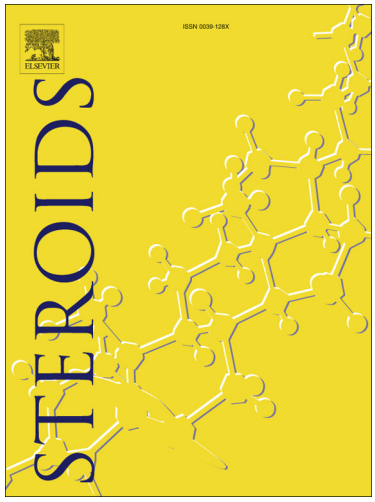

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Biotransformation of testosterone and testosterone heptanoate by four filamentous fungi

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Abstract

The microbial transformations of testosterone and testosterone heptanoate by four fungi: *Absidia griseolla* var. *igachii* PTCC 5260, *Acremonium chrysogenu* PTCC 5271, *Fusarium fujikuroi* PTCC 5144, and *Fusarium solani complex* PTCC 5285 were investigated for the first time. Incubation of testosterone heptanoate with *F. fujikuroi* and *F. solani* yielded three metabolites, which were isolated and characterized as testosterone, androst-4-ene-3,17-dione, and 6β-hydroxy testosterone. 6<beta>-Hydroxy testosterone was the major metabolite obtained from testosterone heptanoate biotransformation by two fungal species. *A. griseolla* and *A. chrysogenu* produced 14<alpha>-hydroxy testosterone as major metabolite, together with testosterone and 6<beta>-hydroxy testosterone in lower yields. The biotransformation of testosterone by *F. fujikuroi* and *A. griseolla* was also investigated in order to examine the influence of the ester group on the course of transformation. Androst-4-ene-3,17-dione was only identified in the biotransformation of testosterone by *F. fujikuroi*. The same product was observed in incubation of testosterone by *A. griseolla*, together with 14<alpha>-hydroxy testosterone in very low yield. Furthermore, time course study was also carried out in order to examine the formation of metabolites as a function of time, which was determined by HPLC. The structures of compounds were determined by their comprehensive spectroscopic analysis and comparison with literature data.

*Keywords***:** Biotransformation; Steroids;Testosterone heptanoate; Filamentous fungi; 14<alpha>-hydroxy testosterone; 6<beta>-Hydroxy testosterone.

1. Introduction

Biotransformation is a broad and growing field of biotechnology and encompasses both enzymatic and microbial biocatalysts for the transformation of natural and synthetic bioactive compounds [1–4].The production of desired chemicals by microbial biotransformation can offer several advantages, including high stereoselectivity, high activity, flexible substrate selectivity, minimal side reaction productivity, mild reaction conditions, and environmentally friendly [5–7].

Since the biological activities of steroids depend on their proper functionalization, microbial transformations represent a unique tool in the production of highly valuable pharmaceuticals from readily available steroid raw materials [8–12]. Microorganisms, especially fungi have remarkable ability to carry out diverse chemical reactions like hydroxylation, oxidation, reduction, hydrolysis, degradation, etc., on steroidal compounds [13–16]. The introduction of hydroxyl functionality into a steroid molecule by fungal biotransformation is still the most efficient method for the preparation of specific derivatives with therapeutic use and commercial value [17–20].

As testosterone is one of the end products of steroid biosynthesis in the human male and is of special importance as a steroid hormone, its microbial transformation is of considerable interest [21,22]. Different microorganisms have been studied with the aim of producing new testosterone derivatives that are not readily accessible by chemical synthesis. The most common transformation of testosterone was hydroxylation at C-2β, -6α, -6β, -7α, -7 β, -11 α, -14 α, -15α, and -15β [22–24]. Oxidation of 17β-OH to the corresponding ketone, which is usually accompanied by hydroxylation, reduction of the double bond, and Baeyer–Villiger lactonization were also reported [21,25].

These results encouraged us to investigate the biotransformation of the testosterone and testosterone heptanoate by using four fungi *Fusarium fujikuroi*, *Fusarium solani, Absidia griseolla*, and *Acremonium chrysogenu.* These fungal species were chosen because of the known capability of the genus *Acremonium, Absidia* and *Fusarium* to hydroxylate of steroids [18,19,26,27]. Furthermore, time course study was also carried out in order to examine the formation of metabolites as a function of time.

2. Materials and methods

2.1. Instrumental methods

Melting points were determined on an Electrothermal 9100 apparatus and were uncorrected. Direct electron ionization mass spectra (EIMS) were obtained by an Agilent 5973 at 70 ev. Infrared spectra were recorded with a Shimadzu IR-470 spectrometer. The ^{1}H and ^{13}C NMR spectra were obtained by using a Bruker AVANCE 300 (Bruker Biospin, Rheinstetten, Germany) spectrometer at 300.13 and 75.47 MHz, respectively. Preparative thin layer chromatography (TLC) was performed on silica gel 60 mesh GF_{254} plates (20 × 20 cm) and observation of plates was carried out under UV. The optical rotation was measured on a polarimeter using a sodium lamp (589 nm) at room temperature. The high performance liquid chromatography (HPLC) system consisted of a Knauer model smart line pump 10000, an online degasser and a K-2600 UV variable-wavelength detector all from Knauer (Berlin, Germany). The HPLC column was a Eurospher 100-5 C18 column (25 cm × 0.46 cm i.d. 5-µm particle size) from Herbert Knauer GmbH (Berlin, Germany).

