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Formation of hydroxysteroid derivatives from androst-4-en-3,17-dione by the filamentous fungus *Mucor racemosus*

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Abstract

This study describes the transformation of androst-4-en-3,17-dione (**I**) into six different hydroxysteroid derivatives by the filamentous fungus *Mucor racemosus* after a 5-day incubation period. The microbial products were purified chromatographically and identified on the basis of their spectral data as 14α-hydroxyandrost-4-en-3,17-dione (**II**), 7α-hydroxyandrost-4-en-3,17-dione (**III**), 14α,17β-dihydroxyandrost-4-en-3-one (**IV**), 6β,14α-dihydroxyandrost-4-en-3,17-dione (**V**), 7β,14α,17β-trihydroxyandrost-4-en-3-one (**VI**), and 6β,11α,17β-trihydroxyandrost-4-en-3-one (**VII**). Observed modifications include hydroxylation at C-6β, C-7α, C-7β, C-11α, C-14α positions and 17-carbonyl reduction. The best fermentation condition was found to be 25 °C and pH 6 for 120 h with a substrate concentration of 1.0 g/L. In substrate concentrations above 6.0 g/L, the biotransformation was completely inhibited.

Keywords: *Mucor racemosus*; Androst-4-en-3,17-dione; Biotransformation; Steroid

1. Introduction

The preferential use of whole cells over enzymes as biocatalysts in the production of useful organic compounds mostly results from the costs of enzyme isolation, purification and stabilization in the latter method. Biotransformation of steroids by microorganisms including bacteria, fungi, and algae has been extensively studied and well documented in recent decades [1]. However, only a small fraction of thousands of microorganism species and steroid substrates studied to date can actually be used in the large-scale manufacture of pharmaceutical steroids. This warrants further studies in order to identify new microorganisms capable of transforming steroids in an efficient and, more importantly, specific way. In addition, microbial models can satisfactorily be used to study mammalian steroid metabolism pathways [2]. The ability of microbial species to produce different metabolites which sometimes are not achievable by chemical synthesis methods has made them an indispensable part of the pharmaceutical industry [3–5].

Fungi are widely used in steroid microbial transformation studies [1,6], since their versatile enzymatic reservoir allow them to modify a wide range of steroids [7]. This, among other reasons, is why fungi have stood in the center of our studies on steroid biotransformation, namely hydroxylation because of its industrial importance, during recent years [8–11].

Mucor racemosus is one of the commonest species of the genus *Mucor* and is widely distributed in nature [12]. Although fungi belonging to the genus *Mucor* have widely been applied in steroid transformation studies [13–16], very limited studies have been done using *M. racemosus*. It has been used in the transformation of some steroid substances such as pregnane [17,18], cardenolide [18], and dehydroepiandrostane [19]. Also, it has been exploited as a microbial model of drug metabolism, e.g. 19-norsteroid substances [2].

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In the present study, we demonstrate the ability of *M. racemosus* to introduce different modifications to androst-4-en-3,17-dione (**I**, AD). We also report the optimum fermentation conditions for this specific process.

2. Materials and methods

2.1. Chemicals and instruments

Androst-4-en-3,17-dione (AD) was purchased from Sigma chemical Co. (St. Louis, MO, USA). Sabouraud-2%-dextrose broth (SDB) and 4%-dextrose agar (SDA) were acquired from Merck (Darmstadt, Germany). All reagents and solvents were of analytical grade.

The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained using a Bruker DRX (Avance 500) spectrometer at 500 and 125 MHz, respectively, with tetramethylsilane (TMS) as internal standard in $CDCl_3$. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS. The coupling constant (J) is given in hertz (Hz). 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 Magna-IR 550 Nicolet FTIR spectrometer. Optical rotations were measured on solution of methanol in 10 cm cells on Perkin-Elmer 142 automatic spectropolarimeter. Melting points (mp) were determined on a melting point apparatus, Gallenkamp, UK, and were uncorrected. 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. Chromatography was performed with acetone/hexane (1:1, v/v) and visualized by spraying the plates with a mixture of phosphoric acid (85%)/distilled water (1:1, v/v) and heating in an oven at 110 °C for 10 min until the colors developed.

