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New metabolites from fungal biotransformation of an oral contraceptive agent: Methyloestrenolone

Salman Zafar [a,b], Marium Bibi [a], Sammer Yousuf [a], M. Iqbal Choudhary [a,c,*]

[a] H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan
[b] Department of Chemistry, Abdul Wali Khan University, Mardan 23200, Pakistan
[c] Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah 21412, Saudi Arabia

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ABSTRACT

Fungal cell cultures were used for the first time for the biotransformation of methyloestrenolone (**1**), an oral contraceptive. Fermentation of **1** with *Macrophomina phaseolina*, *Aspergillus niger*, *Gibberella fujikuroi*, and *Cunninghamella echinulata* produced eleven metabolites **2**–**12**, six of which **2**–**5**, **11** and **12** were found to be new. These metabolites were resulted from the hydroxylation at C-1, C-2, C-6, C-10, C-11, and C-17α-CH_3, as well as aromatization of ring A of the steroidal skeleton of substrate **1**. The transformed products were identified as 17α-methyl-6β,17β-dihydroxyestr-4-en-3-one (**2**), 17α-(hydroxymethyl)-11β,17β-dihydroxyestr-4-en-3-one (**3**), 17α-methyl-2α,11β,17β-trihydroxyestr-4-en-3-one (**4**), 17α-methyl-1β,17β-dihydroxyestr-4-en-3-one (**5**), 17α-methyl-11α,17β-dihydroxyestr-4-en-3-one (**6**), 17α-methyl-11β,17β-dihydroxyestr-4-en-3-one (**7**), 17α-methyl-10β,17β-dihydroxyestr-4-en-3-one (**8**), 17α-(hydroxymethyl)-17β-hydroxyestr-4-en-3-one (**9**), 17α-methylestr-1,3,5(10)-trien-3,17β-diol (**10**), 17α-methyl-3,17β-dihydroxyestr-1,3,5(10)-trien-6-one (**11**), and 17α-methyl-6β,10β,17β-trihydroxyestr-4-en-3-one (**12**).

1. Introduction

Microbial transformation has been extensively employed for the synthesis of fine chemicals for years [1]. Microbial biotransformation of steroids is a well established method for the large scale manufacturing of steroidal drugs [2].

Methyloestrenolone (**1**) belongs to the estrogen agonist pharmacological group. On the basis of mechanism of action, it is also classified as sex hormone. Compound **1** is one of the most effective inhibitors of progesterone formation during pregnancy [3]. No report concerning the microbial transformation of **1** has been published to date.

In the last decade, we have extensively studied the structural modifications of several steroids especially the steroidal drugs [4–23]. Here we report the biotransformation of another steroidal drug, methyloestrenolone (**1**) for the first time. Small scale experiments showed that *Macrophomina phaseolina*, *Aspergillus niger*, *Gibberella fujikuroi*, and *Cunninghamella echinulata* were able to efficiently transform **1** into a diverse array of metabolites. The substrate (**1**) was subjected to a variety of bioactivity screening tests viz. acetylcholinesterase, butyrylcholinesterase, urease, phosphodiesterase, and α-chymotrypsin enzymes inhibition, cytotoxicity (PC-3 cell lines), immunomodulatory, anti-glycation and anti-bacterial activities, but no significant activity was observed. The metabolites **2**–**12** were also evaluated for their acetylcholinesterase, butyrylcholinesterase and urease inhibitory potential but none of them showed any significant activity in available assays.

2. Experimental

2.1. General

Methyloestrenolone (**1**) was purchased from local market in the form of tablets (Gyanecosid, Efroze Chemical Industries, Pakistan). The substrate (**1**) was extracted from the tablets with dichloromethane and purified on silica gel column by using pet. ether/EtOAc (7:3) isocratic solvent system. Pure methyloestrenolone (**1**) was then used for experiments. Precoated TLC (silica gel, PF_{254}; 20 × 20, 0.25 mm) were purchased from Merck, Germany. Column chromatography was carried out by using silica gel (70–230 mesh, Merck). A Buchi-535 melting point apparatus was used to measure the melting points of compounds. A JASCO P-2000 polarimeter was employed to measure optical rotations in methanol. UV Absorption data (in nm) were obtained in methanol with a Hitachi U-3200 spectrophotometer. Infrared (IR) spectra (in cm^{-1})

* Corresponding author at: H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan. Tel.: +92 21 34824924; fax: +92 21 34819018/19.
E-mail address: iqbal.choudhary@iccs.edu (M.I. Choudhary).

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were recorded with an FT-IR-8900 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded in CD_3OD and deuterated DMSO on a Bruker Avance NMR spectrophotometer with solvent signal as the internal standard. Chemical shifts (δ values) were reported as ppm (parts per million), relative to TMS (0 ppm). The coupling constants (J values) were reported in hertz. JEOL JMS-600H mass spectrometer was used to record electron impact mass spectra (EI-MS) and high-resolution mass spectra (HREI-MS) in m/z (rel.%). Single-crystal X-ray diffraction study was performed on a Bruker Smart APEX II, CCD 4-K area detector diffractometer [24]. Data reduction was performed by using SAINT program. The structure was solved by direct method [25] and refined by full-matrix least squares on F2 by using the SHELXTL-PC package [26]. The figures were plotted with the aid of ORTEP program [27].

2.2. Microorganisms and culture medium

The fungi were obtained either from Karachi University Culture Collection (KUCC) or American Type Culture Collection (ATCC).

M. phaseolina (KUCC 730) and *C. echinulata* (ATCC 9244) were grown in a medium, composed of the following ingredients dissolved in distilled H_2O (4.0 L): glucose (40.0 g), glycerol (40.0 mL), peptone (20.0 g), yeast extract (20.0 g), KH_2PO_4 (20.0 g), and NaCl (20.0 g).

The medium (4 L) for *A. niger* (ATCC 10549) was the same as above, except 12 g yeast extract was used.

Four liters of fermentation medium for *G. fujikuroi* (ATCC 10704) was composed of glucose (80.0 g), NH_4NO_3 (4.0 g), KH_2PO_4 (20.0 g), $MgSO_4{\cdot}7H_2O$ (4.0 g) and trace element solution (8.0 mL). The *Gibberella* trace element solution was prepared by dissolving $Co(NO_3)_3$ (0.01 g), $FeSO_4{\cdot}7H_2O$ (0.1 g), $CuSO_4{\cdot}5H_2O$ (0.1 g), $ZnSO_4{\cdot}7H_2O$ (0.16 g), $MnSO_4{\cdot}7H_2O$ (0.01 g), $Mo(NH_4)_3$ (0.01 g) in 100 mL of distilled water.