2.2. Materials

Testosterone and testosterone heptanoate, used in the present study were kindly donated by Caspian Tamin Pharmaceutical Co. (Rasht, Iran). All solvents and reagents were analytical grade. The ingredients of culture media were purchased from Scharlu (Italy) and Merck (Darmstadt, Germany).

2.3. Microorganism and conditions of cultivation

Four fungal species namely *A. griseolla var. igachii* PTCC 5260, *A. chrysogenu* PTCC 5271, *F. fujikuroi* PTCC 5144, and *F. solani* complex PTCC 5285 (deposited in the Persian Type Culture Collection (PTCC), Iranian Research Organization for Science and Technology) were used in the present study. The medium for *A. chrysogenu* was prepared by adding yeast extract (4.0 g), soluble starch (15.0 g), $MgSO_4.7H_2O$ (0.5 g), K_2HPO_4 (1.0 g), and agar (15.0 g) to distilled H_2O (1.0 L). *Fusarium* species and *A. griseolla* were grown on potato-glucose agar plates (20 g glucose, 300 g diced potatoes, 15.0 g agar/L) and stored at 4 °C. The culture media were freshly subcultured before using in the biotransformation experiment.

2.4. Biotransformation condition and product isolation

For each fungal species, ten 250 mL Erlenmeyer flasks, each containing 150 mL of sterilized liquid medium were inoculated with freshly obtained spores from agar slope cultures. The flasks were incubated at 26 °C under constant shaking on an orbital shaker at 120 rpm.

After 3 days of growth, 100 mg of substrate dissolved in 1 mL of absolute ethanol was added to each of the 150 mL cultures. Incubations were continued and the progress of the reaction was monitored by TLC. The cultures were generally sampled every day after addition of substrate. In order to study the catalytic efficiency of microorganisms, substrate controls consisted of sterile medium were similarly incubated but without microorganism. At the end of the incubation period, the products were filtered and extracted three times with 20 mL of chloroform. After evaporating the organic solvent layer, each of the extracts was further purified separately.

2.5. Purification and structural analysis of products

The broth extracts were subjected to repeated preparative thin layer chromatography with *n*-hexane/ethyl acetate as the solvent to yield pure compounds. The structure of compounds was elucidated by their comprehensive spectroscopic analysis including IR, EI-MS, ^{1}H NMR, ^{13}C NMR, melting point, and comparison with literature data.

2.6. HPLC analysis and quantification of the metabolites

The quantitative analysis of testosterone metabolites was carried out by HPLC using a 20µL injection. All chromatography was performed at room temperature, and the UV detector was set at 254 nm. Stock solutions of starting material, androst-4-ene-3,17-dione, and the hydroxylated testosterone standards were prepared in methanol. Aliquots of these solutions were diluted with methanol to prepare five different concentrations for the generation of HPLC calibration curves. All standard solutions were stored at 4 °C until further use. The mobile phase consisted of A

(water–acetonitrile, 47:53) and B, 100% acetonitrile. The HPLC system was operated at a flow rate of 0.5 mL/min with a linear gradient of 100% A to 100% B over 30 min, and then held at 100% B for 20 min. The exact identification of the metabolites and starting materials was established by comparing their retention times with those of authentic standards. The retention times for testosterone, testosterone heptanoate, androst-4-ene-3,17-dione, 6<beta>-, and 14<alpha>-hydroxytestosterones were 19.83, 42, 18.55, 7.66, and 8.76 min, respectively. The quantitative analysis of samples was performed using external standard technique and each analysis was carried out in triplicate.

3. Results and discussion

There are several reports about the biotransformation of testosterone [21,23,25]; however, the incubations of testosterone derivatives by four fungal species *A. griseolla*, *A. chrysogenu*, *F. fujikuroi*, and *F. solani* have not been reported. In the present study, in order to examine the influence of the ester group on the course of transformation [28], testosterone and testosterone heptanoate were chosen as substrate, and were incubated with these four filamentous fungi.

Incubation of testosterone heptanoate (**1**) with *F. fujikuroi* and *F. solani* for 13 days yielded testosterone (**2**), 6<beta>-hydroxy-testosterone (**3**), and androst-4-ene-3,17-dione (**4**). It is worth noting that 6<beta>-hydroxy-testosterone (**3**) was produced as a major metabolite with high yield by these fungal species (Fig. 1). Testosterone (**2**), 6<beta>-hydroxy-testosterone (**3**), and 14<alpha>-hydroxy-testosterone (**5**) were isolated in the transformation by *A. griseolla* and *A. chrysogenu,* (Fig. 2). The latter was a major metabolite and the structure of the metabolites was determined by spectroscopic analysis and comparison with literature data.

The mass spectrum of metabolites **2** and **3** showed a molecular ion peak at *m/z* 304, indicating empirical formulae of $C_{19}H_{28}O_3$. It is supposed that the first step of the reaction was probably hydrolysis of the ester bond, followed by the alcohol resulting from the hydrolysis underwent hydroxylation. Comparison of NMR data of **2** and **3** with that of testosterone heptanoate confirmed this postulate.

