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Original Article

MICROBIAL OXIDATION OF FINASTERIDE WITH *MACROPHOMINA PHASEOLINA*(KUCC 730)

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ABSTRACT

Objective: New microbial oxidative derivatives of finasteride [17β-(*N-tert*-butylcarbamoyl)-4-aza-5α-androst-1-en-3-one] (**1**) has been investigated with *Macrophomina phaseolina* (ATCC730).

Methods: Fermented media of *Macrophomina phaseolina* (ATCC730) was prepared to cultivate the fungal cultures. Substrate 1 was incubated in liquid media for 16 d. After sixteen days, filtration and extraction of the fermented media were carried out with 9 L DCM in three portions. The resulting organic extract was dried using anhydrous (Na2SO4), and evaporated to afford a brown gum (950 mg). This on chromatographic purification with MeOH in CH2Cl2 afforded the metabolites 2-4.

Results: Three oxidised metabolites of finasteride (1) which were identified as 15-oxo-finasteride (2), 11α-hydroxyfinasteride (3), and 15β-hydroxyfinasteride (4). Metabolite 2 was found to be new. The structure of the oxidised metabolites was elucidated by 1-D (^{1}H, ^{13}C) and 2-D NMR (COSY, HMBC, HMQC, NOESY) techniques and MS analyses.

Conclusion: As a result of these study, oxidation at C-7, C-11 and C-15 positions were found. Metabolite 2 was identified as a new metabolite.

Keywords: Microbial transformation, Finateride, Oxidised metabolites, *Macrophomina phaseolina*

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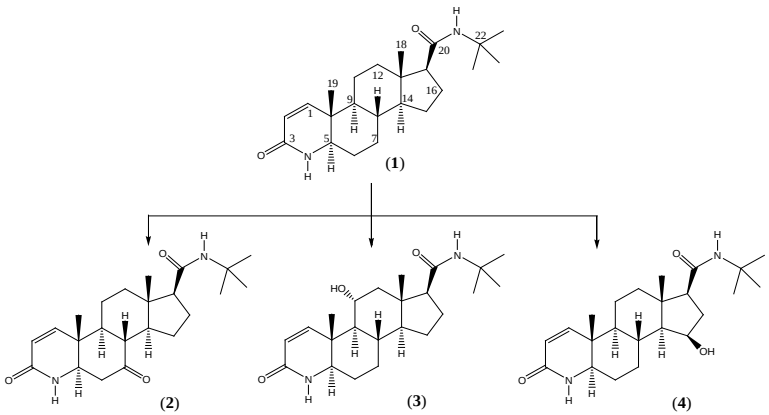
INTRODUCTION

Finasteride [17β-(*N-tert*-butylcarbamoyl)-4-aza-5α-androst-1-en-3-one] (1) is a potent inhibitor of 5α-reductase, an enzyme which catalyzes the conversion of testosterone into dihydrotestosterone in many organs [1]. Finasteride (1) is used in the treatment of hair loss [2], and benign prostate hyperplasia, both these disorders are associated with *in-situ* high levels of increased dihydrotestosterone. It is also employed for the prevention of prostate cancer [3]. Previously, biotransformation of finasteride (1) has been reported by various fungi, including *Selenastrum capricornutum* [4], *Mortierella isabellina*, *Cunninghamella elegans*, and *Bacillus megaterium* [5].

Conversion of steroidal compounds by microbial transformation has turned out to be an important milestone in the research and development of steroidal drug industry. Steroids are also extensively used as anti-inflammatory drugs [6] and antiviral activity has also been reported from a steroidal glycoside Torvoside H, was isolated from the *Solanum turvum* [7]. Since then, steroidal drugs have emerged as the second largest category of drugs in the pharmaceutical industry with about 300 steroidal drugs in the market [8]. Many reports on microbial conversion of steroids have been published during the last half-century [9, 6, 10-21] focusing mainly on steroidal dehydrogenation, hydroxylations, esterification, halogenations, isomerization, methoxylation, and side-chain cleavage [22, 23]. In continuation of our work on micro-biotransformation [24-28] for the first time, we report here the fermentation of finasteride (1) with *Macrophomina phaseolina* (ATCC730) in order to obtain further microbial derivatives of finasteride which can be future candidates of drugs.



