BIOTRANSFORMATION OF TESTOSTERONE AND PROGESTERONE BY *Penicillium digitatum* MRC 500787

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The biotransformation of testosterone and progesterone by *Penicillium digitatum* MRC 500787 for 5 days is described. The biotransformation of testosterone afforded 5α-androstane-3,17-dione, 3α-hydroxy-5α-androstan-17-one, 3β-hydroxy-5α-androstan-17-one and androst-4-ene-3,17-dione. The biotransformation of progesterone afforded 5α-pregnane-3,20-dione.

Keywords: Xenobiotics; Steroids; Biotransformations; *Penicillium digitatum*; Microbial hydrogenation; Whole-cell biocatalysis.

Microbial biotransformation of steroids has found worldwide application for the preparation of more valuable and functionalized compounds due to its high regio- and stereoselectivity[1]. For example, a number of researches on microbial biotransformations of a wide range of steroidal substrates have been reported in recent years[1–3]. There are still enormous efforts to increase the efficiency of microbial biotransformations and to find new useful microorganisms and reactions[1].

Different *Penicillium* species have been used for the biotransformations of some steroids. These biotransformations afforded interesting results, such as 15α-hydroxylation by *P. raistrickii*[4,5], 5α-reduction by *P. decumbens*[6–10], *P. crustosum*[11,12] and *P. chrysogenum*[11] and Baeyer–Villiger oxidation by *P. lilacinum*[13,14], *P. camemberti*[15], *P. notatum*[16] and *P. citroeviride*[17].

As far as biotransformations by *P. digitatum* are concerned, we have not found any literature work on steroids. In this work, we investigated the biotransformation of testosterone and progesterone by *P. digitatum* MRC 500787.

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RESULTS AND DISCUSSION

The incubation of testosterone (**1**) with *P. digitatum* MRC 500787 for 5 days afforded four metabolites. The first metabolite lacked the 17α-H and 4-H resonances of testosterone at δ_H 3.61 ppm (1 H, t, J = 2 Hz) and δ_H 5.72 ppm (1 H, bs), respectively. The ^{1}H NMR spectrum (δ, ppm; J, Hz) of the metabolite demonstrated an upfield shift for the 19 methyl group (Δ 0.17 ppm) when compared to testosterone. The metabolite lacked resonances of testosterone at δ_C 171.31 (C-5), δ_C 123.82 (C-4) and δ_C 81.56 (C-17). These results were in accordance with the microbial hydrogenation of the double bond and an oxidation at C-17. The comparison of the ^{13}C NMR spectrum of the metabolite with the literature[18] suggested that the conjugated system had been reduced from the α-face and the first metabolite was 5α-androstane-3,17-dione (**2**).

The second metabolite contained a new resonance at δ_H 4.05–4.08 (1 H, m, 3-H) and lacked the 17α-H and 4-H resonances of testosterone at δ_H 3.61 (1 H, t, J = 2) and δ_H 5.72 (1 H, bs), respectively. The ^{1}H NMR spectrum of the metabolite demonstrated an upfield shift for the 19 methyl group (Δ 0.33 ppm), showing that some changes had taken place in ring A. The ^{13}C NMR spectrum of the metabolite lacked resonances of testosterone at δ_C 199.62 (C-3), δ_C 171.31 (C-5), δ_C 123.82 (C-4) and δ_C 81.56 (C-17), and contained two new resonances at δ_C 66.42 and δ_C 221.55. These results were consistent with the hydrogenation of the double bond, an oxidation at C-17 and a reduction at C-3. The characteristic resonances[18,19] at δ_C 66.42 and δ_H 4.05 were in accordance with the presence of a 3α-hydroxyl group, showing that the carbonyl of the conjugated system was reduced. When the ^{13}C NMR spectrum of the metabolite was compared with a literature work[18] it was followed that the hydrogenation of the conjugated double bond occurred from the α-face. All these results suggested that the metabolite was 3α-hydroxy-5α-androstan-17-one (**3**).