6<beta>-Hydroxytestosterone (**3**), White amorphous powder; mp 205–207 °C (lit [29] 206–210 °C), $[\alpha]_D^{20}$ +41° (c, 0.5; $CHCl_3$) (lit [29] $[\alpha]_D$ +39° (c, 0.5; $CHCl_3$)); IR (KBr); ν_{max}: 3430, 2926, 2846, 1660 cm^{-1}; MS; *m/z* (%): 304 (2) (M^+, $C_{19}H_{28}O_3$), 302 (6), 287 (6), 185 (2), 152 (32), 91 (48), 67 (51), 55 (100); ^{1}H NMR (300.13 MHz, $CDCl_3$) δ 0.84 (3H, s, H-18), 1.41 (3H, s, H-19), 3.68 (1H, t, J= 8.3 Hz, H-17), 4.37 (1H, brs, H-6), 5.84 (1H, s, H-4); ^{13}C NMR (75.47 MHz, $CDCl_3$) δ 200.4 (C3), 168.2 (C5), 126.4 (C4), 81.7 (C17), 73.1 (C6), 53.7 (C9), 50.5 (C14).

Androst-4-ene-3,17-dione (**4**), White powder; mp: 169–171 °C (lit [30] 170–171 °C), $[\alpha]_D^{20}$ +193° (c, 0.9; $CHCl_3$) (lit [30] $[\alpha]_D$ +190° (c, 0.9; $CHCl_3$)); IR (KBr); ν_{max}: 2933, 2846, 1739, 1673 cm^{-1}; MS; *m/z* (%): 286 (8) (M^+, $C_{19}H_{26}O_2$), 271 (44), 258 (43), 244 (52), 201 (22), 148 (56), 124 (100), 109 (48); ^{1}H NMR (300.13 MHz, $CDCl_3$) δ 0.91 (3H, s, H-18), 1.22 (3H, s, H-19), 5.75 (1H, s, H-4); ^{13}C NMR (75.47 MHz, $CDCl_3$) δ 220.5 (C17), 199.4 (C3), 170.4 (C5), 124.2 (C4), 53.8 (C9), 50.8 (C14), 47.5 (C13), 38.7 (C10).

14<alpha>-Hydroxy testosterone (**5**), White powder; mp: 180–183 °C (lit [31,32] 181–184 °C), $[\alpha]_D^{20}$ +119° (c, 1.0; $CHCl_3$) (lit [31,32] $[\alpha]_D$ +121°(c, 0.974; $CHCl_3$); IR (KBr); ν_{max}: 3410, 2919, 2853, 1733, 1660 cm^{-1}; MS; *m/z* (%): 304 (31) (M^+, $C_{19}H_{28}O_3$), 286 (37), 271 (47), 268 (14), 242 (12), 173 (14), 148 (31), 124 (100), 91 (69);^{1}H NMR (300.13 MHz, $CDCl_3$) δ 0.90

(3H, s, H-18), 1.20 (3H, s, H-19), 4.29 (1H, brs, H-17), 5.71 (1H, s, H-4); ^{13}C NMR (75.47 MHz, $CDCl_3$) δ 199.5 (C3), 170.5 (C5), 124.0 (C4), 83.4 (C14), 78.6 (C17), 46.8 (C9).

The FT-IR spectrum of 6<beta>-hydroxy-testosterone (**3)** showed an absorbance at 3430 cm^{-1}, which was representative of a new hydroxyl group. This was confirmed by the appearance of a new ^{1}H NMR resonance at 4.37 ppm as a broad singlet. The downfield shifts of the Me-19 and H-4 signals were 0.2 and 0.09 ppm relative to testosterone, respectively, by virtue of their spatial proximity to 6β-OH. The H-17α triplet (J= 8.3 Hz), which is characteristic of testosterone, appeared at δ 3.68 ppm. The ^{13}C NMR spectrum for this metabolite also exhibited 19 carbon signals. The appearance of a new CHOH signal at 73.1 ppm confirmed that oxygen insertion had occurred at C-6.

The ^{1}H NMR spectrum of 14α-hydroxy-testosterone (**5**) showed a downfield shift (0.09 ppm) of the Me-18 with slight changes of the chemical shifts of Me-19 and H-4 compared to testosterone (**2**). The hydroxyl group in the 14<alpha>-position caused a downfield shift in the l7α-proton from δ 3.67 ppm in testosterone to δ 4.29 ppm. Analysis of the^{1}H NMR spectrum indicated that the hydroxylation occurred at a tertiary carbon atom. The ^{13}C NMR spectrum displayed the appearance of a resonance for a new oxygenated carbon at C-14 (83.4 ppm). Comparison of its data with that in the literature results identified the structure of this metabolite [31,32].

