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Aspergillus niger-mediated biotransformation of methenolone enanthate, and immunomodulatory activity of its transformed products

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ABSTRACT

Two fungal cultures *Aspergillus niger* and *Cunninghamella blakesleeana* were used for the biotransformation of methenolone enanthate (**1**). Biotransformation with *A. niger* led to the synthesis of three new (**2–4**), and three known (**5–7**) metabolites, while fermentation with *C. blakesleeana* yielded metabolite **6**. Substrate **1** and the resulting metabolites were evaluated for their immunomodulatory activities. Substrate **1** was found to be inactive, while metabolites **2** and **3** showed a potent inhibition of ROS generation by whole blood (IC_{50} = 8.60 and 7.05 μg/mL), as well as from isolated polymorphonuclear leukocytes (PMNs) (IC_{50} = 14.0 and 4.70 μg/mL), respectively. Moreover, compound **3** (34.21%) moderately inhibited the production of TNF-α, whereas **2** (88.63%) showed a potent inhibition of TNF-α produced by the THP-1 cells. These activities indicated immunomodulatory potential of compounds **2** and **3**. All products were found to be non-toxic to 3T3 mouse fibroblast cells.

1. Introduction

Microbial biotransformations have been extensively employed in the drug discovery and development mainly due to their ability to produce regio- and stereo-selective products [1–4]. In many cases, the use of toxic and expensive chemical catalysts has been substituted by biotransformation which is eco-friendly and cost effective, and based on readily available biological catalysts. Diverse classes of organic compounds have been successfully transformed into their structurally novel, and biologically active metabolites by applying biotransformation methods [5,6]. Steroids due to their chemically inactive, and inaccessible hydrocarbon skeleton are difficult to be modified using conventional synthetic methodologies. However, biotransformation techniques have been successfully used for structural modifications of steroids [7–12]. Anabolic-androgenic steroids have attracted the attention of biomedical researchers since decades both because of their ability to enhance strength and mass of muscles in athletes [13], and their potential to treat diseases, such as hormonal and skin disorders [14].

Inflammation is a complex biological response of host against invading microorganisms, including bacteria, viruses, fungi, etc. Diseases such as inflammatory bowel disease, rheumatoid arthritis, and other autoimmune disorders are due to chronic inflammatory conditions. Steroids, particularly glucocorticoids, have been extensively used for the treatment of acute, and chronic inflammatory disorders. However, many of these steroidal anti-inflammatory drugs reported to cause various side effects, including hypertension, gastrointestinal ulcers, insomnia, atherosclerosis, etc [15]. Therefore, there is a need to develop new anti-inflammatory agents with high specificity, and least toxicity.

The anabolic-androgenic drug methenolone enanthate (**1**) has been used in the treatment of advanced breast carcinoma in the postmenopausal women, with no or low hepatotoxic effects [16]. In addition, it has been used by athletes to build the muscle strength. No report on structural biotransformation of **1** using microbial cell cultures is found in literature. However, metabolism of its analogue, methenolone acetate was studied in horses [17]. In continuation of our studies on the biotransformation of bioactive steroids and steroidal drugs [7,9,11], we report here the microbial

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cell cultures catalyzed transformation of **1**, using *C. blakesleeana*, and *A. niger*. The objective of the study was to produce structurally diverse analogues of anabolic-androgenic steroids **1**, and study of their biological activity.

2. Experimental

2.1. Instrumental analysis

Silica gel precoated thin layer chromatography (TLC) plates (E. Merck, Germany), and column chromatography were used for the initial analysis, and purification of metabolites, respectively. Analytical grade solvents were used for this purpose. In addition, preparative reverse phase recycling HPLC–LC-908 with JAEGAL-ODS-L-80 columns were used for the final purification. The molecular formula of the transformed metabolites were deduced using EI-MS (Jeol JMS-600H), HREI-MS (MAT 95XP, Thermo Finnigan), FAB-MS, and HRFAB-MS (JEOL HX110) techniques. Bruker Avance 300, 400, and 500 MHz spectrometers were used to record ^{1}H and ^{13}C NMR spectra. The melting points were determined by using Buchi M-560 instrument, while the JASCO P-200 polarimeter was used to measure the optical rotations. Evolution 300 UV–Visible spectrophotometer was used to record the UV spectra, while the IR data were collected using KBr disks in chloroform on a Bruker Vector 22 FT-IR spectrometer.

2.2. Microbial cultures

Microbial cultures were obtained from the ATCC (American Type Culture Collection). *Aspergillus niger* (ATCC 10549), and *Cunninghamella blakesleeana* (ATCC 8688A) were grown on Sabouraud dextrose agar slant, and maintained at 4 °C.

