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 24 h and 96 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.

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 (Yildirim 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 (Yildirim 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 (Yildirim et al. 2019; Świzdor et al. 2017; Peart et al. 2011; Peart et al. 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 retained on SDA medium at 4°C. It was subcultured before using in the biotransformation process.

Biotransformation process, isolation and identification metabolites

This process was organized in two scales screening and semi-preparative. For the screening scale, spores freshly 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 hours. 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 ^{1}H and ^{13}C 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, 0.5-6 µL per band of the standard solutions with different concentrations for metabolites and substrate were loaded on HPTLC plates. They were applied as 7 mm bands 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). For the analysis of the daily samples, 1 µL of the sample solutions (10 mg/mL) were spotted on the plates and allowed to run. 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 computationally studied for their potential to interact with some steroid hormone receptors (androgen receptor, estrogen receptors α and β, progesterone receptor, and glucocorticoid receptor) using a web-based tool called Endocrine Disruptome (ED) (Kolšek et al. 2014). This program uses AutoDock Vina to carry out the docking process and predicts the binding potentials of the examined compounds with 14 different human nuclear receptors (http://endocrinedisruptome.ki.si). After docking, the binding affinity score is computed for each compound with individual receptors. 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

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. A computational evaluation on the interaction of the metabolites with some steroid hormone receptors was performed using the ED tool. In addition to these three metabolites, some other products were also generated that could not be identified due to their small concentration.

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 medium extract 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* (Fig. 2). 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).

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; ^1H 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.

Table 1

^{13}C NMR signals of substrate (I) and its metabolites (II-IV) (δ in ppm)

Carbon	Substrate (I)	Metabolite (II)	Metabolite (III)	Metabolite (IV)
1	35.77	35.85	35.75	37.62
2	33.95	33.17	33.95	34.32
3	199.3	199.57	199.95	200.40
4	123.93	124.30	123.79	124.69
5	170.97	170.01	171.34	171.20
6	32.79	32.44	32.63	33.73
7	31.57	25.69	28.60	31.25
8	35.71	38.05	38.95	35.46
9	53.95	46.97	46.78	59.33
10	38.77	38.77	38.79	40.14
11	20.67	19.23	19.75	69.07
12	36.45	24.62	31.44	48.71
13	42.84	52.65	47.01	43.72
14	50.53	80.88	83.30	49.97
15	23.36	30.40	26.18	23.40
16	30.49	34.02	29.56	29.83
17	81.61	218.35	78.47	81.21
18	10.90	17.94	15.00	12.43
19	17.44	17.48	17.24	18.61

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; ^1H 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 $C_{19}H_{26}O_3$. On the ^1H NMR spectrum, the lack of H-17 triplet at δ 3.65 ppm along with the significant downfield shifts of H-18 signal from 0.79 ppm to 1.05 ppm showed that there would be a change at the position of C-17. These data were consistent with the ^{13}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}C NMR spectrum, the large shift of C-14 signal from δ 50.53 ppm to 80.88 ppm and also the downfield shifts for C-8 (Δ 2.34 ppm), C-15 (Δ 7.04 ppm), and C-13 (Δ 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; Faramarzi et al. 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; ^1H NMR ($CDCl_3$, 300 MHz): δ 0.89 (3H, s, H-18), 1.19 (3H, s, H-19), 4.28 (1H, t, *J* = 9 Hz, H-17α), 5.70 (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 III revealed $[M + H]^+$ peak at *m/z* 305, agreeing with the formula $C_{19}H_{28}O_3$. The ^{13}C NMR spectrum exhibited a large shift for C-14 signal from 50.53 ppm to 83.30 ppm along with downfield shifts for C-8 (Δ 3.24 ppm), C-15 (Δ 2.82 ppm), and C-13 (Δ 4.17 ppm). These changes suggested that hydroxylation has taken place at C-14. Additionally, the ^1H NMR spectrum displayed a large downfield shift for H-17α from 3.65 ppm to 4.28 ppm (Δ 0.63 ppm). This also confirmed that the hydroxylation has occurred at C-14. The reason for this great shift is the diaxial interaction between H-17α and C-14α hydroxyl groups. Based on the spectral features and comparing them with those reported in the literature, metabolite III was identified as 14α-Hydroxytestosterone (Yildirim et al. 2018; Faramarzi et al. 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; ^1H NMR ($CDCl_3$, 500 MHz): δ 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}C NMR ($CDCl_3$, 75 MHz) spectrum have been presented in Table 1.