The third metabolite contained a new resonance at δ_H 3.53 (1 H, tt, J = 5 and 11, 3-H) and lacked the 17α-H and 4-H resonances of testosterone at δ_H 3.61 (1 H, t, J = 2) and δ_H 5.72 (1 H, bs), respectively. The ^{1}H NMR spectrum of the metabolite demonstrated an upfield shift for the 19 methyl group (Δ 0.37 ppm). The metabolite lacked resonances of testosterone at δ_C 199.62 (C-3), δ_C 171.31 (C-5), δ_C 123.82 (C-4) and δ_C 81.56 (C-17), and contained two new resonances at δ_C 71.06 and δ_C 221.50. These results were in agreement with the reduction of the double bond, the oxidation of the 17β-hydroxyl group to the 17-carbonyl group, and the reduction of the 3-carbonyl group from the β-face. The characteristic resonances[18,19] at

δ_C 71.06 and δ_H 3.53 indicated the presence of a 3β-hydroxyl group. A comparison of the ^{13}C NMR spectrum of the metabolite with that in the literature[18] indicated that the reduction of the α,β-unsaturated system had taken place from the α-face and the metabolite was 3β-hydroxy-5α-androstan-17-one (**4**).

The fourth metabolite contained the 4-H resonance of testosterone at δ_H 5.74 (1 H, s) and lacked the 17α-H resonance of testosterone at δ_H 3.61 (1 H, t, J = 2). The 18-methyl resonance of testosterone at δ_H 0.78 had experienced a downfield shift to δ_H 0.91 (Δ 0.13 ppm). The metabolite maintained resonances of testosterone at δ_C 199.33 (C-3), δ_C 170.35 (C-5) and δ_C 124.07 (C-4), lacked the C-17 resonance at δ_C 81.56 and had a new carbonyl group resonance at δ_C 220.43. These results were in accordance with the presence of an unchanged α,β-unsaturated system and an oxidation at C-17, confirming that the metabolite was androst-4-ene-3,17-dione (**5**).

The incubation of progesterone (**6**) with *P. digitatum* MRC 500787 for five days afforded only one metabolite. The metabolite lacked the 4-H resonance of progesterone at δ_H 5.68 (1 H, bs). The 19-methyl resonance of progesterone at δ_H 1.14 and δ_C 17.23 had experienced upfield shifts to δ_H 0.98 (Δ 0.16 ppm) and δ_C 11.36 (Δ 5.87 ppm), respectively. The ^{13}C NMR spectrum of the metabolite lacked olefinic carbons of progesterone. These results were in agreement with the reduction of the double bond in ring A. A comparison of the ^{13}C NMR spectrum of the metabolite with that in the literature[18] showed that the reduction of the α,β-unsaturated system had taken place from the α-face and the metabolite was 5α-pregnane-3,20-dione (**7**).

Time course experiments were conducted for both substrates. By 8 h, the 18-methyl resonance of testosterone (**1**) shifting from 0.77 to 0.90 ppm and comparison of the methyl group integrations in the ^{1}H NMR spectrum showed that 40% of testosterone had been converted to androst-4-ene-3,17-dione (**5**). After 24 h, the 18-methyl resonances of testosterone shifting from 0.77 to 0.87 and 0.90 ppm and further comparison of the methyl group integrations in the ^{1}H NMR spectrum showed that 20% of androst-4-ene-3,17-dione (**5**) had been converted to 5α-androstane-3,17-dione (**2**). At 48 h, further comparison of the methyl group integrations in the ^{1}H NMR spectrum showed that 60% of androst-4-ene-3,17-dione (**5**) had been converted to 5α-androstane-3,17-dione (**2**). After 72 h, the presence of 3α-H resonance at δ_H 3.53 (1 H, tt, J = 5 and 11) and 3β-H resonance at δ_H 4.05–4.08 (1 H, m) and further comparison of methyl group integrations in the ^{1}H NMR spectrum indicated that 65% of 5α-androstane-3,17-dione (**2**) had been converted to the alcohols **3** and **4**. No further changes were

