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RESEARCH ARTICLE

Microbial transformation of androstenedione by *Cladosporium sphaerospermum* and *Ulocladium chartarum*

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ABSTRACT

In this work, incubations of androstenedione **1** with *Cladosporium sphaerospermum* MRC 70266 and *Ulocladium chartarum* MRC 72584 have been reported. *C. sphaerospermum* MRC 70266 mainly hydroxylated **1** at C-6β, accompanied by a hydroxylation at C-15α, a reduction at C-17, a 5α-reduction and oxidations at C-6 and C-16 following hydroxylations. *U. chartarum* MRC 72584 hydroxylated **1** at C-6β, C-7α, C-7β and C-14α, accompanied by an oxidation at C-6 following its hydroxylation, a reduction at C-17 and a 5α-reduction. 6β,17β-Dihydroxyandrost-4-en-3,16-dione **8**, one of the metabolites from the incubation of **1** with *C. sphaerospermum* MRC 70266, was determined as a new compound.

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Cladosporium sphaerospermum; *Ulocladium chartarum*; androstenedione; biotransformation

Introduction

Steroids are biologically and pharmaceutically important substances as they are involved in many physiological activities. More than 300 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 (Kolet et al. 2013).

The preparation of these types of steroids by conventional synthetic routes usually has some disadvantages, such as being time-consuming, more expensive and environmentally unfriendly processes. However, microbial steroid biotransformations are widely used to prepare the mentioned steroids as they can overcome these disadvantages (Faber 2003; Bhatti and Khera 2012; Donova and Egorova 2012; Kolet et al. 2013; Nassiri-Koopaei and Faramarzi 2015).

Androstenedione **1** is an important metabolite in androgen metabolism and used as a starting material in the preparation of pharmaceutically important androgens, anabolic drugs and some important compounds, such as spironolactone (Malaviya and Gomes 2009).

A number of microorganisms have been used for the biotransformation of androstenedione **1** (Mahato and Garai 1997; Fernandes et al. 2003; Bhatti and Khera 2012; Donova and Egorova 2012; Nassiri-Koopaei and Faramarzi 2015). Most metabolites obtained from microbial biotransformations of androstenedione **1** are either biologically active compounds or important intermediates in the synthesis of steroidal drugs (Heidary and Habibi 2016).

In this work, androstenedione **1** was incubated with *Cladosporium sphaerospermum* MRC 70266 and *Ulocladium chartarum* MRC 72584 for 5 d in order to investigate its metabolism by these fungi.

Materials and methods

Instrumental methods

^{1}H NMR spectra were recorded in deuterochloroform with tetramethylsilane as an internal standard reference at 300 MHz with a Varian Mercury 300 spectrometer (Varian, Palo Alto, CA). ^{13}C NMR spectra were recorded in deuterochloroform at 75 MHz with a Varian Mercury 300 spectrometer (Varian, Palo Alto, CA). Chemical shifts are given in ppm (δ scale), coupling constants (*J*) are given in Hz. Infrared spectra were recorded using a Perkin Elmer Spectrum Two spectrometer (PerkinElmer, Waltham, MA). Elemental analysis was performed using a Thermo Finnigan Flash EA 1112 elemental analyser (Bremen). Melting points were determined by an Electrothermal IA 9200 melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was carried out with 0.2 mm

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Supplemental data for this article can be accessed here.

thick Merck Kieselgel 60 (Istanbul) F254 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 in order to visualize the spots.

Chemicals

Androstenedione **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).

Microorganisms and fermentation conditions

C. sphaerospermum MRC 70266 and *U. chartarum* MRC 72584 were obtained from TUBITAK, Marmara Research Center, Food Science and Technology Research Institute, Culture Collection Unit, Kocaeli, Turkey. Stock cultures were maintained at on PDA slopes at 4 °C.

The liquid medium (1 L) for both fungi was prepared by mixing glucose (20 g), peptone (5 g) and yeast extract (5 g) in distilled water (Shebany 2012). The medium for each fungus was evenly distributed among 10 culture flasks of 250 mL capacity (100 mL in each) and autoclaved for 20 min at 121 °C. Spores of each fungus freshly obtained from PDA slopes were separately transferred into one set of 10 flasks containing sterile medium in a biological safety cabinet. After cultivation of each fungus at 28 °C for 3 d on a rotary shaker (160 rpm), androstenedione **1** (1 g) dissolved in 10 ml of DMF was evenly distributed aseptically among the flasks. The biotransformation of the substrate by each fungus was carried out in 10 flasks for 5 d under the same conditions. Each biotransformation experiment was run with a control flask containing non-inoculated sterile medium and the substrate. After 5 d of incubation, each control was harvested and analysed by TLC. No metabolites were detected in the controls.