2.3. Media preparation

Culture medium (5 L) for *A. niger* (ATCC-10549) was prepared by adding 50 g glucose, 25 g peptone, 25 g KH_2PO_4, 25 g yeast extract, 25 g NaCl, and 25 mL glycerol in distilled H_2O. In the same way for 3 L of *C. blakesleeana* (ATCC-8688A) culture media, glucose (30.0 g), peptone (15.0 g), KH_2PO_4 (15.0 g), yeast extract (15.0 g), NaCl (15.0 g), and glycerol (30.0 mL) were added in distilled H_2O.

2.4. General fermentation and extraction protocol

The specific media cultures were prepared by adding ingredients mentioned above. The media was then transferred to 100 mL Erlenmeyer flasks, and autoclaved at 121 °C, followed by the inoculation of spores of the fungi from mycelia on SDA slants into flasks under sterilized conditions. The fungal cultures were left on the shaker (121 rpm) at 26 ± 2 °C for the fungal growth. When suitable growth was seen in the seed flasks, compound **1** dissolved in methanol, was dispensed to each flask equally, and again left on rotary shaker for 12 days. Negative (without substrate) and positive controls (without fungal culture) were also prepared analogously to identify the fungal metabolites, and the degradation of substrate **1** in the medium, respectively. Incubation for 12 days, followed by filtration of the fungal mass, and extraction with dichloromethane (DCM), yielded an organic phase, which was dried over anhydrous sodium sulfate under reduced pressure to obtain a crude extract. This crude material was then subjected to column chromatography to obtain different fractions. These fractions were subjected to reverse phase recycling HPLC for the final purification.

2.5. Fermentation of methenolone enanthate (**1**) with Aspergillus niger

Starting material **1** (1.2 g) was dissolved in methanol (25 mL), and equally distributed among 50 flasks (0.48 mg/0.5 mL) containing 5 days old *Aspergillus niger* culture. The flasks were then allowed to ferment on rotary shaker at 26 ± 2 °C for 12 days. Fermentation was stopped by adding dichloromethane in each flask, and then filtered to obtain the aqueous extract. Extraction with dichloromethane (3 times), and drying over anhydrous sodium sulfate, followed by the evaporation on rotary evaporator yielded a yellow crude material (1.5 g). The crude extract was then chromatographed over the silica gel column by elution with gradient hexanes-ethyl acetate solvent system. As a result, four main fractions were obtained. Fraction 1 was eluted with 2:8 ethyl acetate: hexanes system, while fractions 2, 3, and 4 were eluted with 3:7, 4:6 and 1:1 ethyl acetate: hexanes solvent systems. These fractions were then subjected to reverse phase recycling HPLC for the purification of metabolites.

2.5.1. 17β-Hydroxy-1-methyl-5α-androst-1-ene-3,16-dione (**2**)

White crystalline solid; m.p.: 155–157 °C; UV λ_{max}: 247 nm (CH_3OH, log ε 1.49); $[\alpha]_D^{25}$ = +39.6° (*c* 0.014, CH_3OH); IR ($CHCl_3$): υ_{max} (cm^{-1}), 3733 (O—H stretching), 1746 (C=O stretching), 1655 (C=C—C=O stretching); HRFAB-MS: *m/z* 315.2006 $[M-H]^+$ ($C_{20}H_{28}O_3$, calcd. 316.2038); ^{1}H NMR (CD_3OD, 300 MHz): Table 1; ^{13}C NMR (CD_3OD, 100 MHz): Table 2.

2.5.2. 15β,17β-Dihydroxy-1-methyl-5α-androstan-1-ene-3-one (**3**)

Brown gummy solid; m.p.: 159–161 °C; UV λ_{max}: 230 nm (CH_3OH, log ε 1.69); $[\alpha]_D^{25}$ = −50.0° (*c* 0.015, CH_3OH); IR ($CHCl_3$): υ_{max} (cm^{-1}), 3374 (O—H stretching), 1658 (C=C—C=O stretching); HREI-MS: *m/z* 318.2211 ($C_{20}H_{30}O_3$, calcd. 318.2195); EI-MS: *m/z* 318 $[M]^+$ (32.6), 276 (19.1), 136 (100.0), 123 (90.1); ^{1}H NMR (CD_3OD, 300 MHz): Table 1; ^{13}C NMR (CD_3OD, 125 MHz): Table 2.

2.5.3. 12β,17β-Dihydroxy-1-methyl-5α-androstan-1-ene-3-one (**4**)

Yellowish solid; m.p.: 271–272 °C; UV λ_{max}: 229 nm (CH_3OH, log ε 2.04); $[\alpha]_D^{25}$ = +39.0° (*c* 0.018, CH_3OH); IR ($CHCl_3$): υ_{max} (cm^{-1}), 3733 (O—H stretching), 1734 (C=O stretching), 1651 (C=C—C=O stretching); HRFAB-MS: *m/z* 317.2100 $[M-H]^+$ ($C_{20}H_{30}O_3$, calcd. 318.2195); ^{1}H NMR (CD_3OD, 300 MHz): Table 1; ^{13}C NMR (CD_3OD, 125 MHz): Table 2.