The molecular ion of the metabolite **4** was *m/z* 286, which indicated the reduction of 2 units as compared to testosterone, and the formula of $C_{19}H_{26}O_2$. In FT-IR spectrum of the least polar metabolite (**4),** the peaks in the region 1739 and 1672 cm^{-1} confirmed the existence of two carbonyl groups in this compound. The ^{1}H NMR data of the androst-4-ene-3,17-dione (**4**)

showed the loss of the resonance, which corresponded to H-17. ^{13}C NMR spectrum showed the appearance of a new carbonyl signal at 220.5 ppm. The remaining spectral data for metabolites **3**–**5** also accord with the structures, which is discussed above

Furthermore, in order to examine the influence of the ester group on the course of transformation, the incubation of testosterone by *F. fujikuroi* and *A. griseolla* was also investigated. The obtained results indicated that testosterone was inert to hydroxylation by *F. fujikuroi* and androst-4-ene-3,17-dione was only obtained (Fig. 3). 6<beta>-Hydroxy derivative, which was formed from the biotransformation of testosterone heptanoate, was not detected in conversion of testosterone. The biotransformation of testosterone by *A. griseolla* resulted in the formation of androst-4-ene-3,17-dione (23%) and 14<alpha>-hydroxy testosterone in very low yield (7%).

3.1. Quantification of testosterone metabolites by HPLC

A simple HPLC method has been developed and optimized for the quantification of testosterone metabolites resulting from testosterone and testosterone heptanoate incubation by four filamentous fungi.

As mentioned previously, there were three products **2**–**4** formed during testosterone heptanoate incubation by *F. fujikuroi* and *F. solani.* Time course study showed the accumulation of the testosterone and androst-4-ene-3,17-dione as major metabolites within 3 days of incubation, after which the concentration of both metabolites were reduced until they approached almost 20% after 13 days of incubation (Fig. 4a and 4b). The yield of 6<beta>-hydroxy testosterone was significantly increased, reaching its maximal level 13 days after the testosterone

heptanoate incubation with *F. fujikuroi* (59%) and *F.solani* (56%). These results suggest that the testosterone heptanoate underwent hydrolysis and the testosterone was transformed.

In comparison, when testosterone heptanoate was incubated with *A. griseolla* and *A. chrysogenu*, metabolites **2**, **3**, and **5** were produced after 24 hrs of incubation. The transformation of 14<alpha>-hydroxytestosterone increased linearly with incubation time up to approximately 3 days, while concentration of compound 2 was gradually decreasing (Fig. 5a and 5b). After 3 days, the amount of metabolites formed became nonlinear and appeared to be constant with further incubation time. The maximum amount of 6β-hydroxytestosterone was obtained after 3 days of incubation [(*A. griseolla* (24%) and *A. chrysogenu* (29%)] and then remained almost constant until the end of the experimental period. In incubation of 1 with *A. griseolla* and *A. chrysogenu*, 14α-hydroxytestosterone was identified as the major product with high yield of 45% and 53%, respectively. For the testosterone heptanoate incubation by these fungal species, time of 3 days was chosen as ideal because the maximum amounts of metabolites were formed in this period.

The last time course study was constructed to examine the biotransformation of testosterone by *F. fujikuroi* and *A. griseolla.* The obtained results indicated that testosterone **2** was not hydroxylated at C-6β by *F. fujikuroi*. After 9 days of incubation, androst-4-ene-3,17-dione (36%) was only obtained as a major product (Fig. 6). *A. griseolla* produced a lower amount of androst-4-ene-3,17-dione (23%) and afforded a minor product (about 7%), which was identified as 14<alpha>-hydroxy testosterone. As shown in Figure 7, the yield of biotransformation by this fungus was increased, reaching its maximal level within 3 days of incubation and then remained almost constant until the end of the experimental period.

It was observed that the presence of the heptanoate group mainly influenced the degree of transformation. It is supposed that the first step of the reaction was probably hydrolysis of the ester bond, followed by the alcohols resulting from the hydrolysis underwent hydroxylation and oxidation at C-17.

4. Conclusions

The topic of microbial transformations of testosterone derivatives was extensively reviewed where biocatalytic hydroxylation, oxidation at C-17, reduction, and hydrolysis were described. However, this is the first reported work on the microbial transformation of testosterone and testosterone heptanoate using four fungi *F. fujikuroi*, *F. solani*, *A. griseolla*, and *A. chrysogenu*.

The obtained results showed that the presence of the heptanoate group mainly influenced the degree of transformation. Bioconversion of testosterone heptanoate resulted in the formation of 6β- and 14α-hydroxytestosterone derivatives with excellent yield. No hydroxylation took place in the biotransformation of testosterone by *F. fujikuroi*, but this fugal species oxidized the 17-hydroxyl group to the corresponding ketone. The similar result was obtained in the biotransformation of testosterone by *A. griseolla* and 14α-hydroxytestosterone derivative was obtained in very low yield. To the best of our knowledge this is the first report on hydroxylation of testosterone by these fungal species.

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Figure legends

Figure 1. Biotransformation of testosterone heptanoate (**1**) with *Fusarium fujikuroi* and *Fusarium solani*

Figure 2. Biotransformation of testosterone heptanoate (**1**) with *Absidia griseolla* and *Acremonium chrysogenu*

Figure 3. Biotransformation of testosterone (**1**) with *Fusarium fujikuroi* and *Absidia griseolla*

Figure 4. Time course profiles of testosterone heptanoate biotransformation by (a) *Fusarium fujikuroi* and b) *Fusarium solani* (mean ± SD; n = 3).

Figure 5. Time course profiles of testosterone heptanoate biotransformation by (a) *Absidia griseolla* and b) *Acremonium chrysogenu* (mean ± SD; n = 3).

Figure 6. Time course profile of testosterone biotransformation by *Fusarium fujikuroi* (mean ± SD; n = 3).

