Biotransformation of testosterone by the filamentous fungus *Penicillium pinophilum*

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Abstract
The microbial biotransformation is a robust procedure in developing steroids and fungi are practical tools in this process; therefore, the fungal modification of testosterone by *Penicillium pinophilum* was investigated. The three prominent metabolites, including 14α-hydroxyandrost-4-en-3,17-dione (II), 14α-hydroxytestosterone (III), and 11α-hydroxytestosterone (IV), were isolated and characterized by chromatographic and spectroscopic methods. The time course profile showed that the content of the metabolites II and III began to decrease after 96 and 24 h, respectively. In comparison, the content of the metabolite IV remained stable after 24 h. In silico studies showed that the probability of binding to the androgen receptor remains high for all three metabolites. However, the probability of binding to the estrogen receptors α and β increased for metabolite IV but decreased for metabolite III. *Penicillium pinophilum* as a potentially viable biocatalyst could hydroxylate C-11α and C-14α positions and oxidize the C-17β hydroxyl group to 17-ketone in testosterone molecule.

Keywords  
Biocatalysts · Fungal transformation · In silico analysis · Steroid bioconversion · Time course study

Introduction

Microbial biotransformation refers to a metabolic process by which a microorganism changes a compound from one chemical to another. This procedure has been extensively used in the pharmaceutical industry, particularly in producing steroid drugs and hormones (Yıldırım et al. 2019; Fernandes and Cabral 2006; Faramarzi et al. 2008b). Generally, the biological activity of steroids is highly dependent on their proper functionalization. Therefore, an efficient and appropriate biotransformation treatment is a key approach in producing active steroids (Wu et al. 2015; Tong and Dong 2009; Świzdor et al. 2017; Karpova et al. 2016; Ghasemi et al. 2014).

Fungi have a special talent among the microorganisms used as biocatalysts for the biotransformation of steroids (Andryushina et al. 2013; Xu and Li 2020; Faramarzi et al. 2008b). One of the advantages of using the fungal biotransformation process is particular changes (chemo-, regio- and stereo-selective) in the steroid molecules (Hüttel and Hoffmeister 2011; Al-Aboudi et al. 2008; Zhang et al. 2013; Leresche and Meyer 2006). Several fungi are known to have the ability to cause specific chemical changes and/or to introduce particular functional groups on the steroidal skeleton at different positions (Świzdor et al. 2017; Fernandes et al. 2003; Hosseinabadi et al. 2015). Although many fungi have been recognized for their capacity to perform a specific reaction, there are still ongoing efforts to find new beneficial microorganisms (Yıldırım et al. 2019; Al-Aboudi et al. 2008; Hegazy et al. 2015; Fernandes and Cabral 2006).

Testosterone, a male sex hormone and a substrate for the production of androgen derivatives, has always been particularly important among researchers (Sambyal and Singh 2020). Several studies have been performed on the microbial modification of testosterone by different microorganisms, especially fungi, to produce its various derivatives. Accordingly, the well-known biocatalytic reactions on testosterone include hydroxylation, oxidation of 17β-OH to the corresponding ketone, dehydrogenation, and ring-D lactonization and hydroxylation is the most common (Yıldırım et al. 2019; Świzdor et al. 2017; Peart et al. 2011, 2013).
Penicillium is a genus of ascomycetous fungi. The members of the genus are widely distributed in the natural environment and are commonly considered non-pathogenic to human (Perrone and Susca 2017; Petit et al. 2009; Ramos-Ponce et al. 2012). Penicillium species are sources for penicillin, mycophenolic acid, pigments, and extracellular enzymes (Nicoletti et al. 2008; Meena et al. 2017). Some members of the genus have been extensively studied for their biocatalytic activities, especially in fungal biotransformation processes of steroids (Bartmańska et al. 2005; Yildirim et al. 2010a; Cabeza et al. 1999; Yang et al. 2014; Holland et al. 1995; Tweit et al. 1962; Panek et al. 2020). However, to our knowledge, there have been no studies on the bioconversion of steroids by P. pinophilum.