Isolation and identification of metabolites

After incubation, each fungal mycelium was separated from the broth by filtration under vacuum, and each mycelium was rinsed with ethyl acetate (500 ml). Each broth was then extracted three times with each 1 L of ethyl acetate. The organic extracts from both incubations were separately dried over anhydrous sodium sulphate and then evaporated in vacuo to give 2 brown gums. The steroids in brown gums were separately chromatographed on silica gel 60 (Merck 107734), eluting with increasing concentrations of ethyl acetate in *n*-hexane. Steroid mixtures from the columns were then separated by column chromatography on aluminium oxide 90 active neutral (activity stage I, Merck 101077), using smaller columns for 2–3 h. The purified steroids were crystallized in appropriate solvents and identified by using melting points and a combination of NMR, IR and elemental analysis.

Biotransformation of androstenedione 1 by C. sphaerospermum MRC 70266

Incubation of androstenedione **1** with *C. sphaerospermum* MRC 70266 for 5 d yielded a brown gum (2021 mg), which was then chromatographed on silica gel. Elution with ethyl acetate in *n*-hexane (3:7) yielded the unreacted substrate (115 mg), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic sample.

Elution with ethyl acetate in *n*-hexane (2:3) yielded 17β-hydroxyandrost-4-en-3-one **2** (20 mg, 2%); crystallized from acetone as prisms; mp 151–152 °C, lit (Świzdor et al. 2017); mp 151–153 °C; IR ν_{max} 3200, 1650 and 1620 cm^{-1}; EA: Calculated for $C_{19}H_{28}O_2$: C 79.12, H 9.79; found: C 79.04, H 9.70%; ^{1}H NMR ($CDCl_3$): δ_H 0.80 (3 H, s, 18-H), 1.18 (3 H, s, 19-H), 3.65 (1 H, t, J= 8.5 Hz, 17α-H) and 5.72 (1 H, s, 4-H); ^{13}C NMR ($CDCl_3$): see Table 1.

Further elution with ethyl acetate in *n*-hexane (2:3) yielded 5α-androstane-3,6,17-trione **3** (32 mg, 3%); crystallized from acetone as plates; mp 193–194 °C, lit (Numazawa et al. 1988); mp 196–197 °C; IR ν_{max}1745, 1715 and 1700 cm^{-1}; EA: Calculated for $C_{19}H_{26}O_3$: C 75.46, H 8.67; found: C 75.33, H 8.55%; ^{1}H NMR ($CDCl_3$): δ_H 0.90 (3 H, s, 18-H), 0.98 (3 H, s, 19-H); ^{13}C NMR ($CDCl_3$): see Table 1.

Elution with ethyl acetate in *n*-hexane (1:1) yielded 6β-hydroxyandrost-4-en-3,17-dione **4** (391 mg, 37%); crystallized from acetone as prisms; mp 191–192 °C, lit (Hanson et al. 1996); mp 190–193 °C; IR ν_{max} 3415, 1740 and 1670 cm^{-1}; EA: Calculated for $C_{19}H_{26}O_3$: C 75.46, H 8.67; found: C 75.28, H 8.47%; ^{1}H NMR ($CDCl_3$): δ_H 0.92 (3 H, s, 18-H), 1.38 (3 H, s, 19-H), 4.37 (1 H, brs, 6α-H) and 5.79 (1 H, brs, 4-H); ^{13}C NMR ($CDCl_3$): see Table 1.

Further elution with ethyl acetate in *n*-hexane (1:1) yielded 17β-hydroxyandrost-4-en-3,16-dione **5** (53 mg, 5%); crystallized from ethyl acetate as prisms; mp 156–157 °C, lit (Yamashita et al. 1976); mp 153–154 °C;

Table 1. ^{13}C NMR data for androstenedione **1** and its metabolites determined in $CDCl_3$.

