

Biotransformation of Progesterone by Whole Cells of Filamentous Fungi *Aspergillus brasiliensis*

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Abstract

Microbial steroid biotransformations have found a wide-reaching application for the production of more precious and functionalized compounds due to their high regio- and stereo selectivity.

In this study, the possibility of using filamentous fungi *Aspergillus brasiliensis* cells in the biotransformation of progesterone, a C-21 steroid hormone was studied for the first time.

The fungal strain was inoculated into the transformation medium supplemented with progesterone as a substrate. Biotransformation of this steroid for 7 days afforded 3 different hydroxylated metabolites: 11 α -hydroxy progesterone; 14 α -hydroxyprogesterone and 21-hydroxyprogesterone.

The metabolites were separated by thin layer chromatography. Structure determinations of the metabolites were performed by comparing NMR, MS and IR spectra of the starting compound with those of metabolites.

These results may be of industrial importance because the metabolites can be used as precursor of some steroid drugs.

Keywords: Progesterone; *Aspergillus brasiliensis*; Microbial transformation; Steroid; Hydroxylation.

Introduction

Steroid drugs are synthesized by chemical or microbial routes, both of which involve conversion of steroid precursors to drug intermediates and final conversion of intermediates to active drugs (1, 2).

Microorganisms have been widely employed for (different products bioconversion and specially) steroids biotransformation to prepare

derivatives, the production of which is difficult to obtain by other methods (3, 4).

The value of microbial biotechnology in the production of steroid drugs was realized for the first time in 1952 when the process of 11 α -hydroxylation of progesterone by *Rhizopus* species was patented by Murray and Peterson (5). Fungi are widely used in steroid microbial transformation studies, since variety of their multipurpose enzyme allow them to transform an extensive range of steroids (6).

Thus far, different *Aspergillus* species have been used for the biotransformation of many

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steroids. These biotransformation evidenced interesting results, such as Baeyer -Villiger oxidations, 5 α -reductions and different microbial hydroxylations on steroid skeleton (6, 7).

There are some reports of biotransformation of progesterone by many strains of *Aspergillus* like *A. wentii*, *A. niger*, *A. nidulans*, *A. ochraceus*, *A. parasiticus*, *A. oryzae*, *A. flavus*, *A. tamari*, *A. parasiticus* and *A. fumigatus* (6, 8-10).

Aspergillus brasiliensis which is described within *Aspergillus* section *Nigri* has very good growth and sporulation. This species can be distinguished from other black aspergilli based on intergenic transcribed region, beta-tubulin and calmodulin gene sequences, by amplified fragment length polymorphism analysis and by extrolite profiles (11, 12).

As far as biotransformations by *A. brasiliensis* are concerned, we have not found any report indicating the biotransformation of steroids by this microorganism. Actually, microbial transformations by this fungus on any substrate have not been reported in literature. The aim of present study was to explore the ability of *A. brasiliensis* in biotransformation of progesterone, as an important steroid for the first time.

Experimental

Materials

Progesterone was purchased from Sigma-Aldrich company and used as substrate for biotransformation.

All other chemicals and reagents were of analytical grade and commercially available.

Instrumental methods

The Infrared spectra (IR) were obtained on a Perkin-Elmer 843 spectrometer with KBr as adjuvant. Electrospray ionization mass spectra (ESI-MS) were obtained using Agilent 6410 Triple Quad mass spectrometer. The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained using a Bruker DRX (Avance 500) spectrometer (Rheinstetten, Germany) at 500 and 125 MHz, respectively, with tetramethyl silane (TMS) as internal standard in CDCl_3 . Chemical shifts (δ) are given in parts

per million (ppm) relative to TMS. Coupling constants (J) are given in hertz (Hz). Thin layer chromatography (TLC) was performed, on 0.25 mm thick layers of silica gel G (Kieselgel 60 HF₂₅₄₊₃₆₆, Merck). Chromatography was performed with chloroform/acetone (7:3) and visualized by spraying the plates with a mixture of methanol-sulfuric acid (6:1) and heating in an oven at 100 °C for 3 min until the colors developed. Melting points (mp) were measured by thermo scientific 9200 apparatus and were uncorrected.

Microorganism

The fungal strain *A. brasiliensis* PTCC 5298 used in the present work was purchased from Iranian Research Organization for Science and Technology (IROST) in lyophilized powder form.

