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BIOTRANSFORMATION OF METHYLTESTOSTERONE BY THE FILAMENTOUS FUNGUS *Mucor racemosus*

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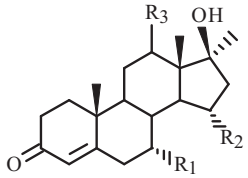
Fungi have proved to be powerful biocatalysts in steroid biotransformations. In the present study, the soil isolate filamentous fungus Mucor racemosus *was applied for bioconversion of methyltestosterone (**1**), an anabolic steroid, in a five-day fermentation. Microbial metabolites were purified chromatographically and identified on the basis of their spectral data as 7α-hydroxymethyltestosterone (**2**), 15α-hydroxymethyltestosterone (**3**), and 12,15α-dihydroxymethyltestosterone (**4**). Observed modifications were hydroxylations at C-7α, C-12, and 15α-positions. Best fermentation condition for production of hydroxylated derivatives was found to be 25°C at 150 rpm for 5 days with a substrate concentration of 1 mg/mL.*

Keywords: *Mucor racemosus*, methyltestosterone, biotransformation, steroid, microbial hydroxylation.

Whole cell biocatalysis using fungi, bacteria, and algae has been extensively applied in steroid biotransformations over the last half century [1–3]. The ability of microorganisms to introduce functional groups into chemically inactive carbons of complex molecules has made microbial transformations an indispensable part of the manufacturing process of most pharmaceutically important steroids. Biocatalysis also provides an environmentally friendly alternative for chemical synthesis methods that are known to produce large amounts of harmful wastes [1, 4]. Owing to their versatile enzymatic pool, fungi have widely been employed as one of the most powerful tools in the biotransformation of a large number of biological compounds, including steroids [5].

Mucor racemosus is a common species of the genus *Mucor* widely distributed in terrestrial ecosystems [6]. The genus *Mucor* has been employed to transform several steroid compounds, including pregnanes [7, 8], cardenolides [8], pregnenolones [9, 10], and dehydroepiandrostanes [9, 11]. The genus is able to modify steroids at the C-7 position [12] to produce important intermediates used in the synthesis of pharmaceutically active steroid derivatives [1]. *Mucor* hydroxylates both 4-en-3-one (androstane and pergnane) [1, 12–14] and 3*β*-OH-5-ene (dehydroepiandrosterone and pregnenolone) systems [9–11].

Methyltestosterone (**1**), an important anabolic steroid derived from testosterone, is used for therapeutic purposes such as weight gain after surgery, treatment of trauma, birth control, regulation of inflammation, and treatment of other diseases [15]. Although some fungi have been used to transform methyltestosterone, the ability of *M. racemosus* to transform this compound has not been evaluated.



1 - 4

1: $R_1 = R_2 = R_3 = H$
2: $R_1 = OH$, $R_2 = R_3 = H$
3: $R_1 = R_3 = H$, $R_2 = OH$
4: $R_1 = H$, $R_2 = R_3 = OH$

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TABLE 1. ^{13}C NMR Signals of Methyltestosterone (**1**) and the Metabolites (δ in ppm, downfield from TMS, in $CDCl_3$)

C atom	**1**	**2**	**3**	**4**	C atom	**1**	**2**	**3**	**4**
1	35.6	35.3	39.8	40.3	11	20.6	20.4	20.7	27.9
2	33.9	33.9	35.4	38.9	12	31.3	31.0	29.7	71.1
3	199.6	199.3	199.0	199.1	13	45.3	45.0	46.3	46.5
4	123.7	126.5	126.2	126.4	14	50.0	45.3	53.0	53.4
5	171.4	168.8	167.9	168.0	15	23.1	22.7	67.7	68.0
6	32.8	44.4	33.9	31.4	16	38.7	38.7	46.2	46.3
7	31.6	67.6	31.3	31.9	17	81.3	81.5	79.6	81.7
8	36.4	41.1	38.3	37.5	18	13.9	13.8	15.3	13.8
9	53.7	45.4	50.1	50.3	19	17.3	17.0	17.3	17.9
10	38.6	40.6	41.0	45.4	20	25.7	25.9	26.3	25.9

In the present study, the potential of an isolated strain of the filamentous fungus *M. racemosus* in the bioconversion of methyltestosterone was investigated. Optimum fermentation conditions were also examined.

