SOLID PHASE MICROBIAL REACTIONS OF SEX HORMONE, TRANS-ANDROSTERONE WITH FILAMENTOUS FUNGI

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ABSTRACT

Objective: A microbial biotransformation study was performed on trans-androsterone (1) using solid phase medium. In the present context, trans-androsterone (1), a sex hormone was fermented with two filamentous fungi, Rhizopus stolonifer (black bread mold) and Fusarium lini.

Methods: Sabouraud-4% glucose-agar were used to cultivate the fungal cultures as solid phase medium. Substrate 1 was incubated with R. stolonifer (ATCC 10404) and F. lini (NRRL 68751) for 8 days. Microbial transformed metabolites were purified by using column chromatographic technique.

Results: The metabolism study of 1 revealed that various metabolites were detected when incubated with filamentous fungi. A total of 3 transformed products were obtained. The reactions occurred that exhibited diversity, including selective hydroxylation at C-6 and C-7 along with oxidation occurs at C-3 positions. Their structure and identified on the basis of extensive spectroscopic data (NMR, HRMS, IR and UV) as 3β,7β-dihydroxy-5α-androst-17-one 2 in a good yield (59%), 6β-hydroxy-5α-androstan-3,17-dione 3, and 3β,6β-dihydroxy-5α-androstan-17-one 4.

Conclusion: Solid phase microbial transformation method can successfully be used for the development of new steroidal drugs. The modified steroidal molecules could favor when compared to their natural counterparts due to several medicinal advantages.

Keywords: Solid phase medium, Microbial Transformation, Trans-androsterone, Rhizopus stolonifer, Fusarium lini, Hydroxylation.

INTRODUCTION

Steroids are widely used as anti-inflammatory, diuretic, anabolic, contraceptive, antiandrogenic, progestational, and anticancer agents as well as in other applications. However, there are various methods of transforming steroids using biocatalyst but filamentous fungi as microbial model are capable of catalyzing stereoselective hydroxylation with greater efficiency [1-5]. The use of microbial models for the transformation of steroids has been incorporated into numerous partial syntheses of new steroids for evaluation as hormones and drugs. These models offers a few advantages over chemical synthesis, because it can be highly enantiomeric, regio-selective and stereo-specific under mild conditions. Microbial transformations have provided adequate tools for the large scale production of natural or modified steroid analogues. Microbial models are relatively easy to maintain and grow, and that scale-up to produce milligram or gram amounts is readily accomplished [5-12]. Furthermore, a variety of metabolites could be obtained by microbial reactions, from which more bioactive metabolites might be found. Yeast and filamentous fungi are very useful microbial models to metabolize a wide variety of xenobiotics using both phase I (oxidative) and phase II (conjugative) biotransformation mechanisms. These fungi are even reported to metabolize a variety of xenobiotics in a region- and stereoselective manners that are similar to those in mammalian enzyme systems [13-28]. Microbial reactions of 3β-hydroxy steroids of androstan-17-one have been extensively investigated, which resulted hydroxylation at various positions on the steroidal skeleton mainly at C-6, C-7, C-11, C-12, C-15, C-16 positions and oxidation at C-3 (fig. 1). 7α-Hydroxylated metabolites of 3β-hydroxysteroids such as androsterone were reported to increase immune response in mice and have anti-gluco corticoid properties [7-10, 13, 14].

In the above context, the solid phase microbial reactions of sex hormone, trans-androsterone (1) were systematically investigated in our group with two filamentous fungi, R. stolonifer (ATCC 10404) and F. lini (NRRL 68751) [4]. Three hydroxylated products 2-4 were isolated and identified in the biotransformation process of 1 (Scheme 2). Herein, we first time reports the solid phase microbial transformations of 1 by filamentous fungi.

