 179 (20), 124 (29), 123 (31), 122 (35), 121 (21), 119 (53), 107 (26), 105 (39)	14α-OH-AD
V	0.59	3.41	M^+302 (100), 287 (34), 152 (29), 136 (5), 121 (8), 105 (9), 91 (15), 79 (12)	6β-OH-AD
VI	0.54	3.17	M^+302 (100), 287 (36), 233 (23), 152 (32), 121 (11), 110 (12), 109 (12), 107 (12), 105 (17), 95 (16), 91 (23), 79 (25)	6α-OH-AD
VII	0.43	3.12	M^+302 (100), 192 (16), 174 (27), 133 (17), 124 (36), 123 (21), 109 (17), 105 (18), 93 (18), 91 (23), 79 (24)	7β-OH-AD
VIII	0.36	3.27	M^+302 (43), 246 (15), 152 (10), 125 (10), 124 (100), 109 (15), 105 (15), 95 (13), 91 (25), 81 (12), 79 (25).	7α-OH-AD
IX	0.32	2.95	M^+318 (100), 176 (14), 148 (16), 137 (14), 136 (39), 125 (17), 124 (39), 123 (23), 122 (22), 109 (24), 91 (16)	9α, 14α-di-OH-AD
X	0.32	2.65	M^+318 (100), 303 (15), 300 (60), 207 (18), 175 (25), 152 (45), 139 (33), 135 (87), 134 (56), 122 (32), 109 (28)	6β, 14α-di-OH-AD
XI	0.59	3.28	M^+300 (16), 282 (32), 148 (30), 147 (36), 134 (79), 133 (49), 122 (45), 121 (100), 119 (53), 105 (29), 91 (69)	14α-OH-ADD
XII	0.51	3.14	M^+300 (100), 255 (35), 228 (41), 147 (33), 138 (55), 134 (64), 122 (46), 121 (65), 120 (36), 119 (43), 115 (29)	6β-OH-ADD
XIII	0.37	3.04	M^+300 (11), 161 (10), 150 (22), 133 (19), 122 (100), 115 (15), 109 (17), 107 (29), 105 (24), 93 (27), 91 (67)	7β-OH-ADD

R_F was determined by TLC in benzene:acetone (3:1, v/v); R_T, by HPLC (acetonitrile:H_2O (60:40, v/v).

at the ionization energy of 70 eV. The temperature of ionization chamber was 150 °C, and heating of sample to 350 °C was of 2.7°/s.

For proton (^{1}H) nuclear magnetic resonance (NMR) analysis, a Varian Unity +400 (Varian, USA) spectrometer was used to record spectra at 400 MHz, using $CDCl_3$ as a solvent. The $CHCl_3$ signal in the solvent (δ 7.24) was used as an internal standard.

3. Results and discussion

3.1. Identification of metabolites

Structures of the isolated metabolites were determined on the basis of mass spectrometry and NMR spectroscopy data. The molecular ions (M^+) of all purified products were shown to be 16 mass units higher than those of the corresponding substrates indicating the attachment of one oxygen atom to one molecule of steroid substrate (Table 1). On the other hand, the obtained NMR spectroscopy data evidenced the presence of additional hydroxyl group in these products as compared with the corresponding substrates. In total, the data confirmed the hydroxylation of substrates **I–III** to products **IV–XIII** (Tables 1 and 2).