observed in the integrations of these resonances at 96 h and later. The results demonstrated that testosterone (**1**) was first converted to androst-4-ene-3,17-dione (**5**) with a 17β-hydroxysteroid oxidase and the resultant dione **5** was then subjected to the activity of a 5α-reductase in order to give 5α-androstane-3,17-dione (**2**). Finally, 5α-androstane-3,17-dione (**2**) was subjected to some reductions at C-3 (Fig. 1).

The metabolism of testosterone (**1**) in *P. digitatum* started with an oxidation at C-17 and was then followed by a 5α-reductase activity. The oxidation at C-17 followed by a 5α-reductase activity has also been reported for the incubation of testosterone by *Ceratocystis paradoxa*[20].

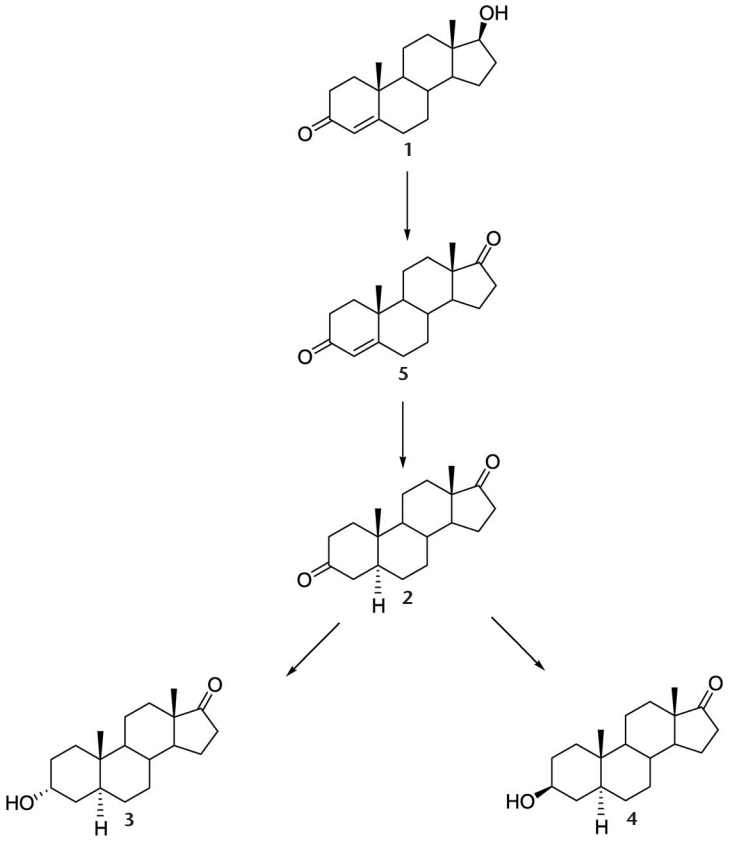

FIG. 1
Metabolism of testosterone (**1**) in *P. digitatum* MRC 500787

The 5α-reductase enzymes converts Δ^4-3-ketosteroids to 5α-3-ketosteroids and requires a 3-keto-4-ene moiety in its substrates[6]. It is also known that some fungi[6–12,20–26] reduce the α,β-unsaturated systems of some Δ^4-3-ketosteroids. No 5β-H steroid was obtained from the incubation of testosterone (**1**) and this indicated that *P. digitatum* catalyzed the hydrogenation of the conjugated double bond from only the α-face as in the incubation of the same substrate by some fungi, such as *P. decumbens*[6] and *P. chrysogenum*[11].