The $[M + H]^+$ peak for the metabolite IV was appeared at *m/z* 305, indicating the empirical formula $C_{19}H_{28}O_3$. The ^1H 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 (Δ 0.12 ppm). As well, the ^{13}C NMR spectrum exhibited a significant shift for C-11 signal from 20.67 to 69.07 (Δ 48.40 ppm) along with downfield shifts for C-12 (Δ 12.26 ppm) and C-9 (Δ 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. 3). 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. 4). 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 III were minimal.

In-silico **assessment of the metabolites**

In this investigation, *in-silico* assessment with the ED program was performed to study the increase or decrease in computed affinities for the produced metabolites due to hydroxylation or oxidation. Based on the obtained results (Table 2), it was determined that the hydroxylation at the 11α-position of testosterone leads to increase the binding affinity of the metabolite (11α-hydroxytestosterone) to the estrogen receptors α and β. In contrast, the hydroxylation at the 14α- position reduces the binding affinity of the metabolite (14α-hydroxytestosterone) to the estrogen receptors α and β. However, both hydroxylation patterns did not affect the binding affinities of the two metabolites to the androgen, progesterone, 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) to the estrogen receptor α. However, it did not affect the binding affinity of the metabolite to the other receptors.

Table 2
Prediction of binding affinities of testosterone and its metabolites with some steroid hormone receptors

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

Discussion

The ability of microorganisms to transform the steroids into other valuable compounds has been studied 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., *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; Świzdor et al. 2017; Yildirim et al. 2011; Yildirim et al. 2010b; Yildirim et al. 2010c; Yildirim and Kuru 2016; Hu et al. 1995; Yildirim et al. 2013; Peart et al. 2013, 2016; Kolet et al. 2013; Kolet et al. 2014; Peart et al. 2011; 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; Holland et al. 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-androstane-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-androstane-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).

In this study, the bioconversion of testosterone by *P. pinophilum* resulted in three steroids, including 11α-hydroxytestosterone, 14α-hydroxytestosterone, and 14α-hydroxyandrostenedione. The chemical structures of these three metabolites showed that *P. pinophilum* has both hydroxylation (at C-11α and C-14α positions) and oxidation (17β-hydroxyl oxidation) capabilities. Although all three metabolites have already been produced by various fungi (such as *Fusarium lini*, *Aspergillus wentii*, and *Mucor hiemalis*) from testosterone as the substrate (Al-Aboudi et al. 2008; Yildirim et al. 2010b; Yildirim et al. 2013), the present work is the first study to show that a member of the genus *Penicillium* can hydroxylate testosterone in a different position.

Generally, hydroxylation is one of the main reactions for functionalizing steroids, and fungi can hydroxylate steroids in a regio- and stereo-selective manner (Nickavar et al. 2019). On the other hand, the time course profile showed that hydroxylation and oxidation reactions occur during the initial stages of fermentation, and 14α-hydroxytestosterone is the main product.

The *in-silico* approach showed a high probability of binding all three metabolites to the androgen receptor like testosterone. However, among the metabolites produced, 11α-hydroxytestosterone showed an increased probability of binding to estrogen receptors α and β, while 14α-hydroxytestosterone revealed a decreased probability of binding to those receptors. On the other hand, 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 14α-hydroxytestosterone is an androgenic compound with modified estrogenic activities.

Declarations

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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 and supervised the findings, carried out the computations, analyzed the data and edited the manuscript. All authors approved the final version of the manuscript.