Carbon atom	1	2	3	4	5	6	7	8	9	10	11	12
1	35.66	35.54	37.43	36.80	35.48	36.29	35.68	36.72	37.88	37.79	35.59	35.38
2	33.88	33.87	36.88	33.96	33.85	34.14	33.86	34.02	37.20	33.73	33.86	33.87
3	199.35	199.75	210.74	200.63	199.32	200.72	199.35	200.54	211.37	199.61	199.12	198.68
4	124.12	123.75	41.16	126.05	124.19	126.17	123.93	126.28	41.10	123.84	125.02	127.16
5	170.34	171.50	57.51	168.41	169.99	168.73	170.26	168.04	57.32	170.39	166.54	166.74
6	32.53	32.73	208.10	72.22	32.45	72.76	32.59	72.31	208.87	32.98	42.49	39.30
7	31.23	31.42	45.30	37.04	31.79	36.97	31.26	38.01	45.92	32.23	74.28	67.10
8	35.11	35.60	37.26	29.20	34.48	29.68	35.41	28.69	36.01	38.51	42.65	40.97
9	53.78	53.78	53.48	53.40	53.65	53.60	53.66	53.37	53.30	46.60	50.72	45.36
10	38.60	38.58	37.97	37.83	38.65	37.90	38.63	38.20	37.88	38.51	38.01	38.52
11	20.27	20.54	20.92	20.05	20.27	20.50	20.21	20.14	21.16	18.98	20.32	20.12
12	30.71	36.30	31.06	31.02	36.19	37.98	31.91	36.07	36.81	35.49	31.16	30.92
13	47.48	42.71	48.05	47.49	42.39	42.81	50.50	42.37	43.29	52.46	48.00	47.29
14	50.80	50.36	51.50	50.63	44.47	50.34	57.38	44.37	51.05	80.38	50.45	45.62
15	21.72	23.25	21.63	21.51	35.36	23.20	70.31	35.38	23.03	24.33	24.95	21.24
16	35.72	30.29	35.58	35.63	216.52	30.31	46.25	216.79	30.02	29.97	35.89	35.67
17	220.45	81.51	219.46	220.88	86.11	81.58	215.77	86.09	81.14	218.64	220.60	220.29
18	13.67	11.00	13.78	13.59	11.33	11.07	15.31	11.37	11.01	17.15	17.33	13.47
19	17.34	17.32	12.58	19.30	17.35	19.46	17.47	19.43	12.45	17.68	13.93	16.98

IR ν_{max} 3455, 1760, 1675 and 1620 cm^{-1}; EA: Calculated for $C_{19}H_{26}O_3$: C 75.46, H 8.67; found: C 75.31, H 8.49%; ^1H NMR ($CDCl_3$): δ_H 0.77 (3 H, s, 18-H), 1.22 (3 H, s, 19-H), 3.76 (1 H, brs, 17α-H) and 5.75 (1 H, s, 4-H); ^{13}C NMR ($CDCl_3$): see Table 1.

Elution with ethyl acetate in *n*-hexane (3:2) yielded 6β,17β-dihydroxyandrost-4-en-3-one **6** (64 mg, 6%); crystallized from ethyl acetate as prisms; mp 222–223 °C, lit (Hanson et al. 1996); mp 215–220 °C; IR ν_{max} 3500, 1655 and 1630 cm^{-1}; EA: Calculated for $C_{19}H_{28}O_3$: C 74.96, H 9.27; found: C 74.82, H 9.12%; ^1H NMR ($CDCl_3$): δ_H 0.82 (3 H, s, 18-H), 1.37 (3H, s, 19-H), 3.64 (1 H, t, J= 8.5 Hz, 17α-H), 4.36 (1 H, brs, 6α-H) and 5.80 (1 H, s, 4-H); ^{13}C NMR ($CDCl_3$): see Table 1.

Elution with ethyl acetate in *n*-hexane (7:3) yielded 15α-hydroxyandrost-4-en-3,17-dione **7** (43 mg, 4%); crystallized from ethyl acetate as needles; mp 203–204 °C, lit (Hosoda et al. 1977); mp 193.5–195 °C; IR ν_{max} 3430, 1730, 1670 and 1620 cm^{-1}; EA: Calculated for $C_{19}H_{26}O_3$: C 75.46, H 8.67; found: C 75.29, H 8.50%; ^1H NMR ($CDCl_3$): δ_H 0.95(3H, s, 18-H), 1.22 (3 H, s, 19-H), 4.40 (1 H, m, 15β-H) and 5.74 (1 H, s, 4-H); ^{13}C NMR ($CDCl_3$): see Table 1.