The present work focuses on P. pinophilum to transform testosterone, the in silico study of the derived metabolites against steroid hormone receptors, and the time course study to analyze the development of the metabolites as a function of the time.

Materials and methods

Chemicals

Testosterone (17β-hydroxyandrost-4-en-3-one, C_{19}H_{28}O_{2}) was acquired from Acros Organics (Geel, Belgium). TLC silica gel 60 F_{254} sheets, HPTLC silica gel 60 F_{254} plates, Sabouraud Dextrose Agar (SDA), and Sabouraud Dextrose Broth (SDB) were procured from Merck (Darmstadt, Germany).

Microorganism

The fungal strain of Penicillium pinophilum PTCC 5168 was obtained from Persian Type Culture Collection (Iranian Research Organization for Science and Technology), and was then subcultured before using in the biotransformation process. For this purpose, the microorganism was initially cultured in a flask containing 50 mL of SDB at 28 °C for 2 days. After this period, the fungus was inoculated on SDA for 5 days to form spore. Then, it was stored at 4 °C.

Biotransformation process, isolation and identification metabolites

This process was organized in two scales, screening and semi-preparative. For the screening scale, spores collected from SDA cultures were inoculated in 250-mL flasks comprising 50 mL of SDB (1 × 10^4 spores/mL in the final medium), and next they were incubated at 28 °C in a shaker at 170 rpm. After 24 h, testosterone solution in methanol was added to the flasks at a final concentration of 0.05%, and fermentation was extended for 6 more days. During the incubation period, sampling was performed at intervals of 24, 48, 72, 96, 120, and 144 h. Each time, the content of one flask was extracted three times with 30 mL of CHCl_3. After evaporating chloroform, the concentrated extract was analyzed by TLC. TLC on silica gel 60 F_{254} with a solvent mixture of CHCl_3–CH_3OH (8.8–1.2 v/v) was applied to separate the metabolites, and UV light 254 nm was used to visualize them. The controls were similarly processed (Nickavar et al. 2019).

The semi-preparative scale was conducted using 1000-mL flasks consisting of 200 mL of SDB and 100 mg of testosterone. The other conditions were the same as those described above. At the end of the process, the reaction mixture was extracted in the manner formerly explained. The repeated preparative layer chromatography on silica gel with the mobile phase of CHCl_3–CH_3OH (8.8–1.2 v/v) was used to isolate the compounds from the extract. Spectral data structurally determined the purified metabolites (Nickavar et al. 2019).

The 1H and 13C NMR spectra were acquired using Bruker Avance 500 and 300 (Bruker BioSpin GmbH, Rheinstetten, Germany) at 500 and 300 MHz, 125 and 75 MHz, respectively in CDCl_3. The ESI–MS spectra were recorded on an Agilent 6410 Triple Quad mass spectrometer in positive ion mode (Agilent Technologies, Inc., Wilmington, DE, USA).

Time course study

This study was performed to assess the amounts of substrate and products throughout the biotransformation reaction by HPTLC. The experimental conditions were similar to those used in the screening process, except that sampling was performed three times in each time step. The content of substrate and metabolites in each sample was determined through an individual five-point calibration curve. For this purpose, the standards and the daily samples were applied as 7 mm bands on HPTLC plates using a CAMAG automatic TLC sampler 4 (CAMAG, Muttenz, Switzerland). The plates were then run with a mixture of chloroform–methanol (8.8–1.2 v/v). Densitometric scanning of the plates was performed at 254 nm using a CAMAG TLC scanner 3 fitted with win CATS software (version 1.4.4). All the quantitative analyses were done in triplicate (Nickavar et al. 2019).

