BIOTRANSFORMATION OF SOME STEROIDS BY *Aspergillus terreus* MRC 200365

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The biotransformations of testosterone, epiandrosterone, progesterone and pregnenolone by *Aspergillus terreus* MRC 200365 for five days were described. The biotransformation of testosterone afforded testolactone. The biotransformation of epiandrosterone afforded 3β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one. The biotransformation of progesterone afforded androst-4-ene-3,17-dione and testolactone. The biotransformation of pregnenolone afforded 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one and testolactone.
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Steroidal lactones are important compounds due to their anticarcinogenic[1,2], antiandrogenic[3,4] and antihypercholesterolemic activites[5]. Although chemical ways of synthesis of these lactones are possible, the microbial biotransformations are much more popular due to environmental concerns. The stereospecific microbial side-chain degradation of C_{21} steroids and microbial biotransformations of some androgens have given some steroidal D lactones[6–27]. The degradation of 17β-acetyl side chain of some C_{21} steroids and ring D oxidation of some androgens are carried out by Baeyer–Villiger monooxygenases (BVMOs). Many bacteria and fungi have these enzymes and they catalyze the insertion of an oxygen atom next to a keto function and convert different ketones to corresponding esters or lactones[28].

Aspergillus terreus Thom is a common soil microorganism[26]. This fungus is used for the production of single cell proteins[29] and a number of important enzymes[26]. *A. terreus* produces some important secondary metabolites, such as lovastatin[30], territrems[31], butyrolactone I and its derivatives[32].

Only a few works on steroid biotransformations with *A. terreus* has been reported in the literature[26,33,34]. In one of these works, the biotransformation of androst-4-ene-3,17-dione by *A. terreus*[26] afforded testosterone (**1**)

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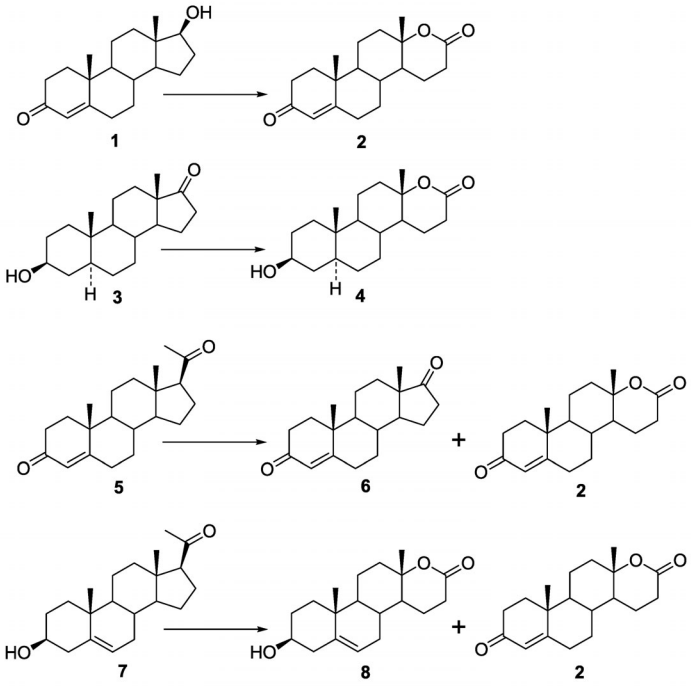
and testolactone (**2**). Furthermore, some *A. terreus* strains showed BVMO activities on cyclic ketones[35]. Therefore, we decided to investigate the biotransformation of testosterone (**1**), epiandrosterone (**3**), progesterone (**5**) and pregnenolone (**7**) by *A. terreus* MRC 200365.

RESULTS AND DISCUSSION

The incubation of testosterone (**1**) with *A. terreus* for 5 days afforded only one metabolite (Scheme 1). The metabolite lacked the resonance of testosterone (**1**) at δ_H 3.61 (1 H, t, J = 2, 17-H). The C-13 resonance at δ_C 42.73 of the starting material had experienced a downfield shift to δ_C 82.58 (Δ 39.85) which was consistent with insertion of an oxygen atom adjacent to this position on the ring D. The 18-methyl resonance of starting material at δ_H 0.77 and δ_C 11.01 had experienced downfield shifts to δ_H 1.37 (Δ 0.60) and δ_C 21.60 (Δ 10.59), respectively. The metabolite lacked the C-17 signal at δ_C 81.52 and had a new signal at δ_C 171.01. All these results suggested that the metabolite was testolactone (**2**).