Comparison of the parameters of ^{1}H NMR spectra of hydroxylation products **IV–XIII** between themselves and with original substrates allows differentiation of these products into compounds with the signals of CH protons of –CH–OH groups in the range of δ 3.5–4.5 ppm (**V–VIII**, **X**, **XII**, **XIII**) and compounds without these signals in their ^{1}H NMR spectra (**IV**, **IX**, **XI**). It should be noted that singlet signals of OH group (two OH groups in the spectrum of **IX**) were observed in the ^{1}H NMR spectra of products **IV**, **IX**, **XI**, which were taken in the aprotic solvent $CDCl_3$. Therefore, metabolites **IV**, **IX** and **XI** are products of oxidation of one of the sp^3-hybridized tertiary carbon atoms of the corresponding substrates. The signal at δ 80 ppm corresponds to this type of oxidized tertiary carbon atom in the ^{13}C NMR spectra of compounds **IX** and **XI**. Chemical shifts of protons of $C^{18}H_3$ and $C^{19}H_3$ groups in metabolites **IV**, **IX**, **XI** differ from the chemical shifts of the same groups in the corresponding substrates almost for equal values: $\Delta\delta \approx 0.14$–$0.15$ ppm for $C^{18}H_3$ and $\Delta\delta \approx 0.01$–$0.02$ ppm for $C^{19}H_3$. Therefore, the same carbon atom could be supposed to be oxidized in metabolites **IV**, **IX** and **XI**, and it cannot be C-9 which has already been oxidized in substrate **II**. Application of the additive scheme for calculation of chemical shifts of $C^{18}H_3$ and $C^{19}H_3$ groups based on the literature data on the increments of substitutes [16] lead to a conclusion about bearing of 14α-OH-substitute by the compounds of this group: 14α-OH-AD (**IV**), 9α,14α-di-OH-AD (**IX**) and 14α-OH-ADD (**XI**) (Table 2).

Three substances: **V**, **X** and **XII**, could be chosen in another group of compounds—products of oxidation of one of the secondary carbons of the original substrates. Their ^{1}H NMR spectra have a number of common features: proton signal of –O–CH is a triplet with spin coupling constant—SCC ~3.0 Hz (hereinafter SCC of $^3J_{C\,H,\,OH}$ is not taken into consideration), signals of protons 4-H and $C^{19}H_3$-groups are shifted downfield by ~0.1 and 0.2 ppm, respectively, and, finally, SCC of $^4J_{4\text{-}H,\,6\text{-}Hax}$ typical of substrates **I–III** and equal to 2.0 Hz for **I**, **II** and 1.5 Hz for **III**, are absent in the spectra of mentioned metabolites. All the above is an evidence of availability of OH-axial-group at C-6 in metabolites **V**, **X** and **XII**; hence, their structures are as follows: 6β-OH-AD, 6β,9α-di-OH-AD and 6β-OH-ADD, respectively. Comparison of experimental values and those calculated by additive scheme of δ values for protons of methyl groups $C^{18}H_3$ and $C^{19}H_3$ brings to the same conclusion concerning the structure of compounds **V**, **X** and **XII** (Table 2).

Comparison of the chemical shift of proton 4-H in the ^{1}H NMR spectra of substrate **I** and products of its oxidation demonstrates that signal of proton 4-H is maximally shifted downfield in compound **VI** ($\Delta\delta = 0.45$ ppm). This indicates the closeness of 4-H in metabolite **VI** to the oxidized carbon atom and, consequently, to the proton of –O–CH, which is axial as it interacts with vicinal protons with SCC $^3J = 12.0$ Hz and 5.6 Hz. Moreover, this proton is coupled to 4-H with SCC $J = 2.0$ Hz which is typical value for $^4J_{4\text{-}H,\,6\text{-}Hax}$. All the above is an evidence of the presence of equatorial OH-group at C-6 in compound **VI**, which is hence 6α-OH-AD. This is in good agreement with the results of chemical shifts of $C^{18}H_3$ and $C^{19}H_3$ calculated by the additive scheme.

Similarly to the above mentioned 14α-OH- and 6β-OH-derivatives, the NMR spectra of the products of bioconversion **VII** and **XIII** have common features: comparatively minor differences in the chemical shifts of $C^{18}H_3$, $C^{19}H_3$ and C^4–H from the same values in the respective substrates, similar values of the chemical shifts of proton of –O–CH (δ 3.57 ppm) and, finally, the same multiplicity of signal of this proton (octet) at identical values of the constants (5.2, 9.7, 11.0 Hz in **VII** and 5.4, 9.7, 10.9 Hz in **XIII**). All this is an evidence of axial orientation of the proton of –O–CH group. It has been established by means of double resonance that the same axial proton of CH_2 group in compounds **VII** and **XIII** interacts with proton of –O–CH (with SCC ≈ 10.5 Hz, which corresponds to the vicinal diaxial interaction) as well as with proton 4-H (with SCC = 2.0 Hz in compound **VII** and SCC = 1.5 Hz in **XIII**; each of them is typical for $^4J_{4\text{-}H,\,6\text{-}Hax}$ in AD and ADD, respectively). Hence it follows that the mentioned axial proton of CH_2 group is C_6H_{ax}, OH-group is located at C-7 being equatorial, and the structure of metabolites **VII** and **XIII** are 7β-OH-AD and 7β-OH-ADD, respectively.

