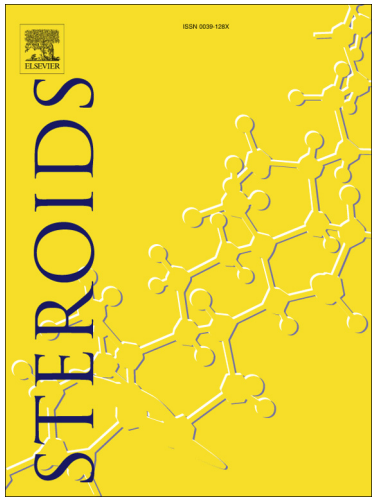

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Biotransformation of progesterone by *Aspergillus nidulans* VKPM F-1069 (wild type)

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Abstract

Biotechnological transformation of steroids using enzyme systems of microorganisms is often the only possible method to modify the molecule in the industrial production of steroid drugs. Filamentous fungus *Aspergillus nidulans* has been little studied as a steroid-transforming microorganism. We studied the ability of the *A. nidulans* VKPM F-1069 strain to transform progesterone (PG) for the first time. This strain converts PG into 3 main products: 11α-hydroxy-PG, 11α-acetoxy-PG and 6β,11α-dihydroxy-PG. It has been established that in the first stage, the hydroxylation of PG occurs into C11α position, then the formed 11α-hydroxy-PG is modified into

11α-acetoxy-PG and 6β,11α-dihydroxy-PG. It was found that changes in the composition of the growth medium, aeration and the duration of the mycelium cultivation do not affect the qualitative composition of PG transformation products, but their ratios have changed. Under conditions of limited aeration, the direction of secondary modification of 11α-hydroxy-PG is shifted towards the formation of 11α-acetoxy-PG.

Keywords: *Aspergillus nidulans,* biotransformation, progesterone, 11α-hydroxyprogesterone, 11α-acetoxyprogesterone, 6β,11α-dihydroxyprogesterone

Highlights:

- *A.nidulans* F-1069 strain converts PG to 11α-OH-PG, 11α-OAc-PG and 6β,11α-diOH-PG
- The medium composition and the growth phase of fungus affect the ratio of products
- Limitation of aeration shifts the direction of 11α-OH-PG modification to acetylation

Abbreviations:

PG (**I**), progesterone, pregn-4-ene-3,20-dione; 11α-OH-PG (**II**), 11α-hydroxyprogesterone, 11α-hydroxypregn-4-ene-3,20-dione; 11α-OAc-PG (**III**), 11α-acetoxyprogesterone, 11α-acetoxypregn-4-ene-3,20-dione; 6β,11α-diOH-PG (**IV**), 6β,11α-dihydroxyprogesterone, 6β,11α-dihydroxypregn-4-ene-3,20-dione; CM, complete medium; MM, minimal synthetic medium; DMSO, dimethyl sulfoxide; DCM, dichloromethane; ER, endoplasmic reticulum; ESI, electrospray ionization; HRMS, high-resolution mass spectrometry; TLC, thin-layer chromatography

1. Introduction

One of the main challenges of industrial corticosteroid synthesis is the introduction of oxygen function at the C11 position of steroid molecule, which is difficult to achieve by chemical methods. However, hydroxylation of the inactive C–H bond of the steroid nucleus in one step with high selectivity is possible only using the enzymatic system of microorganisms [1]. Therefore, 11(α/β)-hydroxylation of steroids by microorganisms is an economically significant method for the production of pharmacologically more active derivatives, including those having a halogen atom at position C9. In addition, biotechnological methods for modifying steroid compounds are more environmentally friendly comparing to chemical methods. Therefore, the search for effective hydroxylating microorganisms remains to be relevant.

Progesterone (pregn-4-ene-3,20-dione, PG) is a natural hormone that regulates reproductive function. It is one of the model compounds for studying the specificity of the hydroxylation of steroids in the series of pregnanes by microorganisms. Microorganisms, including filamentous fungi, are able to hydroxylate PG, introducing a hydroxyl group in different positions of the molecule [2-4]. The efficiency of these processes depends not only on the applied strain of the microorganism but also on the conditions of transformation (medium composition, pH value, aeration, temperature regime of cultivation) [5].

Filamentous fungi of the *Aspergillus* genus are applied in the industrial biotechnology for 11α-hydroxylation of steroids, for example, *A. ochraceus* [6; 7]. Furthermore, it is known that the direction of sterane skeleton hydroxylation by fungi of the genus *Aspergillus* may depend on the strain used: for example, PG can undergo hydroxylation at positions C6β, C11α, C11β, C14α, C17α, C21 [8, 9]. Herewith, it is possible to form not only the monohydroxylated product, but also its mixtures with dihydroxylated and trihydroxylated products. Some species, for example, *A. terreus* [10], *A.tamarii* [11], *A. versicolor* [12], *A. flavus* [13], *A. sojae* [14] transform PG not in the direction of hydroxylation but in the direction of the pregnane side chain elimination with the formation of the androstane series compounds. Thus, the problem of regio- and stereo-selective microbiological oxidation is one of the most important in the chemistry of steroids [1].