2.5.4. Methyl-5α-androst-1-ene-3,17-dione (**5**)

Gummy solid; m.p.: 122–123 °C; UV λ_{max}: 243 nm (CH_3OH, log ε 2.83), (CH_3OH, log ε 3.4, Choudhary et. al. [9,11]); $[\alpha]_D^{25}$ = +26° (*c* 0.043, CH_3OH), (+23°, CH_3OH, Choudhary et. al. [9,11]); IR ($CHCl_3$): υ_{max} (cm^{-1}), 3394 (O—H stretching), 1734 (C=O stretching), 1651 (C=C—C=O stretching); HREI-MS: *m/z* 300.2090 ($C_{20}H_{28}O_2$, calcd. 300.2084); EI-MS: *m/z* 300 $[M]^+$ (31), 258 (20.7), 136 (58.4), 109 (13.5); ^{1}H NMR (CD_3OD, 300 MHz): Table 1; ^{13}C NMR (CD_3OD, 100 MHz): Table 2.

2.5.5. 17β-Hydroxy-1-methyl-5α-androstan-1-ene-3-one (**6**)

Yellow solid; m.p.: 148–150 °C; UV λ_{max}: 231 nm (CH_3OH, log ε 1.90); $[\alpha]_D^{25}$ = +53.5° (*c* 0.014, CH_3OH); IR ($CHCl_3$): υ_{max} (cm^{-1}), 3407 (O—H stretching), 1722 (C=O stretching), 1659 (C=C—C=O stretching); HREI-MS: *m/z* 302.2251 ($C_{20}H_{30}O_2$, calcd. 302.2240); EI-MS: *m/z* 302 $[M]^+$ (15.7), 260 (22.2), 136 (86.5), 123 (100.0); ^{1}H NMR (CD_3OD, 300 MHz): Table 1; ^{13}C NMR (CD_3OD, 125 MHz): Table 2.

2.5.6. 16β,17β-Dihydroxy-1-methyl-5α-androstan-1-ene-3-one (**7**)

White solid; m.p.: 149–151 °C; UV λ_{max}: 235 nm (CH_3OH, log ε 1.46); $[\alpha]_D^{25}$ = +28.6° (*c* 0.012, CH_3OH); IR ($CHCl_3$): υ_{max} (cm^{-1}), 3733 (O—H stretching), 1652 (C=C—C=O stretching), HREI-MS: *m/z* 318.2161 ($C_{20}H_{30}O_3$, calcd. 318.2189); EI-MS: *m/z* 318 $[M]^+$

Table 1
^{1}H NMR (300 MHz) chemical shift assignments of compounds **1–7** (δ in ppm, J in Hz).

Carbon	1	2	3	4	5	6	7
1	–	–	–	–	–	–	–
2	5.70 s	5.72 s	5.70 s	5.65 s	5.71 s	5.70 s	5.70 s
3	–	–	–	–	–	–	–
4	2.42 dd, $J_{4,4}$ = 18.3, $J_{4,5}$ = 13.5	2.44 dd, $J_{4,4}$ = 18.6, $J_{4,5}$ = 13.8	2.40 dd, $J_{4,4}$ = 18.3, $J_{4,5}$ = 13.5	2.93 dd, $J_{4,4}$ = 18.6, $J_{4\beta,5\alpha}$ = 13.8	2.44 dd, $J_{4,4}$ = 18.6, $J_{4,5}$ = 13.8	2.42 dd, $J_{4,4}$ = 18.6, $J_{4,5}$ = 13.8	2.42 dd, $J_{4,4}$ = 18.3, $J_{4,5}$ = 13.5
	2.17 m	2.18 m	2.17 m	2.17 m,	2.15 m	2.17 m	2.18 m
5	1.93 m	1.65 m	1.94 m	1.95 m	1.92 m	1.93 m	1.95 m
6	1.44 m, 1.35 m	1.64 m, 1.08 m	1.46 m, 1.42 m	2.10 m, 1.64 m	1.49 m, 1.44 m	1.58 m, 1.44 m	1.63 m, 0.96 m
7	1.66 m, 1.62 m	2.24 m, 1.65 m	1.65 m, 0.95 m	1.57 m, 1.31 m	1.77 m, 1.06 m	1.67 m, 0.95 m	1.76 m, 1.43 m
8	1.52 m	1.90 m	1.87 m	1.25 m	1.66 m	1.52 m	1.52 m
9	1.22 m	1.97 m	0.98 m	1.97 m	1.29 m	1.22 m	1.26 m
10	–	–	–	–	–	–	–
11	1.40 m, 1.30 m	1.49 m, 1.44 m	1.59 m, 1.52 m	1.82 m, 1.76 m	2.23 m, 1.60 m	1.98 m, 1.48 m	1.57 m, 1.52 m
12	1.75 m, 1.35 m	1.93 m, 1.53 m	2.16 m, 1.26 m	3.77 t, $J_{12,13}$ = 2.4	1.74 m, 1.37 m	1.85 m, 1.18 m	1.80 m, 1.30 m
13	–	–	–	–	–	–	–
14	1.52 m	1.46 m	1.24 m	1.15 m	1.46 m	1.14 m	1.46 m
15	1.56 m, 1.31 m	2.20 m, 1.67 m	4.08 m	1.99 m, 1.49 m	2.42 m, 2.06 m	1.60 m, 1.23 m	1.45 m, 1.42 m
16	1.52 m, 1.47 m	–	1.51 m, 1.21 m	1.21 m, 1.13 m	2.39 m, 2.02 m	1.46 m, 1.42 m	3.98 m
17	4.62 t, $J_{17,16}$ = 8.4	3.77 s	3.33 t, $J_{17,16}$ = 8.4	3.22 t, $J_{17,16}$ = 8.4	–	3.59 t, $J_{17,16}$ = 8.4	3.39 d, $J_{17,16}$ = 5.7
18	0.90 s	0.80 s	0.86 s	0.83 s	0.94 s	0.81 s	0.81 s
19	1.09 s	1.11 s	1.10 s	1.23 s	1.14 s	1.09 s	1.09 s
20	2.12 s	2.16 s	2.12 d, $J_{20,2}$ = 0.9	2.10 s	2.13 d, $J_{20,2}$ = 0.9	2.13 s	2.13 d, $J_{20,2}$ = 0.6
21	–						
22							
	2.32 t, $J_{22,23}$ = 7.2						
23	1.44 m, 1.34 m						
24	1.62 m, 1.75 m						
25	1.63 m, 1.36 m						
26	1.31 m, 0.89 m						
27	0.90 t, overlapped						