Microbial transformation of methyltestosterone by *Mucor racemosus* in 5 days led to formation of three hydroxysteroid compounds (**2** to **4**) as follows. No transformation occurred in the control media. The products were finally characterized using spectral analyses including ^{13}C NMR, ^{1}H NMR, FTIR, and mass spectroscopy.

7α-Hydroxymethyltestosterone (**2**). R_f (chloroform–acetone, 1:1, v/v): 0.60; yield 35%; IR (ν_{max}, cm^{-1}): 3440, 1665, 1620; EI-MS *m/z* (%): 318 (M^+, $C_{20}H_{30}O_3$; 18), 300 (100), 282 (18), 243 (48), 124 (55), 91 (52), 67 (35), 55 (30); ^{1}H NMR ($CDCl_3$, δ): 0.85 (3H, s, H-18), 1.20 (3H, s, H-19), 1.22 (3H, s, H-20), 3.9 (1H, br.s, H-7), 5.75 (1H, s, H-4).

Mass spectrum showed the molecular-ion peak at *m/z* 318 ($C_{20}H_{30}O_3$), which suggested that it incorporated one oxygen atom (increased to 16 units) into the parent compound **1**. IR experiment showed an absorption band at 3440 cm^{-1} for the hydroxyl group. The presence of signals for 18-CH_3 (s, δ 0.85), 20-CH_3 (s, δ 1.22), 19-CH_3 (s, δ 1.20), and H-4 (s, δ 5.75) in the ^{1}H NMR spectrum of compound **2** showed that the main substrate backbone was intact. The appearance of a carbon resonance at δ 67.6 in the ^{13}C NMR of **2** (see Table 1) and a downfield signal for the 7α-proton at δ 3.9 (1H, br.s) in the ^{1}H NMR spectrum confirmed the insertion of a hydroxyl group added to a secondary carbon. In addition, the signal related to the 7-methine group at δ 31.6 in substrate **1** of the ^{13}C NMR spectrum disappeared, which indicated the position of the hydroxyl group at C-7α. The position and stereochemistry of hydroxyl groups was assigned on the basis of the reported data [16].

15α-Hydroxymethyltestosterone (**3**). R_f (chloroform–acetone, 1:1, v/v): 0.48; yield 21%; IR (ν_{max}, cm^{-1}): 3420, 1645, 1624; EI-MS *m/z* (%): 318 (M^+, $C_{20}H_{30}O_3$; 50), 316 (50), 302 (15), 231 (30), 162 (22), 133 (24), 124 (100), 104 (74), 57 (30); ^{1}H NMR ($CDCl_3$, δ): 0.90 (3H, s, H-18), 1.34 (3H, s, Me-17), 1.20 (3H, s, H-19), 5.8 (1H, s, H-4), 4.20 (1H, br.s, H-15).

The infrared analysis showed absorption at 3420 and 1645 cm^{-1} for the hydroxyl and conjugated ketone group. The molecular formula of compound **3** was deduced as $C_{20}H_{30}O_3$ from its mass spectrum (*m/z* 318), which agreed with ^{13}C NMR spectroscopy. The appearance of a CHOH signal at δ 60 up to 77 (60 to 77 interval) in the ^{13}C NMR spectrum confirmed the insertion of the oxygen atom in a secondary carbon. Comparison of the chemical shifts of the 18-, 19-, and 20-methyl with those of methyltestosterone in the ^{1}H NMR spectrum (δ 0.90 for H-18, δ 1.19 for H-19, and δ 1.20 for H-20 in methyltestosterone; δ 0.90 for H-18, δ 1.20 for H-19, and δ 1.34 for H-20 in compound **3**, respectively) and the presence of an additional signal at δ 4.20 suggested that the alteration should be near position 15 of the β-orientation. The stereochemistry of hydroxyl groups in product **3** was determined by its comparison with the chemical shifts of existing metabolites in published data [17].

12,15α-Dihydroxymethyltestosterone (**4**). R_f (chloroform–acetone, 1:1, v/v): 0.38; yield 22%; IR (ν_{max}, cm^{-1}): 3450, 1679, 1627; EI-MS *m/z* (%): 334 (M^+, $C_{20}H_{30}O_4$; 65), 316 (45), 247 (30), 161 (22), 133 (25), 124 (100), 104 (70), 91 (50), 55 (30); ^{1}H NMR ($CDCl_3$, δ): 0.9 (3H, s, H-18), 1.4 (3H, s, Me-17), 1.28 (3H, s, H-19), 5.8 (1H, s, H-4), 4.17 (3H, t, H-12), 4.20 (1H, br.s, H-15).