Scheme 1: Oxidation of finasteride (1) with *Macrophomina phaseolina*

MATERIALS AND METHODS

General

Finasteride (1) was isolated from the drug Proscar, manufactured by Merck Sharp and Dhome Limited of Pakistan. Melting points were determined on a Yanaco MP-S3 apparatus. UV spectra were measured on a Shimadzu UV 240 spectrophotometer. JASCO DIP–360 Digital polarimeters 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 $CHCl_3$. The ^{1}H-NMR and 2D NMR spectra were recorded on a Bruker Avance III 500 spectrometer, while ^{13}C-NMR spectra were recorded on Bruker Avance III 500 spectrometer operating at 125 MHz using $CDCl_3$ as a solvent. chemical shifts were reported in δ (ppm), relative to $SiMe_4$ as an internal standard, and coupling constants (*J*) were measured in Hz. The HREI MS were measured on Jeol HX 110 mass spectrometer. TLC was performed on Si gel precoated plates (PF_{254}, 20 × 20, 0.25 mm, Merck, Germany). Ceric sulphate in 10% H_2SO_4 spraying reagent was used for the staining of compounds on TLC. All reagents used were of analytical grades.

Chemicals and reagents

Silica gel GF-254, preparative TLC (0.5 mm) solvents, Ingredients for media, reagents and substrates were purchased from E. Merck, Aldrich, and Fluka.

General fermentation and extraction procedure

The substrate was dissolved in acetone and the substrate solution was evenly distributed among all the flasks (20-30 mg/0.5 ml). Fermentation was continued for further 12-16 d on a rotary shaker (128 rpm) at 25-28 °C. Time course studies were carried out at regular intervals for analyzing the extent of fermentation on TLC. Fermentation media were harvested by filtration to separate the broth from the mycelium and extracted thrice with organic solvents such as dichloromethane or ethyl acetate. Sodium sulfate (Na_2SO_4) was added in the organic extracts for drying, filtered and then it is concentrated in vaccum to afford a brown gummy crude extract which was analyzed by thin layer chromatographic techniques.

Fungi and culture conditions

Microbial culture *Macrophomina phaseolina* (ATCC730) purchased from American Type Culture Collection (ATCC) from the USA was grown on Potato dextrose agar (PDA) at 25° C and stored at 4° C. *M. phaseolina* (ATCC730) medium was prepared by adding Glucose (10.0 g), peptone (5.0 g), KH_2PO_4 (5.0 g), yeast extract (5.0 g), NaCl (5.0 g) and glycerol (10 ml) into distilled water (1 L) and maintained pH at 5.6.

General stage II fermentation and extraction procedure

Cultures grown on the agar slants were transferred into a broth medium flask containing freshly prepared sterilized medium (100 ml in 250 ml flask). The seed flask was then incubated in a shaker for two days at 25 °C. The remaining flasks were inoculated from seed flasks and incubated for further 2-3 d. After 2 d substrate 1 (450 mg/13 ml acetone) was equally transferred among 25 flasks containing stage II fermentation medium of *Macrophomina phaseolina*. Fermentation was allowed and a time course study was carried out after each 24 h to analyze the degree of transformation on TLC. After four days, filtration and extraction of the fermented media was carried out with 9 L DCM in three portions. Resulting organic extract was dried using anhydrous (Na_2SO_4), and evaporated to afford a brown gum (950 mg). This on chromatographic purification with MeOH in CH_2Cl_2 afforded the metabolites 2-4. 2 (5.4 mg); MeOH/CH_2Cl_2 (3:97), 3 (8.6 mg); MeOH/CH_2Cl_2 (5:95) and 4 (23.4 mg); MeOH/CH_2Cl_2 (7:93).