Elution with ethyl acetate in *n*-hexane (4:1) yielded 6β,17β-dihydroxyandrost-4-en-3,16-dione **8** (33 mg, 3%); crystallized from ethyl acetate as needles; mp 222–224 °C; IR ν_{max} 3455, 1760 and 1665 cm^{-1}; EA: Calculated for $C_{19}H_{26}O_4$: C 71.67, H 8.23; found: C 71.49, H 8.10%; ^1H NMR ($CDCl_3$): δ_H 0.77 (3 H, s, 18-H), 1.38 (3 H, s, 19-H), 3.76 (1 H, brs, 17α-H), 4.34 (1 H, brs, 6α-H) and 5.82 (1H, s, 4-H); ^{13}C NMR ($CDCl_3$): see Table 1.

Biotransformation of androstenedione 1 by U. chartarum MRC 72584

Incubation of androstenedione **1** with *U. chartarum* MRC 72584 for 5 d yielded a brown gum (1987 mg), which was then chromatographed on silica gel. Elution with ethyl acetate in *n*-hexane (3:7) yielded the unreacted substrate (123 mg), which was identified by comparison of its ^1H and ^{13}C NMR spectra with those of an authentic sample.

Elution with ethyl acetate in *n*-hexane (2:3) yielded 5α-androstane-3,6,17-trione **3** (137 mg, 13%), which was identified by comparison of its ^1H and ^{13}C NMR spectra with those of the previously isolated metabolite.

Elution with ethyl acetate in *n*-hexane (1:1) yielded 17β-hydroxy-5α-androstane-3,6-dione **9** (32 mg, 3%); crystallized from acetone as needles; mp 229–230 °C, lit (Numazawa et al. 1988); mp 230–231 °C; IR ν_{max} 3545, 1720 cm^{-1}; EA: Calculated for $C_{19}H_{28}O_3$: C 74.96, H 9.27; found: C 74.81, H 9.11%; ^1H NMR ($CDCl_3$): δ_H 0.81 (3 H, s, 18-H), 1.01 (3 H, s, 19-H) and 3.66 (1 H, t, J= 8.5 Hz, 17αH); ^{13}C NMR ($CDCl_3$): see Table 1.

Further elution with ethyl acetate in *n*-hexane (1:1) yielded 14α-hydroxyandrost-4-en-3,17-dione **10** (148 mg, 14%); crystallized from methanol as plates; mp 256–257 °C, lit (Hanson et al. 1996); mp 255–260 °C; IR ν_{max} 3420, 1735, 1665 and 1615 cm^{-1}; EA: Calculated for $C_{19}H_{26}O_3$: C 75.46, H 8.67; found: C 75.33, H 8.53%; ^1H NMR ($CDCl_3$): δ_H 0.99 (3 H, s, 18-H), 1.21 (3 H, s, 19-H) and 5.73 (1 H, s, 4-H); ^{13}C NMR ($CDCl_3$): see Table 1.

Elution with ethyl acetate in *n*-hexane (3:2) yielded 7β-hydroxyandrost-4-en-3,17-dione **11** (189 mg, 18%); crystallized from acetone as plates; mp 222–223 °C, lit (Holland and Thomas 1982); mp 218–220 °C; IR ν_{max} 3460, 1675 and 1625 cm^{-1}; EA: Calculated for $C_{19}H_{26}O_3$: C 75.46, H 8.67; found: C 75.28, H 8.47%; ^1H NMR ($CDCl_3$): δ_H 0.94 (3 H, s, 18-H), 1.24 (3 H, s, 19-H), 3.58 (1 H, m, 7α-H) and 5.77 (1 H, s, 4-H); ^{13}C NMR ($CDCl_3$): see Table 1.

Further elution with ethyl acetate in *n*-hexane (3:2) yielded 7α-hydroxyandrost-4-en-3,17-dione **12**

(106 mg, 10%); crystallized from methanol as needles; mp 236–237 °C, lit (Holland and Thomas 1982); mp 227–230 °C; IR ν_{max} 3400, 1740, 1660 and 1615 cm^{-1}; EA: Calculated for $C_{19}H_{26}O_3$: C 75.46, H 8.67; found: C 75.39, H 8.55%; ^{1}H NMR ($CDCl_3$): δ_H 0.97(3H, s, 18-H), 1.25 (3 H, s, 19-H), 4.15 (1 H, m, 7β-H) and 5.85 (1 H, s, 4-H); ^{13}C NMR ($CDCl_3$): see Table 1.