Figure 7. Time course profile of testosterone biotransformation by *Absidia griseolla*. Data are shown as mean±SD (n=3).

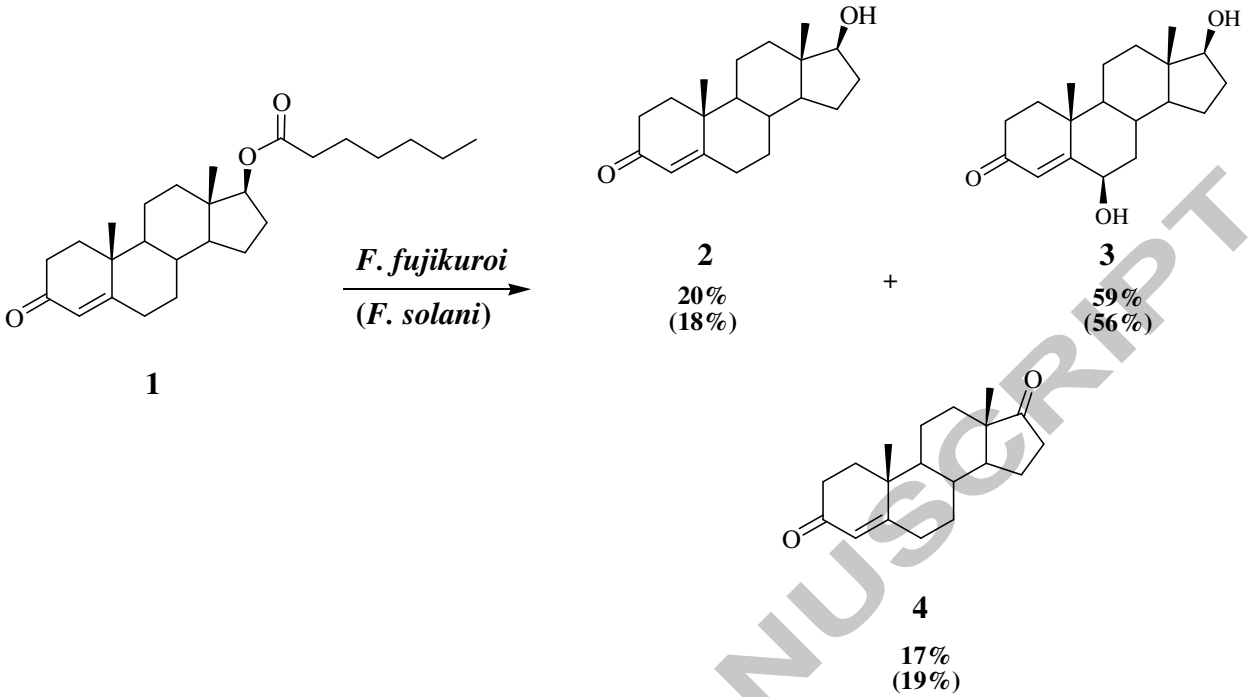

Fig. 1

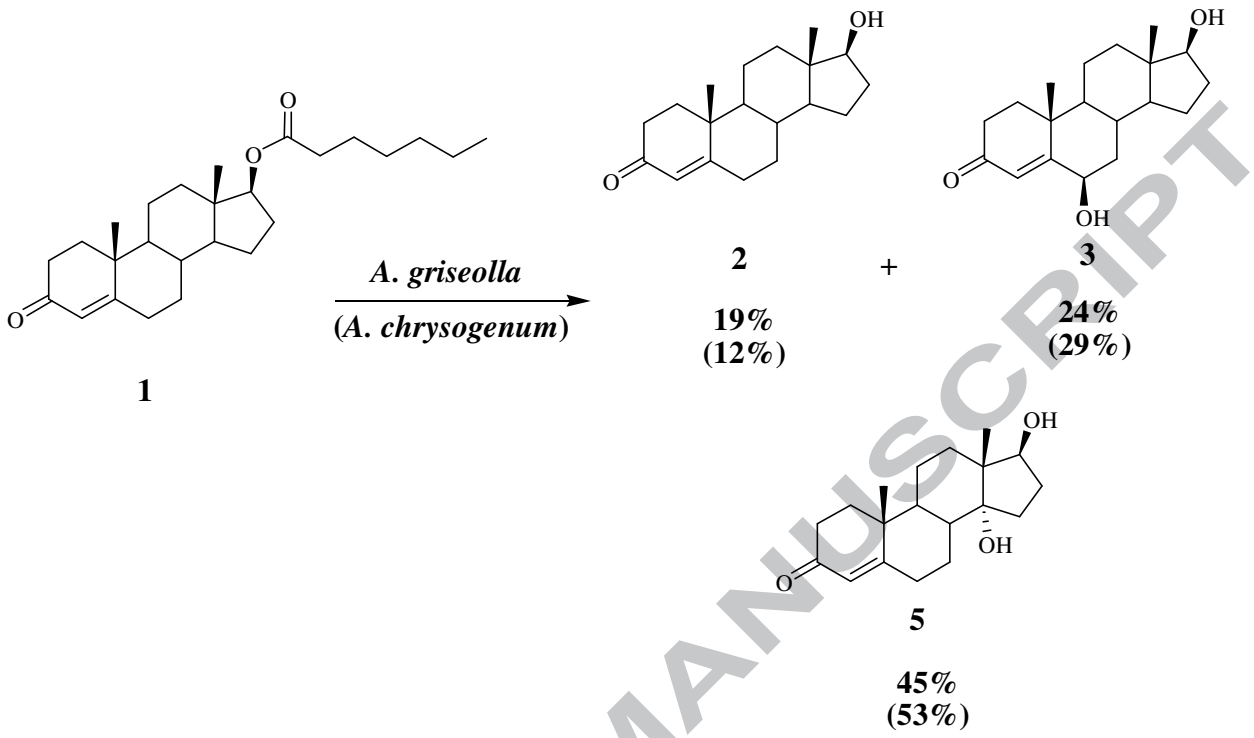

Fig. 2

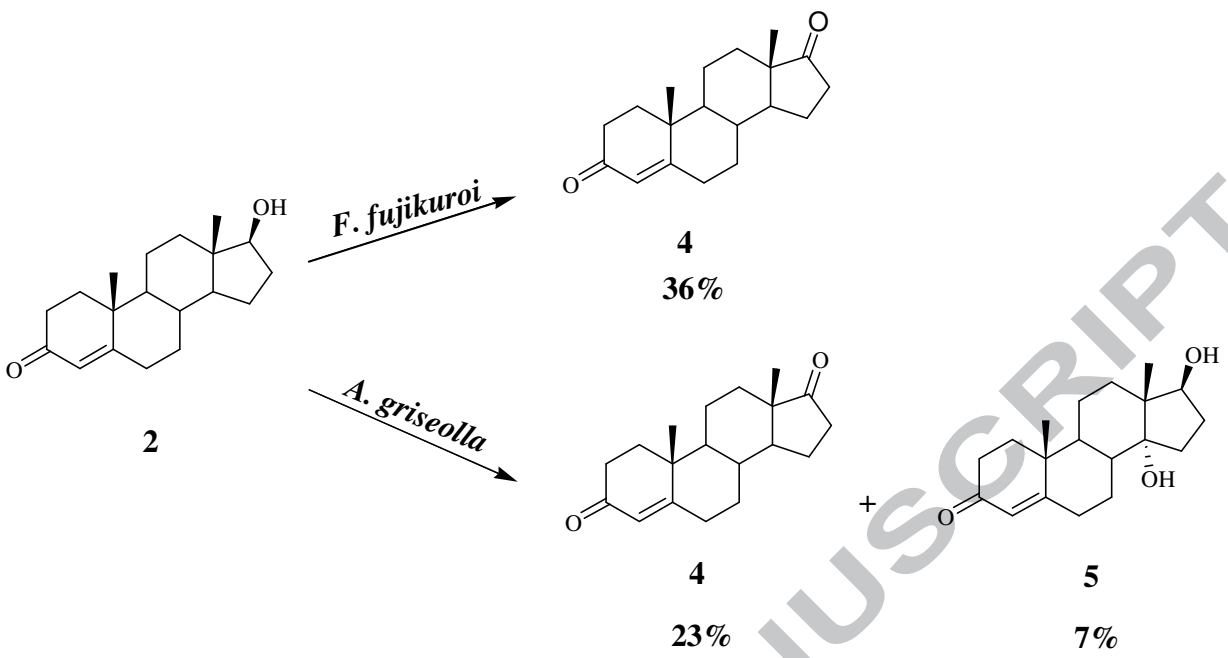

Fig. 3