The quartet with the ratio of intensities of its components 1:3:3:1 and the ~3-Hz interval between them corresponds to the proton of –O–CH in the spectra ^{1}H NMR of the latter metabolite—compound **VIII**. Such multiplicity of the signal points to the equatorial position of the proton of –O–CH group, which interacts with three vicinal protons. Like in the case of compound **VII**, it has been shown by means of double resonance that one of the mentioned protons interacts with –O–CH group with SCC = 3.1 Hz as well as 4-H with SCC = 2.0 Hz. The above is an evidence of axial position of the OH-group at C-7 in compound **VIII** and its structure defined as 7α-OH-AD.

The chemical shifts of protons of $C^{18}H_3$ and $C^{19}H_3$ groups calculated by the additive scheme are in good agreement with the demonstrated structures of metabolites **VII**, **VIII** and **XIII** (Table 2).

Table 2
Chemical shifts in ^{1}H NMR spectra[a] of substrates I–III and metabolites IV–XIII (δ, ppm)

Compound	$C^{18}H_3$[b]	$C^{19}H_3$[b]	1-H	2-H	4-H	–O–CH	OH
I	0.89	1.19	–	–	5.72d[c]	–	–
II	0.88	1.31	–	–	5.84d	–	2.37s
III	0.91	1.22	7.02d	6.20q	6.05t	–	–
IV	1.03 (1.01)[d]	1.21 (1.19)	–	–	5.73d	–	1.23s
V	0.93 (0.93)	1.39 (1.38)	–	–	5.82s	4.39t	1.63s
VI	0.90 (0.90)	1.19 (1.18)	–	–	6.17d	4.36m	...
VII	0.93 (0.92)	1.22 (1.22)	–	–	5.76d	3.57m	...
VIII	0.90 (0.90)	1.20 (1.18)	–	–	5.80d	4.08q	1.36bs
IX	1.03 (1.00)	1.32 (1.31)	–	–	5.88d	–	3.16s 4.06s
X	0.93 (0.92)	1.52 (1.50)	–	–	5.94s	4.40t	...
XI	1.05 (1.03)	1.24 (1.22)	7.03d	6.22q	6.06t	–	1.41s
XII	0.95 (0.95)	1.43 (1.41)	7.02d	6.18q	6.13d	4.56t	...
XIII	0.94 (0.94)	1.27 (1.25)	7.02d	6.24q	6.09t	3.57m	...

[a] Solvent, $CDCl_3$; standard, $CHCl_3$ ($\delta = 7.24$).
[b] All signals are singlets.
[c] The signal is a doublet with ~2 Hz-range between wide components.
[d] In brackets chemical shifts of $C^{18}H_3$ and $C^{19}H_3$ groups calculated by the additive scheme based on the literature data on the increments of substitutes are given.

3.2. Transformation of AD

During *G. butleri* mycelium incubation with AD (**I**), five dominant: **IV**, **V**, **VI**, **VII**, **VIII** (Tables 1 and 2) and two minor: M^+304 and M^+318 metabolites were revealed with major one identified as 7α-OH-AD (**VIII**) (Tables 1–3, Fig. 1). Since *G. butleri* expressed 7α-hydroxylase activity towards DHEA [4], the formation of 7α-OH-AD from AD evidenced that the changing of 3β-OH-5-ene (DHEA) to 3-keto-4-ene (AD) configuration of steroid substrate did not affect the position of hydroxylation. Along with 7α-hydroxylation, the introduction of hydroxyl function to positions 14α, and to a lesser extent—to positions 6β, 6α and 7β was observed thus resulting in the formation of 14α-OH-AD, 6β-OH-AD, 6α-OH-AD and 7β-OH-AD, respectively (Table 3). Other compounds: hydroxytestosterone (M^+304) and di-OH-AD (M^+318) were fixed in traces.