It has been shown that in various fungi including the *Aspergillus* genus hydroxylation is catalyzed by steroid-hydroxylation enzymes, which are a complex of cytochrome P450-dependent monooxygenase with flavoprotein [15, 16]. These enzymes (e.g., progesterone-11α-monooxygenase (EC No. 1.14.99.14), steroid-21-monooxygenase (EC No. 1.14.99.10) et al. [17, 18]) are part of a multienzyme oxidative complex of mycelial fungi. Steroid hydroxylases are highly stereospecific enzymes, often localized on the endoplasmic reticulum (ER) membrane and

are mainly induced by substrates [1]. Their sensitivity to various environmental factors (temperature, pH, etc.) determines the direction of substrate transformation.

The mycelial fungus *Aspergillus nidulans* is one of the most well-known eukaryotic systems, widely used as a model system for decoding cell cycle biology, pathogenicity, drug resistance, human diseases, primary and secondary metabolism of other microorganisms. Despite this, the *A. nidulans* strains are not sufficiently studied as steroid-transforming microorganisms. However, there are reports that this species have 11α-monooxygenase activity and are able to transform PG with the formation of 11α-OH-PG predominantly. For example, M.J. Henry and H.D. Sisler [19] reported that *A. nidulans* (Eidam) Winter fungus (strain 003) hydroxylates PG to form a mixture containing 11α-OH-PG, 6β-OH-PG and 6β,11α-diOH-PG. The culture of the fungus *A. nidulans* (from the collection of the Center of cultures of the laboratory of microbiological chemistry, National Research Center, Cairo, local habitat) transformed PG not only into 11α-OH-PG and 6β,11α-diOH-PG, but also into 21-OH-PG [20, 21].

The purpose of this study was to evaluate the ability of the wild-type *A. nidulans* VKPM F-1069 strain to transform PG, to study the effect of transformation conditions, namely, the composition of the culture medium, the duration of the mycelium cultivation and the aeration conditions, on its direction.

2. Experimental

2.1. Materials

Progesterone (**I**) (CAS No. 57-83-0, $C_{21}H_{30}O_2$, M.w. 314.46; Steraloids Inc., USA) and 11α-hydroxyprogesterone (**II**) (CAS No. 80-75-1, $C_{21}H_{30}O_3$, M.w. 330.46; Steraloids Inc., USA), were used as initial substrates and as standards. 11α-Acetoxyprogesterone (**III**) (CAS № 2268-98-6, $C_{23}H_{32}O_4$, M.w. 372.5; Steraloids Inc., USA) was used as a standard.

Inorganic salts was purchased from companies Fluka (Germany) and Amresco (USA). The yeast extract (Sigma-Aldrich, USA), peptone (Bacto Difco, USA), dimethyl sulfoxide (99.0%; Serva, USA) and agar (Difco Becton Dickinson and Company, Sparks, USA) were used.

Other materials and solvents (chemically pure grade and analytical grade) were purchased from Russian commercial suppliers.

2.2 Microorganism and culture growth

The strain *Aspergillus nidulans* VKPM F-1069 wild type (synonym FGSC A4; ATCC 3863, 12996, 26451; CBS 112.46; NRRL 194) from Russian National Collection of Industrial Microorganisms (VKPM) was used.

To obtain spore inoculation material, *A. nidulans* strain was grown on the agar medium MPA (malt extract - 3 g/L, peptone - 1 g/L, agar - 20 g/L) at 37 °C for 7-10 days. Spores were washed from the agar medium with sterile water. Aqueous suspension of spores was introduced into the 750 ml shaker flasks containing 100 ml of the cultivation medium up to final concentration 1×10^5 spores/ml. Two media for further inoculation were used: complete medium (CM) or minimal synthetic medium (MM).

The CM medium has the following composition (g/L): glucose, 40; $MgSO_4\times7H_2O$, 1.0; KH_2PO_4, 0.74; peptone, 1.0; yeast extract, 1.0; L-asparagine, 0.7 [22], 200 ml of 0.5 M phosphate citrate buffer (pH 6.6), distilled water to 1 L.

The MM medium has the following composition (g/L): glucose, 20; $MgSO_4\times7H_2O$, 0.52; KH_2PO_4, 1.52; $NaNO_3$, 0.85; $CuSO_4\times5H_2O$, 0.13; $CaCl_2$, 0.11; KCl, 0.52; mixture of microelements, 1 ml; 200 ml of 0.5 M phosphate citrate buffer (pH 6.6); distilled water to 1 L. Mixture of microelements consists of (mg/L): $MnSO_4\times5H_2O$, 800; $CuSO_4\times5H_2O$, 400; $ZnSO_4\times7H_2O$, 800; $FeSO_4\times7H_2O$, 800; H_3BO_3, 50; $(NH_4)_2MoO_4\times4H_2O$, 800 [23].

The cultivation of the fungus was carried out on an orbital shaker Brunswick™ Innova® 44/44R at 240–250 rpm (amplitude 5 cm) at 37 °C.

2.3. Biotransformation procedure

After inoculation was over, the initial substrate was loaded into each flask. In all experiments, the substrates were introduced into the cultivation medium as a solution in dimethyl sulfoxide (DMSO). Final DMSO concentration in medium was 4% (v/v) in all experiments. Blank experiment was carried out in the same conditions without *A. nidulans* cells. Control samples were incubated in the absence of substrate. All experiments were performed at 37 °C at least in triplicate.