2.3. General fermentation and extraction conditions

Two stage fermentation protocol was followed for all biotransformation experiments, which involves the preparation of seed flasks, followed by inoculation of all the remaining flasks. Extraction conditions were the same as that used previously in our laboratory [4,5,15].

2.4. Fermentation of methyloestrenolone (1) with M. phaseolina

1.0 g of methyloestrenolone (**1**) was used for fermentation with *M. phaseolina*. The fermentation was continued for 8 days. The crude extract, obtained after filtration and extraction, was fractionated on a silica gel column with pet. ether/ethyl acetate (10% gradient) solvent system to obtain five main fractions. The fractions were subjected to isocratic RP-HPLC (1:1 H_2O/MeOH) to obtain six pure compounds **2**–**7** (Fig. 1).

2.4.1. 17α-Methyl-6β,17β-dihydroxyestr-4-en-3-one (**2**)

Colorless crystalline solid (18 mg, 0.018%). Mp: 197–199 °C. UV (MeOH) λ_{max} nm (log): 237 (4.0). $[\alpha_D^{25}]$: − 76.2 (c = 0.08, MeOH). IR ($CHCl_3$) ν_{max} cm^{-1}: 3406, 1658. ^{1}H NMR (CD_3OD, 500 MHz): See Table 1. ^{13}C NMR (CD_3OD, 125 MHz): See Table 2. HREI-MS m/z (mol. formula, calcd value): 304.2032 ($C_{19}H_{28}O_3$, 304.2038). EI-MS m/z (rel. int.,%): 304 [M^+] (22), 286 (66), 253 (19), 228 (100), 215 (98), 159 (23), 122 (12), 91 (12), 55 (7), 43 (20). Crystal data: $C_{19}H_{88}O_3$, Mr = 304.41, Monoclinic, space group $P2_1$, a = 9.2433(6) Å, b = 9.4562(7) Å, c = 9.2871(6) Å, β = 90.8090(10), V = 1447.41(16) Å^3, Z = 2, ρ_{calc} = 1.246 mg/m^3, $F(000)$ = 332, μ (Mo Kα = 0.71073 Å, max/min transmission 0.9601/0.9539, crystal dimensions 0.58 × 0.50 × 0.50, 1.96° < θ < 25.5°, 4792 reflections were collected, of which 1608 reflections were observed (R_{int} = 0.0128). The R values were: R_1 = 0.0325, wR_2 = 0.0888 for $I > 2\sigma(I)$, and R_1 = 0.0329, wR_2 = 0.0896 for all data; max/min residual electron density: 0.193/−0.241 e Å^{-3}. Crystallographic data for compound **2** has been deposited in the Cambridge Crystallographic Data Center. The crystallographic information can directly be obtained free of charge from CCDC data center (CCDC 836387 reference code).

2.4.2. 17α-(Hydroxymethyl)-11β,17β-dihydroxyestr-4-en-3-one (**3**)

Colorless crystalline solid (8.0 mg, 0.008%). Mp: 114–116 °C. UV (MeOH) λ_{max} nm (log): 243 (4.3). $[\alpha_D^{25}]$: +88.0 (c = 0.1, MeOH). IR ($CHCl_3$) ν_{max} cm^{-1}: 3410, 1657. ^{1}H NMR (CD_3OD, 500 MHz): See Table 1. ^{13}C NMR (CD_3OD, 125 MHz): See Table 2. HREI-MS m/z (mol. formula, calcd value): 320.1992 ($C_{19}H_{28}O_4$, 320.1988). EI-MS m/z (rel. int.,%): 320 [M^+] (21), 302 (18), 289 (13), 271 (32), 228 (29), 215 (34), 159 (13), 105 (13), 85 (64), 83 (95), 44 (100).

2.4.3. 17α-Methyl-2α,11β,17β-trihydroxyestr-4-en-3-one (**4**)

Colorless amorphous solid (6.0 mg, 0.006%). UV (MeOH) λ_{max} nm (log): 242 (4.0). $[\alpha_D^{25}]$: +131.0 (c = 0.1, MeOH). IR ($CHCl_3$) ν_{max} cm^{-1}: 3416, 1674. ^{1}H NMR (CD_3OD, 500 MHz): See Table 1. ^{13}C NMR (CD_3OD, 125 MHz): See Table 2. HREI-MS m/z (mol. formula, calcd value): 320.1986 ($C_{19}H_{28}O_4$, 320.1988). EI-MS m/z (rel. int.,%): 320 [M^+] (28), 302 (100), 284 (45), 276 (91), 244 (96), 232 (48), 226 (61), 200 (73), 185 (42), 159 (31), 121 (60), 105 (37), 91 (42), 43 (50).

2.4.4. 17α-Methyl-1β,17β-dihydroxyestr-4-en-3-one (**5**)

Colorless crystalline material (45.0 mg, 0.045%). Mp: 203–205 °C. UV (MeOH) λ_{max} nm (log): 239 (4.2). $[\alpha_D^{25}]$: +18.3° (c = 0.12, MeOH). IR ($CHCl_3$) ν_{max} cm^{-1}: 3410, 1663. ^{1}H NMR (CD_3OD, 500 MHz): See Table 1. ^{13}C NMR (CD_3OD, 125 MHz): See Table 2. HREI-MS m/z (mol. formula, calcd value): 304.2043 ($C_{19}H_{28}O_3$, 304.2038). EI-MS m/z (rel. int.,%): 304 [M^+] (10), 286 (100), 247 (30), 213 (72), 160 (53), 121 (10), 91 (10), 55 (7), 43 (16).

2.4.5. 17α-Methyl-11α,17β-dihydroxyestr-4-en-3-one (**6**)

Amorphous material (4.5 mg, 0.0045%). UV (MeOH) λ_{max} nm (log): 241 (4.2). $[\alpha_D^{25}]$: +81.4 (c = 0.09, MeOH). IR ($CHCl_3$) ν_{max} cm^{-1}: 3410, 1655. ^{1}H NMR (CD_3OD, 500 MHz): See Table 1. ^{13}C NMR (CD_3OD, 125 MHz): See Table 2. HREI-MS m/z (mol. formula, calcd value): 304.2043 ($C_{19}H_{28}O_3$, 304.2038). EI-MS m/z (rel. int.,%): 304 [M^+] (55), 286 (56), 247 (100), 228 (60), 213 (39), 159 (29), 123 (39), 110 (63), 91 (33), 71 (36), 55 (16), 43 (38).