2.2. Microorganism and fermentation condition

The strain of *M. racemosus* was obtained during a screening program for uricase producing microorganisms [20]. It was maintained on Sabouraud-4%-dextrose agar slope and freshly subcultured before being used in the transformation experiment.

Ten 500-mL Erlenmeyer flasks, filled with 100 mL Sabouraud-2%-dextrose broth, were inoculated with fresh spores from agar slopes and incubated for 24 h at 25 °C on a rotary shaker (150 rpm). AD (1 g) was dissolved in 10 mL of absolute ethanol. One milliliter of the ethanol solution was added to each 500-mL flasks. Incubation continued for 5 days at the same conditions.

2.3. Isolation of AD transformation metabolites

At the end of incubation, the fermentation broth was extracted three times with chloroform. Organic phase was separated, filtered, and the extract was evaporated under reduced pressure. Residue was loaded on preparative TLC using a solvent system of acetone/hexane (1:1, v/v). The purified metabolites were crystallized in appropriate solvents and then identified using spectral data (^{13}C NMR, ^{1}H NMR, FTIR and MS) and their physical constants (melting points and optical rotations).

2.4. Time course experiment and the effect of temperature, pH and substrate concentration

Spores of *M. racemosus* were transferred into a 500-mL Erlenmeyer flask containing 100 mL of SDB medium supplemented with 50 mg of AD dissolved in 2 mL of absolute ethanol and then the incubation continued for 10 days at the same condition described above (see Section 2.2). Sampling was carried out every 24 h. Controls were similarly processed except that no microorganisms were added.

Studies were performed to determine the optimum pH and temperature as well as the maximum amount of substrate that could be transformed to the products. The temperature was varied from 20 to 40 °C at intervals of 5 °C. The effect of pH on biotransformation procedure was studied in non-buffered media by adjusting the pH from 3 to 11 with NaOH and HCl at 0.5 unit intervals. The initial substrate concentration ranged from 0.5 to 6.0 g/L with an interval of 0.5. For each experiment only one parameter was changed at a time. The procedure was carried out in triplicate for each analytical determination. Qualitative studies were performed using TLC and detection was done by UV at 254 nm.

3. Results

Microbial transformation of AD by *M. racemosus* in 5 days led to the formation of six hydroxy-derived androstendione compounds (**II–VII**) (Fig. 1) as follows. No transformation occurred in the control media. Steroid products were characterized using spectral data (^{13}C NMR, ^{1}H NMR, FTIR, MS), melting points and optical rotations. The yields of the bioproducts were calculated according to the percentage of dry weight of each compound. Starting material (**I**) was completely consumed at the end of the fermentation.

3.1. 14α-Hydroxyandrost-4-en-3,17-dione (*II*)

Crystallized from methanol; yield 19%; mp 259–262 °C, $[\alpha]_D + 155°$ ($CHCl_3$), lit [21]; mp 261–263 °C, $[\alpha]_D + 162°$ ($CHCl_3$); IR υ_{max} 3457, 2924, 1734, 1662, 1614, 1144 cm^{-1}; MS (EI) m/z (%) 302 (M^+, $C_{19}H_{26}O_3$; 24), 227 (8), 133 (15), 119 (29), 79 (35), 69 (45), 57 (100); ^{1}H NMR ($CDCl_3$) δ 1.07 (3H, s, H-18), 1.25 (3H, s, H-19), 5.78 (1H, s, H-4); R_f in acetone/hexane (1:1) 0.56.

The infrared analysis of compound **II** showed two carbonyl absorption bands at 1734 and 1662 cm^{-1} and a hydroxyl group at 3457 cm^{-1} for C-17, C-3 and C-14, respectively. The mass spectrum showed the molecular ion peak at m/z 302 ($C_{19}H_{26}O_3$), which suggested that it incorporates one oxygen atom (increased 16 units) into the substrate (**I**). The absences of a CHOH signal at midfield in ^{1}H NMR spectrum confirmed the insertion of the oxygen atom in a tertiary carbon [22]. ^{13}C NMR spectrum of **II**