2.3.1 PG transformation

The initial concentration of PG in the medium was 1 g/L in all experiments (i.e. 100 mg/1 flask). The transformation process was carried out for 66 hours by 96-h mycelium at 240–250 rpm to identify the products of the PG transformation.

To determine the effect of the duration of the mycelium cultivation on the result of PG transformation 24, 48, 96 and 192-h mycelium was used, the transformation process was carried out for 66 hours at 240–250 rpm.

The substrate bioconversion by the *A. nidulans* strain was performed in conditions of intensive aeration (240–250 rpm) or limited aeration (90–100 rpm) under the same cultivation conditions for 216 h by 96-h mycelium to study the effect of aeration. The samples were taken after

14 and 24 h, and then every 24 hours to study the kinetics of products accumulation. The content of steroids was estimated in mol%, based on the initial substrate.

2.3.2 11α-OH-PG transformation

The transformation of 11α-OH-PG was performed under conditions of intensive aeration with initial concentration of substrate in the medium 0.5 g/L (i.e. 50 mg/1 flask) by 96-h mycelium. Two experiments with duration of 24 and 216 h transformation were performed. Upon completion of the processes isolation and identification of the products were carried out.

2.4 Isolation of biotransformation products

To isolate the products of biotransformation, mycelium cells were disintegrated directly in the cultivation medium with a Potter-Elvehjem homogenizer in portions of 10-35 ml, and the resulting homogenate was extracted three times with an equal volume of ethyl acetate. The combined extracts were washed with water, dried with Na_2SO_4, and completely evaporated. The residue was dissolved in 5 ml of dichloromethane (DCM)/ethyl acetate mixture (2:1) and subjected to chromatography on a 16×650-mm Silica gel 60 column (0.043-0.063 mm; Merck) using 30 times the amount of the sorbent to the weight of the dry residue. A mixture of DCM and acetone (0-25%) was used as the eluent. The eluted fractions were analyzed for the content of biotransformation products by thin-layer chromatography (TLC) on Silica gel 60 F_{254} TLC plates (Merck) in the DCM /acetone solvent systems **A** (9:1 v/v) or **B** (4:1 v/v). The biotransformation products were visualized on the plates under UV light (254 nm); the plates were then sprayed with 1% vanillin solution in 10% aqueous solution of $HClO_4$ and developed at 100-120 °C. The fractions showing the simultaneous presence of few metabolites were subjected to preparative TLC on the same plates. Individual substances were eluted from the plates with DCM-acetone mixture (1:1 v/v) and the solvent was evaporated *in vacuo* to dryness. The compounds were crystallized from diethyl ether. Chromatographic purity of the substances was monitored by TLC.

2.5 Identification of biotransformation products

The structure and purity of the isolated compounds were confirmed by TLC and HPLC (in comparison to known standards), ^{1}H NMR and ^{13}C NMR, and high-resolution mass spectrometry (HRMS).

The ^{1}H NMR and ^{13}C NMR spectra were registered on a Bruker Avance-400 spectrometer (Bruker, USA) with the working frequencies of 400 MHz for ^{1}H-NMR and 100.6 MHz for ^{13}C-NMR (with complete proton decoupling) and a Bruker Avance III spectrometer (Bruker, USA) with the working frequencies of 300 MHz for ^{1}H NMR and 75.5 MHz for ^{13}C NMR (with complete

proton decoupling) at 300K. Chemical shifts in ppm were measured relative to the residual solvent signals as internal standards ($CDCl_3$, δ ^{1}H: 7.26 ppm, ^{13}C: 77.1 ppm; DMSO-d_6, δ ^{1}H: 2.50 ppm, ^{13}C: 39.5 ppm) and relative to tetramethylsilane, spin–spin coupling constants (*J*) are given in Hz.

High-resolution mass spectra (HRMS) were registered on a Bruker Daltonics micrOTOF-Q II hybrid quadrupole time-of-flight mass spectrometer using electrospray ionization (ESI); measurements were done for positively charged ions. The voltage on the capillary was 4500 V; range of scanned masses, *m/z* 50-3000; external calibration (Electrospray Calibrant Solution; Fluka, Germany); nebulizer pressure: 0.4 bar; flow rate: 3 µl/min; nitrogen as dry gas (6 liters/min); interface temperature: 180 °C. The samples were injected into the mass spectrometer chamber from the Agilent 1260 HPLC system equipped with an Agilent Poroshell 120 EC-C18 column (3.0×50 mm; 2.7 µm) and an identically packed security guard using an autosampler. The samples were in 50% acetonitrile (LC-MS grade; Panreac, Spain) in water (MilliQ ultrapure water; Merck Millipore KGaA, Germany). The column was eluted with a gradient of acetonitrile (A) concentrations in water (B) with a flow rate of 400 µl/min in the following gradient parameters: 0-15% A for 6 min, 15-85% A for 1.5 min, 85-0% A for 0.1 min, and 0% A for 2.4 min. The retention times were: 6β,11α-diOH-PG – 4.2 min; 11α-OH-PG – 5.2 min; 11α-OAc-PG – 6.0 min, PG – 6.7 min.

Melting points of the isolated compounds were determined with a Melting Point M-565 instrument (Büchi Labor Technik AG, Switzerland).