Cultures of fungi were grown at 26 °C for 5 days until good sporulation was obtained on Czapek. Stock cultures were maintained at 4 °C on Czapek medium slopes and freshly subcultured before use in transformation experiments. The organism was transferred to fresh medium and refreshed every two weeks.

Inoculum preparation and biotransformation process

Five day old spores were washed from slants with distilled water containing Tween-80 and aseptically inoculated in 500 mL Erlenmeyer flask containing 100 mL of the growth medium under aseptic condition (pH of the medium was adjusted to 7.4 before sterilization).

Volume of inoculums, containing 1×10^6 spores, was used in all experiments unless otherwise stated. The used biotransformation medium was the same as that of growth medium. The flasks were incubated on a rotary shaker at 125 rpm, 26 ± 1 °C for 48 h until pellet formation.

100 mg of progesterone dissolved in 1 mL acetone was added to a 48 h-grown culture and incubated to continue the transformation. A parallel control that received no progesterone and also a culture medium without microorganism, containing the substrate were run concurrently (Control cultures).

Biotransformation was carried out under aseptic condition for further 7 days (13).

Sampling was carried out every 24 h. The

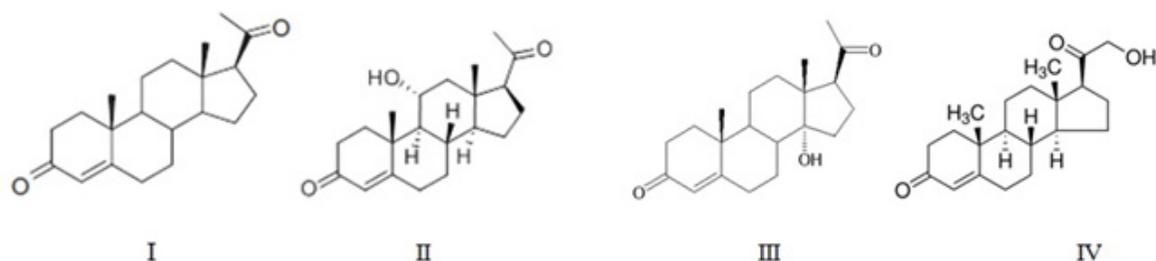


Figure 1. The Structure of progesterone and its metabolites.

samples (5cc) were extracted with three volumes of chloroform and analyzed by thin layer chromatography (TLC).

Product isolation and analyses

At the end of incubation after the transformation, both mycelia and filtrate were separately extracted with chloroform (3 volumes), the chloroform extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure. Complete evaporation of the solvent gave a semi-solid mass from the extract of the filtrate. The residue was then loaded on TLC plates and fractionated with chloroform/acetone (7:3, v/v) solvent system and the metabolites were separated from silica gel by a mixture of methanol/chloroform/acetone (three times). The fractions thus obtained were further purified by further chromatography. Purified metabolites were identified by the spectral data (^{13}C NMR, ^1H NMR, FTIR and MS).

Result and Discussion

Incubation of progesterone by *A. brasiliensis* for 7 days afforded 3 main products II-IV.

Products of progesterone (I) bioconversion were recovered from TLC plates and their

chemical structures were determined mainly based on ^1H -NMR (Table I) and ^{13}C -NMR (Table II) spectra, together with data obtained from mass spectral and FTIR spectral data. Further support for the identification of the compounds was obtained by comparison of the reported spectral values for the compounds. The molecular structures of metabolites are shown in Figure 1.

R_f of progesterone and metabolites in chloroform: acetone (7:3) were 0.9, 0.53, 0.74, 0.8 respectively.

The mass fragmentation profile of the products revealed molecular ion peaks at m/z 330 for all the compounds II-IV, which is at 16 mass units higher than that of the parent compound, progesterone, indicating the possible insertion of one oxygen atom in the structure of each of the products.

Compound II: 11 α -hydroxy progesterone

The purified material had m.p. of 165-167 °C. Its IR spectrum showed an absorption bands at 3439 cm^{-1} (OH) and 1699-1660 cm^{-1} (conjugated CO).

The ^1H NMR spectrum of the metabolite II demonstrated a downfield shift (δ 0.08 ppm) for the 19-methyl group. The metabolite had a characteristic resonance at δ_{H} 4.07 ppm due

Table 1. ^1H NMR data for compounds 1-4.