Metabolite **4** exhibited the molecular formula $C_{20}H_{30}O_4$, as determined by a combination of mass spectrum (*m/z* 334) and ^{13}C NMR analyses. It contained 36 units more than methyltestosterone. The infrared spectrum indicated absorption frequencies at 3450 and 1679 cm^{-1} for the hydroxyl group and 3-ketone conjugated with 4-ene, respectively. The ^{13}C NMR spectrum of **4** indicated two additional signals at δ 68.0 and 71.1 as compared to the substrate, which strongly suggested the presence of two hydroxyl groups. The ^{1}H NMR chemical shifts of **4** exhibited two oxygen-bearing methine proton signals at δ 4.17 (1H, t, H-12) and 4.20 (1H, br.s, H-15), corresponding to H-12 and C-15, respectively.

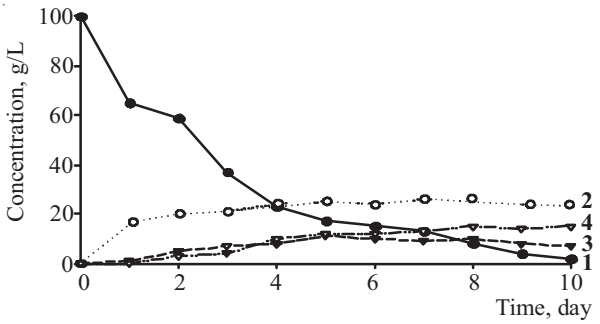

Fig. 1. Time course profile for the biotransformation of methyltestosterone (**1**) by *M. racemosus*.

For the time course study, production of **2** to **4** as a function of incubation time was detected by thin layer chromatography. The starting material, methyltestosterone, 1 g/L, was transformed into various metabolites within 5 days. According to TLC profile (Fig. 1), compound **2** appeared in the broth from the first day while compounds **3** to **4** were produced from the 2nd day. After 10 days of incubation, the methyltestosterone concentration fell below detection limits, and the concentration of metabolite **4** reached a plateau.

The effect of substrate concentration in the range 0.5 to 6 g/L on methyltestosterone biotransformation by *M. racemosus* was also studied. The optimum substrate concentration was 1 g/L, and in concentrations above 6 g/L, methyltestosterone was not converted to any metabolite.

The highest bioconversion rate of methyltestosterone was obtained at 150 rpm. The rate and yield of compound **2** did not increase with higher shaking speeds. For compounds **3** and **4**, although higher speeds resulted in higher production rates, this was accompanied by a concomitant decrease in the overall yield.

The highest bioconversion rate was obtained within the pH range 5.5–6.5. The optimal pH for production of nearly all metabolites was 6. The rate of biomass production increased with higher pH (starting at pH 3.5), and after reaching a maximum at pH 5–6 again decreased gradually, and transformation eventually stopped at pH 10.5.

The bioconversion reaction proceeded well at 25°C for the production of all the metabolites, and the substrate was totally consumed in this temperature during 5 days. At 40°C, the substrate remained unconverted in the broth.

It has already been shown that some bacterial and fungal species are capable of metabolizing methyltestosterone. There are some reports on hydroxylation of methyltestosterone at the C-6β [18], C-7α [16], C-7β [19], C-9α [20], C-11α [21], C-12β [21], and C-15α [17] positions as well as the production of 6β,11α- [18] and 6β,12β-dihydroxy compounds [21]. In this study, it was demonstrated that a 5-day incubation of *M. racemosus* in the presence of methyltestosterone under appropriate conditions results in the formation of metabolites **2** to **4**. Some of these modifications may be of pharmaceutical importance [15]. For example, 7α-hydroxymethyltestosterone (**2**) shows antagonist properties on androgen receptors [22]. These properties may be significantly improved by substituting the 7-OH group with small steric groups [23].

The transformation pattern of steroids by *M. racemosus* seems to be influenced by substrate structure. In a previous study it was demonstrated that *M. racemosus* is able to induce 7- and 14-hydroxylation on androst-4-en-3,17-dione [13]. On the other hand, this microorganism fails to carry out 14-hydroxylation on methyltestosterone. These results indicate that hydroxylation at C-14 may be inhibited by the presence of a 17-methyl group. However, this does not seem to be the case for other positions available for hydroxylation on the D ring, as indicated by the isolation of compounds **3** and **4** from the cultures of *M. racemosus* in the present study. For most other fungi, hydroxylation on the D ring does not appear to be directly influenced by the presence of a 17-methyl group [21, 24].