SCHEME 1
Biotransformation of steroidal substrates by *Aspergillus terreus* MRC 200365

The incubation of epiandrosterone (**3**) with *A. terreus* for 5 days afforded only one metabolite (Scheme 1). The metabolite had a resonance at δ_H 3.53 (1 H, tt, J = 5 and 11, 3-H), confirming that the 3β-OH group maintained. The C-13 resonance of epiandrosterone (**3**) at δ_C 47.76 had experienced a downfield shift to δ_C 83.40 (Δ 35.64). The 18-methyl resonance of **3** at δ_H 0.84 and δ_C 13.76 had experienced downfield shifts to δ_H 1.29 (Δ 0.45) and δ_C 20.11 (Δ 6.35), respectively. Absence of the 17-carbonyl group signal at δ_C 221.50 and presence of a new signal at δ_C 171.64 demonstrated that a steroidal D lactone occurred. These results indicated that the metabolite was 3β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**4**).

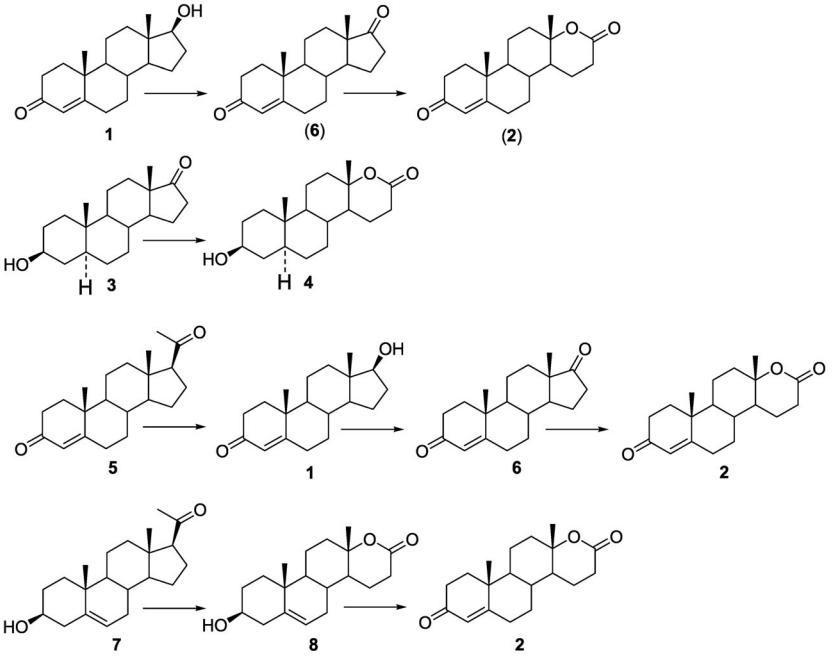
The incubation of progesterone (**5**) with *A. terreus* for 5 days afforded two metabolites (Scheme 1). The first metabolite contained the 4-H resonance of progesterone (**5**) at δ_H 5.74 (1 H, s). The metabolite did not contain the methyl resonance at δ_H 2.12 and the 18-methyl resonance of **5** at δ_H 0.66 had experienced a downfield shift to δ_H 0.90 (Δ 0.24). The metabolite lacked the carbonyl group resonance of progesterone (**5**) at δ_C 209.08 and had a new carbonyl group resonance at δ_C 220.41. These results suggested that the side chain of **5** had been cleaved and the substrate was then converted to androst-4-ene-3,17-dione (**6**). The second metabolite was identified as testolactone (**2**) by comparison of its spectra with those of an authentic sample.