Hydrogen atom	I	II	III	IV
4-H	5.73	5.72	5.72	5.74
17 α -H	2.53 (1H, t)	2.6(1H, t)	3.2(1H, t)	2.9(1H, t)
18-CH ₃	0.67	0.68(3H, s)	0.78(3H, s)	0.69(3H, s)
19-CH ₃	1.2	1.31	1.22	1.18
21-CH ₃	2.13(3H, s)	2.12 (3H, s)	2.18(3H, s)	4.21 (dd, 21 α -H)
Other significant H signal	-	4.03 (1H, dd, 11 β -H)	3.23 (t, 12 α -H)	-

Carbon atom	I	II	III	IV
1	35.46	33.58	35.72	35.68
2	33.72	34.17	33.92	33.89
3	199.18	200.36	199.93	199.58
4	123.66	124.51	123.95	123.98
5	170.8	171.14	170.55	170.87
6	32.55	33.56	32.57	32.72
7	31.65	31.51	31.92	31.85
8	35.28	37.46	35.72	35.52
9	53.38	55.32	46.28	53.54
10	38.34	39.93	38.26	38.35
11	20.78	68.79	21.32	20.90
12	38.40	50.35	38.61	38.55
13	43.68	44.13	46.28	44.66
14	55.76	55.32	85.15	56.60
15	24.13	24.22	24.73	24.46
16	22.57	22.90	22.69	22.69
17	63.23	63.11	59.42	63.67
18	13.11	14.48	17.23	13.45
19	17.13	18.27	17.02	17.35
20	209.08	208.93	210.45	210.17
22	31.29	31.38	31.47	59.02

to the signal of 11β proton, in the shape of a triplet splitted into doublets (1H, td, J = 10.0 and 5.0 Hz) (14) and absence of the characteristic C-11 at δ 20.78 in ^{13}C NMR spectrum replaced with δ 68.54 ppm suggesting that hydroxylation had taken place at the equatorial proton at C-11, generating an 11α -hydroxy group. The comparison of the metabolite's melting point and spectral data with those in the literature (15, 16) confirmed that it was 11α -hydroxyprogesterone.

Compound III: 14α -hydroxy progesterone

This compound had m.p. of 195-197 °C. Increased polarity of the metabolite III compared to progesterone, and its IR and mass data suggested the insertion of an oxygen atom as tertiary hydroxyl functional group in the molecular structure. The infrared analysis of compound III showed two carbonyl absorption bands at 1693 and 1644 cm^{-1} and a hydroxyl group at 3474 cm^{-1} . Among the 8β -, 9α -, 14α - and 17α -tertiary positions available in progesterone, hydroxylation at 8β -position should furnish a triplet for 9α -proton while that at 9α -position would cause a

down-field shift of 19-CH_3 by ca. 0.13 ppm and small but significant shift of 4-H signal to ca. δ 5.9 without affecting the 17-H signal. In fact, no such situation was observed with the spectral profile of III. In contrast, a distinctive shift of 0.71 ppm (ca. δ 2.5 to 3.2) for $17\alpha\text{-H}$ signal without any change in the triplet, an appreciable down-field shift (by 0.13 ppm) for 18-CH_3 but no change for 19-CH_3 signal are characteristic features of 14α -hydroxylation (13, 17).

Compound IV: 21 -hydroxyprogesterone (11-Deoxycorticosterone)

Melting point of this product was 142-144 °C. The IR spectrum showed two carbonyl absorption bands at 1691 and 1662 cm^{-1} and a hydroxyl group at 3479 cm^{-1} .

In case of metabolite IV, absence of characteristic C-21 at δ 31.29 in ^{13}C NMR spectrum proposes the substitution of hydroxyl group in C -21 which appeared at δ 59.2. The ^1H NMR spectrum demonstrated a large downfield shift ($\Delta\delta$: 2.07 ppm) for the 21-methyl group. The characteristic resonances at δ_{H} 4.20

ppm the shape of a doublet, also a downfield shift of 17 α -methyl protons singlet ($\Delta\delta = 0.4$ ppm) without any change in the triplet, and no significant changes for 18-CH₃ and 19-CH₃ signals confirmed this metabolite as 21-hydroxyprogesterone.

Thus far the important biotransformations which have been reported for progesterone were 11 β , 16 α , 17 α and 21 α hydroxylation for producing corticosteroids (18, 19). Steroidal hydroxylase system of filamentous fungi is usually presented by mono oxygenase of microsomal localization which contains cytochrome P-450 (Cyt P-450) as a terminal oxidase. The cytochrome P-450 binds a substrate and flavoprotein NADP(H)-P450-reductase that provides an electron transport from reduced coenzyme to Cyt P-450 enzyme (20, 21).