In silico evaluation of metabolites

The identified metabolites were studied for their potential to interact with some steroid hormone receptors (androgen receptor, estrogen receptors α and β, progesterone receptor, and glucocorticoid receptor) using Endocrine Disruptome (ED) (http://endocrinedisruptome.ki.si). This free Web-based platform performs molecular docking analysis with
Autodock Vina against 14 well-validated human nuclear receptors. Then, the application predicts the binding affinity potential for each examined compound with individual targets. The gradation of the potentials is performed based on the probability of binding. The results fall into one of the four color-coded categories as follows: red color represents compounds with a high probability of binding, the orange color corresponds to compounds with the sub-high probability of binding, yellow color reflects compounds with a medium probability of binding, and green color denotes compounds with a low probability of binding (Kolšek et al. 2014).

**Results and discussion**

In this investigation, the capability of *Penicillium pinophilum* in the transformation of testosterone during a 6-day period was studied, and three metabolites were identified and assessed by spectroscopic analysis and HPTLC. An in silico evaluation on the interaction of the metabolites with some steroid hormone receptors was also performed using the ED tool.

**Structure elucidation of compounds**

Based on the daily TLC pattern obtained from the biotransformation process in the screening scale, it was found that 96 h after adding substrate (testosterone) is the suitable extraction time for the semi-preparative scale. The TLC profile of the extracted medium showed the presence of three distinct and clear bands with $R_f$ values of 0.33, 0.45, 0.75, respectively (Fig. 1). This means that testosterone (I) has been transformed into three significant metabolites (II–IV) by *Penicillium pinophilum*. Accordingly, the fungi produced the three metabolites, then they were isolated by preparative layer chromatography technique and finally were identified using the spectral data. All the corresponding spectra have been represented in supplementary material (Fig. S1–S9). However, in addition to these metabolites, some other products were also generated that could not be identified due to their small concentration.

17β-Hydroxyandrost-4-en-3-one (Testosterone) (substrate I): White solid; $R_f$ in Chloroform–Methanol (8.8–1.2, v/v): 0.80; ESI-MS, $m/z$ [M + H]$^+$: 289; $^1$H NMR (CDCl$_3$, 500 MHz): δ 0.79 (3H, s, H-18), 1.20 (3H, s, H-19), 3.65 (1H, t, $J = 8.5$ Hz, H-17α), 5.72 (1H, s, H-4). The chemical shift values of carbon atoms in $^{13}$C NMR (CDCl$_3$, 125 MHz) spectrum have been presented in Table 1.

14α-Hydroxyandrost-4-en-3,17-dione (metabolite II): White solid; $R_f$ in Chloroform–Methanol (8.8–1.2, v/v): 0.75; ESI-MS, $m/z$ [M + H]$^+$: 303; $^1$H NMR (CDCl$_3$, 300 MHz): δ 1.05 (3H, s, H-18), 1.22 (3H, s, H-19), 5.75 (1H, s, H-4). The chemical shift values of carbon atoms in $^{13}$C NMR (CDCl$_3$, 75 MHz) spectrum have been presented in Table 1.
The mass spectrum of metabolite II exhibited [M + H]+ peak at m/z 303, corresponding to the formula C_{15}H_{26}O_{3}. On the \(^1\text{H}\) NMR spectrum, the lack of H-17 triplet at \(\delta\) 3.65 ppm along with the significant downfield shifts of H-18 signal from 0.79 to 1.05 ppm showed that there would be a change at the position of C-17. These data were consistent with the \(^{13}\text{C}\) NMR spectrum, which revealed that the signal at 81.61 ppm had been replaced by a signal at 218.35 ppm for C-17. Collectively, these data indicated the oxidation of the C-17β hydroxyl group to C-17 carbonyl group. Furthermore, on the \(^{13}\text{C}\) NMR spectrum, the large shift of C-14 signal from \(\delta\) 50.53 to 80.88 ppm and also the downfield shifts for C-8 (\(\Delta\) 2.34 ppm), C-15 (\(\Delta\) 7.04 ppm), and C-13 (\(\Delta\) 9.81 ppm) confirmed the presence of a hydroxyl group at C-14α position. The data were in agreement with those reported in the literature for 14α-hydroxyandrost-4-en-3,17-dione (Faramarzi et al. 2008a, 2008b).