2.6 Preparation of 11α-acetoxyprogesterone by chemical method

A solution of 11α-OH-PG (34 mg) in acetic anhydride (0.2 ml) containing one drop of 60% $HClO_4$ was kept at room temperature for 1 h. After completion of the reaction, the reaction mixture was added dropwise in 5 ml of water containing 1 ml of 25% ammonia solution, and stirred for 30 min (pH ~ 7.5). Then the reaction mass was extracted with DCM three times, the extract was washed with water until neutral, evaporated to dryness. A dry residue (39 mg) was obtained, and 11α-OAc-PG (27 mg) was recovered by preparative chromatography. The yield was 70.5%, mp. 173-174 °C (published mp. 175-177 °C [24]).

2.7 Characterization of biotransformation products

11α-Hydroxypregn-4-ene-3,20-dione (11α-hydroxyprogesterone, 11α-OH-PG, II),

Mp. 164-166 °C (published mp. 164-165 °C [25]). M.w. 330.46.

HRMS spectrum $C_{21}H_{30}O_3$ (*m/z*): calculated for $[M+H]^+$ 331.2268, found 331.2264; calculated for $[M+Na]^+$ 353.2087**,** found 353.2088**.**

^{1}H NMR (400 MHz, $CDCl_3$, *δ*): 5.73 (s, 1H, CH-4), 4.04 (ddd (*pseudo*-dt), $^3J_{11,\,9}$ = 10,3 Hz $^3J_{11,\,12\alpha}$ 4.8 Hz $^3J_{11,\,12\beta}$ 4.6 Hz 1H, CH-11β), 2.66 (dt, $^2J_{1\beta,\,1\alpha}$ 13.7 Hz $^3J_{1\beta,\,2}$ 4.4 Hz 1H, CH-1β), 2.55

(dd (*pseudo*-t), $^{3}J_{17,\ 16\alpha}$ 8.7 Hz $^{3}J_{17,\ 16\beta}$ 9.1 Hz 1H, CH-17), 2.48-2.26 (m, 5H, CH_2-2 and CH_2-6 and CH-12β), 2.22-2.10 (m, 1H, CH-16β), 2.13 (s, 3H, CH_3-21), 2.02 (td, $^{2}J_{1a,\ 1b}$ 13.7 Hz $^{3}J_{1\alpha,\ 2}$ 4.5 Hz 1H, CH-1α), 1.87-1.81 (m, 1H, CH-7β), 1.78-1.64 (m, 3H, CH-8 and CH-15α and CH-16α), 1.58-1.47 (2H (t, $^{3}J_{12\alpha,\ 12\beta}$ 11.3 Hz 1H, CH-12α), 1.31 (s, 3H, CH_3-19), 1.29-1.20 (m, 2H, CH-12α and CH-14), 1.14 (t $^{3}J_{9,\ 8}$ 10.3 Hz 1H, CH-9), 1.14-1.04 (2×ddd, $^{3}J_{7\alpha,\ 7\beta}$ 13.1 Hz $^{3}J_{7,\ 6}$ 13.0 Hz $^{3}J_{7\alpha,\ 8}$ 3.8 Hz 2H, CH_2-7α), 0.69 (s, 3H, CH_3-18).

^{13}C NMR (100.6 MHz $CDCl_3$, *δ*): 208.91 (s, CO-20), 200.23 (s, CO-3), 170.96 (s, C-5), 124.65 (s, CH-4), 68.93 (s, CH-11), 63.21 (s, CH-17), 59.08 (s, CH-9), 55.44 (s, CH-14), 50.53 (s, CH_2-12), 44.19 (s, C-13), 40.02 (s, C-10), 37.59 (s, CH_2-1), 35.04 (s, CH_2-2), 34.23 (s, CH_2-6), 33.65 (s, CH-8), 31.65 (s, CH_2-7), 31.37 (s, CH_3-21), 24.31 (s, CH_2-15), 23.07 (s, CH_2-16), 18.40 (s, CH_3-19), 14.55 (s, CH_3-18).

The ^{1}H NMR and ^{13}C NMR spectrums were identical to the standard sample.

11α-Acetoxypregn-4-ene-3,20-dione (11α-acetoxyprogesterone, 11α-OAc-PG, III)

Mp. 172-174 °C (published mp. 175-177 °C [24]. M.w. 372.5;

HRMS spectrum $C_{23}H_{32}O_4$ (*m/z*): calculated for $[M+H]^+$ 373.2373, found 373.2361; calculated for $[M+Na]^+$ 395.2193, found 395.2183.