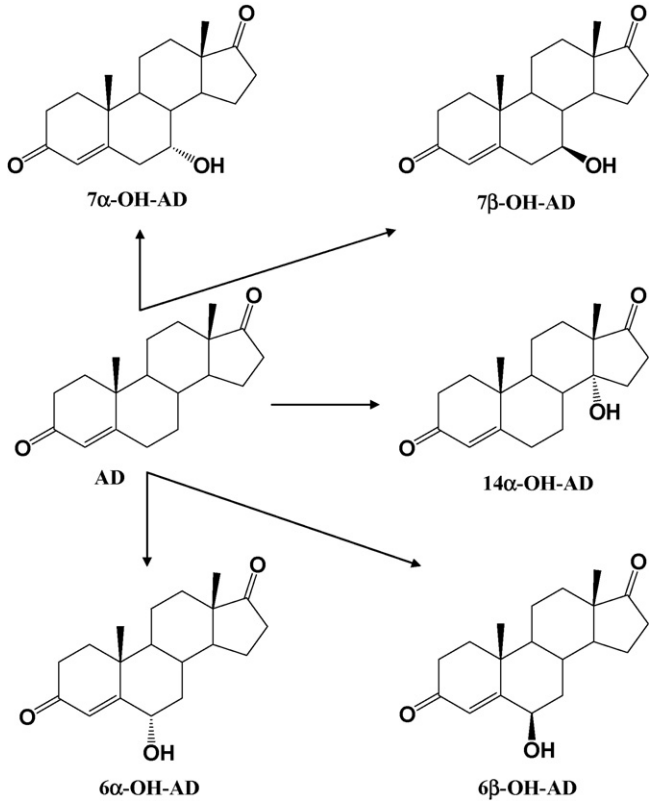

Fig. 1. Major products of AD transformation by *Gongronella butleri* F-1033.

It should be noted that *G. butleri* almost fully converted AD with no residual substrate after 120 h of incubation. This differed from the data obtained for *P. blakesleeanus* which poorly converted AD forming T and 14α-OH-T, while actively transformed progesterone [11].

Proceed from the predicted assumption on the mechanism of enzymatic hydroxylation, AD should be transformed mainly to 7α-OH-AD or 6β-OH-AD, as well as to 14α-OH-AD or 11β-OH-AD [16]. Along with the formation of these compounds (excepting for the latter), we observed also the formation of epimers: 7β-OH-AD and 6α-OH-AD. Epimer formation from β → α and vice versa from α → β in positions 6 and 7 may be proposed and also attributed to the dehydrogenase activity as its known for the microbial transformation of the bile acids and relative compounds [1].

In a number of cases mycelial fungi were also able to form 7α-, 14α- and 6β-hydroxy derivatives as major metabolites

Table 3
Product accumulation at the conversion of AD by *G. butleri* F-1033

Time (h)	Steroids, molar yield (%)					
	AD	Hydroxyderivatives of AD				
		14α	6β	7α	6α	7β
0	100	0	0	0	0	0
24	56.8	14.2	4.3	12.3	1.4	1.3
48	23.3	20.4	5.8	22	2.3	2.1
72	2.4	21.8	4.4	26.6	2.7	2.4
96	0.3	18.3	1.8	26.6	2.8	2.0

from AD. The formation of 7α-OH-AD as a major product from AD was described for *B. obtusa* which was known as 7β-hydroxylator of progesterone [12]. Culture of *A. coerulea* transformed AD to 14α-OH-AD [9]. Mycelium of *F. culmorum* demonstrated 15α-hydroxylase activity towards progesterone and formed 6β-OH-AD as major metabolite at AD transformation [17,18]. Notably, bacteria of *Bacillus* genus (HA-V6-3 and HA-V6-11) being 7α-hydroxylators of pregnenolone were also able to hydroxylate AD in positions 7α, 6β and 14α [19].

It is of interest that products formed at AD transformation by *G. butleri* differed from products of AD bioconversion by *Fusarium oxysporum var. cubense* and *Colletotrichum musae*. Similar to *G. butleri,* these strains expressed 7α-hydroxylase activity towards 3β-OH-5-ene-steroids, but at the incubation with AD accumulated mainly 12β- and 15α-OH-derivatives [20], but not 6β-OH-AD as *G. butleri* did.

Hydroxylation of AD by *G. butleri* was accompanied by 17β-reduction—the reaction typical for many microorganisms [3]. For instance, *C. lunata* (*Cochliobolus lunatus*) [7,21,22], *P. blakesleeanus* [11], *Taenia crassiceps* and *Taenia solium* [23] were able to reduce AD and its derivatives in position 17 while *B. bassiana* accumulated C-17-reduced products from AD in pH-dependent manner (dehydrogenase activity was revealed at a neutral pH and absent at pH 6.0) [11].