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

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

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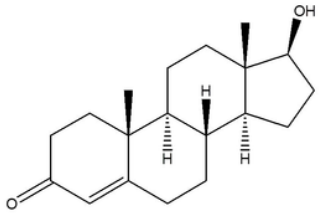
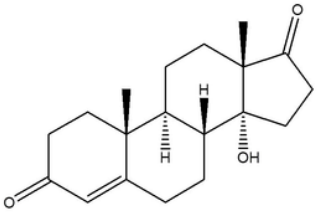
Figures

Figure 1

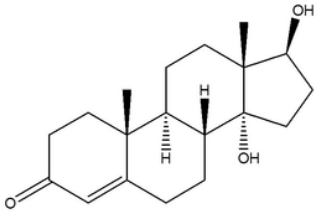
TLC fingerprinting profile obtained from the biotransformation of testosterone by *Penicillium pinophilum*



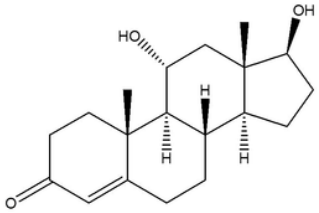
Testosterone
(substrate I)



14α-Hydroxyandrost-4-en-3,17-dione



14α-Hydroxytestosterone



11α-Hydroxytestosterone

Figure 2

The structure of testosterone and its metabolites

Figure 3

Three-dimensional HPTLC densitogram obtained from the biotransformation of testosterone by *Penicillium pinophilum*

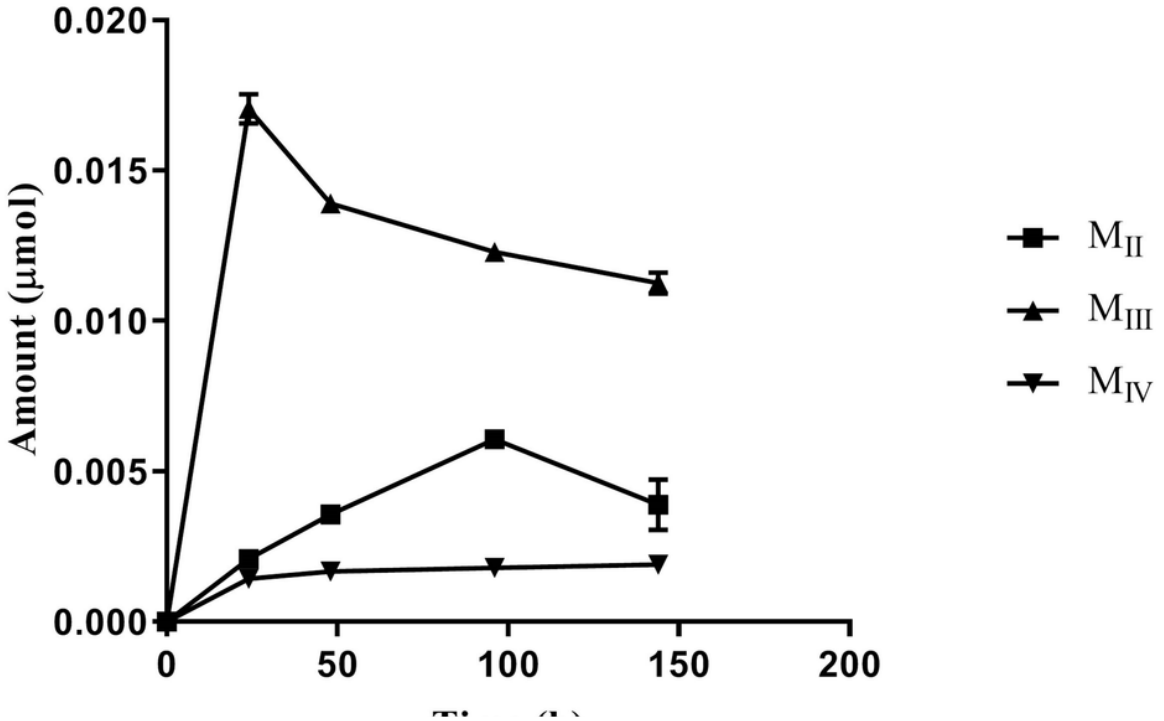


Figure 4

Time course changes of metabolites during the biotransformation of testosterone by *Penicillium pinophilum*. Each point represents the mean of three experiments and the vertical bar shows the standard deviation

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