2.4.6. 17α-Methyl-11β,17β-dihydroxyestr-4-en-3-one (**7**)

Colorless crystalline material (14.0 mg, 0.014%). Mp: 224–225 °C (Reported 219–224 °C [28]). UV (MeOH) λ_{max} nm (log): 239 (4.1). $[\alpha_D^{25}]$: +61.4 (c = 0.14, MeOH). IR ($CHCl_3$) ν_{max} cm^{-1}: 3398, 1657. ^{1}H NMR (CD_3OD, 500 MHz): See Table 1. ^{13}C NMR (CD_3OD, 125 MHz): See Table 2. HREI-MS m/z (mol. formula, calcd value): 304.2043 ($C_{19}H_{28}O_3$, 304.2038). EI-MS m/z (rel. int.,%): 304 [M^+] (12), 286 (71), 268 (17), 228 (100), 215 (45), 159 (18), 121 (12), 91 (42), 55 (7).

2.5. Fermentation of methyloestrenolone (1) with A. niger

Incubation of 1 g (in 20 mL acetone/40 flasks) of compound **1** with a fully grown culture of *A. niger* was carried out for 14 days. 2.5 g of a brown gum was obtained after extraction. The extract was subjected to fractionation on silica gel column with pet. ether–ethyl acetate (10% gradient) solvent system to obtain three main fractions which on repeated isocratic RP-HPLC (1:1 H_2O/MeOH) yielded three pure compounds **2**, **8** and **9** (Fig. 2). Metabolite **2** had also been obtained by fermentation with *M. phaseolina*, as discussed above (Fig. 1).

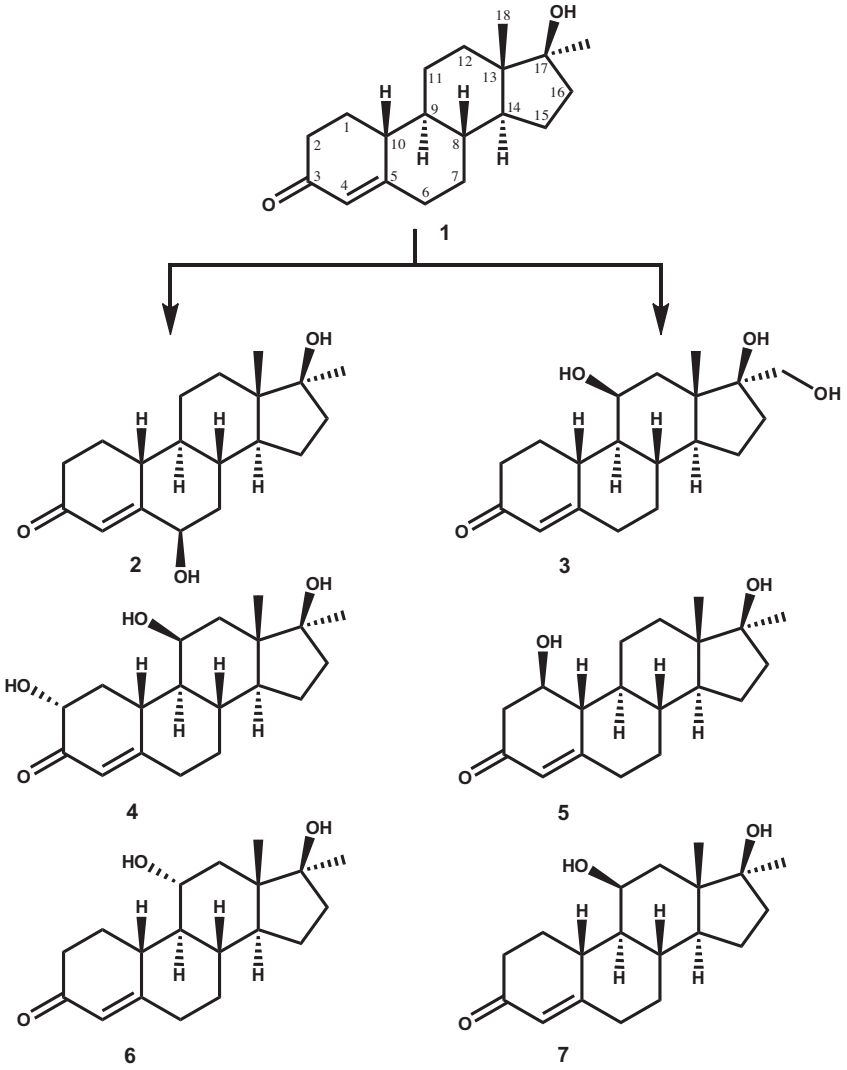

Fig. 1. Biotransformation of methyloestrenolone (**1**) with *Macrophomina phaseolina*.

*2.5.1. 17α-Methyl-10β,17β-dihydroxyestr-4-en-3-one (**8**)*

Colorless crystalline material (8.0 mg, 0.008%). Mp: 190–192 °C (Reported 182–190 °C [29]). UV (MeOH) λ_{max} nm (log): 230 (4.1). $[\alpha_D^{25}]$: +51.2 (c = 0.08, MeOH) (Reported = $[\alpha_D^{25}]$ = + 44.6 (c 0.269, dioxane) [29]). IR ($CHCl_3$) ν_{max} cm^{-1}: 3433, 1662. ^1H NMR (DMSO-d_6, 500 MHz): See Table 1. ^{13}C NMR (DMSO-d_6, 125 MHz): See Table 2. HREI-MS m/z (mol. formula, calcd value): 304.2043 ($C_{19}H_{28}O_3$, 304.2038). EI-MS m/z (rel. int.,%): 304 [M^+] (87), 286 (67), 228 (83), 216 (100), 215 (87), 160 (37), 124 (43), 91 (26), 55 (24), 43 (43).

*2.5.2. 17α-(Hydroxymethyl)-17β-hydroxyestr-4-en-3-one (**9**)*

Colorless crystalline material (12.0 mg, 0.012%). Mp: 136–142 °C. (Reported Mp: 140 °C) $[\alpha_D^{25}]$ +26.5 ($CHCl_3$) [30]) UV (MeOH) λ_{max} nm (log): 239 (3.9). IR ($CHCl_3$) ν_{max} cm^{-1}: 3406, 1658. ^1H NMR (CD_3OD, 500 MHz): See Table 1. ^{13}C NMR (CD_3OD, 125 MHz): See Table 2. HREI-MS m/z (mol. formula, calcd value): 304.2043 ($C_{19}H_{28}O_3$, 304.2038). EI-MS m/z (rel. int.,%): 304 [M^+] (87), 286 (44), 273 (100), 255 (73), 231 (25), 215 (85), 159 (24), 91 (35), 55 (15), 41 (17). Single-crystal X-ray diffraction analysis [31].