Table 2
^{13}C NMR chemical shift assignments of compounds **1–7** (δ in ppm).

Carbon	1[a]	2[a]	3[b]	4[b]	5[a]	6[b]	7[b]
1	176.8	176.4	176.9	171.1	176.4	176.9	176.9
2	128.9	129.0	128.9	128.3	129.0	128.9	128.9
3	202.3	202.1	202.3	202.3	202.1	202.3	202.3
4	42.1	42.1	42.1	39.8	42.1	42.1	42.1
5	46.1	41.6	48.3	40.6	46.1	46.2	46.2
6	29.7	31.5	29.7	26.2	29.5	29.7	31.3
7	31.2	28.6	31.5	24.5	30.5	31.3	35.3
8	39.1	38.3	38.9	51.0	39.0	39.5	39.0
9	51.2	46.6	49.3	33.0	51.2	51.5	51.5
10	44.2	43.5	44.2	44.1	44.2	44.2	44.7
11	32.6	29.5	26.3	38.8	26.1	30.6	26.0
12	38.4	37.0	36.1	71.0	32.8	38.1	38.2
13	43.8	44.3	43.5	44.2	48.8	44.0	44.2
14	52.5	51.4	51.6	52.6	53.3	52.8	50.5
15	26.4	37.8	70.6	30.6	22.7	36.0	29.7
16	28.5	218.3	38.8	38.1	36.5	24.4	78.6
17	84.0	87.3	82.3	75.5	223.3	82.4	90.9
18	13.1	12.5	12.3	14.2	14.2	12.2	13.3
19	14.3	14.2	14.2	14.9	14.6	14.4	14.2
20	25.5	25.5	25.5	26.2	25.4	25.5	25.5
21	175.6						
22	35.3						
23	29.8						
24	26.2						
25	24.6						
26	23.5						
27	14.2						

a = 100 MHz.
b = 125 MHz.

(37.0), 276 (20.9), 136 (100.0), 123 (92.8); ^{1}H NMR (CD_3OD, 300 MHz): Table 1; ^{13}C NMR (CD_3OD, 125 MHz): Table 2.

2.6. Fermentation of methenolone enanthate (*1*) with Cunninghamella blakesleeana

Substrate **1** (0.6 g) was dissolved in methanol (15 mL), and equally distributed among 30 flasks (20 mg/0.5 mL), containing 3 days old culture of *C. blakesleeana*. Fermentation for 12 days, followed by mass filtration, extraction with DCM, drying over anhydrous sodium sulfate, and evaporation under vacuum yielded 1.1 g of crude material. The crude extract was then chromatographed over the silica gel column by elution with gradient hexanes- ethyl acetate solvent system to obtain a single fraction, which on purification yielded metabolite **6**.

