Introduction

Microbial biotransformation of steroids has found worldwide application for the preparation of more valuable and functionalized compounds due to its high regio- and stereoselectivity (Fernandes et al., 2003). Therefore, a number of researches on microbial biotransformations of a wide range of steroidal substrates have been reported in recent years (Fernandes et al., 2003; Holland, 1999; Mahato and Garai, 1997). There are still enormous efforts to increase the efficiency of microbial steroid biotransformations and to find new useful microorganisms and reactions (Fernandes et al., 2003).

Aspergillus wentii Wehmer is a toxigenic, ubiquitous, soil-inhabiting fungus usually found on decayed vegetation and moist grains (Wells et al., 1975; Wu et al., 1974). It produces several toxic metabolites such as aflatoxins (Schroeder and Verrett, 1969), emodin (Wells et al., 1975), and ochratoxin A (Varga et al., 1996) as well as other secondary metabolites such as kojic acid (Brian, 1951), hexadecylitaconic acid (Selva et al., 1980), 3-nitropropionic acid (Burrows et al., 1965), 1-amino-2-nitrocyclopentanecarboxylic acid (Burrows and Turner, 1966), wentilactone A and B (Dorner et al., 1980).

As far as biotransformations by Aspergillus wentii are concerned, we have not found any literature references on steroids. Actually, the only biotransformations by this fungus reported in literature are on some aromadendrane-type sesquiterpenoids (Miyazawa et al., 2008). In our work we have investigated the biotransformation of testosterone (1) and progesterone (2).

Experimental

General

Testosterone and progesterone were purchased from Fluka (Istanbul, Turkey). Solvents were of analytical grade and were purchased from Merck (Istanbul, Turkey). The steroids were separated by column chromatography on silica gel 60 (Merck 107734) with 50% ethyl acetate in hexane as eluent. Thin layer chromatography (TLC) was carried out with 0.2 mm thick Merck Kieselgel 60 F254 TLC plates using ethyl acetate/n-hexane (1:1, v/v) as eluent. TLC plates were dipped into an anisaldehyde/H2SO4 reagent and heated to 120 °C for 3 min in order to visualize the spots. Infrared spectra were recorded using a Shimadzu IR Prestige-21 instrument. 1H NMR spectra were recorded in deuteriochloroform with tetramethylsilane as an internal standard reference at 300 MHz with a Varian Mercury 300 spectrometer. 13C NMR spectra were recorded in deuteriochloroform at 75 MHz with a Varian Mercury 300 spectrometer. Chemical shifts are given in ppm (δ scale), coupling constants (J) are given in Hz. Melting points were determined by an Electrothermal IA 9200 melting point apparatus and are uncorrected.

Microorganism

Aspergillus wentii MRC 200316 was obtained from TUBITAK, Marmara Research Center, Food Science and Technology Research Institute, Culture Collection Unit, Kocaeli, Turkey. Stock cultures were maintained at 4 °C on PDA slopes and refreshed every two weeks.
Culture medium

The liquid medium for *Aspergillus wentii* MRC 200316 was prepared by mixing sucrose (15 g), glucose (15 g), polypeptone (5 g), MgSO₄·7 H₂O (0.5 g), KCl (0.5 g), K₂HPO₄ (1 g), and FeSO₄·7 H₂O (10 mg) in distilled water (1 l). Then the pH value was adjusted to 7.2 (Miyazawa et al., 2008). The medium was evenly distributed among 10 culture flasks of 250 ml capacity (100 ml in each) and autoclaved for 20 min at 121 °C.

