Biotransformation of testosterone by *Ulocladium chartarum* MRC 72584

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The incubation of testosterone **1** with *Ulocladium chartarum* MRC 72584 has been reported. *U. chartarum* MRC 72584 hydroxylated testosterone **1** at C-7β, C-6β, C-14α and C-12β, accompanied by a 5α-reduction and oxidations at C-6 and at C-17.

Keywords: testosterone, *Ulocladium chartarum*, hydroxylation, 5α-reduction

Steroids are biologically and pharmaceutically important substances that possess many physiological activities. Steroid-based drugs are now extensively used for some health problems due to their anti-inflammatory, anti-microbial, anti-tumour, anti-estrogenic, anti-allergenic, anti-diabetic, anti-HIV and anti-convulsant properties.[1] The preparation of steroids by conventional chemical synthetic routes has some disadvantages, and microbial steroid biotransformations are widely used to overcome these.[1,2]

Ulocladium species are widely distributed and are mostly saprotrophs, including some plant pathogens and food spoilage organisms. A few *Ulocladium* species have attracted attention as potential sources of enzymes and as bio-control agents.[3] In this study, testosterone **1** was incubated with *U. chartarum* MRC 72584 to investigate its metabolism.

Results and discussion

Incubation of testosterone **1** with *U. chartarum* MRC 72584 for 5 d afforded seven metabolites (Table 1). The first metabolite was identified as androst-4-ene-3,17-dione **2** (Fig. 1). The ^{13}C NMR spectrum of **2** lacked the C-17 resonance of **1** at δ_C 81.25 ppm and showed a new resonance at δ_C 220.50 ppm, indicating an oxidation at C-17 (Table 2). The absence of the characteristic 17αH resonance (1H, t, J = 8.5 Hz) of **1** at δ_H 3.65 ppm confirmed the oxidation of C-17. NMR data of **2** were comparable to the literature.[4]

The second metabolite was identified as 5α-androstane-3,6,17-trione **3**. Its ^{1}H NMR spectrum, **3** lacked the 4-H resonance (1H, s) of **1** at δ_H 5.74, indicating that the hydrogenation of the double bond in ring A had taken place. The alkene resonances of **1** at δ_C 123.87 and 171.52 ppm were replaced by signals for C-4 and C-5 (δ_C 41.09 and 57.39 ppm), respectively. A comparison of the ^{13}C NMR spectrum of **3** with that in literature showed that the reduction of the α,β-unsaturated system had taken place from the α-face.[5] In the ^{13}C NMR spectrum of **3**, the C-17 resonance of **1** at δ_C 81.25 ppm was replaced by a signal at δ_C 219.54 ppm, indicating that an oxidation had taken place at C-17, and the C-6 resonance of **1** at δ_C 32.63 ppm was replaced by resonance at δ_C 208.16 ppm, suggesting that hydroxylation followed by oxidation to a ketone had taken place at C-6. NMR data of **3** were in accordance with the literature.[5]

The third metabolite was identified as 17β-hydroxy-5α-androstane-3,6-dione **4**. The ^{1}H NMR and ^{13}C NMR spectra of **4** revealed the same changes as for compound **3**, except that the metabolite retained the C-17 resonance of **1** at δ_C 81.07 ppm. NMR data of **4** were consistent with the literature.[5]

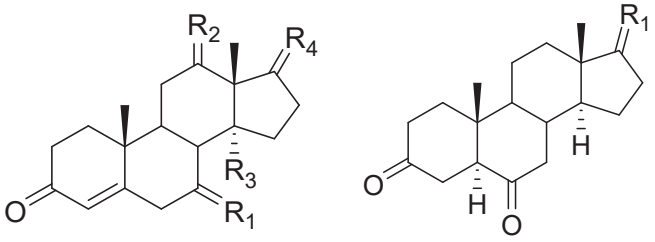
The fourth metabolite was identified as 7β-hydroxyandrost-4-ene-3,17-dione **5**. The NMR spectrum of **5** had characteristic resonances at δ_H 3.53 ppm (1H, m) and δ_C 74.21 ppm, indicating the presence of a 7β-hydroxyl group.[6] The ^{13}C NMR spectrum of **5** showed a downfield shift for C-8 (Δ 7.08 ppm) while there was a γ-gauche upfield shift for C-9 (Δ 2.98 ppm), indicating the presence of a 7β-hydroxyl group. The ^{13}C NMR spectrum of **5** lacked the C-17 resonance of **1** at δ_C 81.25 and had a new carbon atom resonance at δ_C 220.70 ppm, suggesting that an oxidation had taken place at C-17. NMR data of **5** were comparable to the literature.[6]