2.7. Oxidative burst inhibition assay

Fresh human blood for isolation of polymorphonuclear cells (PMNs) was collected from healthy volunteers (ages 22–35 years), and mixed with equal amount of Hank's balanced salt solution without Ca^{2+} and Mg^{2+} ($HBSS^{2-}$) at pH of 7.3 (Sigma, St. Louis, USA) and lymphocyte separation medium (LSM). This mixture was allowed to settle RBCs for 45 min. Supernatant was layered onto LSM, and spun at 400 g for 20 min at room temperature. The remaining RBCs were lysed by hypotonic treatment, and washed with $HBSS^{2-}$ at 300 g for 15 min at 4 °C. The cells were then re-suspended in cold $HBSS^{2-}$, and stored on ice. Viability of PMNs (>95%) was determined by trypan blue exclusion method [18]. The oxidative burst assay on whole blood, as well as on isolated PMNs was performed by the chemiluminescence technique, described in the literature [19].

2.8. Cytokine inhibition assay

The cytokine inhibition assay for all the compounds were performed by using the protocols described in the literature [19]. Briefly THP-1 cells from the European Collection of Cell Cultures (ECACC, UK) were maintained in a RPMI-1640 machine having 50 μmol/L mercaptoethanol (Merck, Darmstadt, Deutschland), 5.5 mmol/L glucose (BioM Laboratories, Chemical Division, Malaysia), 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine (PAA Laboratories, GmbH, Pasching, Austria), 1 mmol/L sodium pyruvate (GIBCO, New York, USA), and 10 mmol/L HEPES (MP Biomedicals,

Illkirch, France). Upon reaching 70% confluency, 2.5×10^5 cells/mL were plated in 24-well tissue culture plates, and differentiated into macrophage mimicking cells with the addition of 20 ng/mL phorbol myristate acetate (PMA), (SERVA, Heidelberg, Germany). The plates were then incubated for 24 h at 37 °C in 5% CO_2. The cells were then stimulated with 50 ng/mL of *Escherichia coli* lipopolysaccharide B (DIFCO Laboratories, Michigan, USA), followed by treatment with the test compounds at a concentration of 25 μg/mL. The plates were again left for incubation at 37 °C in 5% CO_2 for duration of 4 h. The level of TNF-α in supernatants was analyzed by performing ELISA using human Duo Set kit (R & D Systems, Minneapolis, USA) kit, according to instructions prescribed by the manufacturer.

2.9. Cytotoxicity assay

Cytotoxic activity of the transformed products was evaluated by using the standard MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric assay in the 96-well flat-bottomed microplates [20]. For this assay, the Dulbecco's Modified Eagle Medium, supplemented with 5% of fetal bovine serum (FBS), along with the 100 IU/mL of penicillin, and 100 μg/mL of streptomycin in 75 cm^2 flasks, were used to culture the 3T3 (mouse fibroblast) cells in the incubator at 37 °C in 5% CO_2. Cells were harvested, and 5×10^4 cells/mL were plated in the 96-well plates (100 μL/well), and allowed to incubate overnight. After incubation, the medium was removed, followed by the addition of 200 μL of fresh medium with different concentrations of the test compounds (1–30 μM) in triplicates. The plate was then further incubated for 48 h. It was preceded with the addition of 200 μL MTT (0.5 mg/mL) to each well, and incubated for 4 h. Finally, 100 μL of dimethyl sulphoxide (DMSO) was added to each well. The microplate reader (Spectra Max plus, Molecular Devices, CA, USA) was used to calculate the extent of the MTT reduction to formazan within cells by measuring the absorbance at 540 nm. The cytotoxicity was recorded as concentration causing 50% growth inhibition (IC_{50}) of 3T3 cells. The percent inhibition was calculated by using the following formula, and the results were processed with the help of *EZ-Fit*™ enzyme kinetics program (Perellela Scientific, Inc., Amherst, Mars, USA).

$$\%\ \text{Inhibition} = \frac{100 - (\text{OD of test compound} - \text{OD of negative control})}{(\text{OD of positive control} - \text{OD of negative control})} \times 100$$