Biotransformation of testosterone (1) by *A. wentii* MRC 200316

Spores freshly obtained from PDA slopes were transferred aseptically into each flask containing sterile medium in a biological safety cabinet. After cultivation at 27 °C for 2 d on a rotary shaker (150 rpm), testosterone (1) (500 mg) dissolved in 10 ml of dimethylformamide (DMF) was evenly distributed aseptically among the flasks. The biotransformation of the substrate was carried out in 10 flasks for 5 d under the same conditions. The fungal mycellium was separated from the broth by filtration under vacuum, and the mycellium was rinsed with ethyl acetate (500 ml). The broth was saturated with NaCl and then extracted three times each with 1 l of ethyl acetate. The organic extract was dried over anhydrous sodium sulfate, and the solvent evaporated in vacuo to give a brown gum (713 mg) which was then chromatographed on silica gel. Elution with 50% ethyl acetate in hexane afforded 6α-hydroxytestosterone (3) (401 mg), which was identified by comparison of its melting point, IR, ¹H NMR, and ¹³C NMR spectra with those in the literature (Hu et al., 1995).

Further elution with 50% ethyl acetate in hexane afforded 14α-hydroxytestosterone (4) (37 mg), which was also identified by comparison of its melting point, IR, ¹H NMR, and ¹³C NMR spectra with those in the literature (Hu et al., 1995).

Biotransformation of progesterone (2) by *A. wentii* MRC 200316

Under the same conditions, the incubation of progesterone (2) (500 mg) with *A. wentii* MRC 200316 for 5 d afforded a brown gum (705 mg) which was then chromatographed on silica gel. Elution with 50% ethyl acetate in hexane afforded 11α-hydroxyprogesterone (5) (457 mg), which was identified by comparison of its melting point, IR, ¹H NMR, and ¹³C NMR spectra with those in the literature (Farooq et al., 1994).

Time course experiments

Time course experiments were conducted for both substrates (Hunter et al., 2009). Conditions were identical to those in main biotransformation experiments except that each individual steroidal substrate (300 mg) dissolved in DMF (6 ml) was evenly distributed between 6 flasks (each containing 100 ml of medium). One flask was harvested after 8 h. Then every 24 h one flask was harvested and extracted. TLC analysis of the isolated mixture was performed immediately. Following 6 h under high vacuum, the product ¹H NMR spectra were determined in CDCl₃ to confirm the steroidal nature of the extracts.

Results and Discussion

Testosterone (1) and progesterone (2) were incubated with *A. wentii* MRC 200316 for 5 d. The results of the biotransformations are presented in Fig. 1. The chemical structures of the biotransformation products were assigned mainly based on ¹H NMR (Table I) and ¹³C NMR (Table II) spectra. The incubation of testosterone (1) with *A. wentii* MRC 200316 for 5 d afforded two metabolites. The characteristic resonances of the first metabolite at δH 4.35 ppm (1H, bs) (Kirk et al., 1990) and δC 72.86 ppm (Blunt and Stothers, 1977) suggested that hydroxylation had taken place at the axial proton at C-6 and the metabo-

| Table I. ¹H NMR data determined in CDCl₃ at 300 MHz for compounds 1 – 5. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Compound | 4-H | 17α-H | 18-CH₃ | 19-CH₃ | 21-CH₃ | Other significant resonances |
| 1 | 5.72 | 3.61 (1H, t, J = 8.5 Hz) | 0.78 | 1.19 | - | - |
| 2 | 5.73 | 2.53 (1H, t, J = 8.5 Hz) | 0.67 | 1.20 | 2.13 | - |
| 3 | 5.81 | 3.66 (1H, t, J = 8.5 Hz) | 0.81 | 1.38 | - | 4.35 (1H, bs, 6α-H) |
| 4 | 5.71 | 4.29 (1H, t, J = 8.2 Hz) | 0.90 | 1.20 | - | - |
| 5 | 5.69 | 2.50 (1H, t, J = 9.0 Hz) | 0.65 | 1.28 | 2.10 | 3.98 (1H, td, J = 10.0 and 5.0 Hz, 11β-H) |
lite was 6β-hydroxytestosterone (3). The structure of 3 was confirmed by comparison of its melting point and spectral data with those in the literature (Hu et al., 1995).

The second metabolite showed a new carbon atom resonance at δC 83.29 ppm, indicating the presence of a hydroxy group. The 1H NMR spectrum of the metabolite had a downfield shift for the 17α-H resonance (Δ 0.68 ppm), suggesting its diaxial interaction with a 14α-hydroxy group. The comparison of the metabolite’s melting point and spectral data with those in the literature (Hu et al., 1995) demonstrated that it was 14α-hydroxytestosterone (4).