^{1}H NMR (300 MHz, DMSO-d_6) , δ: 5.67 (s, 1H, CH-4), 5.14 (dt, $^{3}J_{11,\ 9}$ = 10.6 Hz, $^{3}J_{11,\ 10}$ 5.0 Hz, 1H, CH-11), 2,62 (dd (*pseudo*-t), $^{3}J_{17,\ 16\alpha}$ 8.9 Hz, $^{3}J_{17,\ 16\beta}$ 9.1 Hz, 1H, CH-17), 2.47-2.35 (m, 2H, CH-2β and CH-6β),2.29-2.12 (m, 3H, CH-6α and CH-12β and CH-2α), 2.10-1.96 (m, 1H, CH-16β), 2.05 (s, 3H, CH_3-21), 2.01 (s, 3H, CH_3-23), 1.91-1.78 (m, 3H, CH_2-1 and CH-7α), 1.72-1.60 (m, 3H, CH-16α and CH-15α and CH-8), 1.50 (t, $^{3}J_{12\alpha,\ 12\beta}$ 11.6 Hz, 1H, CH-12α), 1.44 (t, $^{3}J_{9,\ 8}$ 10.6 Hz, 1H, CH-9), 1.39-1.29 (ddd, $^{3}J_{14,\ 8}$ 10.7 Hz, $^{3}J_{14,\ 15\beta}$ 6.4 Hz, $^{3}J_{14,\ 15\alpha}$ 5.6 Hz, 1H, CH-14), 1.29 (wd.s, 4H, CH_3-19 and CH-15β), 1.17-1.00 (2×ddd, $^{3}J_{7\alpha,\ 7\beta}$ 12.6 Hz, $^{3}J_{7,\ 6\alpha} \sim {}^{3}J_{7,\ 6\beta}$ 12.2 Hz, $^{3}J_{7\alpha,\ 8}$ 3.1 Hz, 2H, CH_2-7), 0.64 (s, 3H, CH_3-18).

^{13}C NMR (300 MHz, DMSO-d_6) , δ: 208.61 (c, CO-20), 198.40 (c, CO-3), 170.14 (c, C-5), 170.10 (c, CO-22) 124.48 (c, CH-4), 70.58 (c, CHOH-11), 62.30 (c, CH-17), 55.34 (c, CH-9), 54.39 (c, CH-14), 45.02 (c, CH_2-12), 43.54 (c, C-13), 39.75 (c, C-10), 36.79 (c, CH_2-1), 34.72 (c, CH-8) 34.19 (c, CH_2-2), 33.03 (c, CH_2-6), 31.67 (c, CH_2-7), 31.37 (c, CH_3-21), 24.16 (c, CH_2-15), 22.96 (c, CH_2-16), 22.02 (c, CH_3-23), 18.20 (c, CH_3-19), 14.24 (c, CH_3-18).

The ^{1}H NMR and ^{13}C NMR spectrums were identical to the standard sample, as well as the spectra of the sample obtained by the chemical method of 11α-OH-PG acetylation.

6β,11α-Dihydroxypregn-4-ene-3,20-dione (6β,11α-dihydroxyprogesterone, 6β,11α-di-OH-PG, IV)

Mp. 242-244 °C (published mp. 244-246 °C [26]. M.w. 346.46.

HRMS spectrum $C_{21}H_{30}O_4$ (*m/z*): calculated for $[M+H]^+$ 347.2217, found 347.2212; calculated for $[M+NH_4]^+$ 364.2482, found 364.2482.

^{1}H NMR (300 MHz DMSO-d_6): 5.65 (s, 1H CH-4), 5.08 (br. s, 1H, OH), 4.35 (wd. s, 1H, OH), 4.14 (br. s, 1H, CH-6), 3.86 (ddd (*pseudo*-dt), $^3J_{11,\,9}$ 10.4 Hz $^3J_{11,\,12\alpha}$ 4.6 Hz $^3J_{11,\,12\beta}$ 4.4 Hz 1H, CH-11), 2.75 (ddd (*pseudo*-td of protons X in the ABXY system), $^2J_{1a,\,1b}$ 13.7 Hz $^3J_{1a,\,2a}$ 3.6 Hz $^3J_{1a,\,2b}$ 3.3 Hz, 1H, CH-1β), 2.61 (dd (*pseudo*-t), $^3J_{17,\,16a}$ 8.5 Hz $^3J_{17,\,16b}$ 8.8 Hz,1H, CH-17), 2.43 (ddd, $^2J_{2a,\,2b}$ 14.7 Hz $^3J_{2a,\,1a}$ 4.5 Hz $^3J_{2a,\,1b}$ 3.6 Hz, 1H, CH-2β), 2.18 (m, 2H, CH-2α and CH-12β), 2.08 (br. s, 4H, CH_3-21 and CH-16β), 1.92-1.86 (m, 1H, CH-8), 1.86-1.84 (m, 2H, CH-1α and CH-7β), 1.67-1.57 (m, 2H, CH-16α and CH-15α), 1.47 (dd (*pseudo*-t), $^3J_{12\alpha,\,11}$ 10.4 Hz $^3J_{12\alpha,\,12\beta}$ 11.6 Hz, 1H, CH-12α), 1.39 (s, 3H, CH_3-19), 1.30-1.08 (m, 3H, CH-7α and CH-15β and CH-14), 1.00 (m (*pseudo*-t), 1H, CH-9), 0.60 (s, 3H, CH_3-18).

^{13}C NMR (300 MHz DMSO-d_6): 208.14 (s, CO-20), 199.63 (s, CO-3), 169.56 (s, C-5), 125.51 (s, CH-4), 71.22 (s, CHOH-6), 67.12 (s, CHOH-11), 62.37 (s, CH-17), 58.20 (s, CH-9), 54.72 (s, CH-14), 49.33 (s, CH_2-12), 43.61 (s, C-13), 38.87 (s, C-10), 38.55 (s, CH_2-1), 37.98 (s, CH_2-7), 34.05 (s, CH_2-2), 30.92 (s, CH_3-21), 27.94 (s, CH-8), 23.83 (s, CH_2-15), 22.25 (s, CH_2-16), 19.57 (s, CH_3-19), 14.16 (s, CH_3-18).