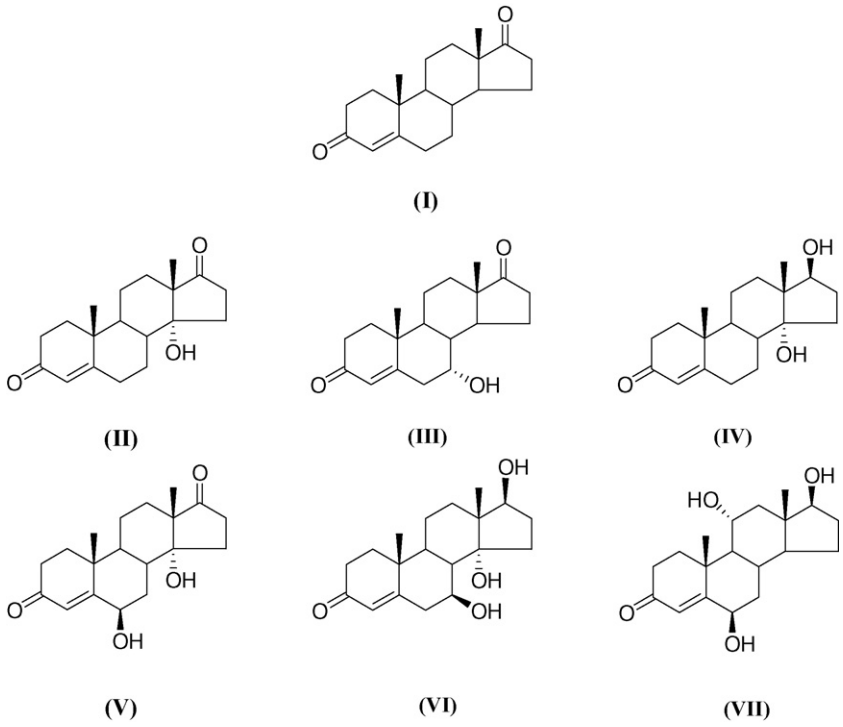

Fig. 1. Biotransformation of AD (**I**) by *Mucor racemosus* and the chemical structures of its bioproducts **II–VII**. Androst-4-en-3,17-dione (**I**), 14α-hydroxyandrost-4-en-3,17-dione (**II**), 7α-hydroxyandrost-4-en-3,17-dione (**III**), 14α,17β-dihydroxyandrost-4-en-3-one (**IV**), 6β,14α-dihydroxyandrost-4-en-3,17-dione (**V**), 7β,14α,17β-trihydroxyandrost-4-en-3-one (**VI**), and 6β,11α,17β-trihydroxyandrost-4-en-3-one (**VII**).

Table 1
^{13}C NMR signals of the substrate (**I**) and the biometabolites **II–VII** (δ in ppm downfield from TMS, in $CDCl_3$)

Carbon atom	I	II	III	IV	V	VI	VII
1	35.6	36.1	35.8	36.2	34.6	35.6	36.8
2	33.8	33.5	34.3	34.4	32.7	34.0	34.1
3	199.0	200.0	199.2	200.2	201.5	198.6	201.0
4	124.0	124.5	127.6	124.3	126.5	127.2	124.9
5	170.1	170.6	167.3	171.3	169.0	166.3	171.9
6	32.4	32.8	39.8	33.0	73.1	41.2	69.2
7	31.2	26.0	67.5	29.0	37.5	69.3	39.1
8	35.0	38.4	41.4	39.3	32.7	40.0	28.6
9	53.7	47.3	45.8	47.2	47.1	41.1	59.6
10	35.8	39.1	39.0	39.2	38.6	38.7	39.3
11	20.2	19.5	20.6	20.1	19.91	19.6	66.9
12	30.6	24.9	31.4	33.1	24.9	32.5	48.8
13	47.3	53.0	47.7	47.4	53.3	47.3	44.0
14	50.7	81.1	46.1	83.8	81.2	84.1	50.2
15	21.6	30.7	21.7	30.0	30.4	29.8	23.7
16	35.6	34.3	36.1	26.5	33.6	28.6	29.4
17	220.0	218.9	220.7	79.0	219.7	78.6	79.3
18	13.5	18.3	13.9	15.3	18.3	14.7	11.5
19	17.2	17.7	17.4	17.7	19.50	17.2	18.8

Androst-4-en-3,17-dione (**I**), 14α-hydroxyandrost-4-en-3,17-dione (**II**), 7α-hydroxyandrost-4-en-3,17-dione (**III**), 14α,17β-dihydroxyandrost-4-en-3-one (**IV**), 6β,14α-dihydroxyandrost-4-en-3,17-dione (**V**), 7β,14α,17β-trihydroxyandrost-4-en-3-one (**VI**), and 6β,11α,17β-trihydroxyandrost-4-en-3-one (**VII**).