3α-Hydroxy-5α-androstan-17-one (**3**) and 3β-hydroxy-5α-androstan-17-one (**4**) obtained from the the incubation of testosterone (**1**) demonstrated that reductions of the 3-carbonyl group of compound **2** from the α- and β-faces had taken place and afforded 3α-hydroxy-5α-androstan-17-one (**3**) and 3β-hydroxy-5α-androstan-17-one (**4**), respectively. Reduction from the β-face (8.9%) was more effective than that from the α-face (3.6%). 3β-Hydroxy-5α-androstan-17-one (**4**) was isolated for the first time from a fungal biotransformation of testosterone (**1**). 3α-Hydroxy-5α-androstan-17-one (**3**) has been isolated from the incubations of testosterone (**1**) by some other fungi, such as *P. crustosum*[11] and *Ceratocystis paradoxa*[20].

During the time course experiment for progesterone (**6**), the 18-methyl resonances of progesterone shifting from 0.66 to 0.60 ppm and comparison of the methyl group integrations in the ^{1}H NMR spectra indicated that 5α-pregnane-3,20-dione (**7**) appeared by 24 h and no more reaction was observed after 72 h (Fig. 2).

5α-Pregnane-3,20-dione (**7**) obtained from the incubation of progesterone (**6**) suggested the activity of a 5α-reductase on the conjugated double bond from only the α-face as in the incubations of the substrate **6** by some other fungi, such as *Penicillium decumbens*[7], *Ceratocystis paradoxa*[20], *Aspergillus fumigatus*[21] and *Rhizopus nigricans*[24,25].

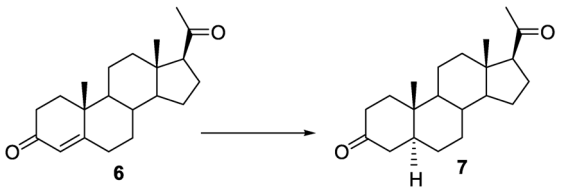

FIG. 2
Metabolism of progesterone (**6**) in *P. digitatum* MRC 500787

In conclusion, we have shown that *P. digitatum* MRC 500787 has the ability to metabolize testosterone (**1**) and progesterone (**6**) and given their possible metabolic pathways. The metabolism of testosterone (**1**) by *P. digitatum* had taken place as the oxidation of the substrate at C-17 followed by the 5α-reduction of the α,β-unsaturated system from the α-face. The 5α-reduction was then accompanied by some reductions at C-3 from both α- and β-faces. 3β-Hydroxy-5α-androstan-17-one (**4**) was first obtained from a fungal incubation of testosterone (**1**). The metabolism of progesterone (**6**) by *P. digitatum* had taken place as a 5α-reduction of the α,β-unsaturated system from the α-face.

EXPERIMENTAL

Testosterone and progesterone were purchased from Fluka. *Penicillium digitatum* MRC 500787 was obtained from TUBITAK, Marmara Research Center, Food Science and Technology Research Institute, Culture Collection Unit, Turkey. Stock cultures were maintained at 4 °C on PDA slopes. Potato dextrose agar and agar for PDA slopes and the ingredients for liquid medium were purchased from Merck. Solvents were of analytical grade and were also purchased from Merck.

The steroids were separated by column chromatography on silica gel 60 (Merck 107734) with increasing concentrations of ethyl acetate in hexane as eluent. TLC was carried out with 0.2 mm thick Merck Kieselgel 60 F_{254} TLC plates using ethyl acetate/hexane (1:1, v/v) as an eluent. In order to develop the chromatograms, TLC plates were dipped into an anisaldehyde–H_2SO_4 reagent and heated to 120 °C for 3 min. IR spectra (ν, cm^{-1}) were recorded using a Shimadzu IR Prestige-21. Optical rotation mesurements were carried out on a WXG-4 polarimeter. Elemental analysis was performed using a Thermo Finnigan Flash EA 1112 elemental analyser. ^{1}H NMR spectra were recorded in deuteriochloroform with tetramethylsilane as an internal standard reference at 300 MHz on a Varian Mercury 300 spectrometer. ^{13}C NMR spectra were recorded in deuteriochloroform at 75 MHz on a Varian Mercury 300 spectrometer. Chemical shifts are given in ppm (δ-scale), coupling constants *J* in Hz. Melting points were determined by an Electrothermal IA 9200 melting point apparatus and are uncorrected. Experiments were run with control flasks containing non-inoculated sterile medium and one of the substrates. After five days of incubation, all controls were also harvested and analyzed by TLC. No metabolites were detected in controls.