2.6. Fermentation of methyloestrenolone (1) with G. fujikuroi

Incubation of **1** (1 g/20 mL acetone) with *G. fujikuroi* culture in 40 flasks for 11 days produced three metabolites **7**, **10** (4:6 Pet. ether/EtOAc), and **11** (7:3 Pet. ether/EtOAc) (Fig. 3) which were purified by column chromatography (silica gel).

*2.6.1. 17α-Methylestr-1,3,5(10)-trien-3,17β-diol (**10**)*

Colorless crystalline solid (14.0 mg, 0.014%). Mp: 190–191 °C (Reported 184–186 °C [32]). $[\alpha_D^{25}]$: +37.2 (c = 0.11, MeOH). IR ($CHCl_3$) ν_{max} cm^{-1}: 3344, 3300, 1610, 1498. ^1H NMR (CD_3OD, 500 MHz): See Table 1. ^{13}C NMR (CD_3OD, 125 MHz): See Table 2. HREI-MS m/z (mol. formula, calcd value): 286.1937 ($C_{19}H_{26}O_2$, 286.1933). EI-MS m/z (rel. int.,%): 286 [M^+] (35), 272 (100), 244 (6), 213 (51), 186 (18), 172 (33), 160 (40), 146 (28), 133 (20), 107 (98), 91 (6).

*2.6.2. 217α-Methyl-3,17β-dihydroxyestr-1,3,5(10)-trien-6-one (**11**)*

Colorless crystalline solid (10.0 mg, 0.01%). Mp; 219–221 °C. UV (MeOH) λ_{max} nm (log): 245 (4.2). $[\alpha_D^{25}]$: −28.0 (c = 0.1, MeOH). IR ($CHCl_3$) ν_{max} cm^{-1}: 3410, 1664, 1606. ^1H NMR (CD_3OD, 500 MHz): See Table 1. ^{13}C NMR (CD_3OD, 125 MHz): See Table 2. HREI-MS m/z (mol. formula, calcd value): 300.1782 ($C_{19}H_{24}O_3$, 300.1773). EI-MS m/z (rel. int.,%): 300 [M^+] (33), 272 (100), 244 (7), 213 (51), 186 (18), 172 (33), 160 (40), 146 (28), 133 (20), 107 (98), 91 (6).

Table 1
Selected ^{1}H NMR chemical shifts of compounds **1–12** (δ in ppm; J in Hz; $W_{1/2}$ in Hz).

	COMPOUNDS											
C	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[a]	**6**[a]	**7**[a]	**8**[b]	**9**[a]	**10**[a]	**11**[a]	**12**[a]
1	2.26, m; 2.37, m	1.36[c], m; 2.29[c], m	1.57, m; 2.32[c], m	1.42[c], m; 2.52[c], m	4.41, q (3.0)	2.28, m; 2.43, m	2.29, m; 2.35, m	1.77, m; 2.04, m	1.36, m; 1.96, m	7.06, d (8.0)	7.33, d (8.5)	1.88, m; 2.18, m
2	1.73, m; 2.21, m	2.31, m; 2.36, m	2.28, m; 2.37, m	4.13, m	2.57, d (3.0)	2.23, m; 2.41, m	1.75, qd (14.0, 3.5); 2.24, m	2.12, m; 2.49, m	2.28, m; 2.37, m	6.52, dd (8.5, 2.5)	7.00, dd (8.5, 2.5)	2.28, dt (8.2, 2.2), 2.64, ddd (8.7, 6.2, 2.5)
3	–	–	–	–	–	–	–	–	–	–	–	–
4	5.80, m	5.85, d (2.0)	5.82, s	5.80, t (1.5)	5.83, t (2.0)	5.78, s	5.82, s	5.61, s	5.78, s	6.46, d (2.5)	7.36, d (3.0)	5.81, s
5	–	–	–	–	–	–	–	–	–	–	–	–
6	2.30, m; 2.45, m	4.28, t (2.5)	2.31[c], m; 2.47, dt (14.0, 3.0)	2.28[c], td (14.0, 5.0); 2.47[c], dt (14.5, 3.0)	2.35[c], m; 2.53, dt (15.0, 3.5)	2.38, m; 2.47, dq (13.5, 2.0)	2.31, m; 2.48, dt (6.0, 3.0)	2.18[c], m; 2.56[c], m	2.27, m; 2.38, m	2.73, m; 2.79, m	–	4.38, t (1.5)
7	1.01, m; 1.82, m	1.25[c], m; 1.96[c], m	1.05, qd (13.5, 4.0); 1.88, m	1.05, qd (13.0, 4.0); 1.91, m	1.05, m; 1.80, m	1.07, m; 1.93, m	1.05, qd (13.0, 4.0); 1.92, m	0.89[c], m; 1.73[c], m	1.61, m; 1.96, m	1.26, m; 1.85, m	2.24, dd (16.5, 13.0); 2.63, dd (17.0, 3.5)	1.25, m; 2.02, dt (10.5, 7.0)
8	1.75, m	1.92, m	1.75, qd (11.0, 3.5)	1.75, qd (11.5, 3.5)	1.46, m	1.52, qd (11.0, 3.0)	2.34, m	1.72, m	2.19, m	1.39, m	1.91, m	2.14, m
9	0.82, m	0.85, m	0.98, td (11.0, 3.5)	0.96, td (11.0, 3.5)	1.34[c], m	1.03, m	0.96, td (11.0, 3.0)	0.93, m	0.85, qd (10.5, 5.0)	2.10, m	2.45, m	1.05, td (5.7, 2.2)
10	2.10, m	2.55, m	2.63, br. t (10.5)	2.82[c], m	2.33[c], m	2.42, m	2.62, m	4.93,s (OH)	2.50, dt (14.5, 3.0)	–	–	–
11	1.62, m; 1.87, m	1.62[c], m; 1.87[c], m	4.16, q (3.0)	4.15, m	1.38[c], m; 1.96, m	3.83, td (10.5, 5.5)	4.18, m ($W_{1/2}$ = 10 Hz)	1.53, m; 1.55, m	1.05, m; 1.55, m	1.45, m; 2.30, m	1.58, m; 2.40, m	1.60, m; 1.70, m
12	1.37, m; 1.54, m	1.37, m; 1.54, m	1.42, m; 1.55, m	1.48, dd (14.0, 3.5); 1.82, dd (14.0, 2.5)	1.39[c], m; 1.55, m	1.30, m; 1.85, m	1.48, dd (14.0, 3.5); 1.82, dd (14.0, 2.5)	1.18, m; 1.43, m	1.36, m; 1.64, m	1.47, m; 1.63, m	1.57, m; 1.70, m	1.35, m; 1.57, m
13	–	–	–	–	–	–	–	–	–	–	–	–
14	0.82, m	1.27, m	1.35, m	1.26, m	1.33[c], m	1.35, m	1.28, m	1.15, m	1.37, m	1.40, m	1.64, m	1.32, m
15	1.28, m; 1.55, m	1.28, m; 1.57, m	1.39, m; 1.58, m	1.37, m; 1.62, m	1.34[c], m; 1.62, m	1.32, m 1.62, m	1.40, m; 1.62, m	1.15, m; 1.47, m	1.37, m; 1.62, m	1.34, m; 1.65, m	1.34, m; 1.65, m	1.32, m; 1.62, m
16	1.64, m; 1.85, m	1.67[c], m; 1.85[c], m	1.59, m; 1.92, m	1.65[c], m; 1.88[c], m	1.66, m; 1.88, m	1.69, m; 1.88, m	1.65, m; 1.87, m	1.48, m; 1.74, m	1.84, m; 1.94, m	1.70, m; 1.89, m	1.72, m; 1.89, m	1.66, m; 1.86, m
17	–	–	–	–	–	–	–	4.03, s (OH)	–	–	–	–
18	0.90, s	0.93, s	1.14, s	1.12, s	0.93, s	0.91, s	1.12, s	0.77, s	0.95, s	0.88, s	0.88, s	0.93, s
17α-CH_3	1.20, s	1.18, s	3.38, d (11.0) 3.57, d (11.5)	1.16, s	1.19, s	1.20, s	1.16, s	1.05, s	3.42, d (11.0); 3.60, d (11.0)	1.23, s	1.26, s	1.18, s