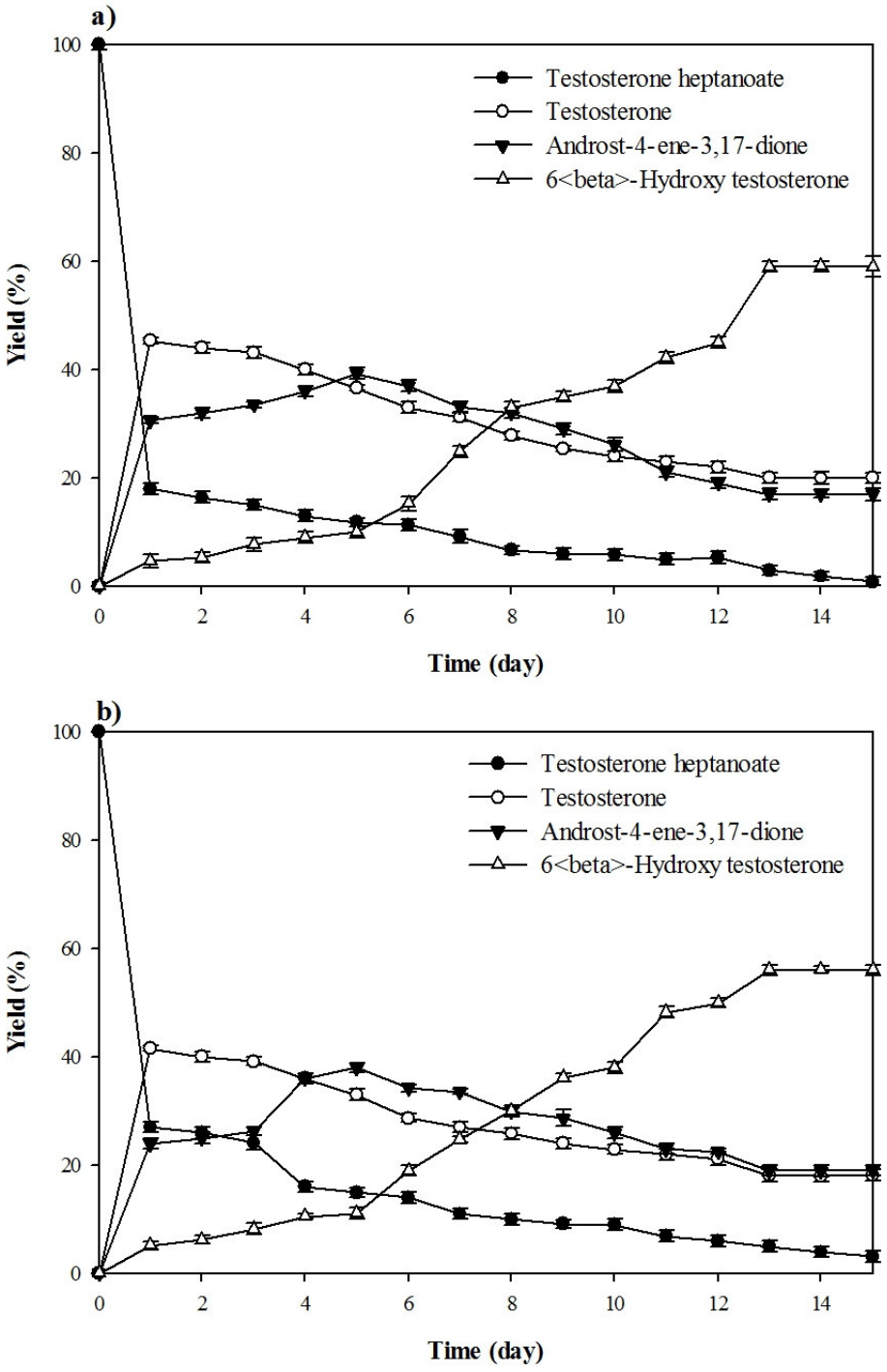
a)
Testosterone heptanoate
Testosterone
Androst-4-ene-3,17-dione
6<beta>-Hydroxy testosterone
Yield (%)
Time (day)
b)
Testosterone heptanoate
Testosterone
Androst-4-ene-3,17-dione
6<beta>-Hydroxy testosterone
Yield (%)
Time (day)

Fig. 4