Results and discussion

Incubation of androstenedione **1** with *C. sphaerospermum* MRC 70266 for 5 d yielded 7 metabolites (Figure 1). The first metabolite was identified as 17β-hydroxyandrost-4-en-3-one **2**. The ^{13}C NMR spectrum of **2** lacked the C-17 resonance of **1** at δ_C 220.45 ppm and had a new carbon atom resonance at δ_C 81.51 ppm (see Supplemental data), suggesting the presence of a 17β-hydroxyl group. The ^{1}H NMR spectrum of **2** had a new resonance (1 H, t, J = 8.5 Hz) at δ_H 3.65 ppm, further suggesting the presence of a 17β-hydroxyl group. NMR data of **2** were in accordance with literature (Świzdor et al. 2017).

The second metabolite was identified as 5α-androstane-3,6,17-trione **3**. The ^{1}H NMR spectrum of 3 lacked the 4-H resonance (1 H, s) of **1** at δ_H 5.75, indicating the reduction of a double bond in ring A had taken place. 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 (Al-Awadi et al. 2005). The ^{13}C NMR spectrum of **3** lacked the C-6 resonance of **1** at δ_C 32.53 ppm and showed a new resonance at δ_C 208.10 ppm, suggesting that a hydroxylation had taken place at C-6 and the resulting alcohol was then oxidized to a ketone. NMR data of **3** were consistent with prior literature (Al-Awadi et al. 2005).

The third metabolite was identified as 6β-hydroxyandrost-4-en-3,17-dione **4**. NMR spectra of **4** showed new resonances at δ_H 4.37 ppm (1 H, brs) and δ_C 72.22 ppm, which were characteristic for a 6β-hydroxyl group (Hanson et al. 1996). The ^{13}C NMR spectrum of **4** showed a downfield shift for C-7 ($\Delta\delta_C$ 5.81 ppm) and a γ-carbon up-field shift for C-8 ($\Delta\delta_C$ 5.91 ppm), further supporting the presence of a 6β-hydroxyl group. NMR data of **4** were in agreement with literature (Hanson et al. 1996).

The fourth metabolite was identified as 17β-hydroxyandrost-4-en-3,16-dione **5**. The ^{13}C NMR spectrum of **5** lacked the C-17 resonance of **1** at δ_C 220.45 ppm and showed a new resonance at δ_C 86.11 ppm, indicating the presence of a hydroxyl group at C-17. The ^{13}C NMR spectrum of 5 also lacked the C-16 resonance of **1** at δ_C 35.72 ppm and had a new carbon atom resonance at δ_C 216.52 ppm, suggesting the presence of a carbonyl group at C-16. In its ^{1}H NMR spectrum, **5** had a typical broad singlet resonance at δ_H 3.76 ppm, showing the presence of a 17β-hydroxyl group next to a carbonyl group at C-16 (Kirk et al. 1990; Peart et al. 2013). All these results suggested that a reduction at C-17 and an oxidation at C-16 following its hydroxylation had taken place. NMR data of **5** were in accordance with literature (Kirk et al. 1990; Peart et al. 2013).

The fifth metabolite was identified as 6β, 17β-dihydroxyandrost-4-en-3-one **6**. The ^{13}C NMR spectrum of **6** had two characteristic resonances (Hanson et al. 1996) at δ_C 72.76 ppm and δ_C 81.58 ppm, suggesting the presence of 6β- and 17β-hydroxyl groups, respectively. The ^{1}H NMR spectrum of **6** showed two new resonances at δ_H 3.64 ppm (1 H, t, J = 8.5 Hz) and δ_H 4.36 ppm (1 H, brs), further suggesting the presence of 17β- and 6β-hydroxyl groups, respectively. NMR data of **6** were consistent with prior literature (Hanson et al. 1996).

