Biotransformation of Some Steroids by *Aspergillus wentii*

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First steroid biotransformations performed by *Aspergillus wentii* MRC 200316 are reported. Testosterone (**1**) yields 6β-hydroxytestosterone (**3**) and 14α-hydroxytestosterone (**4**) while progesterone (**2**) yields 11α-hydroxyprogesterone (**5**).

Key words: Steroids, Biotransformation, *Aspergillus wentii*

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), hexadecylcitraconic 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 F_{254} TLC plates using ethyl acetate/*n*-hexane (1:1, v/v) as eluent. TLC plates were dipped into an anisaldehyde/H_2SO_4 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. ^{1}H NMR spectra were recorded in deuteriochloroform with tetramethylsilane as an internal standard reference at 300 MHz with a Varian Mercury 300 spectrometer. ^{13}C 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.

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Culture medium

The liquid medium for *Aspergillus wentii* MRC 200316 was prepared by mixing sucrose (15 g), glucose (15 g), polypeptone (5 g), $MgSO_4 \cdot 7\ H_2O$ (0.5 g), KCl (0.5 g), K_2HPO_4 (1 g), and $FeSO_4 \cdot 7\ H_2O$ (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 6β-hydroxytestosterone (**3**) (401 mg), which was identified by comparison of its melting point, IR, ^{1}H NMR, and ^{13}C 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, ^{1}H NMR, and ^{13}C 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, ^{1}H NMR, and ^{13}C 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 ^{1}H NMR spectra were determined in $CDCl_3$ 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 biotransfromations are presented in Fig. 1. The chemical structures of the biotransformation products were assigned mainly based on ^{1}H NMR (Table I) and ^{13}C 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 δ_H 4.35 ppm (1H, bs) (Kirk *et al.*, 1990) and δ_C 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. ^{1}H NMR data determined in $CDCl_3$ at 300 MHz for compounds **1** – **5**.

Compound	4-H	17α-H	18-CH_3	19-CH_3	21-CH_3	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 = 8.8 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)

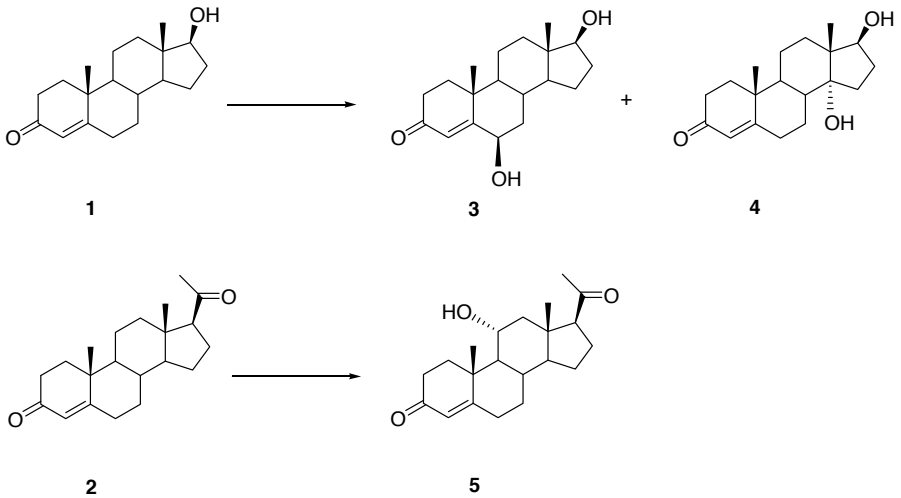

Fig. 1. Biotransformation of testosterone (**1**) and progesterone (**2**) by *A. wentii* to 6β-hydroxytestosterone (**3**), 14α-hydroxytestosterone (**4**), and 11α-hydroxyprogesterone (**5**).

Table II. ^{13}C NMR data determined in $CDCl_3$ at 75 MHz for compounds **1**–**5**.

C	**1**	**2**	**3**	**4**	**5**
1	35.61	35.46	36.31	35.60	37.26
2	33.91	33.72	34.15	33.82	34.03
3	199.62	199.18	200.73	199.94	200.49
4	123.82	123.66	126.23	123.71	124.27
5	171.31	170.80	168.63	171.19	171.47
6	32.76	32.55	72.86	32.49	33.48
7	31.49	31.65	37.00	28.45	31.37
8	38.62	35.28	29.70	38.78	34.77
9	53.86	53.38	53.62	46.66	58.67
10	36.37	38.34	37.92	38.66	39.81
11	20.59	20.78	20.52	19.60	68.54
12	35.68	38.40	38.00	32.55	50.09
13	42.77	43.68	42.84	46.86	44.00
14	50.42	55.76	50.36	83.29	55.16
15	23.29	24.13	23.22	26.02	24.08
16	30.38	22.57	30.31	29.42	22.73
17	81.56	63.23	81.63	78.49	62.98
18	11.02	13.11	11.07	14.86	14.36
19	17.37	17.13	19.47	17.14	18.12
20	-	209.08	-	-	209.02
21	-	31.29	-	-	31.28

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 δ_C 83.29 ppm, indicating the presence of a hydroxy group. The ^{1}H 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

Table III. The results from time course experiments.

Compound	Time [h]					
	8	24	48	72	96	120
1	+++++	+++++	+++++	++	-	-
3	-	-	-	+++	+++++	+++++
4	-	-	-	-	+++++	+++++
2	+++++	+++++	+++++	++	-	-
5	-	-	-	+++	+++++	+++++

-, None; ++, 40%; +++, 60%; +++++, 100%.

Table IV. Product yields following chromatography.

Substrate	Product	Yield (%)
Testosterone (**1**)	6β-Hydroxytestosterone (**3**)	76
	14α-Hydroxytestosterone (**4**)	7
Progesterone (**2**)	11α-Hydroxyprogesterone (**5**)	87

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 showed that 60% of testosterone (**1**) had been converted to 6β-hydroxytestosterone (**3**) within 72 h. After 96 h, the 19-methyl resonance of testosterone (**1**) at δ_H 1.19 ppm disappeared whereas the 19-methyl resonance of 6β-hydroxytestosterone (**3**) at δ_H 1.38 ppm remained and a new 18-methyl resonance at δ_H 0.90 ppm emerged. These results indicated that most of testosterone (**1**) was first converted only to 6β-hydroxytestosterone (**3**), and the rest of the substrate was then converted to 6β-hydroxytestosterone (**3**) and 14α-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 (Δ 0.08 ppm) for the 19-methyl group. The metabolite had characteristic resonances at δ_H 3.98 ppm (1H, td, J = 10.0 and 5.0 Hz) (Kirk *et al.*, 1990) and δ_C 68.54 ppm (Blunt and Stothers, 1977) suggesting that hydroxylation had taken place at the equatorial proton at C-11, generating an 11α-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 11α-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 11α-hydroxyprogesterone (**5**) within 72 h. After 96 h, the disappearance of the 19-methyl resonance of progesterone (**2**) at δ_H 1.20 ppm and the presence of the 19-methyl resonance of 11α-hydroxyprogesterone (**5**) at δ_H 1.28 ppm suggested that all of the substrate was converted to 11α-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 efficent way (Table IV). *A. wentii* hydroxylated testosterone (**1**) mainly at 6β-position and an independent minor hydroxylation took place at 14α-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 11α-hydroxyprogesterone (**5**) in a very efficent 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.

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