Distinctive feature of the transformation process of AD by *G. butleri* was lack of 1(2)-dehydrogenase activity which was common for some other hydroxylators (*Nectria haematococca*, *C. lunata*, *Bacillus sphaericus*) [8,24,25].

Accumulation of di-hydroxylated derivative of AD by *G. butleri* is in agreement with the data described. Strain of *B. bassiana* CCTCC AF206001 transformed AD with formation of 6β,11α-di-OH-AD in traces [15]. Similarly, conversion of T by *Absidia glauca* was carried out with accumulation of 6β,11α-di-OH-AD as a by-product of 7α-hydroxylation of T [26].

3.3. Transformation of ADD

In the course of ADD (**III**) conversion four products were revealed: **XI**, **XII**, **XIII** and M^+286 (Tables 1 and 2). The compounds **XI**, **XII**, **XIII** were detected in approximately equal quantities, and identified as 14α-OH-ADD, 6β-OH-ADD and 7β-OH-ADD, correspondingly. 1(2)-Dehydro-T (M^+286) was detected in traces. The concentration of hydroxylated products increased during 72 h of incubation reached 16.5, 13.3 and 14.5%, respectively (Table 4). No further product accumulation was observed after 72 h, while more than 39% of the substrate (ADD) remained non-converted.

No formation of AD, T or their derivatives was observed during ADD transformation. Unlike *G. butleri*, *Cephalosporium aphidicola* and *Fusarium lini* accumulated AD, 1(2)-dehydro-T, 11α-OH-ADD, 11α-OH-AD, 11α-OH-T and 11α-OH-1(2)-dehydro-T at ADD conversion [27]. The products formed at ADD transformation by *G. butleri* are shown in Fig. 2.

The comparison of product composition obtained at the conversion of ADD and AD by *G. butleri* allowed to propose that the presence of 1(2)-double bond in ADD structure hindered the introduction of hydroxyl-group to the positions 6α and 7α, as well as promoted a shift in a product ratio towards preferable accumulation of 14α-OH-, 6β-OH- and 7β-OH-ADD. Similar effects were described for *A. glauca* at the transformation of T and 1(2)-dehydro-T. The former was hydroxylated mainly in positions 7α, 11α and 6β, while the presence of additional double bond in 1(2)-dehydro-T promoted accumulation of β-epimers: 6β-, 7β- and 15β-OH-derivatives [26].

As compared with AD, the presence of additional 1(2)-double bond in ADD molecule probably provides its abridgement on C-3–C-17-direction with flattened ring A, and imparted certain additional rigidity to this ring [28]. It is possible that these changes hinder introduction of hydroxyl function to some positions of the molecule.

Table 4
Metabolites of ADD conversion by *G. butleri* F-1033

Time (h)	Steroids, molar yield (%)			
	ADD	Hydroxyderivatives of ADD		
		14α	6β	7β
0	100	0	0	0
24	52.1	12.8	10.3	11.0
48	41.8	16.0	12.8	14.1
72	39.9	16.5	13.3	14.5
96	39.0	16.6	13.3	14.8

3.4. Transformation of 9α-OH-AD

During incubation of *G. butleri* with 9α-OH-AD (**II**) only two products were detected (**IX** and **X**) identified as 9α,14α-di-OH-AD and 6β,9α-di-OH-AD, correspondingly (Tables 1 and 2). Therefore, the sites available for action of hydroxylating system were tertiary carbons C-6(β) of B ring and C-14(α) of C ring.

The concentration of 9α,14α-di-OH-AD and 6β,9α-di-OH-AD reached level of 52 and 28.9%, correspondingly, for 96 h

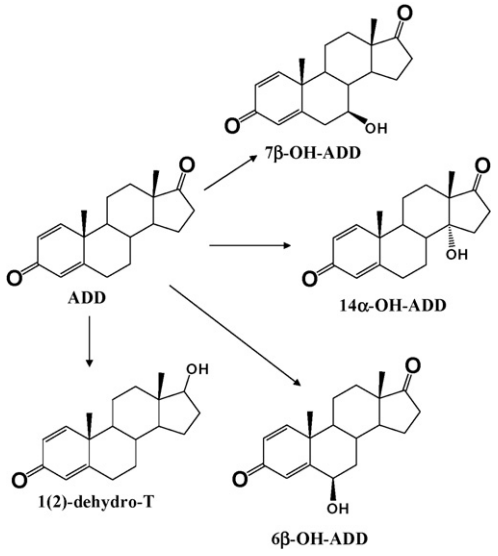

Fig. 2. Products of ADD transformation by *G. butleri* F-1033.