The incubation of pregnenolone (**7**) with *A. terreus* for 5 days afforded two metabolites (Scheme 1). The first metabolite had resonances at δ_H 3.54 (1 H, tt, J = 5 and 11 Hz) and δ_H 5.35 (1 H, d, J = 5 Hz), indicating the presence of the 3β-OH group and a double bond at C-5, respectively. The C-13 resonance at δ_C 43.94 of pregnenolone (**7**) had experienced a downfield shift to δ_C 83.26 (Δ 39.32) while the 18-methyl resonance of **7** at δ_H 0.60 and δ_C 13.16 had experienced downfield shifts to δ_H 1.34 (Δ 0.74) and δ_C 20.01 (Δ 6.85), respectively. These downfield shifts were consistent with insertion of an oxygen atom on the ring D. Absence of the carbonyl group signal at δ_C 209.72 and presence of a new signal at δ_C 171.76 suggested a steroidal D lactone formation. All these results indicated that the metabolite was 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**8**). The second metabolite was identified as testolactone (**2**) by comparison of its spectra with those of an authentic sample.

Time course experiments monitored by TLC and ^{1}H NMR spectroscopy were conducted for all substrates in order to determine the metabolic pathway (Scheme 2). Following 8 h of incubation, the 18-methyl signal of testosterone (**1**) shifting from 0.77 to 0.90 ppm and comparison of the methyl group integrations in the ^{1}H NMR spectrum indicated that 20% of **1** was

converted to **6**. At 24 h, the presence of the 18-methyl signal of **1** shifting from 0.77 to 1.37 ppm and further comparison of the methyl group integrations in the ^{1}H NMR spectrum indicated that 100% of **6** had been converted to **2**. Further comparison of the methyl group integrations in the ^{1}H NMR spectra suggested that no more reactions took place after 24 h. These results suggested that testosterone (**1**) was first converted to androst-4-ene-3,17-dione (**6**) by a 17β-hydroxysteroid oxidoreductase and the resultant dione **6** was then converted to testolactone (**2**) by a BVMO as in the incubation of the substrate with *Penicillium lilacinum*[27].

At 24 h, the 18-methyl signal of epiandrosterone (**3**) shifting from 0.84 to 1.29 ppm and comparison of the methyl group integrations in the ^{1}H NMR spectrum indicated that 15% of **3** had been converted to **4**. Further comparison of the methyl group integrations in the ^{1}H NMR spectra suggested that lactonization had been completed at first 24 h and no more reactions were observed after 48 h. These results indicated that epiandrosterone (**3**) was only converted to the corresponding lactone **4** by a BVMO as in the enzymatic Baeyer–Villiger oxidation of this compound[36].



SCHEME 2
Metabolism of steroidal substrates by *Aspergillus terreus* MRC 200365

At 8 h, the 18-methyl signals of progesterone (**5**) shifting from 0.66 to 0.77 ppm and δ_H 0.90 and comparison of the methyl group integrations in ^{1}H NMR spectrum indicated that 35% of **5** had been converted to **1** and **6**. After 24 h, the presence of three 18-methyl signals at δ_H 0.66, 0.90 and 1.37 and further comparison of the methyl group integrations in ^{1}H NMR spectrum indicated that 100% of **1** and 80% of **6** had been consumed and lactonization occurred. After 48 h, further comparison of the methyl group integrations in the ^{1}H NMR spectrum suggested that lactonization had been completed in the first 24 h. No further changes were observed in the methyl group integrals of the ^{1}H NMR spectra after 72 h. These results suggested that a BVMO first converted some of progesterone (**5**) to testosterone acetate, which was not observed during the time course experiment due to the presence of high levels of an esterase enzyme[7]. This was then hydrolyzed to give testosterone (**1**). The oxidation of testosterone (**1**) at C-17 gave androst-4-ene-3,17-dione (**6**). Finally, some of this dione was converted to testolactone (**2**) by the activity of a BVMO. Apart from a minor 11β-hydroxylation pathway, the metabolism of progesterone (**5**) by *Aspergillus tamarii* took place as the same way[6].