14α-Hydroxytestosterone (metabolite III): White solid; \(R_f\) in Chloroform–Methanol (8.8–1.2, v/v): 0.45; ESI–MS, m/z [M + H]+: 305; \(^1\text{H}\) NMR (CDCl\(_3\), 300 MHz): \(\delta\) 0.89 (3H, s, H-18), 1.19 (3H, s, H-19), 3.69 (1H, t, \(J = 10.3\) Hz, H-17α), 4.28 (1H, td, \(J = 8.5\) Hz, H-17α), 5.73 (1H, s, H-4). The chemical shift values of carbon atoms in \(^{13}\text{C}\) NMR (CDCl\(_3\), 75 MHz) spectrum have been presented in Table 1.

The mass spectrum of metabolite III revealed [M + H]+ peak at m/z 305, agreeing with the formula C\(_{19}\)H\(_{28}\)O\(_3\). The \(^{13}\text{C}\) NMR spectrum exhibited a large shift for C-14 signal from 50.53 to 83.30 ppm along with downfield shifts for C-8 (\(\Delta\) 3.24 ppm), C-15 (\(\Delta\) 2.82 ppm), and C-13 (\(\Delta\) 4.17 ppm). These changes suggested that hydroxylation has taken place from \(\delta\) 50.53 to 80.88 ppm along with downfield shifts for C-8 (\(\Delta\) 2.34 ppm), C-15 (\(\Delta\) 7.04 ppm), and C-13 (\(\Delta\) 9.81 ppm) confirmed the presence of a hydroxyl group at C-14α position. The data were in agreement with those reported in the literature for 14α-hydroxyandrost-4-en-3,17-dione (Faramarzi et al. 2008a, 2008b).

11α-Hydroxytestosterone (metabolite IV): White solid; \(R_f\) in Chloroform–Methanol (8.8–1.2, v/v): 0.33; ESI–MS, m/z [M + H]+: 305; \(^1\text{H}\) NMR (CDCl\(_3\), 500 MHz): \(\delta\) 0.82 (3H, s, H-18), 1.32 (3H, s, H-19), 3.69 (1H, t, \(J = 8.5\) Hz, H-17α), 4.04 (1H, td, \(J = 10.3\) Hz and \(J = 4.6\) Hz, H-11β), 5.73 (1H, s, H-4). The chemical shift values of carbon atoms in \(^{13}\text{C}\) NMR (CDCl\(_3\), 75 MHz) spectrum have been presented in Table 1.

The mass spectrum of metabolite IV was appeared at m/z 305, indicating the empirical formula C\(_{19}\)H\(_{28}\)O\(_3\). The \(^1\text{H}\) NMR spectrum revealed a new signal as a triplet of doublet at 4.04 ppm (\(J = 10.3\) Hz and \(J = 4.6\) Hz) and a mild downfield shift for H-19 signal (\(\Delta\) 0.12 ppm). As well, the \(^{13}\text{C}\) NMR spectrum exhibited a significant shift for C-11 signal from 20.67 to 69.07 ppm (\(\Delta\) 48.40 ppm) along with downfield shifts for C-12 (\(\Delta\) 12.26 ppm) and C-9 (\(\Delta\) 5.38 ppm). These data supported the presence of a hydroxyl group at the C-11 position. On the other hand, the splitting pattern and coupling constants for the new proton signal displayed an alpha configuration. Comparison of the spectral data with those reported in the literature supported that the metabolite IV is 11α-hydroxytestosterone (Peart et al. 2013, 2016).