Table 5
Metabolites of 9α-OH-AD conversion by *G. butleri* F-1033

Time (h)	Steroids, molar yield (%)		
	9α-OH-AD	Hydroxyderivatives of 9α-OH-AD	
		14α	6β
0	100	0	0
24	70.5	17.0	7.30
48	46.6	31.7	14.5
72	22.6	45.0	22.8
96	8.1	52.0	28.9

(Table 5). No further product accumulation was observed after this time, while 8.1% of the substrate remained non-converted (Table 5).

As demonstrated earlier for *C. lunata,* the presence of 9α-hydroxyl-group can impede microbial 14α-hydroxylation of androstane steroid [28]. Unlike, *G. butleri* actively hydroxylated 9α-OH-AD in position 14α forming 9α,14α-dihydroxy-AD as a major product (Fig. 3).

No 7α-hydroxylation was observed, thus indicating that the presence of 9α-hydroxyl function can screen 7α-position from the enzyme attack.

Position of hydroxylation is determined not only by the presence of hydroxyl-substitutes in the nucleus of steroid substrate but also by the type of enzyme which is responsible for the reaction. 11β-Hydroxylase of *C. lunata* (*C. lunatus*) catalyzed introduction of OH-group to position 14α [29,30], whereas 7α-hydroxylase of *G. butleri* may be responsible for 14α-hydroxylation of androstane steroids.

Identifying methods for obtaining hydroxy-derivatives of androstanes is of importance not only for the syntheses of novel drugs, but also on account of their own pharmacologic activity. For instance, 7α-, 7β- and 17β-hydroxyderivatives of DHEA express antiglucocorticoid and antioxidant effects, play significant role for immune reactions, etc. [4]. Testosterone 7α-OH-derivatives are known to be the important precursors in the chemical synthesis of physiologically active steroids. Products

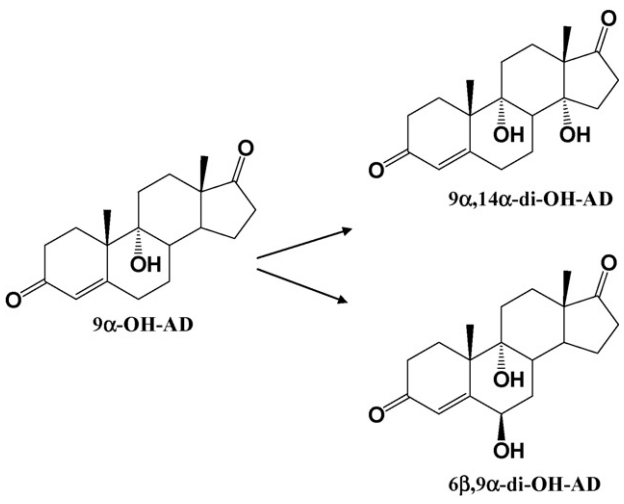

Fig. 3. Major products of 9α-OH-AD transformation by *G. butleri* F-1033.

of hydroxylation of 17α-ethynyl- and 17α-ethyl-T and namely 17α-ethyl-11α-OH-T produced by *Cunninghamella elegans* was shown to demonstrate inhibitory activity towards tyrozinase [31]. Hydroxyderivatives of AD obtained with *C. lunata*—11α-OH-AD, 11α-OH-T and 15α-OH-ADD were tested for their activity towards of tyrozinase and prolyl endopeptidase [8].

In the present work we found that *G. butleri* was able to effectively carry out hydroxylation of 3-keto-4-ene-androstane steroids which could be of significance as intermediates in the synthesis of pharmaceutical preparations. Besides, the effect of 1(2)-double bond and 9α-OH-group presence in steroid nucleus on the position of hydroxylation was studied.

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