Time course experiments[27] were conducted in order to determine the metabolic pathway. 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). The first flask was harvested after 8 h. Then every 24 h one flask was harvested and extracted. TLC analysis was performed immediately on the isolated mixture. Following 6 h under high vacuum, ^{1}H NMR spectra of the products were determined in $CDCl_3$ to confirm the steroidal nature of the extracts.

Biotransformation of Testosterone (**1**) by *P. digitatum* MRC 500787

Spores freshly obtained from PDA slopes were transferred aseptically into 10 Erlenmeyer flasks of 250 ml containing sterile MYB medium[28] (100 ml; malt extract 2%, glucose 1%, bacteriological peptone 1% and yeast extract 0.3%) in a biological safety cabinet. After cultivation at 24 °C for 2 days on a rotary shaker (150 rpm), testosterone (**1**; 500 mg, 1.734 mmol) dissolved in DMF (10 ml) was evenly distributed aseptically among the flasks. The biotransformation of the substrate was carried out in 10 flasks for five days under the same conditions. The fungal mycellium was separated from the broth by filtration under the vacuum and the mycellium was rinsed with ethyl acetate (500 ml). The broth was then extracted 3× with 1 l of ethyl acetate. The organic extract was dried over sodium sulfate anhydrous and the solvent evaporated in vacuo to give a brown gum (687 mg), which was then chromatographed on silica gel. Elution with 15% ethyl acetate in hexane afforded 5α-androstane-3,17-dione (**2**; 32 mg, 6.4%) crystallized from ethyl acetate as needles, m.p. 129–131 °C, $[\alpha]_D^{20}$ +105, *c* 0.1, $CHCl_3$, (ref.[29] gives m.p. 130–132 °C, $[\alpha]_D$ +102, *c* 1, CH_2Cl_2). For $C_{19}H_{28}O_2$ (288.43) calculated: 79.12% C, 9.79% H; found: 79.03% C, 9.68% H. IR: 1730 (C=O), 1716 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 0.87 s, 3 H (H-18); 1.02 s, 3 H (H-19). ^{13}C NMR (75 MHz, $CDCl_3$): 221.00, 211.69, 53.81, 51.16, 47.69, 46.54, 44.53, 38.38, 38.02, 35.78, 35.75, 35.17, 31.42, 30.47, 28.55, 21.73, 20.65, 13.75, 11.41.

Elution with 20% ethyl acetate in hexane afforded 3α-hydroxy-5α-androstan-17-one (**3**; 18 mg, 3.6%) crystallized from ethyl acetate as needles, m.p. 180–182 °C, $[\alpha]_D^{20}$ +95, *c* 0.1, $CHCl_3$, (ref.[30] gives m.p. 182–183 °C, $[\alpha]_D$ +97, *c* 1, C_2H_5OH). For $C_{19}H_{30}O_2$ (290.45) calculated: 78.57% C, 10.41% H; found: 78.50% C, 9.43% H. IR: 3440 (O–H), 1720 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 0.80 s, 3 H (H-18); 0.86 s, 3 H (H-19); 4.05–4.08 m, 1 H (H-3). ^{13}C NMR (75 MHz, $CDCl_3$): 221.55, 66.42, 54.39, 51.46, 47.80, 39.10, 36.23, 35.85, 35.75, 35.02, 32.11, 31.53, 30.83, 28.98, 28.22, 21.73, 20.02, 13.81, 11.16.