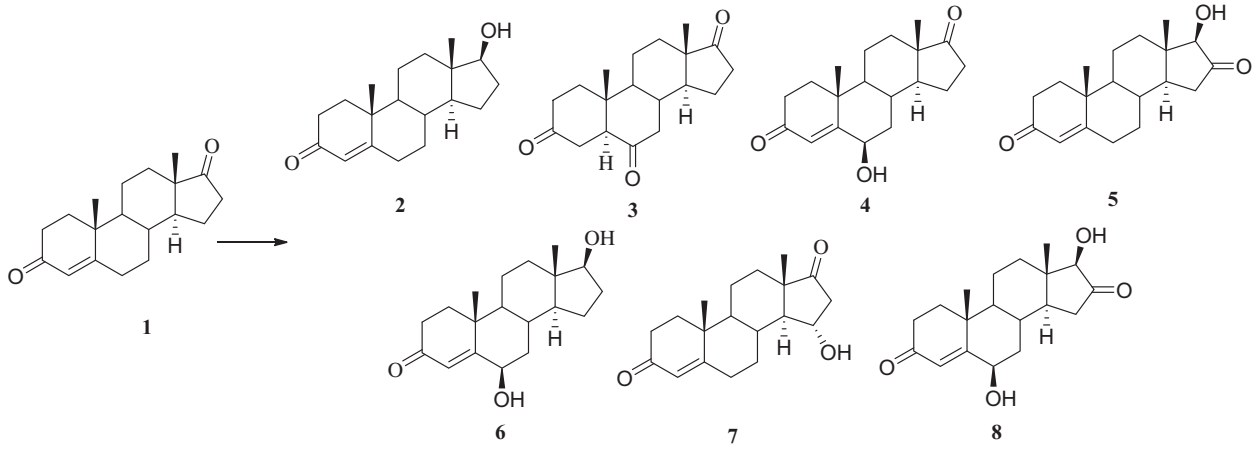

Figure 1. Biotransformation of androstenedione **1** by *C. sphaerospermum* MRC 70266.

The sixth metabolite was identified as 15α-hydroxyandrost-4-en-3,17-dione **7**. The metabolite showed characteristic resonances at δ_H 4.40 ppm (1 H, m) and δ_C 70.31 ppm, indicating the presence of a 15α-hydroxyl group (Huang et al. 2010). The ^{13}C NMR spectrum of **7** showed downfield shifts for C-14 ($\Delta\delta_C$ 6.58 ppm) and C-16 ($\Delta\delta_C$ 10.53 ppm), further indicating the presence of a 15α-hydroxyl group. NMR data of **7** were in agreement with literature (Huang et al. 2010).

The seventh metabolite was identified as 6β,17β-dihydroxyandrost-4-en-3,16-dione **8**. The ^{13}C NMR spectrum of the meabolite showed a characteristic resonance at δ_C 72.31 ppm suggesting the presence of a 6β-hydroxyl group (Hanson et al. 1996). The ^{13}C NMR spectrum of **8** lacked the C-17 resonance of **1** at δ_C 220.45 ppm and had a new resonance at δ_C 86.09 ppm, indicating the presence of a hydroxyl group at C-17. The ^{13}C NMR spectrum of **8** had a new carbon atom resonance at δ_C 216.79 ppm, suggesting the presence of a carbonyl group in ring D. The ^{1}H NMR spectrum of **8** had a characteristic broad singlet resonance at δ_H 3.76 ppm, indicating the presence of a 17β-hydroxyl group in close proximity to a carbonyl group at C-16 (Kirk et al. 1990; Peart et al. 2013). These results indicated that a hydroxylation and subsequent oxidation at C-16 and a reduction at C-17 had taken place. The presence of 2 methyl, 6 methylene, 6 methine and 5 quaternary carbons in the DEPT spectra of **8** were in agreement with the proposed structure of the metabolite. 6β,17β-Dihydroxyandrost-4-en-3,16-dione **8** was determined as a new metabolite.

Incubation of androstenedione **1** with *U. chartarum* MRC 72584 for 5 d yielded 5 metabolites (Figure 2). The first metabolite was identified as 5α-androstane-3,6,17-trione **3**, which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of the previously isolated metabolite.

The second metabolite was identified as 17β-hydroxy-5α-androstane-3,6-dione **9**. The ^{1}H NMR spectrum of **9** lacked the 4-H resonance (1 H, s) of **1** at δ_H 5.75, indicating that the hydrogenation of double bond in ring A had taken place. The ^{13}C NMR spectrum of **9** lacked the C-6 resonance of **1** at δ_C 32.53 ppm and had a new resonance at δ_C 208.87 ppm, indicating that a hydroxylation had taken place at C-6 and the resulting alcohol was then oxidized to a ketone. The ^{13}C NMR spectrum of **9** had another resonance at δ_C 81.14 ppm, which was typical for the presence of a 17β-hydroxyl group. NMR data of **9** were in agreement with literature (Al-Awadi et al. 2005).