After 24 h, the 18-methyl signal of pregnenolone (**7**) shifting from 0.60 to 1.34 ppm and comparison of the methyl group integrations in the ^{1}H NMR spectrum indicated that 50% of **7** had been converted to **8**. A new signal at δ_H 5.78 (1 H, bs, 4-H), three 18-methyl signals at δ_H 0.60, 1.34 and 1.37 and further comparison of the methyl group integrations in the ^{1}H NMR spectrum indicated that 50% of **8** had been converted to **2** by 48 h. No further changes were observed in the methyl group integrals of the ^{1}H NMR spectra after 72 h. The results indicated that 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**8**) occurred as a first lactone and it was then isomerized to testolactone (**2**) by a 3β-hydroxysteroid oxidoreductase/Δ^5-Δ^4 isomerase enzyme as in the incubation of pregnenolone (**7**) by *Aspergillus tamarii*[14].

In conclusion, we have shown that *A. terreus* MRC 200365 converted testosterone (**1**), epiandrosterone (**3**), progesterone (**5**) and pregnenolone (**7**) into some steroidal lactones and given their possible metabolic pathways. The results suggested that this fungus produces BVMO(s), which can carry out degradation of 17β-acetyl side chain of steroidal 4-ene-3-ketones and 5-en-3β-alcohols. The enzyme(s) can also carry out ring D oxidation of 4-ene-3-ketoandrogens and 3β-hydroxyandrogens.

EXPERIMENTAL

Testosterone, epiandrosterone, progesterone and pregnenolone were purchased from Fluka. *A. terreus* Thom MRC 200365 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. Solvents were of analytical grade and were purchased from Merck. Potato dextrose agar and agar for PDA slopes and malt extract for liquid medium 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. Infrared spectra (wavenumbers in cm^{-1}) were recorded using a Shimadzu IR Prestige-21. Optical rotation mesurements were carried out on a WXG-4 polarimeter, $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. 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 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*) in Hz. Melting points were determined by an Electrothermal IA 9200 melting point apparatus and are uncorrected. Biotransformation experiments were run with control flasks containing non-inoculated sterile medium and one of the substrates. After 5 days of incubation, all controls were also harvested and analysed by TLC. No metabolites were detected in controls.

Time course experiments[14] 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). One 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, the product ^{1}H NMR spectra were determined in $CDCl_3$ to confirm the steroidal nature of the extracts.

Biotransformation of Testosterone (**1**) by *A. terreus* MRC 200365

Spores freshly obtained from PDA slopes were transferred aseptically into 10 Ehrlenmayer flasks of 250 ml containing 100 ml of sterile 2% malt extract medium[35] in a biological safety cabinet. After cultivation at 32 °C for 3 days on a rotary shaker (160 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 5 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 three times 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 (726 mg), which was then chromatographed on silica gel. Elution with 30% ethyl acetate in hexane afforded the unchanged starting material (320 mg, 64%) which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic material.

Elution with 60% ethyl acetate in hexane afforded testolactone (**2**; 96 mg, 18.3%), which was crystallized from methanol as needles, m.p. 206–207 °C, $[\alpha]_D^{20}$ +48 (*c* 0.1, $CHCl_3$). Literature[8] gives m.p. 210–212 °C, $[\alpha]_D^{23}$ +43 (*c* 1.0, $CHCl_3$). For $C_{19}H_{26}O_3$ (302.4) calculated: 75.46% C, 8.67% H; found: 75.41% C, 8.39% H. IR: 1740 (C=O), 1640 (C=C). ^{1}H NMR: 1.17 s, 3 H (H-19); 1.37 s, 3 H (H-18); 5.78 bs, 1 H (H-4). ^{13}C NMR: 198.98, 171.01, 169.32, 123.82, 82.58, 52.24, 45.43, 38.75, 38.24, 37.70, 35.26, 33.62, 32.13, 30.17, 28.31, 21.60, 19.87, 19.61, 17.18.

Biotransformation of Epiandrosterone (**3**) by *A. terreus* MRC 200365

Under similar conditions, the incubation of epiandrosterone (**3**; 500 mg, 1.72 mmol) with *A. terreus* afforded a brown gum (760 mg), which was then chromatographed on silica gel. Elution with 30% ethyl acetate in hexane afforded the unreacted starting material (330 mg, 66%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic material.