Further elution with 20% ethyl acetate in hexane afforded 3β-hydroxy-5α-androstan-17-one (**4**; 45 mg, 8.9%) crystallized from ethyl acetate as needles, m.p. 175–177 °C, $[\alpha]_D^{20}$ +90, *c* 0.1, $CHCl_3$, (ref.[31] gives m.p. 174–176 °C, $[\alpha]_D^{18}$ + 88, *c* 0.7, CH_3OH). For $C_{19}H_{30}O_2$ (290.45) calculated: 78.57% C, 10.41% H; found: 78.51% C, 9.40% H. IR: 3470 (O–H), 1726 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 0.82 s, 3 H (H-19); 0.84 s, 3 H (H-18); 3.53 tt, 1 H, *J*(3,2) = 5.0, *J*(3,4) = 12.0 (H-3). ^{13}C NMR (75 MHz, $CDCl_3$): 221.50, 71.06, 54.35, 51.34, 47.76, 44.76, 37.98, 36.87, 35.80, 35.57, 34.97, 31.47, 31.35, 30.83, 28.32, 21.72, 20.43, 13.76, 12.25.

Elution with 25% ethyl acetate in hexane afforded androst-4-ene-3,17-dione (**5**; 81 mg, 16.3%) crystallized from ethyl acetate as prisms, m.p. 172–173 °C, $[\alpha]_D^{20}$ +197, *c* 0.1, $CHCl_3$, (ref.[32] gives m.p. 174–176 °C, $[\alpha]_D^{23}$ +194, *c* 0.8695, $CHCl_3$). For $C_{19}H_{26}O_2$ (286.42) calculated: 79.68% C, 9.15% H; found: 79.65% C, 8.93% H. IR: 1730 (C=O), 1712 (C=O), 1640 (C=C). ^{1}H NMR (300 MHz, $CDCl_3$): 0.90 s, 3 H (H-18); 1.20 s, 3 H (H-19); 5.74 s, 1 H (H-4). ^{13}C NMR (75 MHz, $CDCl_3$): 220.43, 199.33, 170.35, 124.07, 53.74, 50.76, 47.45, 38.58, 35.69, 35.62, 35.07, 33.84, 32.50, 31.20, 30.68, 21.68, 20.24, 17.31, 13.64.

Further elution with 25% ethyl acetate in hexane afforded the unreacted starting material (240 mg, 48%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic material.

Biotransformation of Progesterone (**6**) by *P. digitatum* MRC 500787

Under the same conditions, the incubation of progesterone (**6**; 500 mg, 1.59 mmol) by *P. digitatum* MRC 500787 for 5 days afforded a brown gum (694 mg), which was then chromatographed on silica gel. Elution with 15% ethyl acetate in hexane afforded 5α-pregnane-3,20-dione (**7**; 62 mg, 12.3%) crystallized from ethyl acetate as needles, m.p. 199–200 °C, $[\alpha]_D^{20}$ +148, *c* 0.1, $CHCl_3$, (ref.[20] gives m.p. 198–201 °C, $[\alpha]_D$ +144, *c* 1.6, $CHCl_3$). For $C_{21}H_{32}O_2$ (316.49) calculated: 79.70% C, 10.19% H; found: 79.66% C, 10.10% H. IR: 1728 (C=O), 1710 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 0.60 s, 3 H (H-18); 0.98 s, 3 H (H-19); 2.09 s, 3 H (H-21). ^{13}C NMR (75 MHz, $CDCl_3$): 211.83, 209.46, 63.62, 56.34, 53.53, 46.54, 44.54, 44.08, 38.81, 38.43, 38.03, 35.57, 35.25, 31.53, 31.44, 28.71, 24.31, 22.70, 21.33, 13.35, 11.36.

Elution with 20% ethyl acetate in hexane afforded the unreacted starting material (**6**; 280 mg, 56%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic material.

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