(see Table 1) suggested that the location of hydroxyl group was at C-14 (δ 81.1) which is comparable to the published data [23].

3.2. 7α-Hydroxyandrost-4-en-3,17-dione (*III*)

Crystallized from methanol; yield 21%; mp 249–250 °C, $[\alpha]_D + 160^\circ$ ($CHCl_3$), lit [21]; mp 248–250 °C, $[\alpha]_D + 155^\circ$ ($CHCl_3$); IR υ_{max} 3395, 2923, 1738, 1656, 1610, 1140 cm^{-1}; MS (EI) *m/z* (%) 302 (M^+, $C_{19}H_{26}O_3$; 20), 149 (15), 124 (40), 91 (40), 67 (50), 55 (100); ^{1}H NMR ($CDCl_3$) δ 0.96 (3H, s, H-18), 1.26 (3H, s, H-19), 4.14 (1H, m, H-7), 5.86 (1H, s, H-4); R_f in acetone/hexane (1:1) 0.45.

The mass spectrometry indicated a molecular ion at *m/z* 302 ($C_{19}H_{26}O_3$). The IR spectra showed absorption band for a hydroxyl group at 3395 cm^{-1} compared to **I**. In ^{1}H NMR spectrum of **III** showed an additional signal at δ 4.14 as multiplet, characteristic of a 7α-proton [22]. The appearance of a carbon resonance at δ 67.5 in the ^{13}C NMR spectra of **III** confirmed the insertion of a hydroxyl group added to a secondary carbon, position of the hydroxyl group at C-7α was assigned on the basis of published data [24].

3.3. 14α,17β-Dihydroxyandrost-4-en-3-one (*IV*)

Crystallized from methanol; yield 15%; mp 181–184 °C, $[\alpha]_D + 121^\circ$ ($CHCl_3$), lit [21]; mp 183–186 °C, $[\alpha]_D + 124^\circ$ (c, 0.974 in $CHCl_3$); IR υ_{max} 3434, 2941, 1656, 1612, 1148 cm^{-1}; MS (EI) *m/z* (%) 304 (M^+, $C_{19}H_{28}O_3$; 14), 286 (35), 284 (56), 240 (14), 173 (15), 161 (20), 148 (29), 123 (50), 91 (47), 56

(100); ^{1}H NMR ($CDCl_3$) δ 0.94 (3H, s, H-18), 1.24 (3H, s, H-19), 4.34 (1H, t, J = 7.7 Hz, H-17), 5.76 (1H, s, H-4); R_f in acetone/hexane (1:1) 0.4.

Compound **IV** exhibited the molecular formula of $C_{19}H_{28}O_3$, which was deduced from its mass spectrum. It contained 18 units more than AD. IR spectrum showed an absorption band at 3434 cm^{-1} for hydroxyl group. It was also showed that the band for 17-carbonyl group (1738 cm^{-1} in AD) was disappeared in **IV**. The ^{13}C NMR spectrum showed an oxygen-bearing methine carbon signal at δ 79.0 for 17-carbonyl group and a downfield signal for 17α-proton at δ 4.34 (1H, t) in ^{1}H NMR spectrum. In addition, the signal related to 17-carbonyl group at δ 220 in ^{13}C NMR spectrum was disappeared which indicated that it was reduced. Signal at δ 83.8 in ^{13}C NMR was for C-14 [25]. No related signal was found in ^{1}H NMR spectrum for its proton which indicated the insertion of a hydroxyl group added to a tertiary carbon. The position and stereochemistry of hydroxyl groups was assigned on the basis of the reported data [22].