Fig. 1: Structure of androsterone and microbial target positions of substituents

MATERIALS AND METHODS

General

Trans-androsterone (1) was purchased from sigma-aldrich (USA). Melting points were determined on a Yanaco MP-S3 apparatus. UV spectra were measured on a Shimadzu UV 240 spectrophotometer. JASCO DIP–360 Digital polarimeter was used to measure the optical rotations in chloroform by using 10 cm cell tube. FTIR-8900 Spectrophotometer was used to record IR spectra in CHCl3. The 1H-NMR and 2D NMR spectra were recorded on a Bruker Avance III 500 spectrometer, while [13C]-NMR spectra were recorded on Bruker Avance III 500 spectrometer operating at 125 MHz using CDCl3 as solvent. Chemical shifts were reported in (δ ppm), relative to SiMe4, as internal standard, and coupling constants (J) were measured in...
H2. The HREI MS was measured on Jeol HX 110 mass spectrometer. TLC was performed on Si gel precoated plates (PF 254, 20 x 20, 0.25 mm, Merck, Germany). Ceric sulphate in 10% H2SO4 spraying reagent was used for the staining of compounds on TLC. All reagents used were of analytical grades.

**Fungal culture and medium**
Solid phase media for filamentous fungi, R. stolonifer (ATCC 10404) and F. lini (NRRL 68751), was prepared by adding Sabouraud 4% glucose-agar (Merck) (180 g) in 3L. The solution was boiled on a hot plate until a transparent solution was obtained and then poured in 42 flasks of 100 mL and autoclaved at 123°C. Fungi was inoculated on the solid phase media and allowed to grow for two days at 28°C [4, 13].

**General fermentation and extraction conditions**
The trans-androsterone (1) (1 g) was dissolved in acetone (20 mL) and fed in each flask (0.5 mL), which was kept for 8 days. After 8 days, content of all the flasks was filtered with CH2Cl2. The extracts were dried over anhydrous sodium sulfate and concentrated in vacuo to afford a gum that was adsorbed on equal quantities of Si gel (70-230 mesh, E. Merck), and eluted with solvent gradients of petroleum ether and EtOAc. Metabolite 2 was obtained from the transformation by R. stolonifer (ATCC 10404), while metabolites 3 and 4 were obtained from fermentation with F. lini (NRRL 68751) (Scheme 1).

**RESULTS AND DISCUSSION**
We report here the solid phase microbial transformation of trans-androsterone (1) (C19H28O3, m/z 290.32) with two filamentous fungi, R. stolonifer (ATCC 10404) and F. lini (NRRL 68751) for the first time (Scheme 2).

### Scheme 1: Fermentation and extraction conditions

3β,7β-dihydroxy-5α-androstan-17-one (1) (1 g) was dissolved in acetone (20 mL) and subjected to solid phase microbial transformation of trans-androsterone (1) (C19H28O3, m/z 290.32) with two filamentous fungi, R. stolonifer (ATCC 10404) and F. lini (NRRL 68751) for the first time (Scheme 2). The metabolite 2 was found more polar on TLC as compared to substrate 1. The HREI-MS of metabolite 2 exhibited the molecular ion (M+) at m/z 306.2208, corresponding to the formula C19H30O3, which indicated that a new oxygen functionality was introduced during the fermentation period. Metabolite 2 was found to be 7β-hydroxy derivative of 1, based on 1H and 13C-NMR signals at δH 3.45/3.48 Hz. Hydroxylation at C-7 position was further supported by COSY and HMBC interactions. The β (equator) stereochemistry of the C-7 OH group was deduced on the basis of 2D NOESY correlations of H-7 (δH 3.69 Hz) with H-3′ (δH 1.40) [19]. The metabolite 2 was deduced as 3β,7β-dihydroxy-5α-androstan-17-one (fig. 2). Metabolite 2 (3β,7β-dihydroxy-5α-androstan-17-one) was previously obtained by metabolism of trans-androsterone in the human brain [29].