3. Results and discussion

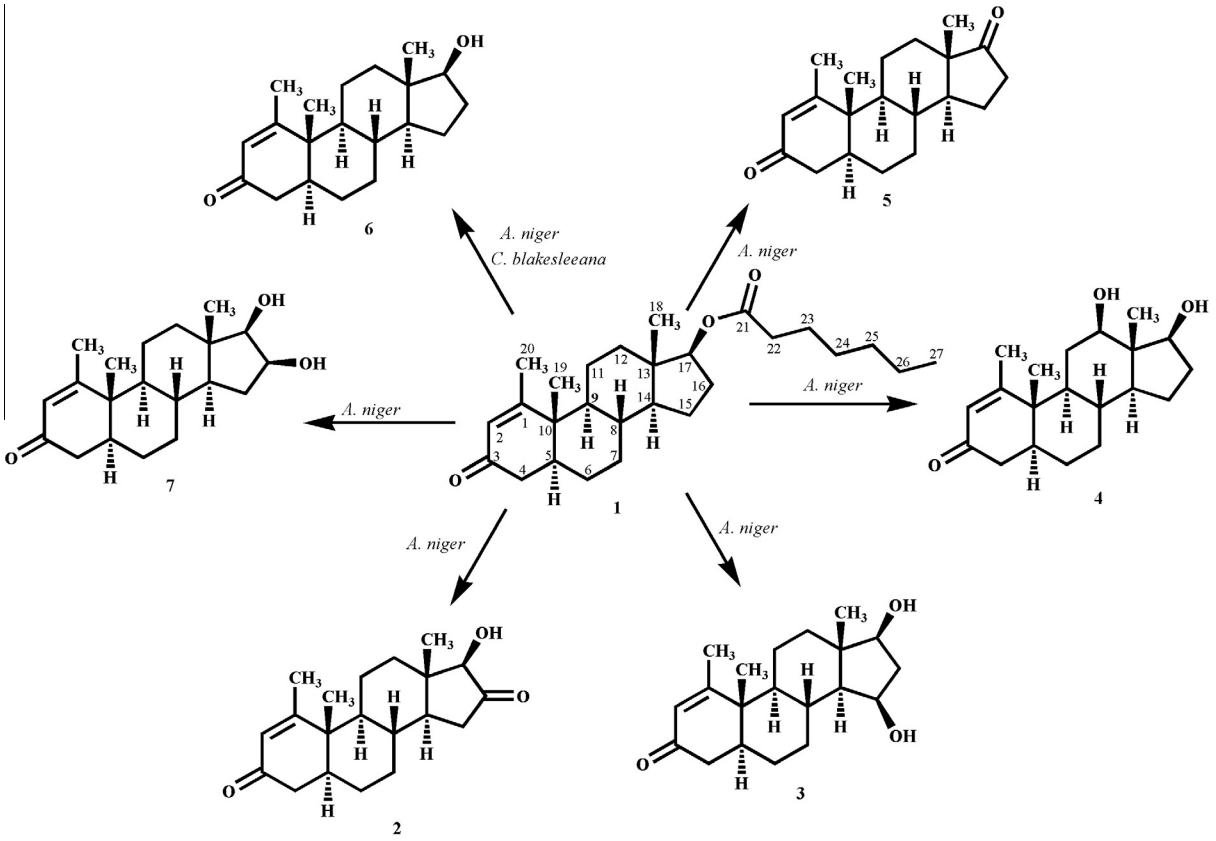
First microbial biotransformation of methenolone enanthate (**1**) with *Aspergillus niger*, and *Cunninghamella blakesleeana* is being reported here. Fermentation of **1** with *A. niger* afforded six metabolites **2–7**, three of them (i.e., **2–4**) were found to be new, whereas *C. blakesleeana* yielded a known metabolite **6** (Scheme 1). Structures of compounds **2–7** were deduced by comparing their spectroscopic data with the substrate **1**, and other related metabolites reported earlier.

Compound **2** (6 mg) was obtained from fraction 2 as a white crystalline material from the reverse phase recycling HPLC with an isocratic elution of MeOH and H_2O (75:25). Compounds **7** and **2** were obtained with retention times (T_R) 21, and 27 min, respectively. The molecular formula for **2** was deduced as $C_{20}H_{28}O_3$, based on its HRFAB-MS at *m/z* 315.2006 $[M-H]^+$ (calc. 316.2038); 96 amu less than **1**, indicating the hydrolytic removal of the ester side chain along with the addition of a ketonic moiety in the steroidal skeleton. In the IR spectrum, peaks at υ_{max} (cm^{-1}) 3733, 1746, and 1655 indicated the presence of hydroxyl group, a carbonyl, and α,β-unsaturated carbonyl functionalities, respectively. The presence of α,β-unsaturated carbonyl was also inferred from the UV–visible spectrum (λ_{max} 247 nm). The ^1H NMR spectrum showed a downfield methine singlet at δ 3.77 which was assigned to a geminal proton to a hydroxyl group, whereas in the ^{13}C NMR spectrum, only 20 signals appeared, clearly indicating the loss of the ester side chain. An additional downfield quaternary carbon signal at δ 218.3 indicated a ketonic group. The OH group was placed at C-17 (δ 87.3) based on the HMBC experiment (Scheme 2). Key HMBC correlations of H-17 (δ 3.77 s) with C-16 (δ 218.3), C-13 (δ 44.3), and C-18 (δ 12.5) supported the presence of a ketonic carbonyl at C-16, and an OH at C-17 of ring D. The relative stereochemistry at C-17 was deduced by the NOESY interactions between H-14 (δ 1.46 m, *axial*), and H-17 (δ 3.77 s, *axial*), suggesting a β orientation of the geminal OH group (Scheme 3). Thus metabolite **2** was identified as 17β-hydroxy-1-methyl-5α-androst-1-ene-3,16-dione.