3.4. 6β,14α-Dihydroxyandrost-4-en-3,17-dione (V)

Crystallized from methanol; yield 9%; mp 255–258 °C, $[\alpha]_D$ + 106° (MeOH), lit [23]; mp 256–257 °C, $[\alpha]_D$ + 104° (c, 0.1 in MeOH); IR υ_{max} 3444, 2937, 1738, 1674, 1610, 1160, 1110 cm^{-1}; MS (EI) m/z (%) 318 (M^+, $C_{19}H_{26}O_4$; 6), 302 (25), 270 (35), 245 (15), 171 (12), 161 (19),133 (26), 91 (65), 123 (100), 55 (86); ^{1}H NMR($CDCl_3$) δ 1.06 (3H, s, H-18), 1.40 (3H, s, H-19), 4.43 (1H, brs, H-6), 5.78 (1H, s, H-4); R_f in acetone/hexane (1:1) 0.33.

The presence of signals for 18-CH_3 (s, δ 1.06), 19-CH_3 (s, δ 1.40) and H-4 (s, δ 5.78) in the ^{1}H NMR spectra of compound **V** showed that the fundamental of androst-4-en-3,17-dione backbone kept intact. ^{13}C NMR spectrum (see Table 1) showed two additional signals at δ 73.1 and δ 81.2 for C-6 and C-14, respectively [23]. ^{1}H NMR showed a proton signal at δ 4.43 for H-6. This position of hydroxyl group at C-6β was proved in agree with the published data for H-6α in 6β-hydroxy steroids [23]. Second hydroxyl group in compound **V** was assigned at C-14 on the basis of published data [26]. Mass spectral fragmentation of compounds **V** was in full agreement for proposed molecular formula $C_{19}H_{26}O_4$ with molecular ion peak appearing at m/z 318 (M^+) and other ion peak at m/z 302, representing loss of an oxygen atom. The IR spectrum showed characteristic absorption at 3443, 1737 and 1659 cm^{-1} for hydroxyl groups, a 17-carbonyl on cyclopentane and 3-carbonyl group conjugated with double bond, respectively.

3.5. 7β,14α,17β-Trihydroxyandrost-4-en-3-one (VI)

Crystallized from methanol; yield 24%; mp 225–230 °C, $[\alpha]_D$ + 43° (MeOH); IR υ_{max} 3400, 2952, 1654, 1620, 1160, 1088 cm^{-1}; MS (EI) m/z (%) 320 (M^+, $C_{19}H_{28}O_4$; 4), 302 (6), 180 (15), 163 (29), 123 (36), 109 (41), 83 (100), 45(15); ^{1}H NMR ($CDCl_3$) δ 0.85 (3H, s, H-18), 1.26 (3H, s, H-19), 4.29 (1H, brs, H-17), 4.31 (1H, brs, H-7), 5.80 (1H, s, H-4); R_f in acetone/hexane (1:1) 0.28.

Metabolite **VI** provided ^{1}H NMR, ^{13}C NMR (see Table 1) and DEPT-90 and -135 spectrums data which strongly suggested the presence of three hydroxyl groups, two secondary and a tertiary. In the mass spectrum, a molecular ion peak at m/z 320 was found ($C_{19}H_{28}O_4$). The IR spectrum of **VI** showed peaks for hydroxyl and carbonyl group conjugated with double bond at 3400 and 1654 cm^{-1}, respectively. In ^{13}C NMR spectrum, the characteristic absorption for the 17-carbonyl disappeared and a signal at δ 78.6 was found instead of δ 220.0 as compared to the parent compound which confirmed the presence of a hydroxyl group at C-17. Two additional signals at δ 69.3, δ 84.1 in ^{13}C NMR spectrum were related to C-7 and C-14, respectively. In ^{1}H NMR spectrum, the signals at α 4.29 and α 4.31 indicated H-7α and H-17α, respectively. Obtained data was supported with DEPT experiment. The stereochemistry of C-7 in the product **VI** was determined by its comparison with the chemical shift for H-7α and H-7β in compounds having α-hydroxyl and β-hydroxyl groups at C-7 (for α-hydroxyl δ 4.03–4.27 and for β-hydroxyl δ 4.13–4.43) [27]. There is a report on the formation of compound **VI** by *Mucor piriformis* in the literature [28] with no spectral analyses.