The third metabolite was identified as 14α-hydroxyandrost-4-en-3,17-dione **10**. The ^{13}C NMR spectrum of **10** showed a new carbon atom resonance at δ_C 80.38 ppm, indicating the presence of a hydroxyl group. The ^{1}H NMR spectrum of **10** showed a downfield shift ($\Delta\delta_H$ 0.07 ppm) for the 18-methyl proton resonance with the absence of any new proton signals, suggesting that a tertiary hydroxyl group was present at C-14. The ^{13}C NMR spectrum of **10** revealed a downfield shift for C-8 ($\Delta\delta_C$ 3.40 ppm) whilst it showed a γ-gauche upfield shift for C-16 ($\Delta\delta_C$ 5.75 ppm). These shifts suggested the presence of the 14α-hydroxyl group. The position and configuration of the introduced hydroxyl group were confirmed by comparison its NMR data with those reported in the literature (Hanson et al. 1996).

The fourth metabolite was identified as 7β-hydroxyandrost-4-en-3,17-dione **11**. NMR spectra of **11** had characteristic resonances at δ_H 3.58 ppm (1 H, m) and

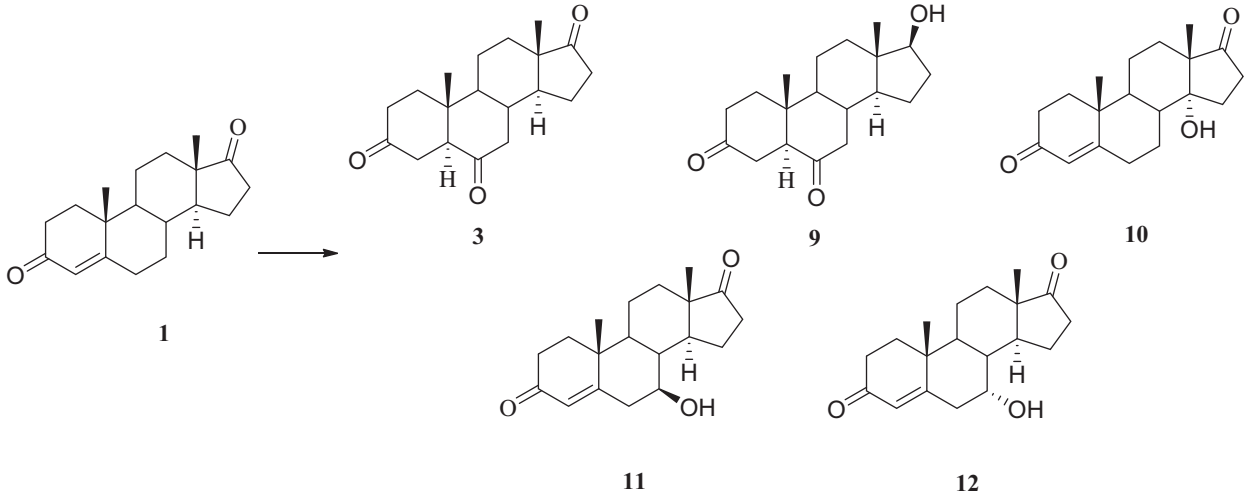

Figure 2. Biotransformation of androstenedione **1** by *U. chartarum* MRC 72584.

δ_C 74.28 ppm, indicating the presence of a 7β-hydroxyl group (Holland and Thomas 1982). The ^{13}C NMR spectrum of **11** showed a downfield shift for C-8 ($\Delta\delta_C$ 7.54 ppm) whereas it showed a γ-gauche up-field shift for C-9 ($\Delta\delta_C$ 3.06 ppm), further indicating the presence of a 7β-hydroxyl group. NMR data of **11** were consistent with literature (Holland and Thomas 1982).

The fifth metabolite was identified as 7α-hydroxyandrost-4-en-3,17-dione **12**. NMR spectra of **12** had characteristic resonances at δ_H 4.15 ppm (1 H, m) and δ_C 67.10 ppm, indicating the presence of a 7α-hydroxyl group (Holland and Thomas 1982). The ^{13}C NMR spectrum of **12** showed a downfield shift for C-8 (Δ 5.86 ppm) whereas it showed a γ-gauche up-field shift for C-9 (Δ 8.42 ppm), further indicating the presence of a 7α-hydroxyl group. NMR data of **12** were in agreement with literature (Holland and Thomas 1982).