15-Oxofinasteride (2): colourless solid (5.4 mg) (MeOH: CH_2Cl_2, 3:97), M. p.: 132-133 °C

UV (MeOH) λmax nm (log ∈ε): 243 (3.9), $[\alpha][25]_{D:+}$73.3° (*c* = 0.12, $CHCl_3$); R_f: 0.55 (DCM/MeOH = 85:15); IR (CHCl3) λmax cm^{-1}: 1672, 2934, and 1726 EI-MS *m/z* (rel. int., %): *m/z* 387 [*M+H+*] (100), 386 (10), 368 (18), 353 (28), 266 (12), 244 (23), 212 (16), 199 (15), 180 (55), 168 (14), 159 (31), 105 (42), 81 (61), 67 (12), 53 (22). HREI-MS *m/z* (mol. formula, calcd value): 386.2545 ($C_{23}H_{34}N_2O_3$, 386.2543). ^{1}H-NMR: table 1 ^{13}C-NMR: : δ 150.5 (C-1), 166.3 (C-3), 59.6 (C-5), 25.6 (C-6), 27.5 (C-7), 47.5 (C-9), 39.5 (C-10), 38.2 (C-12), 42.3 (C-13), 64.2 (C-14), 212.8 (C-15), 37.4 (C-16), 52.9 (C-17), 14.3 (C-18), 12.1 (C-19), 168.8 C-20), 51.7 (C-22), 29.0 (C-23).

11α-Hydroxyfinasteride (3): colourless solid (8.6 mg), M. p.: 141-143 °C UV (MeOH) λmax nm (log ∈ε): 207 (3.6), $[\alpha][25]_{D:+}$69.3° (*c* = 0.14, $CHCl_3$); R_f: 0.63 (DCM/MeOH = 85:15); IR (CHCl3) λmax cm^{-1}: 3336, 3311, 1730, 1678, 1600; EI-MS: *m/z* (rel. int., %): 389 [*M+H+*] (100), 388 (16), 370 (8), 355 (7), 270 (31), 256 (32), 240 (13), 222 (46), 180 (35), 168 (54), 159 (21), 109 (39), 105 (22), 81 (21), 67 (2), 53 (29). HREI-MS *m/z* (mol. formula, calcd value): 388.2744 (C23H36N2O3, 388.2726). ^{1}H-NMR: table 1; ^{13}C-NMR: δ 167.0 (C-3), 53.5 (C-9), 44.0 (C-10), 69.2 (C-11), 49.9 (C-12), 40.5 (C-13).

15β-Hydroxyfinasteride (4): colourless solid (23.4 mg), M. P.: 142-145 °C UV (MeOH) λmax nm (log ∈ε): 202 (3.8), $[\alpha][25]_{D:+}$65.3° (*c* = 0.14, $CHCl_3$); R_f: 0.42 (DCM/MeOH = 80:20); IR (CHCl3) λmax cm^{-1}: 3342, 2935, 1666, 1595; EI-MS: *m/z* (rel. int., EI-MS: *m/z* (rel. int., %): 389 [*M+H+*] (18), 388 (55), 370 (58), 355 (7), 288 (12), 270 (37), 260 (12), 256 (10), 232 (100), 157 (53), 128 (85), 115 (24), 110 (72), 72 (39), 58 (46). HREI-MS *m/z* (mol. formula, calcd value): 388.4848 ($C_{20}H_{36}N_2O_3$, 388.4823). ^{1}H-NMR: table 1;^{13}C-NMR: δ 166.9 (C-3), 31.6 (C-8), 43.5 (C-13), 59.7 (C-14), 69.6 (C-15), 40.9 (C-16), 57.7 (C-17).

