Steroids 112 (2016) 68–73

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

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

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ARTICLE INFO

Article history:
Received 20 February 2015
Received in revised form 12 April 2016
Accepted 21 April 2016
Available online 29 April 2016

Keywords:
Biotransformation
Aspergillus niger
Cunninghamella blakesleeana
Anabolic-androgenic steroids
Methenolone enanthate
Immunomodulatory
Anti-inflammatory
TNF-α inhibitors

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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http://dx.doi.org/10.1016/j.steroids.2016.04.007
0039-128X/

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 $[M]^+$