In this work, as can be seen from Table 2, *C. sphaerospermum* MRC 70266 hydroxylated **1** mainly at C-6β, accompanied by a hydroxylation at C-15α, a reduction at C-17, a 5α-reduction and oxidations at C-6 and C-16 following hydroxylations. In a recent work, however, *C. sphaerospermum* MRC 70266 metabolized 17β-hydroxyandrost-4-en-3-one **2** in a different way (Yildirim et al. 2019). The same fungus mainly hydroxylated **2** at C-6β, accompanied by a minor oxidation at C-17, a minor 5α-reduction and some minor hydroxylations at C-7β, C-12β, C-15α and C-16β. These results might have suggested that *C. sphaerospermum* MRC 70266 metabolized **1** and **2** in some different ways due to the lack of a 17β-hydroxyl group in **1** as in the biotransformations of some 4-ene-3-oxo steroids by *Fusarium culmorum* (Kołek and Swizdor 1998). In another recent work, *Cladosporium cladosporioides* MRC 70282 (Yildirim, Kuru, and RF Yılmaz 2018), a different *Cladosporium* isolate, metabolized **1** and **2** in different ways from how *C. sphaerospermum* MRC 70266 did. *C. cladosporioides* MRC 70282 reduced **1** at C-17, hydroxylated it at C-16β and most of this hydroxyl group was then oxidized to a ketone. In addition to this, a minor epimerization at C-17 had taken place. In the same work, this fungus hydroxylated **2** at C-16β and most of this hydroxyl group was then oxidized at C-16. This was accompanied by a minor epimerization and a minor independent oxidation at C-17.

It can also be seen from Table 2, U. *chartarum* MRC 72584 metabolized **1** different from how *C. sphaerospermum* MRC 70266 did. *U. chartarum* MRC 72584 hydroxylated **1** at C-6β, C-7α, C-7β and C-14α, accompanied by an oxidation at C-6 subsequent to its hydroxylation, a 5α-reduction and a reduction at C-17. In a recent work, *U. chartarum* MRC 72584 hydroxylated 17β-hydroxyandrost-4-en-3-one **2** at C-6β, C-7β, C-14α and C-12β, accompanied by a 5α-reduction and oxidations at C-6 and at C-17 (Yildirim, Kuru, and Ş Yılmaz 2018). These results might have suggested that *U. chartarum* MRC 72584 metabolized **1** and **2** in some different ways due to the presence of a 17β-hydroxyl group in **2** (Kołek and Swizdor 1998).

Reports of fungal 5α-reduction, oxidations of hydroxyl groups following hydroxylations, the reduction of **1** at C-17 and hydroxylations of **1** at C-6β and C-7α are very common (Mahato and Garai 1997; Fernandes et al. 2003; Bhatti and Khera 2012; Donova and Egorova 2012; Nassiri-Koopaei and Faramarzi 2015). However, fungal hydroxylations of **1** at C-7β and C-15α are rare. For example, *Mucor racemosus* hydroxylated **1** at C-6β, C-7α, C-7β and C-11α, accompanied by a reduction at C-17 (Faramarzi et al. 2008). *Gongronella butleri* (Kollerov et al. 2008) hydroxylated **1** at C-6β, C-6α, C-7α, C-7β and C-14α whilst *Botryosphaeria obtusa* (Smith et al. 1990) hydroxylated **1** at C-6β and C-7β. In another work, *Gibberella saubinetti* hydroxylated **1** at C-6β and C-15α (Urech et al. 1960). In a recent work, *Colletotrichum lini* hydroxylated **1** at C-11α and C-15α (Wu et al. 2015).

In short, it was shown that *C. sphaerospermum* MRC 70266 and *U. chartarum* MRC 72584 metabolized **1** in different ways and 6β,17β-dihydroxyandrost-4-en-3,16-dione **8** was determined as a new metabolite. Our

Table 2. Metabolite yields following chromatography.

Fungus	Metabolite	%Yield
C. sphaerospermum MRC 70266	17β-Hydroxyandrost-4-en-3-one **2**	2
	5α-Androstane-3,6,17-trione **3**	3
	6β-Hydroxyandrost-4-en-3,17-dione **4**	37
	17β-Hydroxyandrost-4-en-3,16-dione **5**	5
	6β,17β-Dihydroxyandrost-4-en-3-one **6**	6
	15α-Hydroxyandrost-4-en-3,17-dione **7**	4
	6β,17β-Dihydroxyandrost-4-en-3,16-dione **8**	3
U. chartarum MRC 72584	5α-Androstane-3,6,17-trione **3**	13
	17β-Hydroxy-5α-Androstane-3,6-dione **9**	3
	14α-Hydroxyandrost-4-en-3,17-dione **10**	14
	7β-Hydroxyandrost-4-en-3,17-dione **11**	18
	7α-Hydroxyandrost-4-en-3,17-dione **12**	10

work on biotransformations of some other steroids by *C. sphaerospermum* MRC 70266, *U. chartarum* MRC 72584 and some other fungi is in progress.

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