Solid phase fermentation of 1 with R. stolonifer for 8 days afforded one regio- and stereoselective hydroxyl product, 2 (Scheme 2). The metabolite 2 was found more polar on TLC as compared to substrate 1. The HREI-MS of metabolite 2 exhibited the molecular ion (M+) at m/z 306.2208, corresponding to the formula C19H30O3, which indicated that a new oxygen functionality was introduced during the fermentation period. Metabolite 2 was found to be 7β-hydroxy derivative of 1, based on 1H and 13C-NMR signals at δH 3.45/3.48 Hz. Hydroxylation at C-7 position was further supported by COSY and HMBC interactions. The β (equator) stereochemistry of the C-7 OH group was deduced on the basis of 2D NOESY correlations of H-7 (δH 3.45) with H-3′ (δH 1.40) [19]. The metabolite 2 was deduced as 3β,7β-dihydroxy-5α-androstan-17-one (fig. 2). Metabolite 2 (3β,7β-dihydroxy-5α-androstan-17-one) was previously obtained by metabolism of trans-androsterone in the human brain [29].

Solid phase microbial reaction of 1 with F. lini for 8 days yielded two regio- and stereoselective hydroxyl products, 3 and 4 (Scheme 2). Metabolite 3 was obtained as a colorless solid. Its molecular formula was determined as C19H30O3 according to the HREI MS (m/z
303.9814: calcd for C_{19}H_{28}O_{3}, 303.9824), indicated that one oxygen had been incorporated into the molecule, as compared to 1. Compound 3 was found to be 6β-hydroxy derivative of 5α-androstan-3,17-dione (5), based on NMR signals at δ 3.99/δC 71.9. The position of the newly introduced hydroxyl at C-6 position was inferred from the HMBC coupling of C-6 proton with C-5 (δ 45.5) and C-7 (δ 39.1). The stereochemistry of C-6 OH was deduced to be β (axial), based on the 2D NOESY correlations between H-6 (δ 3.99) and H-9 (δ 1.92) (fig. 3) [14]. The metabolite 3 was deduced as 6β-hydroxy-5α-androstan-3,17-dione. Metabolite 3 (6β-hydroxy-5α-androstan-3,17-dione) was previously obtained by hydrogenation and dehydration of Δ4-3-keto steroids having 6-hydroxy group by Mycobacterium smegmatis [30]. Compound 4 was isolated as colorless solid from solid phase culture of F. lini (Scheme 2). The metabolite 4 was found more polar on TLC as compared to substrate 1. The molecular composition of polar metabolite 4 [M]+ at m/z 306.2166 (C_{19}H_{30}O_{3}, calc. 306.2194) was similar to metabolite 2 as deduced from HREI-MS. Thus indicated that a new oxygen functionality into was introduced the molecule during the fermentation period. Transformed product 2 was found to be 6β-hydroxy derivative of 1, based on 1H and [13]C-NMR signals at δH 3.95/δC 71.9. Hydroxylation at C-6 position was further supported by COSY and HMBC interactions. The stereochemistry of C-6 OH was deduced to be β (axial), based on the 2D NOESY correlations between H-6 (δ 3.99) and H-9 (δ 1.92) (fig. 3) [14]. The metabolite 4 was deduced as 3β,6β-dihydroxy-5α-androstan-17-one. Compound 4 (3β,6β-dihydroxy-5α-androstan-17-one) was previously reported as the metabolic product of androstenedione [31].

CONCLUSION
In summary, the solid phase biotransformation method of sex hormone, trans-androsterone to its regio- and stereoselective hydroxyl products with filamentous fungi was high-efficiency. Present research has been conducted for the first time. Metabolism of 1 for 8 days with filamentous fungi, R. stolonifer (ATCC 10404) and F. lini (NRRL 68751), yielded three hydroxylated transformed products 2-4. Structures of all metabolites were elucidated by using spectroscopic techniques. These modified steroidal molecules could favor when compared to their natural counterparts due to several medicinal advantages. Moreover, microbial-based biotransformations in connection with conventional organic synthesis can provide novel routes for the development of new steroidal drugs.

CONFLICT OF INTERESTS
Declared None

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