RESULTS AND DISCUSSION

Metabolism of finasteride (1) with *Macrophomina phaseolina* KUCC730 for 16 d resulted in three oxidative products, 2-4 (Scheme 1). Structures of the metabolites deduced through the comparative spectroscopic studies with the substrate(1). The HREI MS of metabolite 2 exhibited the molecular ion (M^+) at *m/z* 316.1536, corresponding to the formula $C_{20}H_{28}O_3$, The EI-MS of the compound 2 exhibited the M+at *m/z* 386, 14 amu. higher than the substrate 1, indicating the introduction of an oxygen atom, as carbonyl functionality. The HREI-MS of metabolite 2 exhibited a M+at *m/z* 386.2545, corresponding to the formula $C_{23}H_{34}N_2O_3$ (Calcd 386.2543). The IR spectrum of 2 showed the presence of an amide, an olefinic, and carbonyl functionalities by showing absorptions at 1672, 2934, and 1726 cm-1, respectively. The UV spectrum exhibited an strong absorption at 243 nm, indicating an α,β-unsaturated amidic moiety. The ^{1}H-NMR spectrum of the metabolite 2 was almost similar to the parent compound 2, except a downfield shift of the H-14 signal from δ 1.08 to 1.64, indicating an oxidation at its vicinal carbon. The ^{13}C-NMR spectrum of 2 was also quite similar to the substrate 1, and showed resonances for all 23 carbons, including seven methine, five methylene, five methyl, and six quaternary carbons. Additionally, it also exhibited a new quaternary ketonic carbon signal, resonated at δ 212.7 (C-15). The position of the newly introduced ketonic moiety was deduced to be at C-15 through the HMBC correlations of H-14 (δ 1.64) and H-16 (δ 2.91, 2.71) with δ 212.7 (C-15). Based on the above mentioned spectral details the metabolite 2 was deduced to be a new compound and characterized as 15-oxo-finasteride (2).

Metabolite 3 was obtained as a colorless solid (Scheme 1). The HREI-MS analysis of metabolite 3 displayed an M+at *m/z* 388.2744, which is corresponded to the formula $C_{23}H_{36}N_2O_3$ (Calcd 388.2726), indicating the introduction of an oxygen atom in the metabolite. The ^{1}H-NMR spectrum of 3 was quite similar to the substrate 1, except for a new downfield hydroxyl-bearing methine proton signal at δ 4.04 (m, *W*1/2 = 23.7 Hz). A downfield shift of H-1 from δ 6.80 to 7.94 was also observed, which indicated the appearance of a newly introduced OH functionality at C-11. The ^{13}C-NMR spectrum of 3 also exhibited a new methine signal resonating at δ 69.2 (C-11), whereas a β-downfield shift of C-9 (δ 53.5), and C-12 (δ 49.9) suggested the introduction of the hydroxyl group in their vicinity. Two-dimensional NMR techniques (HMBC and COSY 45 °) were further employed to assign the location of the new hydroxyl group. In the HMBC spectrum, 2*J* correlations of H-11 (δ 4.04) with C-9 (δ 53.5), and C-12 (δ 49.9), and 3*J* correlation of H-11 with C-13 (δ 40.5) was observed. The COSY 45° showed the cross peaks between H-11 (δ 4.04) and H-9 (δ 1.17), and H-12 (δ 1.31, 2.20). The stereochemistry

of this newly introduced hydroxyl group was assigned through NOESY correlations between H-11 (δ 4.04), and Me-18 (δ 0.68), and Me-19 (δ 1.04). Based on the above mentioned spectral data, the structure of the known metabolite 3 was identified as 11α-hydroxy finasteride. Metabolite 3 was earlier obtained by biotransformation of finasteride with *Selenastrum capricornutum* [4].

Metabolite 4 was obtained as a crystalline solid (Scheme 1). The formula of the metabolite 4 was deduced as $C_{23}H_{36}N_2O_3$ (Calcd 388.4848), through HREI-MS (*m/z* 388.4823). This indicated a mono hydroxylation in the substrate 1.