[a] 500 MHz, CD_3OD.
[b] 500 MHz, DMSO-D_6.
[c] Exchangeable assignments.

Table 2
^{13}C NMR Chemical shifts of compounds **1–12**.

	COMPOUNDS											
C	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[a]	**6**[a]	**7**[a]	**8**[b]	**9**[a]	**10**[a]	**11**[a]	**12**[a]
1	26.9	27.1	27.0	35.7	66.3	28.5	27.1	33.0	27.0	127.2	127.8	34.0
2	37.3	37.0	37.3	73.0	46.9	36.5	37.1	33.5	37.0	113.8	122.5	34.8
3	199.6	203.9	203.0	202.1	201.7	204.1	203.1	198.5	202.5	156.0	157.0	202.8
4	124.5	125.6	124.8	122.7	124.7	124.5	124.5	123.1	125.1	116.0	113.2	126.0
5	166.6	169.1	172.1	171.0	166.1	172.1	172.1	165.0	171.0	139.0	134.5	162.5
6	36.4	72.6	36.4	35.5	36.2	37.8	36.2	31.5[c]	36.5	30.5	200.8	73.8
7	32.4	39.3	32.4	31.9	30.7	33.5	32.2	31.2[c]	33.8	28.6	45.0	39.5
8	37.1	35.7	37.1	36.9	41.9	42.2	37.2	35.5	43.9	41.2	42.2	31.2
9	55.1	50.8	55.1	55.8	43.9	55.8	55.1	52.7	50.9	45.1	43.8	54.2
10	42.5	39.6	39.0	39.2[c]	48.4	45.3	39.1	69.1	35.5	132.5	140.0	72.0
11	27.2	27.2	67.4	67.1	26.5	73.0	68.0	19.5	27.3	27.2	26.8	21.0
12	32.6	32.6	40.1	40.0	32.5	44.0	40.0	30.7	32.5	32.8	32.4	32.4
13	45.6	47.0	46.4	46.4	46.8	47.2	46.0	45.0	46.5	47.0	46.8	46.8
14	49.5	50.7	52.2	51.8	50.8	50.0	51.9	49.0	51.2	51.0	50.6	50.8
15	23.9	23.9	24.3	23.9	24.0	24.1	24.2	23.5	24.4	24.0	23.7	24.2
16	39.0	39.2	33.5	39.1[c]	39.1	39.1	39.0	38.1	32.2	39.0	39.1	39.2
17	81.3	82.3	84.5	82.5	82.3	81.5	82.0	79.5	84.0	82.0	82.0	82.0
18	14.8	14.7	17.5	17.1	14.6	15.5	17.0	14.0	15.0	14.8	14.2	14.5
17α-CH_3	26.2	26.1	67.8	26.4	26.0	25.6	26.2	26.5	67.8	26.0	26.0	25.8

[a] 125 MHz, CD_3OD.
[b] 125 MHz, DMSO-D_6.
[c] Exchangeable assignments.

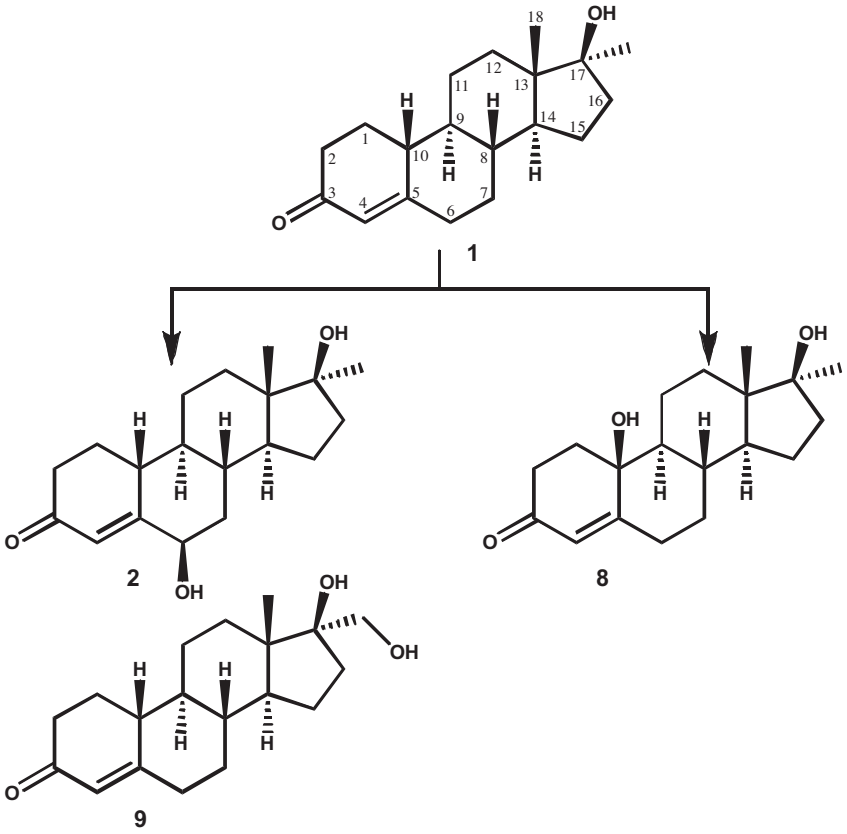

Fig. 2. Biotransformation of methyloestrenolone (**1**) with *Aspergillus niger*.