The fifth metabolite was identified as 7β,17β-dihydroxyandrost-4-ene-3-one **6**. The ^{13}C NMR spectrum of **6** had two characteristic resonances at δ_C 74.92 and 81.09 ppm, suggesting the presence of 7β- and 17β-hydroxyl groups, respectively. The ^{13}C NMR spectrum of **6** showed a downfield shift for C-8 (Δ 7.48 ppm) and a γ-gauche upfield shift for C-9 (Δ 3.81 ppm), which were in agreement with the presence of a 7β-hydroxyl group. The ^{1}H NMR spectrum of **6** possessed a resonance at δ_H 3.63 (1H, t, J = 8.5 Hz), assigned to a 17β-hydroxyl group. NMR data of **6** were consistent with the literature.[7]

The sixth metabolite was identified as 14α,17β-dihydroxyandrost-4-ene-3-one **7**. The metabolite **7** had a

Table 1 Yields of metabolites

Substrate	Metabolite	Yield (%)
Testosterone **1**	Androst-4-ene-3,17-dione **2**	3
	5α-Androstane-3,6,17-trione **3**	4
	17β-Hydroxy-5α-androstane-3,6-dione **4**	9
	7β-Hydroxyandrost-4-ene-3,17-dione **5**	3 11
	7β,17β-Dihydroxyandrost-4-ene-3-one **6**	11
	14α,17β-Dihydroxyandrost-4-ene-3-one **7**	12
	12β,17β-Dihydroxyandrost-4-ene-3-one **8**	9



1 R_1=R_2=H_2, R_3=H, R_4=βOH,αH
2 R_1=R_2=H_2, R_3=H, R_4=O
3 R_1=O
4 R_1=βOH,αH
5 R_1=βOH,αH, R_2=H_2, R_3=H, R_4=O
6 R_1=βOH,αH, R_2=H_2, R_3=H, R_4=βOH,αH
7 R_1=R_2=H_2, R_3=OH, R_4=βOH,αH
8 R_1=H_2, R_2=βOH,αH, R_3=H, R_4=βOH,αH

Fig. 1 Chemical structures of testosterone **1** and its metabolites.

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Table 2 ^{13}C NMR data determined in $CDCl_3$ for compounds **1–8**

C atom	1	2	3	4	5	6	7	8
1	35.48	35.44	37.35	37.84	35.56	35.65	35.59	35.45
2	33.74	33.69	36.82	37.16	33.84	33.91	33.81	33.77
3	199.64	199.40	210.78	211.35	199.17	199.36	199.89	199.72
4	123.87	123.58	41.09	41.07	124.94	124.72	123.67	123.97
5	171.52	170.54	57.39	57.27	166.75	167.48	171.24	170.86
6	32.63	32.38	208.16	208.86	42.45	42.24	32.48	32.62
7	31.33	31.04	45.22	45.88	74.21	74.92	28.44	30.83
8	35.58	34.89	37.21	35.98	42.66	43.06	38.64	34.57
9	53.69	53.57	53.34	53.25	50.71	49.88	46.65	52.49
10	38.45	38.48	37.87	37.84	38.01	38.02	38.78	38.48
11	20.10	20.44	20.84	21.12	20.30	20.55	19.60	29.27
12	36.21	30.07	30.97	36.77	31.15	36.28	32.53	78.75
13	42.61	47.33	48.00	43.26	48.00	43.51	46.85	46.88
14	50.24	50.58	51.39	51.02	50.46	50.69	83.19	48.50
15	23.14	21.55	21.56	23.00	24.92	26.33	26.01	23.01
16	29.51	35.44	35.54	29.98	35.90	30.72	29.44	29.81
17	81.25	220.50	219.54	81.07	220.70	81.09	78.41	81.57
18	10.93	13.52	13.72	10.99	17.32	11.10	14.83	6.0
19	17.17	17.21	12.52	12.42	13.92	17.32	17.12	17.19