The results of present study indicate that *A. brasiliensis* hydroxylated progesterone in position C₁₁ and C₁₄ which have been previously reported in some microbial transformations (3, 17, 22, 23). The ability of different species of *Aspergillus* such as *A. fumigatus*, *A. niger*, *A. fischeri* and *A. wentii* in bioconversion of progesterone to 11 α -hydroxyl progesterone has already been reported in literature (15, 24-26). 11 α -hydroxylation is an essential step in corticosteroid synthesis. Thus, the ability of *A. brasiliensis* to convert I to II exemplifies the potential of this fungi for this specific purpose. In addition, 14 α -hydroxylation is an important process since its product possesses anticancer properties (6). Consequently, the 14 α -hydroxylation capacity of *A. brasiliensis* may be of commercial importance.

Hydroxylation at C-21 position of the steroid skeleton was previously recorded in literature (3, 27). This substitution is an important structural feature for the biological activity of the adrenal cortical hormones (28). 21-hydroxyprogesterone or 11-Deoxycorticosterone, is a steroid hormone produced by the adrenal gland that possesses mineralocorticoid activity and acts as a precursor for aldosterone.

Conclusion

The results obtained in this study indicate that *A. brasiliensis* is capable of hydroxylating

the different sites of progesterone. It produces, in acceptable yields, three valuable steroidal transformation products, 11 α -hydroxy progesterone as a major compound, 14 α -hydroxy progesterone and 21-hydroxyprogesterone or deoxycorticosterone. To the best of our knowledge, there have been no reports of steroid biotransformation by *A. brasiliensis* in the literature. Therefore understanding the process of biotransformation by this microorganism could be of applied importance where fungi are used for production of valuable steroids with different pharmacological properties.

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References

- (1) Rao SM, Thakkar K and Pawar K. Microbial transformation of steroids: current trends in cortical side chain cleavage. *Quest.* (2013) 1: 16.
- (2) Tabatabaei Yazdi M, Faramarzi M, Ghostrinoudi H, Amini M and Ghasemi Y. Biotransformation of AD and ADD using *Nostoc muscorum*. *Iran. J. Pharm. Res.* (2010) 3: 84-85.
- (3) Bhatti HN and Khera RA. Biological transformations of steroidal compounds: A review. *Steroids* (2012) 77: 1267-1290.
- (4) Korbekandi H, Ashari Z, Iravani S and Abbasi S. Optimization of biological synthesis of silver nanoparticles using *fusarium oxysporum*. *Iran. J. Pharm. Res.* (2013) 3: 289-298.
- (5) Murray HC and Peterson H. *Oxygenation of steroids by mucorales fungi*. US Patent, US2602769 A (1952).
- (6) Fernandes P, Cruz A, Angelova B, Pinheiro HM and Cabral JMS. Microbial conversion of steroid compounds: Recent developments. *Enzy. Microbial. Technol.* (2003) 32: 688-705.
- (7) Choudhary MI, Batool I, Shah SAA, Nawaz SA and Atta ur R. Microbial hydroxylation of pregnenolone derivatives. *Chem. Pharmaceutic. Bulletin.* (2005) 53: 1455-1459.
- (8) Eman Mostafa M and Zohri AA. Progesterone side-chain degradation by some species of *Aspergillus flavus* group. *Folia Microbiologica.* (2000) 45: 243-247.