2.7. Fermentation of methyloestrenolone (1) with C. echinulata

900 mg of compound **1** (20 mL acetone) was fed equally to 40 cultured flasks of *C. echinulata,* yielding metabolites **6** (6:4 Pet. ether/EtOAc) and **12** (1:1 Pet. ether/EtOAc) after 12 days of fermentation (Fig. 4). Metabolite **12** was found to be a new compound.

2.7.1. 17α-Methyl-6β,10β,17β-trihydroxyestr-4-en-3-one (**12**)

Colorless amorphous solid (5.0 mg, 0.006%). $[\alpha_D^{25}]$: +15 (c = 0.0018, MeOH). UV (MeOH) λ_{max} nm (log): 239 (4.2). IR ($CHCl_3$) ν_{max} cm^{-1}: 3399, 1658. ^{1}H NMR (CD_3OD, 500 MHz): See Table 1. ^{13}C NMR (CD_3OD, 125 MHz): See Table 2. HREI-MS m/z (mol. formula, calcd value): 320.2033 ($C_{19}H_{28}O_4$, 320.2046). EI-MS m/z

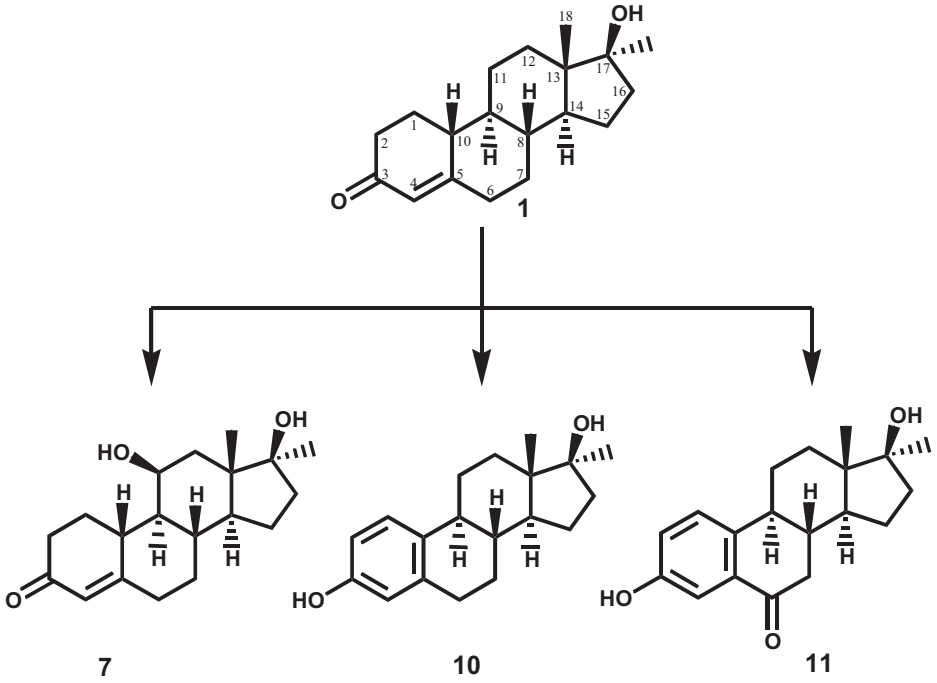

Fig. 3. Biotransformation of methyloestrenolone (**1**) with *Gibberella fujikuroi*.

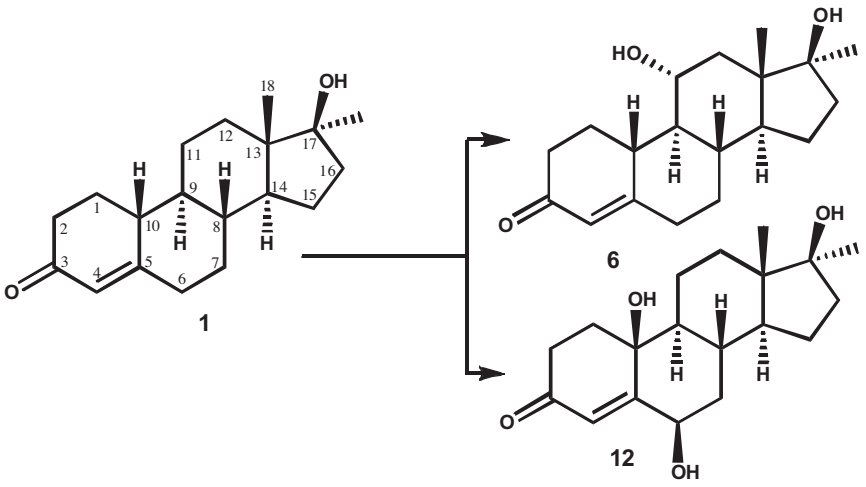

Fig. 4. Biotransformation of methyloestrenolone (**1**) with *Cunninghamella echinulata*.

(rel. int.,%): 320 [M^+] (35), 286 (71), 268 (17), 228 (100), 215 (45), 159 (18), 121 (12), 91 (42), 55 (7).

3. Results and discussion

Microbial transformation of the oral contraceptive, methyloestrenolone (**1**), $C_{19}H_{28}O_4$, was investigated for the first time. Fermentation of **1** with *M. phaseolina*, *A. niger*, *G. fujikuroi* and *C. echinulata* yielded eleven metabolites **2**–**12** (Figs. 1–4). Metabolites **2**–**5**, **11** and **12** were found to be new which are discussed below in detail.

The composition of metabolite **2** was deduced as $C_{19}H_{28}O_3$ (M^+ *m*/*z* 304.2032, calcd 304.2038), suggesting the incorporation of an oxygen to the substrate **1**. The UV analysis indicated the retention of chromophore (λ_{max} = 241 nm), while hydroxyl (3410 cm^{-1}) and conjugated ketone (1660 cm^{-1}) functionalities were deduced from the IR spectrum.