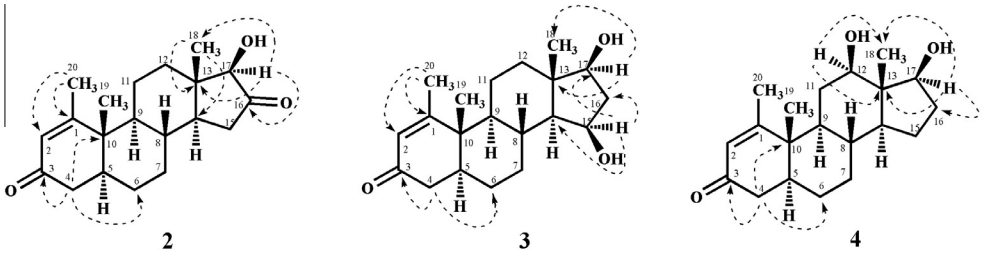
Metabolite **3** (7 mg, brown gummy solid, T_R = 30 min) was obtained from the reverse phase recycling HPLC, using isocratic methanol and water system (7:3). The HREI-MS data of **3** supported the molecular formula $C_{20}H_{30}O_3$ *m/z* 318.2211 $[M^+]$ (calcd. 318.2195) with six degrees of unsaturation. The IR absorptions at υ_{max} (cm^{-1}) 3374, 1658, and UV absorption at λ_{max} 230 nm, supported the presence of hydroxyl, and α,β-unsaturated carbonyl groups. In the ^1H NMR spectrum, an additional methine multiplet at δ 4.08, and a signal at δ 70.6 in the ^{13}C NMR spectrum, suggested hydroxylation. The HMBC (Scheme 2) correlations of H-14 (δ 1.24, m) with C-9 (δ 49.3), C-13 (δ 43.5), and C-16 (δ 38.8), supported the presence of an OH group at C-15. Besides this, H-17 (δ 3.33, t) also showed HMBC correlations with C-15 (δ 70.6), C-12 (δ 36.1), C-13 (δ 43.5), C-16 (δ 38.8), and C-18 (δ 12.3). The NOESY (Scheme 3) spectrum showed cross peaks between H-14 (m 1.24, *axial*) and H-15, and H-17. Thus the relative stereochemistry of OH groups at C-15, and C-17 was deduced as β. These data permitted to establish the structure of metabolite **3** as 15β, 17β-dihydroxy-1-methyl-5α-androstan-1-ene-3-one.

Fraction 4 afforded metabolite **4** (12 mg, T_R = 22 min) as a yellowish solid through reverse phase recycling HPLC. The HRFAB-MS of **4** ($[M+H]^+$, *m/z* 317.2100) was consistent with the formula $C_{20}H_{30}O_3$ (calc. 318.2195). The IR showed absorption at υ_{max} (cm^{-1}) at 3733, 1734, 1651 for OH, carbonyl and α,β-unsaturated carbonyl group and UV spectra showed absorptions λ_{max} (nm) 229. The ^1H NMR showed an additional methine proton triplet at δ 3.77 ($J_{12,13}$ = 2.4 Hz). Broad-band decoupled ^{13}C NMR spectrum of compound **4** was distinctly similar to that of **3**. The HMBC (Scheme 2) spectrum showed the correlations of H-18 (δ 0.83, s) with C-12 (δ 71.0), C-13 (δ 44.2), C-14 (δ 52.6), and C-17 (δ 75.5), supporting the OH groups at C-12 (δ 71.0), and C-17 (δ 75.5). In addition, H-17 (3.22, t, $J_{17,16}$ = 8.4 Hz) also showed HMBC correlations with C-12 (δ 71.0), C-13 (δ 44.2), and C-18 (δ 14.2), indicating an OH at C-12 (δ 71.0). The geminal H-12 (δ 3.77, t) showed NOESY (Scheme 3) interactions with H-9 (δ 1.97, m), H-14 (δ 1.15, m), and H-17 (δ 3.22, t), suggesting a β disposition of the OH groups at C-12 (*equatorial*) (δ 71.0), and at C-17. In addition to the NOESY spectrum, the key COSY correlations between H-11 and H-12 and H-16 and H-17, led to the deduction of the structure of **4** as 12β,17β-dihydroxy-1-methyl-5α-androstan-1-ene-3-one.