new carbon atom resonance at δ_C 83.19 ppm in its ^{13}C NMR spectrum although it did not show any new signals in the range 3–5 ppm in its ^1H NMR spectrum, indicating the presence of a tertiary hydroxyl group. The downfield shifts for C-8 (Δ 3.06 ppm) and C-15 (Δ 2.87 ppm) and γ-gauche upfield shifts for C-7 (Δ 2.89 ppm) and C-16 (Δ 0.07 ppm) were in accordance with the presence of a hydroxyl group at C-14. In its ^1H NMR spectrum, the metabolite **7** showed a typical downfield shift (Δ 0.63 ppm) for the 17α-H resonance and this suggested its diaxial interaction with a 14α-hydroxyl group.[8] This shift confirmed the presence of a 14α-hydroxyl group. NMR data of **7** were in accordance with the literature.[8]

The seventh metabolite was identified as 12β,17β-dihydroxyandrost-4-ene-3-one **8**. The ^{13}C NMR spectra of **8** showed a typical resonance at δ_C 78.75 ppm, indicating the presence a 12β-hydroxyl group.[9] The downfield shifts for C-11 (Δ 9.17 ppm) and C-13 (Δ 4.27 ppm) and γ-gauche upfield shifts for C-9 (Δ 1.20 ppm) and C-14 (Δ 1.74 ppm) were in accordance with the presence of a 12β-hydroxyl group. The ^{13}C NMR spectra of **8** showed a resonance at δ_C 81.57 ppm, suggesting that the 17β-hydroxyl group of **1** was retained. NMR data[9] and the melting point[10] of **8** were comparable to literature.

As can be seen from Table 1, testosterone **1** was hydroxylated by *U. chartarum* MRC 72584 at C-6β, C-7β, C-12β and C-14α. An oxidation at C-6 following the hydroxylation of **1** at C-6β, a 5α-reduction before or after this hydroxylation and an oxidation at C-17 were also observed.

Reports of 5α-reduction and hydroxylation of testosterone **1** at C-6β and C-14α by fungi are very common.[2,11,12] However, fungal hydroxylations of **1** at C-7β and C-12β are very rare.[9,10,13,14]

In a previous work, *Botryosphaerica obtusa* hydroxylated **1** at C-7β, C-11α, C-12β and C-15α positions.[13] In another work, *Botrytis cinerea* hydroxylated **1** at C-7β and reduced its C4–C5 double bond from the α-face.[14] *Rhizomucor tauricus*[9] hydroxylated **1** at C-6β and C-12β while a *Penicillium* isolate[10] hydroxylated **1** at C-1α, C-6β, C-12β and C-15β. We have now shown that *U. chartarum* MRC 72584 hydroxylated **1** at C-6β, C-7β, C-12β and C-14α and in addition to this, a 5α-reduction and oxidations at C-6 and C-17 were also observed. Our work on steroid biotransformations by *U. chartarum* MRC 72584 and some other fungi is in progress.

Experimental

Testosterone **1** was purchased from Sigma-Aldrich (Istanbul, Turkey). Solvents were of analytical grade and were purchased from Merck (Istanbul, Turkey). Potato dextrose agar (PDA) and agar for PDA slopes and ingredients for liquid medium were also purchased from Merck (Istanbul, Turkey). The steroids were separated by column chromatography on silica gel 60 (Merck 107734), and eluted with increasing concentrations of ethyl acetate in *n*-hexane. Steroid mixtures from the column were then separated by column chromatography on aluminium oxide 90 active neutral (activity stage I, Merck 101077), using smaller columns for 2–3 h. 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) as eluent. TLC plates were dipped into an anisaldehyde/H_2SO_4 reagent and heated to 120 °C for 3 min to visualise the spots. Infrared spectra were recorded using a Perkin Elmer Spectrum Two spectrometer. ^1H 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 deuterochloroform at 75 MHz with a Varian Mercury 300 spectrometer and are reported in Table 2. Chemical shifts are given in ppm (δ scale) and coupling constants (*J*) are given in Hz. Melting points were determined by an Electrothermal IA 9200 melting point apparatus and are uncorrected.

U. chartarum MRC 72584 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. The biotransformation experiment was performed in duplicate and run with a control flask containing non-inoculated sterile medium and the substrate. After 5 d of incubation, the control was harvested and analysed by TLC. No metabolites were detected in the control.