The ^{1}H NMR spectrum (Table 1) of **2** showed a downfield methine signal at δ 4.28 as a triplet ($J_{6e,7a,e}$ = 2.5 Hz), with corresponding carbon at δ 72.6 in the ^{13}C NMR spectrum (Table 2). The C-4 olefinic proton resonated as a close doublet at δ 5.85 ($J_{4,6e}$ = 2.0 Hz), due to its weak allylic couplings with C-6 allylic proton (δ 4.28), which is geminal to the hydroxyl group. The proton at δ 4.28 also showed HMBC correlations with C-4 (δ 125.6), and C-10 (δ 39.6). It showed vicinal coupling with H_2-7 (δ 1.25, δ 1.96) in COSY spectrum. The carbon at δ 72.6 was HMBC correlated with H-4 (δ 5.85) and H-8 (δ 1.92). From the above observations, the hydroxyl-bearing methine carbon was identified as C-6. H-6 did not show any NOESY interaction with H-10 (δ 2.55) and H-8 (δ 1.92), therefore it was assigned an α-orientation. Finally the structure of **2** was identified as a new compound, 17α-methyl-6β,17β-dihydroxyestr-4-en-3-one (**2**). The assigned structure was unambiguously deduced by single-crystal X-ray diffraction studies

(Fig. 5). The ORTEP diagram of compound **2** (Fig. 5) showed that molecule consists of four fused rings A (C1–C5/C10), B (C5–C10), C (C8–C9/C11–C14) and D (C13–C17). Ring A exists in a half chair conformation, whereas *trans* fused rings B and C are in chair conformations. Ring D is folded like an envelope. The hydroxy substituents at C-6 and C-17 adopt *axial* and *pseudo equatorial* orientations, respectively. In the crystal structure, the molecules are linked by O_2–$H_{2A}\cdots O_3$ and O_3–$H_{2A}\cdots O_2$ interactions to form infinite chains running parallel to *a*-axis (Fig. 6). The bond dimensions (bond lengths and angles) were in normal range and similar to those found in structurally related steroidal compounds [31,33].

The HREI-MS analysis of metabolite **3** supported the molecular composition $C_{19}H_{28}O_4$ [$M^+ = m/z$ 320.1992 (calcd 320.1988)], with 32 amu increment from substrate **1,** suggesting the incorporation of two oxygen atoms. An enone (1657 cm^{-1}, λ_{max} 238 nm), and hydroxyl functionality (3410 cm^{-1}) were deduced from the UV and IR spectral analyses, respectively.

The ^{1}H NMR spectrum of **3** displayed a hydroxyl-bearing methine proton signal at δ 4.16 (q, $J_{9a,12a,e}$ = 3.0 Hz) and downfield methylene protons at δ 3.38 (d, $J_{20a,20b}$ = 11.0 Hz) and 3.57 (d, $J_{20b,20a}$ = 11.5 Hz), while the ^{13}C NMR spectrum (Table 2) displayed corresponding peaks at δ 67.4 (CH) and 67.8 (CH_2), respectively. The spectrum showed only a single methyl carbon signal at δ 17.5. This suggested the hydroxylation at the other methyl group, most probably C-17α-CH_3. This initial inference was further supported by the HMBC correlations of the methylene protons (δ 3.38 and 3.57) with C-16 (δ 33.5).

The other hydroxyl-bearing methine proton (δ 4.16) showed HMBC cross peaks with C-9 (δ 55.1) and C-13 (δ 46.4). It was therefore assigned to C-11 proton. H-11 (δ 4.16) also showed vicinal couplings in COSY spectrum with H-9 (δ 0.98) and H-12 (δ 1.42, δ 1.55), further confirming the assignment. The stereochemistry at C-11 was deduced by 2D-NOE techniques. H-10 (δ 2.63) showed NOE interaction with H-8 (δ 1.75), which in turn showed NOE with H-18 (δ 1.14). All the above protons are axially oriented, and none showed any NOE correlation with H-11 (δ 4.16). This suggested that H-11 is probably equatorially oriented (α). The new compound was thus identified as 17α-(hydroxymethyl)-11β,17β-dihydroxyestr-4-en-3-one (**3**).

Molecular composition of **4** was deduced as $C_{19}H_{28}O_4$ (M^+ m/z 320.1986, calcd 320.1988), which also suggested the incorporation of two oxygen atoms in substrate **1**. The presence of hydroxyl functional group (3416 cm^{-1}) and an enone system (λ_{max} = 242 nm, 1674 cm^{-1}) was inferred from the IR and UV spectral analyses.

The ^{1}H NMR spectra of metabolite **4** (Table 1) showed two hydroxyl-bearing methine proton signals at δ 4.13 (m) and 4.15 (m). Their corresponding carbons were resonated at δ 73.0 and 67.1, respectively, in the ^{13}C NMR spectrum (Table 2). The chemical shift and multiplicity of the methine proton signal at δ 4.15 was similar to the H-11 of the metabolite **3**. Thus after careful examination of the HMBC and COSY spectra, the –OH was placed at C-11 with a α-oriented geminal methine proton. The other hydroxyl-bearing methine C-2 (δ 73.0) was HMBC correlated with H_2-1 (δ 1.42, δ 2.52) and H-4 (δ 5.80). On the other hand, H-2 (δ 4.13) showed HMBC cross peaks with C-3 (δ 202.1) and C-10 (δ 39.2). Based on these observations, this -OH was placed at C-2. The COSY cross peaks of H-2 (δ 4.13) with H_2-1 (δ 1.42, 2.52) further supported this inference. The H-2 (δ 4.13) showed NOE interaction with H-10 (δ 2.82), which in turn showed NOE with H-8 (δ 1.75). Thus H-2 was deduced to be β-oriented. The structure of this new metabolite **4** was elucidated as 17α-methyl-2α,11β,17β-trihydroxyestr-4-en-3-one.

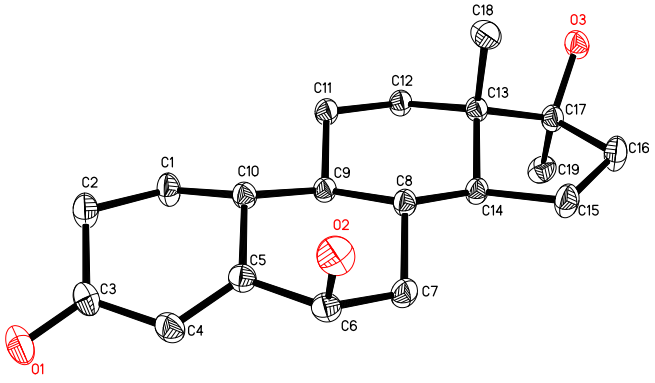

Fig. 5. The molecular structure of the compound **2** with displacement ellipsoids drawn at 30% probability level. Hydrogen atoms are omitted for clarity.