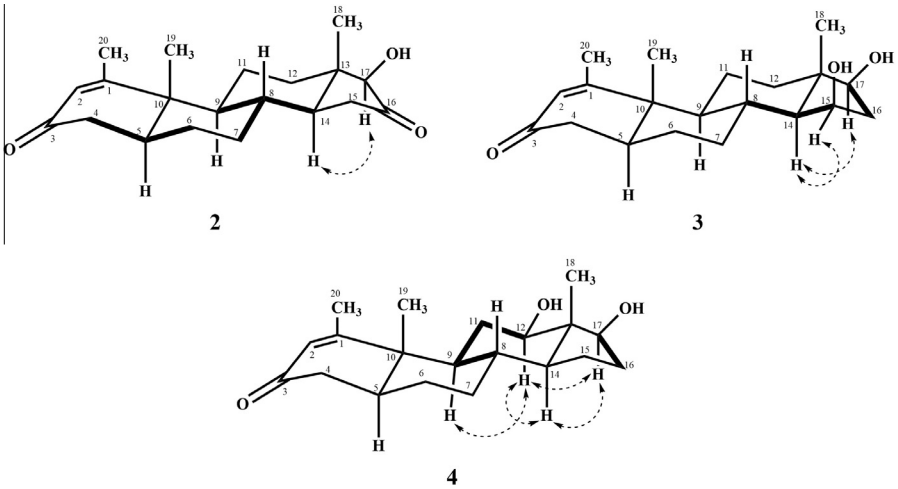
Structures of metabolites **5** (7 mg), and **6** (14 mg) were identified as 1-methyl-5α-androstan-1-ene-3,17-dione, and 17β-hydroxy-1-methyl-5α-androstan-1-ene-3-one, respectively, based on their HREI-MS, and by comparing their spectral data with the reported data for metabolites of methenolone acetate in horses [17], and fungal transformation of mesterolone by *Fusarium lini*, and *Cephalosporium aphidicola* [9]. Metabolite **7** (7 mg) was identified as 16β, 17β-dihydroxy-1-methyl-5α-androstan-1-ene-3-one by comparison of its spectral data with the data reported for a metabolite of methenolone acetate in horses [17].



Scheme 1. Biotransformation of methenolone enanthate (**1**) by *Aspergillus niger* and *Cunninghamella blakesleeana*.



Scheme 2. Key HMBC correlations in compounds **2**, **3**, and **4**.



Scheme 3. Key COSY (▬) and NOESY (⇢) correlations.

Table 3
Effect of compounds on oxidative burst using whole blood phagocytes, isolated PMNs and on proinflammatory cytokine TNF-α production.

Compound	Oxidative burst (IC_{50} μg/mL)		TNF-α% inhibition (μg/mL) THP-1
	Whole blood	PMNs	
1	−41.0 ± 4.0	–	–
2	8.60 ± 1.0	14.0 ± 1.7	88.63 ± 13.1
3	7.05 ± 1.3	4.70 ± 0.5	34.21 ± 9.1
4	>100	–	–
5	98.2 ± 4.8	–	–
6	31.1 ± 1.3	–	–
7	47.6 ± 2.4	–	–
Ibuprofen	11.3 ± 2.9	2.50 ± 0.6	–

Each compound was tested in triplicates, and the experiments were repeated twice. The data from one representative experiment were presented as mean ± SD.

Methenolone enanthate (**1**), and its biotransformed metabolites were evaluated for their anti-inflammatory effect on the ROS production using whole blood, as well as isolated phagocytes. In this assay, substrate **1** showed no inhibition of ROS, production by whole blood and by isolated phagocytes, while all biotransformed products, except compound **4**, showed anti-inflammatory potential in the oxidative burst assay (Table 3). Compounds **5–7** showed a moderate to low inhibition of ROS generation with IC_{50} values 31.1–98.2 μg/mL, whereas new metabolites **2** and **3** showed a potent inhibition of ROS on whole blood (IC_{50} = 8.60 and 7.05 μg/mL, respectively), as well as on isolated PMNs (IC_{50} = 14.0 and 4.70 μg/mL), respectively. Potent compounds **2** and **3** were further evaluated for their effects on the production of proinflammatory cytokine TNF-α by THP-1 cells. Metabolite **2** (88.63%) potently inhibited the production of TNF-α, whereas compound **3** (34.21%) caused a moderate inhibition. All transformed metabolites were found to be non-toxic to 3T3 mouse fibroblast cells.

4. Conclusion

The biotransformation of methenolone enanthate (**1**) by *A. niger* and *C. blakesleeana* led to the synthesis of six metabolites, three of which (**2–4**) were found to be new. Metabolites **2** and **3** exhibited a potent immunomodulatory activity against ROS production by whole blood, as well as by isolated PMNs. Metabolite **2** (88.63%) was also found to be a potent inhibitor of proinflammatory cytokine TNF-α production. Metabolites **2** and **3** showed their potential as new anti-inflammatory agent. Metabolite **2** can be further studied for the treatment of various chronic anti-inflammatory diseases in which blockade of TNF-α proved to be beneficial. The exact mode of action and *in vivo* metabolism of these compounds required to be studied.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2016.04.007.

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