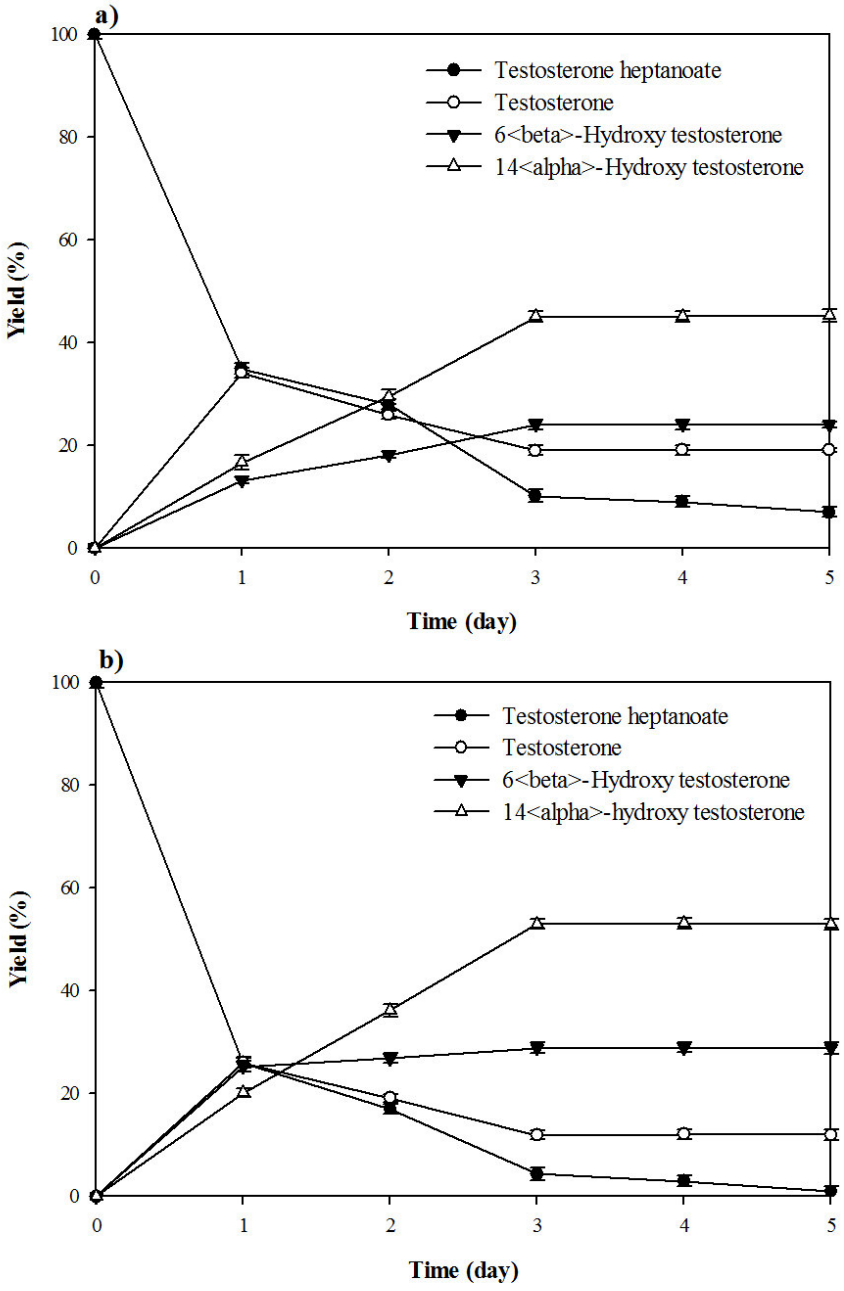
a)
Testosterone heptanoate
Testosterone
6<beta>-Hydroxy testosterone
14<alpha>-Hydroxy testosterone
Yield (%)
Time (day)
b)
Testosterone heptanoate
Testosterone
6<beta>-Hydroxy testosterone
14<alpha>-hydroxy testosterone
Yield (%)
Time (day)