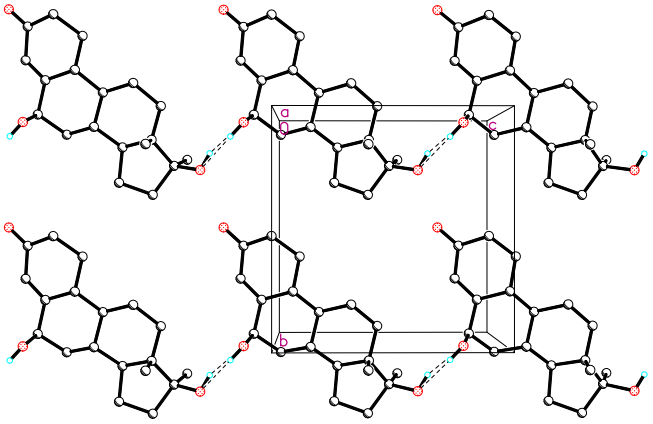

Fig. 6. The crystal packing of compound **2**.

Metabolite **5** with M^+ at m/z 304.2043 (calcd 304.2038) was in agreement with the formula $C_{19}H_{28}O_3$. This suggested the incorporation of an oxygen atom into substrate **1**. The presence of an enone moiety in ring A was inferred from the UV spectrum (λ_{max} = 239 nm), while the IR spectrum exhibited absorption bands at 3410 (OH) and 1663 cm^{-1} (C=C–C=O).

The ^{1}H NMR spectrum of **5** (Table 1) showed a methine proton signal at δ 4.41 (q, $J_{1a,2a,e,10a}$ = 3.0 Hz), with corresponding carbon at δ 66.3 in the ^{13}C NMR spectrum (Table 2). From the HMQC, COSY and HMBC spectral analyses, the methine signals (δ 4.41/δ 66.3) were assigned to H/C-1. The H-1 (δ 4.41) showed HMBC correlations with C-3 (δ 201.7), C-5 (δ 166.1) and C-10 (δ 48.4). Moreover, the position of this methine proton (δ 4.41) was deduced by COSY cross peaks of H-1 (δ 4.41) with H_2-2 (δ 2.57) and H-10 (δ 2.33). The stereochemistry at C-1 was determined by NOESY spectral analyses. The H-1 showed strong NOESY interaction with H-9 (δ 1.34), which indicated that H-1 is *axial* and α-oriented. This new metabolite **5** was thus identified as 17α-methyl-1β,17β-dihydroxyestr-4-en-3-one.

The HREI-MS supported the molecular composition of **11** as $C_{19}H_{24}O_3$. The M^+ appeared at m/z 300.1782 (calcd 300.1773). The compound showed a UV absorption (λ_{max}) at 245 nm. The IR spectrum showed major absorptions at 3410 (OH), 1664 (C=O) and 1606 (C=C) cm^{-1}.

The ^{1}H NMR spectrum of metabolite **11** (Table 1) was distinctly similar to metabolite **10**. The spectrum showed protons in the aromatic region at δ 7.00 (dd, $J_{1,2}$ = 8.5 Hz, $J_{2,4}$ = 2.5 Hz), 7.33 (d, $J_{2,1}$ = 8.5 Hz) and 7.36 (d, $J_{4,2}$ = 3.0 Hz). The ^{13}C NMR spectrum (Table 2) showed the corresponding aromatic carbon signals at δ 122.5, 127.8, and 113.2, respectively. An additional carbon signal at δ 200.8 was for ketonic carbonyl group. Comparison with the NMR data of metabolite **10** indicated the aromatization of ring A.

These aromatic carbons were identified as C-1 (δ 127.8), C-2 (δ 122.5), and C-4 (δ 113.2), based on COSY and HMBC interactions. H-1 (δ 7.33) and H-8 (δ 1.91) showed HMBC cross peaks with the ketonic carbonyl carbon (δ 200.8), therefore it was assigned to the C-6. The rest of the structural features were distinctly similar with metabolite **10**. Thus the new metabolite **11** was characterized as 17α-methyl-3,17β-dihydroxyestr-1,3,5(10)-trien-6-one.

The molecular composition of **12** was found to be $C_{19}H_{28}O_4$ [$M^+ = m/z$ 320.2033, calcd 320.2046], suggesting dihydroxylation, as in cases of metabolites **3** and **4**. The UV spectrum showed absorption maximum at 239 nm. The IR spectrum displayed absorptions at 3399 (OH) and 1658 (C=O) cm^{-1}.

The ^{1}H NMR spectrum of compound **12** (Table 1) showed a deshielded triplet at δ 4.38 ($J_{6e,7a,e}$ = 1.5 Hz) with corresponding carbon appearing at δ 73.8 in the ^{13}C NMR spectrum (Table 2). This observation was very similar to metabolite **2**. The hydroxylation was thus proposed to have occurred at C-6 (α-oriented). Another downfield quaternary carbon also appeared at δ 72.0. Rest of the spectrum did not show any major difference with the substrate **1**. The downfield methine carbon (δ 73.8) and the quaternary carbon (δ 72.0) were HMBC correlated with the C-4 olefinic proton (δ 5.81). The HMBC of H-6 (δ 4.38) with the downfield quaternary carbon (δ 72.0) suggested that the other hydroxyl group was substituted at C-10 of the steroidal skeleton. C-10 (δ 72.0) was also HMBC correlated with H_2-2 (δ 2.28, dt, $J_{2a,2e}$ = 8.2 Hz, $J_{2a,1a/1e}$ = 2.2 Hz, δ 2.64, ddd, $J_{2e,2a}$ = 8.7 Hz, $J_{2e,1a}$ = 6.2 Hz, $J_{2e,1e}$ = 2.5 Hz) which also supported the assignment. The structure of a new metabolite **12** was finally deduced as 17α-methyl-6β,10β,17β-trihydroxyestr-4-en-3-one.

Metabolites **6**–**10** were characterized as known compounds based on detailed spectral analyses. Compound **6** has been earlier obtained *via* the oxygenation of steroids by Mucorales fungi, reported in a patent obtained by the Upjohn Co. in 1955 [34]. Metabolite **7** was prepared by Magerlein et. al., in 1961 through several step synthetic manipulation of 3-methoxy-1,3,5(10)-estratrien-17-one [28]. Sasaki synthesized metabolite **8** from 3,3-ethylenedioxy-5(10)-estren-17β-ol through several synthetic steps [29]. 3,17- and 20 steroid ketones produced metabolite **9** upon synthetic functional group modifications, which was patented in 1964 [30]. Metabolite **10** was a synthetic product of estrone, reported by Haack et al. [32].

In conclusion, we report here an efficient method for the transformation of an oral contraceptive steroid, methyloestrenolone (**1**), by using fungal cultures. This strategy can be used effectively for the synthesis of libraries of new oral contraceptive steroids by the *in vitro* metabolism.

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