During the time course experiment (Table III) for testosterone (1), the 19-methyl resonance
of testosterone (1) shifted from 1.19 ppm to 1.38 ppm, and comparison of the methyl group integrations in the $^1$H NMR spectrum indicated that 60% of the substrate was converted to 6$\beta$-hydroxytestosterone (3) within 72 h. After 96 h, the 19-methyl resonance of testosterone (1) at $\delta_H$ 1.19 ppm disappeared whereas the 19-methyl resonance of 6$\beta$-hydroxytestosterone (3) at $\delta_H$ 1.38 ppm remained and a new 18-methyl resonance at $\delta_H$ 0.90 ppm emerged. These results indicated that most of testosterone (1) was first converted only to 6$\beta$-hydroxytestosterone (3) and the rest of the substrate was then converted to 6$\beta$-hydroxytestosterone (3) and 14$\alpha$-hydroxytestosterone (4).

The incubation of progesterone (2) with A. wentii MRC 200316 for 5 d afforded only one metabolite. The $^1$H NMR spectrum of the metabolite demonstrated a downfield shift (A 0.08 ppm) for the 19-methyl group. The metabolite had characteristic resonances at $\delta_H$ 3.98 ppm (1H, td, $J = 10.0$ and 5.0 Hz) (Kirk et al., 1990) and $\delta_C$ 68.54 ppm (Blunt and Stothers, 1977) suggesting that hydroxylation had taken place at the equatorial proton at C-11, generating an 11$\alpha$-hydroxy group. The comparison of the metabolite’s melting point and spectral data with those in the literature (Farooq et al., 1994) confirmed that it was 11$\alpha$-hydroxyprogesterone (5).

During the time course experiment (Table III) for progesterone (2), the 19-methyl resonance of progesterone (2) shifted from 1.20 ppm to 1.28 ppm, and comparison of the methyl group integrations in the $^1$H NMR spectra indicated that 60% of the substrate was converted to 11$\alpha$-hydroxyprogesterone (5) within 72 h. After 96 h, the disappearance of the 19-methyl resonance of progesterone (2) at $\delta_H$ 1.20 ppm and the presence of the 19-methyl resonance of 11$\alpha$-hydroxyprogesterone (5) at $\delta_H$ 1.28 ppm suggested that all of the substrate was converted to 11$\alpha$-hydroxyprogesterone (5). These results confirmed that the fungus hydroxylated progesterone (2) only at C-11 and no starting material remained.

We have shown that A. wentii has the ability to hydroxylate testosterone (1) and progesterone (2) in an efficient way (Table IV). A. wentii hydroxylated testosterone (1) mainly at 6$\beta$-position and an independent minor hydroxylation took place at 14$\alpha$-position. Some fungi such as Cephalosporium aphidicola (Hanson et al., 1996) and Mucor plumbeus (Lamm et al., 2007) have afforded these two metabolites with lower yields. A. wentii converted progesterone (2) to 11$\alpha$-hydroxyprogesterone (5) in a very efficient way like in the incubations of this substrate with some fungi such as Rhizopus nigricans and Aspergillus ochraceus strains (Sedlaczek, 1988). Our work on steroid biotransformation by A. wentii and some other fungi is in progress.

Acknowledgements

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<th>96</th>
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-, None; ++, 40%; ++++, 60%; ++++, 100%.

Table III. The results from time course experiments.

Table IV. Product yields following chromatography.

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<th>Substrate</th>
<th>Product</th>
<th>Yield (%)</th>
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<td>Testosterone (1)</td>
<td>6$\beta$-Hydroxytestosterone (3)</td>
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<td>14$\alpha$-Hydroxytestosterone (4)</td>
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<tr>
<td>Progesterone (2)</td>
<td>11$\alpha$-Hydroxyprogesterone (5)</td>
<td>87</td>
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Holland H. L. (1999), Recent advances in applied and mechanistic aspects of the enzymatic hydroxylation of steroids by whole-cell biocatalysts. Steroids 64, 178–186.