3. Results and discussion

It is known that the steroid-hydroxylation activity of the mycelial fungus may depend on the duration of mycelium cultivation before substrate loading [27]. The kinetics of *A. nidulans* F-1069 mycelium growth in CM medium was studied. This medium was previously described by A.H. El-refai and K.M. Ghanem [20], as the most favorable for the conversion of PG to 11α-hydroxy-derivative by *A. nidulans* culture. The kinetics of fungal biomass accumulation in the process of growing is presented (Figure 1).

Figure 1

On 96 h of cultivation the *A. nidulans* F-1069 reached the end of the logarithmic growth phase accompanied with the significant biomass production. Therefore, a 96-h mycelium was used for identification of the PG transformation products.

3.1. Biotransformation of PG

After growing mycelium for 96 h, the PG was loaded as a solution in DMSO into the growth medium without mycelium separation. DMSO not only increases the substrate solubility in aqueous medium [28] but also leads to an increase in the yield of 11α-hydroxylated product [29].

It has been found that the strain *A. nidulans* VKPM F-1069 has steroid-hydroxylation activity. PG (**I**) was converted to three major metabolites: 11α-OH-PG (**II**), 11α-OAc-PG (**III**) and 6β,11α-diOH-PG (**IV**) with the following R*f* values 0.43, 0.84 and 0.24, respectively, (PG R*f* - 0.89) in system **B** (eluted twice) (Figure S1). The products were identified *via* HPLC-HRMS (Figure 2).

Figure 2

The formation of monohydroxylated product (**II**) was supported by accurate mass determination of $C_{21}H_{30}O_3$ in ESI HRMS, *m/z*: $[M+H]^+$ found 331.2264 (calcd. 331.2268) and $[M+Na]^+$ found 353.2088 (calcd. 353.2087). 11α-OH-PG (**II**) was identified by its ^{1}H NMR and ^{13}C NMR spectra and in comparison with that of the PG (**I**) and the spectra of the 11α-OH-PG sample (**II**), obtained by us earlier [4] (spectra can be found in the Supplementary material, S2-S4).

The structure of the acetylated product (**III**) was determined by several analytical methods. Formation of **III** was supported by accurate mass determination of $C_{23}H_{32}O_4$ in ESI HRMS, *m/z*: $[M+H]^+$ found 373.2361 (calcd. 373.2373) and $[M+Na]^+$ found 395.2183 (calcd. 395.2193).). NMR spectra of the obtained 11α-OAc-PG (**III**) were identical to the spectra of the commercial standard sample and the chemically synthesized one (section 2.6 of this article). In addition, 11α-OAc-PG (**III)** obtained during the PG transformation was hydrolyzed by a known method of chemical solvolysis under base catalysis conditions: by KOH in methanol medium [30] to form 11α-OH-PG. (spectra can be found in the Supplementary material, S5-S13).

The formation of dihydroxylated poduct (**IV**) was confirmed by the data of ^{1}H NMR, ^{13}C NMR, 2D NMR spectroscopy and HPLC-HRMS, and matched with 6β,11α-diOH-PG sample obtained by us earlier [4] (spectra can be found in the Supplementary material, S14-S22). There are peculiarities in the observed signals that haven't been discussed earlier in literature. For example, the proton at CH-6 is expected as a part of the ABX system, nevertheless, it appears unresolved as a broadened singlet. This can be explained by the long-range coupling interaction $^4J_{CH\text{-}6,\ CH\text{-}4}$ through space, since these hydrogen atoms are coplanar, *via* the so called W-pathways. The effect is additionally confirmed in HMBC spectrum where H-4 / C-6 cross-peak demonstrates a pronounced interaction (as well as H-4/C-2 or H-6/C-8 cross-peak). Several CH_2-signals are found in ABXY patterns in ^{1}H NMR, e.g. CH_2-1, CH_2-7, CH_2-12, as well as in ABX patterns, e.g. CH_2-15 and CH_2-16 groups. Their assignment is challenging; due to the conformational restrictions they are vastly splitted in ^{1}H NMR with the second-order effects, overlap with other signals and may be misplaced

in the attribution. We carried out an independent thorough investigation with 2D NMR experiments in DMSO-d_6 and compared our assignment with previous studies on NMR of the steroid systems [31]. Our data confirm the shifts assignments of earlier reports of ^{13}C NMR of 6β,11α-dihydroxy-PG [32], and clarify the missing characteristic CH_2-7 and CH_2-10 signals of earlier papers [26], as well as previous lack of fine structure description of ^{1}H NMR spectrum.

All the ^{1}H, ^{13}C, HSQC and HMBC NMR spectra, HRMS spectral data as well as HPLC profiles of the formed progesterone derivatives can be found in the Supporting information.

3.2. The effect of the duration of mycelium cultivation on the transformation products accumulation

To determine the effect of the duration of the mycelium cultivation on the result of PG transformation, we used the mycelium at the beginning, middle and at the end of the logarithmic growth phase (24, 48 and 96 h, respectively), as well as mycelium at the stationary growth phase (192 h). The processes were performed with intensive aeration under the conditions described above in section 2.3.1.