- (9) Hunter AC, Coyle E, Morse F, Dedi C, Dodd HT and Koussoroplis SJ. Transformation of 5-ene steroids by the fungus *Aspergillus tamarii* KITA: Mixed molecular fate in lactonization and hydroxylation pathways with identification of a putative 3 β -hydroxy-steroid dehydrogenase/ Δ 5- Δ 4 isomerase pathway. *Biochimica et Biophysica Acta - Molecul. Cell Biol. Lipids* (2009) 1791: 110-117.
- (10) Dutta TK and Samanta TB. Bioconversion of progesterone by the activated immobilized conidia of *Aspergillus ochraceus* TS. *Current Microbiol.* (1999) 39: 309-312.
- (11) Varga J, Kocsu \acute{b} e S, T \acute{o} th B, Frisvad JC, Perrone G, Susca A, Meijer M and Samson RA. *Aspergillus brasiliensis* sp. nov., a biseriata black *Aspergillus* species with world-wide distribution. *Int. J. System. Evolution. Microbiol.* (2007) 57: 1925-1932.
- (12) Niknejad F, Moshfegh M, Najafzadeh MJ, Houbrakend J, Rezaei S, Zarrini G, Faramarzi MA and Nafissi-Varcheh N. Halotolerant ability and α -amylase activity of some saltwater fungal isolates. *Iran. J. Pharm. Res.* (2013) 12: 113-119.
- (13) Habibi Z, Yousefi M, Ghanian S, Mohammadi M and Ghasemi S. Biotransformation of progesterone by *Absidia griseoella* var. *igachii* and *Rhizomucor pusillus*. *Steroids* (2012) 77: 1446-1449.
- (14) Harold C and Robert W. A survey of the high-field 1H NMR spectra of the steroid hormones, their hydroxylated derivatives, and related compounds. *J. Chem. Soc. Perkin Transactions 2* (1990) 1567-1594.
- (15) Yildirim K, Kupcu I and Gulsan F. Biotransformation of some steroids by *Aspergillus wentii*. *Zeitschrift fur Naturforschung - Section C. J. Biosci.* (2010) 65: 688-692.
- (16) Farooq A, Hanson JR and Iqbal Z. Hydroxylation of progesterone by *Cephalosporium aphidicola*. *Phytochem.* (1994) 37: 723-726.
- (17) Kolet SP, Haldar S, Niloferjahan S and Thulasiram HV. *Mucor hiemalis* mediated 14 α -hydroxylation on steroids: *In-vivo* and *in-vitro* investigations of 14 α -hydroxylase activity. *Steroids* (2014) 85: 6-12.
- (18) Faramarzi MA, Tabatabaei Yazdi M, Amini M, Zarrini G and Shafiee A. Microbial hydroxylation of progesterone with *Acremonium strictum*. *FEMS Microbiol. Let.* (2003) 222: 183-186.
- (19) Teshima S and Kanazawa A. *In-vitro* bioconversion of progesterone to 17- α -hydroxyprogesterone and testosterone by sliced testes of crab, *portunus-trituberculatus*. *Bulletin of the Japanese Society of Scientific Fisheries* (1971) 37: 524.
- (20) Bernhardt R. Cytochromes P450 as versatile biocatalysts. *J. Biotechnol.* (2006) 124: 128-145.
- (21) Kollerov VV, Shutov AA, Fokina VV, Sukhodol'skaia GV, Gulevskaia SA and Donova MV. Bioconversion of C19- and C21-steroids with parent and mutant strains of *Curvularia lunata*. *Prikladnaia Biokhimiia Mikrobiologiya* (2010) 46: 212-220.
- (22) El-Kadi IA and Eman Mostafa M. Hydroxylation of progesterone by some *Trichoderma* species. *Folia Microbiologica.* (2004) 49: 285-290.
- (23) Lamm AS, Chen ARM, Reynolds WF and Reese PB. Steroid hydroxylation by *Wetzelinia sclerotiorum*, *Phanerochaete chrysosporium* and *Mucor plumbeus*. *Steroids* (2007) 72: 713-722.
- (24) Fouad WA, Abbas IH, Elwan KM, Swellum MA and El-DougDoug KA. Biotransformation of progesterone by microbial steroids. *J. Applied Sci. Res.* (2009) 5: 137-143.
- (25) Smith KE, Ahmed F, Williams RAD and Kelly SL. Microbial transformations of steroids - VIII. Transformation of progesterone by whole cells and microsomes of *Aspergillus fumigatus*. *J. Steroid Biochem. Molecular Biol.* (1994) 49: 93-100.
- (26) Sallam LAR, El Refai AMH, Nada S and Abdel Fattah AF. Enzymic hydroxylation and side chain degradation of progesterone by *Aspergillus fischeri*. *J. General Applied. Microbiol.* (1973) 19: 155-160.
- (27) Zaffaroni A, Casas Campillo C, Cordoba F and Rosenkranz G. Steroids. LXVI. Microbiological hydroxylation of steroids in position 21. *Cellular and Molecular Life Sci.* (1955) 11: 219.
- (28) Zohri A and Abdel-Galil M. Progesterone transformations by three species of *Humicola*. *Folia Microbiologica* (1999) 44: 277-282.