Time-course experiments demonstrated that 7α-hydroxylation occurred during the early stages of incubation, as indicated by the detection of compound **2** at appreciable levels from the first day of incubation, while 15α-hydroxylation and 12,15-dihydroxylation took place in later stages. It seems that 7α-hydroxylation is the common pattern of steroid bioconversion in the genus *Mucor* [11].

EXPERIMENTAL

Chemicals and Instruments. Methyltestosterone was kindly donated by Abu-Reihan Pharmaceutical Company (Tehran, Iran). Sabouraud-2% dextrose broth (SDB) and 4% dextrose agar (SDA) were purchased from Merck (Darmstadt, Germany). All reagents and solvents were of analytical grade.

Thin layer chromatography (TLC) and preparative TLC were performed, respectively, on 0.25 and 0.5 mm layers of silica gel G (Kieselgel 60 $HF_{254+366}$, Merck). Layers were prepared on glass plates and activated at 105°C 1 h before use. Chromatograms were developed using the chloroform–acetone (1:1, v/v), and detection was achieved by spraying the plates with phosphoric acid (85%)–distilled water (1:1, v/v) followed by heating at 110°C for 10 min.

Chemical structures of pure fungal products were determined on the basis of ^{1}H NMR and ^{13}C NMR spectra obtained using a Bruker DRX (Avance 500) spectrometer at 500 and 125 MHz, respectively. Samples were dissolved in $CDCl_3$ with TMS as internal standard. Chemical shifts (δ) were reported in parts per million (ppm) and coupling constants (J) in hertz. Mass spectra (MS) were obtained with a Finnigan MAT TSQ-70 instrument by electron impact (EI) at 70 eV. Infrared (IR) spectra were recorded using KBr disks on a Magna-IR 550 Nicolet FTIR spectrometer.

Maintenance of the Fungus and Fermentation Conditions. The filamentous fungus of *Mucor racemosus* [13, 14] was maintained on Sabouraud-4% dextrose agar slant and freshly subcultured before use in the transformation experiment. Fresh spores from agar slopes were transferred to ten 500 mL Erlenmeyer flasks, each containing 100 mL of Sabouraud-2% dextrose broth and incubated at 25°C for 24 h on a rotary shaker (150 rpm). Methyltestosterone (1 g) was dissolved in 10 mL of absolute ethanol. One milliliter of the prepared ethanol solution was added to each 500 mL flask to reach a concentration of 1 mg/mL. The flasks were placed on a rotary shaker and incubation was continued for 5 days at the same conditions.

Product Isolation and Analysis. After incubation, the culture broth and mycelia were separately extracted three times with chloroform. The organic phase was separated and filtered, and the extract was concentrated under reduced pressure. The residue was loaded on preparative TLC using a solvent system of chloroform–acetone (1:1, v/v). The purified metabolites were crystallized in appropriate solvents and then identified using spectral analyses, including ^{13}C NMR, ^{1}H NMR, FTIR, and MS.

Time Course Experiment and the Effect of Temperature, Aeration Rate, pH, and Substrate Concentration. Spores of *M. racemosus* were cultivated in a 500 mL Erlenmeyer flask containing 100 mL of SDB medium supplemented with 100 mg of methyltestosterone (dissolved in 1 mL of absolute ethanol) and then incubated at 25°C and 150 rpm for 10 days. Sampling was carried out every 24 h. Controls were similarly processed except that no microorganism was added.

Studies were also performed to determine the optimum temperature, aeration rate, pH, and substrate concentration for the production of metabolites. The temperature was varied from 20 to 40°C with increments of 5°C. The effect of aeration rate on biotransformation procedure was studied in the range 50 to 250 rpm. The effect of pH on biotransformation procedure was studied in nonbuffered media by adjusting the pH from 3.5 to 11 with NaOH and HCl with 0.5 unit increments. The initial substrate concentration ranged from 0.5 to 6 g/L with increments of 0.5 g/L. For each experiment only one parameter was changed at a time. The procedure was carried out in triplicate for each analytical determination.

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