Regardless of the duration of the fungus cultivation, the component composition of the major products did not change, however their ratios have changed (Figure 3, Supplementary material Table S23).

Figure 3

With an increase of the duration of the mycelium cultivation up to 96 h, the PG conversion increased. The most intensive accumulation of products **III** and **IV** occurred when using 96-h mycelium (peak of the logarithmic growth phase). The transformation of PG (**I**) by stationary growth phase mycelium (192 h) resulted in lower PG conversion with reduced quantities of products **III** and **IV**, and increased content of (**II**) comparing with 96 h mycelium. Reduction of 6β-hydroxylating activity of *A. nidulans* strain with the increase of the duration of the mycelium cultivation was observed earlier [20].

3.3. The kinetics of products accumulation during the PG biotransformation

Some authors note that at pH values close to neutral, 6β-hydroxylation is a secondary process, and 6β,11α-diOH-PG is produced from the initially formed 11α-OH-PG [20, 33, 34]. Here we assume that 11α-OAc-PG is also a product of its secondary modification. To confirm this, we studied the kinetics of products accumulation during PG transformation process. The biotransformation was performed under the conditions described above (section 3.1) for 216 hours. The kinetics of products accumulation is shown in Figure 4 (solid lines).

Figure 4

It is important to note, that TLC and HPLC chromatograms of the sample taken after 14 h of transformation showed the presence of untransformed PG (98.5% of loaded) and 11α-OAc-PG (0.8 mol %). During further transformation the formation of 6β,11α-diOH-PG and 11α-OH-PG was observed.

After 216 h of transformation from 300 mg of loaded PG (3 flasks) three main products were observed. The dry residue after the evaporation of the extract contained 123 mg (34.6 mol %) of 11α-OAc-PG, 6.3 mg (2.0 mol %) of 11α-OH-PG, and 150 mg (45.4 mol %) 6β,11α-diOH-PG according to HPLC analysis. The untransformed PG was absent.

3.4. Transformation of 11α-OH-PG

Additionally, experiments on the transformation of 11α-OH-PG under similar conditions were performed. 200 mg of 11α-OH-PG was loaded for the transformation (4 flasks).

After 24 hours of transformation 178 mg (88.0 mol %) of untransformed 11α-OH-PG and 19.8 mg (8.8 mol %) of 11α-OAc-PG were found in the dry residue after evaporation of the extract according to TLC and HPLC data.

After 216 hours of transformation the dry residue contained 192 mg (85.2 mol %) of 11α-OAc-PG and 26 mg (12.4 mol %) of 6β,11α-diOH-PG. According to HPLC data 11α-OH-PG was practically absent. The yield of crystalline 11α-OAc-PG was 180 mg (80%, counting on the loaded 11α-OH-PG).

Thus, the complete conversion both PG and 11α-OH-PG was achieved after 216 hours of transformation. However, the selectivity of 11α-OAc-PG formation in the first case was 34.6 mol%, and in the second it reached 85 mol%. Consequently, products **III** and **IV** are competitive and can be obtained both from PG and from 11α-hydroxy-PG. However, the appearance of 6β,11α-diOH-PG occurs earlier when using PG as an initial substrate (Figure 4B). This may be due to the presence of PG, which is known to be an inducer of 6β-hydroxylase [20]. The scheme of the studied transformations is shown in Figure 5.

Figure 5

In the first stage, the hydroxylation of PG occurs into C11α position, and then the formed 11α-hydroxy-PG is modified into 11α-acetoxy-PG and 6β,11α-diOH-PG.

It is known that some microorganisms can perform acetylation of secondary alcohols, including steroid compounds. For example, the ability of yeast and yeast-like organisms (*Saccharomyces fragilis, S. lactis, Candida pseudotropicalis* and *Torulopsis sphaerica*) to acetylate testosterone in the CM medium was previously described [22]. However, acetylation occurred only

with 17β-hydroxy derivative, whereas the corresponding 17α-epimer remained intact. In addition, the authors noted that under these conditions acetylation of the steroid molecule with the hydroxy group at the 11α, 11β, 20β and 21 positions does not occur. Therefore, acetylation of the secondary hydroxyl groups is usually carried out chemically, using, for example, acetic anhydride as the acetylating agent. For example, 11α-OAc-PG is obtained from 11α-OH-PG by the chemical method using acetic anhydride in pyridine medium [24].The ability of mycelial fungi, in particular, the *Aspergillus* genus, to acetylate steroid alcohols has not been described previously. Here we report about the ability of the *A. nidulans* VKPM F-1069 strain to acetylate 11α-OH-PG for the first time.

It is known that esters are the products of an enzyme-catalyzed condensation reaction between acyl-CoA and alcohols. The biochemical esterification reaction is catalyzed by an ester synthase and the ester synthesis rate is determined by the concentration of the available substrate and the total enzymatic activity [35].