**Time course profile of the biotransformation process**

For this study, a simple HPTLC method has been designed to measure testosterone and its metabolites during the biotransformation process (Fig. S10). The final obtained profile showed that the conversion of the substrate (testosterone) started from day one, and all the three identified products were detectable from that day onward (Fig. 2). According to this, the amount of testosterone was continuously reduced so that, at the end of the process (i.e., after 144 h), about 97.4% of testosterone was metabolized. The primary product (i.e., metabolite III) was detected in 0.017 μmol on the first day and gradually decreased to 0.011 μmol on the sixth day. The metabolite II reached the maximum level of 0.006 μmol on the fourth day and then reduced. The highest amount of metabolite IV was about 0.002 μmol on day six. However, the daily changes in the amount of metabolite IV were minimal.

Based on the results obtained from the time course experiments, especially the trend of changes in the content of the metabolites II (14α-hydroxyandrostenedione) and III (14α-hydroxytestosterone), and also due to the absence of androst-4-en-3,17-dione (androstenedione, AD) during the biotransformation process (tested via Co-TLC)
with AD standard), it can be assumed that the metabo-
lite III was gradually transformed into the other products
including the metabolite II. However, the metabolite IV
(11α-hydroxytestosterone) has been derived directly from
testosterone in a separate pathway (Fig. 3).

**In silico assessment of the metabolites**

In this investigation, in silico assessment with the ED pro-
togram was performed to study the increase or decrease in
computed affinities for the produced metabolites due to
hydroxylation or oxidation. One of the important functional
features of this application, which distinguishes it from other
tools for screening studies, is that the program is developed
to predict the binding affinity of a compound to several tar-
gets at a time. This approach is in contrast to the mode of
the common software which predicts binding affinities of several
ligands to one target at once (Kolšek et al. 2014).

The in silico approach showed a high probability of
binding all three metabolites to the androgen receptor. Moreover, it was determined that the hydroxylation at the
11α-position of testosterone leads to increase the binding
affinity of the metabolite (IV, 11α-hydroxytestosterone) to
the estrogen receptors α and β, while the hydroxylation at
the 14α- position of testosterone reduces the affinity of the
metabolite (III, 14α-hydroxytestosterone) to those receptors. However, both hydroxylation patterns did not affect the bind-
ing affinities of the two metabolites to the androgen, pro-
gesterone, and glucocorticoid receptors. On the other hand,
the oxidation of 14α-hydroxytestosterone at the 17β-position
enhanced the binding affinity of the produced metabolite
(i.e., 14α-hydroxyandrostenedione, metabolite II) to the
estrogen receptor α, whereas it did not affect the binding
affinity of the metabolite to the other receptors (Table 2).

Previous studies have shown that some hydroxylated
androgens have inhibitory activity on estrogen biosynthesis
(Heidary and Habibi 2016; Kolet et al. 2014). Therefore,
it can theoretically be considered that metabolites III and
IV are androgenic compounds with modified estrogenic
activities.

In general, the ability of microorganisms to transform
the steroids into other valuable compounds has been stud-
ied for many years and numerous fungi have been used
for this purpose. Among steroids, testosterone is one of
the valuable steroid substrates that many efforts have
been made to transform it into different derivatives with
pharmaceutical capacities and various fungal strains (such
as *Aspergillus* spp., *Cephalosporium* spp., *Mucor* spp.,

![Fig. 3](image_url)

**Fig. 3** The proposed biotransformation pathway of testosterone into corresponding metabolites by *Penicillium pinophilum*
Fusarium spp., Penicillium spp., corynespora spp., and Rhizopus spp.) have been used for this purpose. Hydroxylation, hydroxyl group oxidation, reduction, and lactonization are the major changes in testosterone caused by fungi (Hanson et al. 1996; Świdzor et al. 2017; Yildirim et al. 2011, 2010b, 2010c, 2013; Yildirim and Kuru 2016; Hu et al. 1995; Peart et al. 2013, 2016, 2011; Kolet et al. 2013, 2014; Al-Aboudi et al. 2008; Bartmańska et al. 2005; Yang et al. 2014; Hunter et al. 2011).