3.6. 6β,11α,17β-Trihydroxyandrost-4-en-3-one (VII)

Crystallized from chloroform; yield 7%; mp 235–240 °C, $[\alpha]_D$ + 31° (MeOH), lit [29]; mp 235–236 °C, $[\alpha]_D$ + 34° (c, 1 in MeOH); IR υ_{max} 3405, 2928, 1654, 1609, 1138, 1058 cm^{-1}; MS (EI) m/z (%) 320 (M^+, $C_{19}H_{28}O_4$; 4), 286 (16), 275 (18), 180 (16), 163 (37), 106 (58), 90 (62), 78 (74), 54 (100); ^{1}H NMR ($CDCl_3$) δ 0.85 (3H, s, H-18), 1.35 (3H, s, H-19), 3.70 (1H, t, J = 8.6 Hz, H-17), 4.06 (1H, m, H-11), 4.30 (1H, t, J = 7.8 Hz, H-6), 5.76 (1H, s, H-4); R_f in acetone/hexane (1:1) 0.25.

The molecular formula of compound **VII** was deduced as $C_{19}H_{28}O_4$ from its mass spectrum (m/z 320). The IR spectrum showed absorption at 3404 and 1654 cm^{-1} for hydroxyl and conjugated ketone group. ^{1}H NMR data showed three oxygen bearing methine protons at 3.70 (1H, t, J = 8.6 Hz, H-17), 4.06 (1H, m, H-11) and δ 4.30 (1H, t, J = 7.8 Hz, H-6), which indicated this compound had three hydroxyl groups at C-6, C-11 and C-17, respectively [29]. The ^{13}C NMR chemicals shifts of **VII** (see Table 1) exhibited signals at δ 66.9, 69.2 and 79.3 correspondences to C-11, C-6 and C-17, respectively. The chemical shifts and stereochemistry of H-17 and H-6 supported α-orientation of both hydroxyl groups [29].

For a time course study, production of **II–VII**, as a function of incubation time, was detected by thin layer chromatography. The starting material, AD 1.0 g/L, was transformed into various metabolites within 5 days. According to TLC profile (Fig. 2), compound **III** appeared in the broth from the first day while compounds **II**, **IV** and **VI** were produced from the second day. Subsequently, the rest of the metabolites were increasingly formed in the medium within the following days. Increasing the incubation period up to 10 days did not result in higher product concentrations. Indeed compound **V** and **VII** concentrations fell below detection limits after this prolonged period. The effect of substrate concentration in the range of 0.5–6.0 g/L on AD

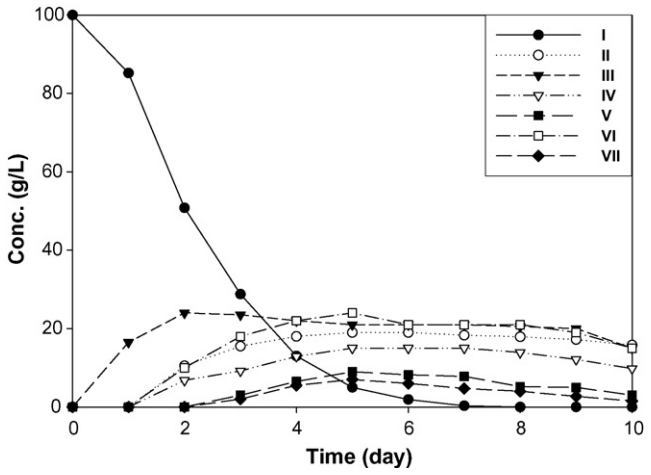

Fig. 2. Time course profile for the biotransformation of AD (**I**) by *M. racemosus*. Androst-4-en-3,17-dione (**I**), 14α-hydroxyandrost-4-en-3,17-dione (**II**), 7α-hydroxyandrost-4-en-3,17-dione (**III**), 14α,17β-dihydroxyandrost-4-en-3-one (**IV**), 6β,14α-dihydroxyandrost-4-en-3,17-dione (**V**), 7β,14α,17β-trihydroxyandrost-4-en-3-one (**VI**), and 6β,11α,17β-trihydroxyandrost-4-en-3-one (**VII**).

biotransformation by *M. racemosus* was also studied. Based on the TLC profile, the optimum substrate concentration was 1.0 g/L, and in concentrations above 6.0 g/L, AD was not converted to any metabolite.