The ^{1}H-NMR spectrum of compound 4 was quite similar to the substrate 1, except an additional downfield methine proton signal, resonated at δ 4.14 (dt, *J* 15e,16a = 7.3 Hz, *J* 15e,14a = 2.0 Hz), indicating the introduction of a hydroxyl group at a secondary carbon. The ^{13}C-NMR spectrum showed an additional downfield methine signal resonated at δ 69.6 (C-15), and downfield shifts of C-14 (δ 59.7), and C-16 (δ 40.9), suggested the hydroxylation at C-15. The presence of the hydroxyl functionality was further deduced by HMBC and COSY 45 ° spectrum. In HMBC spectrum, 2*J*-heteronuclear couplings of H-15 (δ 4.14) with C-14 (δ 59.7) and C-16 (δ 40.9), and 3*J*-heteronuclear couplings C-13 (δ 43.5) were observed indicating the hydroxylation at C-15.

In COSY 45° homonuclear interactions between H-15 (δ 4.14) and H2-16 (δ 2.01, 1.95), and H-14 (δ 0.83), further encouraged the assigned position of hydroxylation at C-15. The stereochemistry of C-15 hydroxyl group was assigned on the basis of NOESY correlations of H-15 (δ 4.14) and H-14 (δ 0.83), and on the comparison with the reported data [5] as β (*axial*) hydroxyl group.

Metabolite 4 (15β-hydroxyfinasteride) was previously obtained by the microbial transformation of finasteride with *Mortierella isabellina* [5].

Table 1: **^{1} H-NMR (600 MHz, $CDCl_3$) chemical shifts assignments of Compounds 2-4**

Carbon No	2 δ_H (*J* = Hz)	3 δ_H (*J* = Hz)	4 δ_H (*J* = Hz)
1	6.79, d (9.9)	7.94, d (10.2)	6.74, d (9.9)
2	5.81, d (9.8)	5.68, d (10.2)	5.69, d (9.9)
3	-	-	-
4	5.53, br. s	5.26, br. s	5.36, br. s
5	3.33, t (8.2)	3.33, dd (11.4, 4.5)	3.26, m
6	1.60, m; 1.61, d (6.2)	1.20, m; 2.05, d (6.20)	1.55, m; 1.53, d (6.2)
7	2.81, m; 1.71, m	1.71, m; 1.01,m	2.04, m; 1.20, m
8	1.78, m	1.37, m	1.77, m
9	1.01, m	1.17, m	0.98, m
10	-	-	-
11	2.03, m; 1.80, m	4.04, m ($W_{½}$ = 23.7)	1.66, m; 1.38, m
12	3.10, d (6.2); 1.99, m	2.20 dd (11.4, 4.5); 1.31	1.80, m; 1.79 m
13	-	-	-
14	1.64, m	1.35, m	0.83, m
15	-	2.01, m; 1.22, m	4.14, dt (7.3, 2.0)
16	2.91, m; 2.71, m	1.63, m; 1.52, m	2.01, m; 1.95, m
17	2.41,m	2.06, m	1.92, m
18	0.76, s	0.68, s	0.84, s
19	0.96, s	1.04, s	0.89
20	-	-	-
21	5.16, br. s	5.13, br. s	6.13, br. s
22	-	-	-
23	1.36, s	1.32	1.24, s

CONCLUSION

In conclusion, the biotransformation of finasteride (**1**) by fungal culture *Macrophomina phaseolina* yielded metabolites **2**-**4**. The main oxidation and hydroxylations occurred in rings B C and D especially at C-7, C-11 and C-15 positions. The metabolite **2** was identified as the new metabolites of the fermentation. As a result of these studies, the regio-and stereoselective hydroxylation at C-11α and C-15β positions were found. These transformed products provide accesses to regions of the steroidal skeletons, which are difficult to be functionalized by conventional chemical methods.

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CONTRIBUTION

The first author has carried out the research. Second third fourth and fifth authors have provided study conception, the design of work, drafting of the manuscript and critical revision.

CONFLICT OF INTERESTS

Declared none

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