Fig. 5

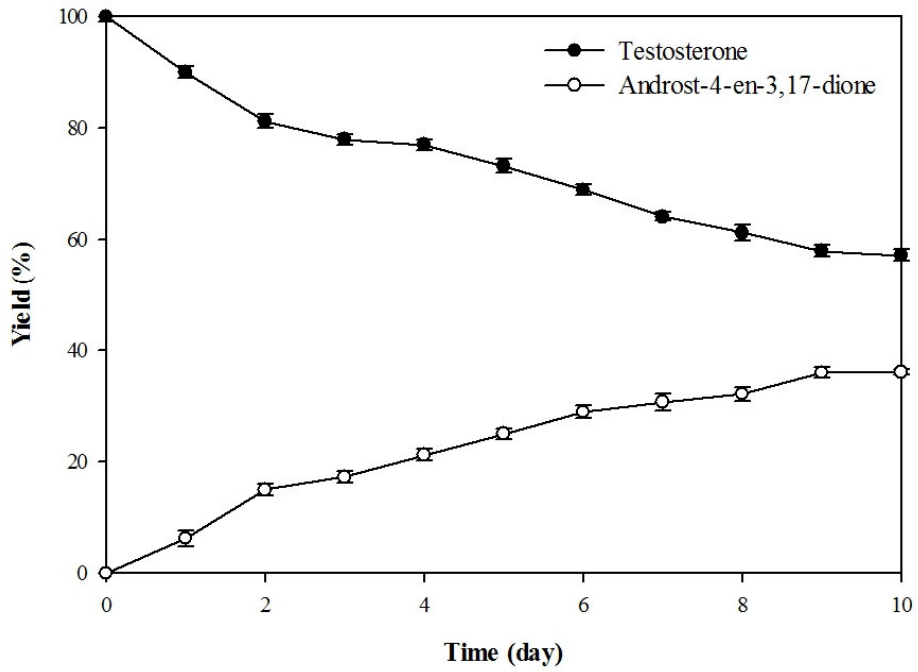

Fig. 6

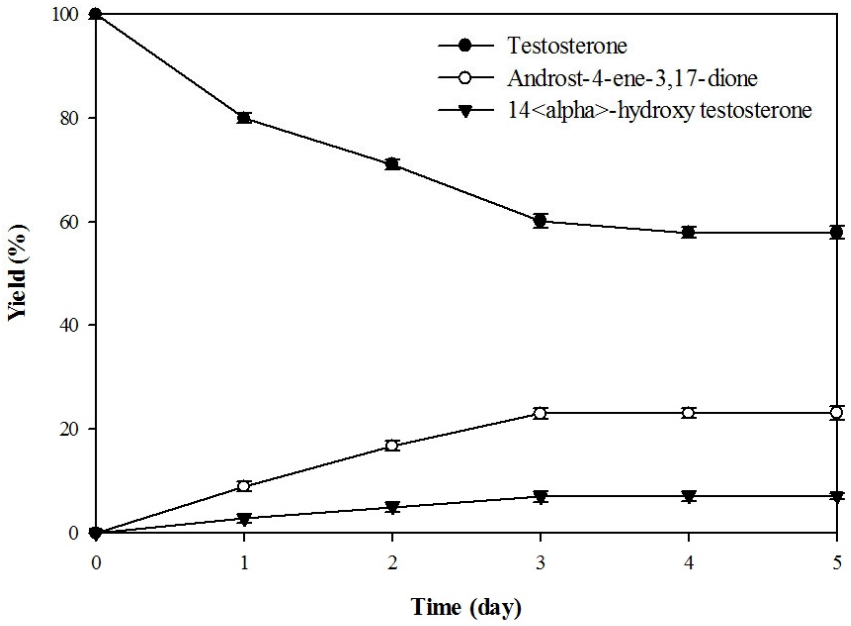

Fig. 7

Highlights

- Biotransformation of testosterone and testosterone heptanoate was investigated.
- Four filamentous fungi were used for the biotransformation.
- 6β and 14α-hydroxytestosterone were obtained in biotransformation of testosterone heptanoate.
- Androst-4-ene-3,17-dione was the major product in the biotransformation of testosterone.
- Time course study was also carried out with four fungal species.