The highest bioconversion rate of AD 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 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. Compounds **II** and **IV**, the 14α-hydroxylated metabolites, accumulated in the fermentation liquid at 20 and 35 °C. At 40 °C, the substrate remained unconverted in the broth.

4. Discussion

A 5-day incubation of *M. racemosus* with AD in appropriate conditions resulted in the formation of metabolites **II–VII** (see Fig. 1). It has already been shown that some bacterial and fungal species are capable of metabolizing androstendione. There are some reports on the hydroxylation of AD at C-6β [30], C-7α, C-7β [28,31], C-9α [30], C-11α, C-11β [31], C-14α [26,28], C-15α [32], C-17β [28] positions and also the production of androst-1,4-dien-3,17-dione (ADD) [32]. AD is among the most important intermediates in the production of some valuable pharmaceutical steroid compounds [1].

The present work shows that *M. racemosus* is able to convert AD into six steroid metabolites. The biotransformation characteristics observed included C-6β, C-7α, C-7β, C-11α, C-14α hydroxylations and 17-carbonyl reduction into the related C-17β hydroxyl form according to spectral analyses.

Several fungi, especially from the order of Mucorales are able to introduce a hydroxyl group at the 14α-position of progesterone and some other steroids at acceptable yields [25]. Considering the low yields and nonspecific nature of existing chemical synthesis methods [33] such organisms have received much attention from the pharmaceutical industry. In this work we demonstrated that *M. racemosus* produced a 14α-hydroxylated compound (**II**). 6β,14α-Dihydroxyandrost-4-en-3,17-dione (**V**) has some inhibitory effect on the estrogen biosynthesis pathway [34]. Treatment of compound **V** with chromium trioxide in sulfuric acid afforded monohydroxylated compound which was characterized as 14α-hydroxyandrost-4-en-3,6,17-trione. This compound can potentially be used in medical situation where estrogen serves as a major player such as ovulation and the growth of estrogen-dependent tumors [35]. Also, it is one of the most potent inhibitors of human placental aromatase [23]. Compound **III** is a 7α-hydroxy derivative of AD. Such derivatives of steroids are also difficult to synthesize by chemical methods and can be used in the preparation of diuretic compounds [36].

The rate of hydroxylation at C-7 and C-14 is much greater than that of hydroxylation at C-6 and C-11 under the applied condition of cultivation and transformation.

We also presented here that *M. racemosus* was able to transform the substrate (**I**) into two trihydroxy steroid derivates (**VI** and **VII**). The hydroxylation at C-6β and C-7β occurred subsequent to the reduction of 17-carbonyl group.

Time course experiments clearly indicated that 7α-hydroxylation occurred during the early stages of incubation to produce metabolites **III**, while 14α-hydroxylation and 17-carbonyl reduction happened at the second day to give products **II**, **IV** and **VI**. Products of the other bio-reactions including 6β- and 11α-hydroxylations (compounds **V** and **VII**) did not appear before the third day of incubation. It seems that 7α- and 14α-hydroxylations are two common patterns of steroid bioconversion in the genus *Mucor* [24,25]. The highest bioconversion rate of AD was obtained within pH values from 5.5 to 6.5. The bioconversion reaction proceeded well at 25 °C for production of all the metabolites. Compounds **II** and **IV**, the 14α-hydroxylated metabolites, accumulated in the fermentation liquid at 20 and 35 °C. At 40 °C, the substrate remained unconverted in the broth. Increasing concentration of AD from 0.5 to 6.0 g/L showed that higher concentration of the substrate (≥6.0 g/L) decrease microbial conversion. The optimum substrate concentration, which gave the maximum efficiency in microbial conversion of AD by *M. racemosus*, was 1.0 g/L.

To sum up, the result shows that *M. racemosus* is a suitable biocatalyst for hydroxylations at some specific sites in the molecule of AD. It is also an efficient 7- and/or 14-hydroxylator and may be used as a model in steroidal drug metabolism studies.

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