Biotransformation of testosterone **1**

The liquid medium for *U. chartarum* MRC 72584 was prepared by mixing glucose (20 g), peptone (5 g) and yeast extract (5 g) in 1 L of distilled water.[15] 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. Spores freshly obtained from a PDA slope were transferred aseptically into each flask containing sterile medium in a biological safety cabinet. After cultivation at 28 °C for 3 d on a rotary shaker (160 rpm), testosterone **1** (1 g) dissolved in DMF (10 mL) was evenly distributed aseptically among the flasks. The biotransformation of

1 was carried out in 10 flasks for 5 d under the same conditions. The fungal mycelium was separated from the broth by filtration under vacuum and the mycelium was rinsed with ethyl acetate (500 mL). The broth was extracted three times each with ethyl acetate (1 L). The organic extract was dried over anhydrous sodium sulfate and the solvent evaporated *in vacuo* to give a brown gum (1343 mg), which was then chromatographed on silica gel.

Elution with 30% ethyl acetate in *n*-hexane afforded androst-4-ene-3,17-dione **2**, which was crystallised from acetone as prisms; yield 30 mg (3%); m.p. 173–174 °C (lit.[4] 170–172 °C); IR (ν_{max} cm^{-1}): 1736 and 1440; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.91 (3H, s, 18H), 1.21 (3H, s, 19H), 5.75 (1H, s, 4H).

Elution with 40% ethyl acetate in *n*-hexane afforded the unreacted starting material (80 mg), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic sample.

Further elution with 40% ethyl acetate in *n*-hexane afforded 5α-androstane-3,6,17-trione **3**, which was crystallised from ethyl acetate as plates; yield 44 mg (4%); m.p. 193–194 °C (lit.[16] 196–197 °C); IR (ν_{max} cm^{-1}): 1740, 1720 and 1700; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (3H, s, 18H), 0.97 (3H, s, 19H).

Elution with 50% ethyl acetate in *n*-hexane afforded 17β-hydroxy-5α-androstane-3,6-dione **4** which was crystallised from acetone as needles; yield 95 mg (9%); m.p. 233–234 °C (lit.[16] 230–231 °C); IR (ν_{max} cm^{-1}): 3550 and 1710; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.80 (3H, s, 18H), 1.00 (3H, s, 19H), 3.67 (1H, d, J = 8.5 Hz, 17αH).

Further elution with 50% ethyl acetate in *n*-hexane afforded 7β-hydroxyandrost-4-ene-3,17-dione **5**, which was crystallised from acetone as plates; yield 32 mg (3%); m.p. 222–223 °C (lit.[6] 218–220 °C); IR (ν_{max} cm^{-1}): 3465, 1670 and 1620; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.94 (3H, s, 18H), 1.23 (3H, s, 19H), 3.53 (1H, m, 7αH), 5.76 (1H, s, 4H).

Elution with 60% ethyl acetate in *n*-hexane afforded 7β,17β-dihydroxyandrost-4-ene-3-one **6**, which was crystallised from acetone as prisms; yield 117 mg (11%); m.p. 200–201 °C, (lit.[7] 197.5–199.5 °C); IR (ν_{max} cm^{-1}): 3415, 1660 and 1610; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.80 (3H, s, 18H), 1.21 (3H, s, 19H), 3.44 (1H, m, 7αH), 3.63 (1H, t, J = 8.5 Hz, 17αH), 5.75 (1H, s, 4H).

Further elution with 60% ethyl acetate in *n*-hexane afforded 14α,17β-dihydroxyandrost-4-ene-3-one **7**, which was crystallised from acetone as needles; yield 128 mg (12%); m.p. 180–181 °C (lit.[8] 181–185 °C); IR (ν_{max} cm^{-1}): 3450, 1660 and 1640; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (3H, s, 18H), 1.18 (3H, s, 19H), 4.28 (1H, t, J = 7.8 Hz, 17αH), 5.70 (1H, s, 4H).

Elution with 70% ethyl acetate in *n*-hexane afforded 12β,17β-dihydroxyandrost-4-ene-3-one **8**, which was crystallised from acetone as needles; yield 95 mg (9%); m.p. 120–121 °C (lit.[10] 121–125 °C); IR (ν_{max} cm^{-1}): 3370, 1735 and 1655; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.80 (3H, s, 18H), 1.16 (3H, s, 19H), 3.48 (1H, dd, J = 5.0 and 11.0 Hz, 12αH), 3.85 (1H, t, J = 8.5 17αH), 5.71 (1H, s, 4H).

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