Moreover, fungal steroid hydroxylases are often localized on the ER membrane [1, 16]. The synthesis of fatty acids with the participation of acetyl-coenzyme A (acetyl-CoA) occurs on the membranes of the ER in the cytoplasm as well [36]. Acetyl-CoA is the sole donor of acetyl groups for acetylation [37, 38]. It is known to be also involved in *O*-acetylation of polysaccharides, alkaloids, anthocyanins, isoprenoids and phenols in plants [39].

It can be assumed that acetylation of 11α-OH-PG occurs with the participation of acetyl-CoA, which can act as a donor of the acetyl group. However, the amount of 11α-OAc-PG depends not only on the concentration of 11-OH-PG (i.e. 11α-hydroxylation activity of a strain) but also on the activity of the enzyme 6β-hydroxylase, under the action of which 11-OH-PG is converted into 6β,11α-diOH-PG - a product of the competitive direction of its transformation. It is known that the efficiency of hydroxylation processes depends on the aeration [40].

Therefore, we have assumed that the direction of secondary modification of the primary formed 11-OH-PG can be dependent on the aeration intensity.

3.5. The aeration effect on the accumulation of PG transformation products

The aeration effect on the PG biotransformation was studied in shake flasks with varying agitation regimes: intensive aeration and limited aeration. The substrate was loaded after 96 hours of strain cultivation in CM. Figure 4A shows the dependence of PG conversion rate on the agitation regime, and Figure 4B shows the kinetics of the products accumulation under different aeration.

As can be seen from Figure 4A, the PG conversion rate decreases when aeration is limited. At the same time, during the transformation under limited aeration conditions, there is a decrease in the accumulation of compound **IV**, and an increase in the content of the monohydroxylated product

(**II**) in comparison with the conditions of intensive aeration (Figure 4B). The maximum amount of product **IV** is reached by 96 h of transformation under the intensive aeration (45.4 mol %), and by 120 h under limited aeration (36.3 mol %), and does not change further. It should also be noted that the aeration regime has little effect on the accumulation rate and the amount of 11α-OAc-PG. After the 6β-hydroxylation process has stopped, the process of 11α-OH-PG acetylation continues in both cases, as can be seen from Figure 4B. Moreover, it proceeds more intensively under conditions of limited aeration.

3.6. The medium composition effect on the accumulation of transformation products

To evaluate the effect of medium composition on the result of PG transformation we used CM and MM media. The diagram of PG biotransformation products accumulation by 96-h culture of the fungus on different media under conditions of intensive aeration is shown in Figure 6.

Figure 6

The biotransformation proceeds to form the same composition of products with both media for inoculation and PG transformation. Herewith, PG conversion in MM medium slows down more than twice as compared with the CM medium. However, the quantity of the monohydroxylated product (**II**) on the MM medium is twice higher, due to the secondary transformation suppression: 6β-hydroxylating activity is halved, and acetylating activity is 6 times lower. A similar decrease in the activity of 6β-hydroxylase during the PG transformation by the *A. niger* strain in MM medium at 37 °C was observed earlier by us [4].

Conclusion

In this study, we demonstrated that filamentous fungus *A. nidulans* VKPM F-1069 hydroxylates the PG at the position C11α with subsequent 6β-hydroxylation and acetylation of the formed 11α-OH-PG *in situ*. The possibility of biocatalytic acetylation of the steroid secondary hydroxyl group by filamentous fungus is shown by us for the first time. It has been found that changes in the duration of the mycelium cultivation and the composition of the growth medium do not affect the qualitative composition of PG transformation products but changes their ratio. Under limited aeration, the direction of secondary modification of 11α-OH-PG was shifted towards the formation of 11α-OAc-PG by reducing the 6β-hydroxylase activity.

One of the advantages of the *A. nidulans* strain used is the absence of the side-chain cleavage at the C17-C20 bond with the formation of the androstane series compounds (androstenedione, testosterone and its acetate) during the transformation of PG.

The obtained data can serve as a basis for the development of the medical drugs synthesis technology alternative to chemical synthesis, where *A. nidulans* strain can be used as a biocatalyst for biotechnological modification of steroids. Compounds **II** and **III** obtained by biocatalytic transformation are of interest not only as precursors of highly active steroid preparations but also as independent drugs with gestagenic and antiandrogenic activity [41-43].

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Figures

Figure 1 – Kinetics of fungal biomass accumulation

Figure 2 – HPLC profile of PG biotransformation major metabolites. HPLC conditions: Agilent Poroshell 120 EC-C18 (3.0 × 50 mm; 2,7 µm) column; flow rate 400 µL/min in a gradient of concentrations of A (CH_3CN) in B (H_2O): 0-15% A for 6.0 min, 15%-85% A for 1.5 min, 85%-0% A for 0.1 min, 0% A for 2.4 min.

Figure 3 – Effect of the duration of the mycelium cultivation on the ratio of PG transformation products

Figure 4 – Kinetics of the PG conversion under different aeration: **A**- content of the residual amount of PG, **B**- content of transformation products. Designations: solid line –intensive aeration, dashed line – limited aeration; ■- PG (**I**), ▲ – 11α-OH-PG (**II**), ● – 11α-OAc-PG (**III**), **X**– 6β,11α-diOH-PG (**IV**).

Figure 5 – Scheme of PG transformation

Figure 6 – Effect of medium composition on the ratio of PG transformation products

Appendix A. Supplementary data

Supplementary data to this article can be found online at ….

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