The genus Penicillium is one the most studied fungi in the biotransformation processes of steroids, including testosterone. Bartmańska et al. studied the biotransformation of testosterone by P. notatum. The primary metabolite was testolactone, with a yield of 98% (Bartmańska et al. 2005). Fermentation of testosterone by P. decumbens produced two major metabolites, including 5α-dihydrotestosterone and 5α-androstandione (Holland et al. 1995, 1994). Cabeza et al. examined the biotransformation of testosterone with P. chrysogenum and P. crustosum. The identified metabolites were 5α-dihydrotestosterone, 3α-hydroxy-5β-androstan-17-one, 3α-hydroxy-5α-androstan-17-one, 4-androstene-3,17-dione, and 5α-androstan-3,17-dione (Cabeza et al. 1999). Incubation of testosterone with P. vinaceum gave testololactone as the main metabolite (Panek et al. 2020). Yildirim et al. described the biotransformation of testosterone by P. digitatum. This fungal strain afforded four metabolites, including 5α-androstan-3,17-dione, 3α-hydroxy-5α-androstan-17-one, 3β-hydroxy-5α-androstan-17-one, and androst-4-ene-3,17-dione (Yildirim et al. 2010a). Fungal transformation of testosterone by P. simplicissimum resulted in testololactone as the main metabolite (Yang et al. 2014).

This investigation indicated that P. pinophilum has the hydroxylation capacity of testosterone in C-11α and C-14α positions in addition to having the ability of oxidizing the hydroxyl group at the position C17β of testosterone. The hydroxylation reaction is one of the main reactions for functionalizing steroids, and P. pinophilum revealed that it can do this in a practical and efficient manner.

**Conclusion**

In the present study, the bioconversion of testosterone by P. pinophilum resulted in three products, including 14α-hydroxyandrostenedione, 14α-hydroxytestosterone, and 11α-hydroxytestosterone. The chemical structures of these three metabolites showed that P. pinophilum has both hydroxylation and oxidation capabilities in a regio- and stereo-selective fashion. The time course profile demonstrated that, first, these reactions occur in the early stages of fermentation and, second, 14α-hydroxyandrostenedione is produced as a result of the biotransformation of 14α-hydroxytestosterone. In silico study exhibited that the hydroxylation pattern specifically affects the binding affinity of each metabolite to the estrogen receptors in such a way that 11α-hydroxylation increases this affinity but 14α-hydroxylation decreases it.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00203-022-03191-3.

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**Author contributions** MM performed the experiments, data acquisition and analysis, and wrote the first draft of the manuscript. BN designed the study, analyzed the data and edited the manuscript. All authors approved the final version of the manuscript.

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**Data availability** The datasets used and/or analyzed during the present study are available from corresponding author on reasonable request.

| Compound | Color-coded probability binding classes (and computed binding affinity score) |
|----------|--------------------------------------------------------------------------------|
|          | Androgen receptor | Estrogen receptor α | Estrogen receptor β | Glucocorticoid receptor | Progesterone receptor |
| Testosterone (I) | (-9.9) | (-8.4) | (-9.2) | (-9.2) | (-2.8) |
| 14α-Hydroxyandrostenedione (II) | (-9.9) | (-9.0) | (-9.0) | (-8.5) | (-2.8) |
| 14α-Hydroxytestosterone (III) | (-9.8) | (-8.2) | (-8.5) | (-8.8) | (-2.8) |
| 11α-Hydroxytestosterone (IV) | (-9.4) | (-9.2) | (-10.0) | (-8.6) | (-2.8) |
Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare that they have no conflict of interest regarding this article.

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