Elution with 50% ethyl acetate in hexane afforded 3β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**4**; 56 mg, 10.6%), which was crystallized from ethyl acetate as needles, m.p. 173–174 °C, $[\alpha]_D^{20}$ –45 (*c* 0.1, $CHCl_3$). Literature[37] gives m.p. 169–170 °C, lit.[38] $[\alpha]_D^{25}$ –40 (*c* 2.0, $CHCl_3$). For $C_{19}H_{30}O_3$ (306.4) calculated: 74.47% C, 9.87% H; found: 74.42% C, 9.23% H. IR: 3440 (O–H), 1720 (C=O). ^{1}H NMR: 0.78 s, 3 H (H-19); 1.29 s, 3 H (H-18); 3.53 tt, 1 H, *J*(3,2) = 5, *J*(3,4) = 11 (H-3). ^{13}C NMR: 171.64, 83.40, 71.04, 53.04, 46.24, 44.15, 39.22, 37.87, 37.77, 36.72, 35.47, 31.28, 30.57, 28.61, 28.24, 22.00, 20.11, 19.76, 12.14.

Biotransformation of Progesterone (**5**) by *A. terreus* MRC 200365

Under similar conditions, the incubation of progesterone (**5**; 500 mg, 1.59 mmol) with *A. terreus* afforded a brown gum (725 mg), which was then chromatographed on silica gel. Elution with 30% ethyl acetate in hexane afforded the unreacted starting material (300 mg, 60%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic material.

Elution with 30% ethyl acetate in hexane afforded androst-4-ene-3,17-dione (**6**; 25 mg, 5.5%), which was crystallized from ethyl acetate as prisms, m.p. 172–173 °C, $[\alpha]_D^{20}$ +192 (*c* 0.1, $CHCl_3$). Literature[8] gives m.p. 174–176 °C, $[\alpha]_D^{23}$ +194 (*c* 1.0, $CHCl_3$). For $C_{19}H_{26}O_2$ (286.4) calculated: 79.68% C, 9.15% H; found: 79.62% C, 8.96% H. IR: 1715 (C=O), 1616 (C=C). ^{1}H NMR: 0.90 s, 3 H (H-18); 1.20 s, 3 H (H-19); 5.74 s, 1 H (H-4). ^{13}C NMR: 220.41, 199.33, 170.34, 124.09, 53.76, 50.77, 47.46, 38.59, 35.70, 35.64, 35.09, 33.86, 32.51, 31.21, 30.69, 21.70, 20.26, 17.32, 13.66.

Elution with 60% ethyl acetate in hexane afforded testolactone (**2**; 120 mg, 25%) which was identified by comparison of its spectra and melting point with those of an authentic sample.

Biotransformation of Pregnenolone (**7**) by *Aspergillus terreus* MRC 200365

Under similar conditions, the incubation of pregnenolone (**7**; 500 mg, 1.58 mmol) with *A. terreus* afforded a brown gum (747 mg), which was then chromatographed on silica gel. Elution with 30% ethyl acetate in hexane afforded the unreacted starting material (200 mg, 40%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic material.

Elution with 50% ethyl acetate in hexane afforded 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**8**; 100 mg, 20.8%), which was crystallized from ethyl acetate as needles, m.p. 234–235 °C, $[\alpha]_D^{20}$ –95 (*c* 0.1, $CHCl_3$). Literature[17] gives m.p. 227–230 °C, $[\alpha]_D^{20}$ –93.9 (*c* 0.1, $CHCl_3$). For $C_{19}H_{28}O_3$ (304.4) calculated: 74.96% C, 9.27% H; found: 74.95% C, 9.23% H. IR: 3440 (O–H), 1720 (C=O), 1650 (C=C). ^{1}H NMR: 0.98 s, 3 H (H-19); 1.34 s, 3 H (H-18); 3.54 tt, 1 H, *J*(3,2) = 5, *J*(3,4) = 11 (H-3); 5.35 d, 1 H *J*(6,7) = 5.0 (H-6). ^{13}C NMR: 171.76, 140.53, 120.52, 83.26, 71.41, 48.86, 46.60, 41.70, 38.80, 36.81, 36.50, 34.35, 31.28, 30.98, 28.71, 21.88, 20.01, 19.78, 19.23.

Elution with 60% ethyl acetate in hexane afforded testolactone (**2**; 110 mg, 23%), which was identified by comparison of its spectra and melting point with those of an authentic sample.

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