Journal of Sciences, Islamic Republic of Iran 17(4): 309-312 (2006) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

Biotransformation of Hydrocortisone by *Neurospora crassa*

E. Gharaee Fathabad,[1] M. Tabatabaei Yazdi,[1,*] M.A. Faramarzi,[1] and M. Amini[2]

[1] *Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Tehran University of Medical Sciences, P.O. Box 14155-6451,Tehran 14174, Islamic Republic of Iran*
[2] *Department of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, P.O. Box 14155-6451,Tehran 14174, Islamic Republic of Iran*

Abstract

The ability of *Neurospora crassa* FGSC 4335 in the biotransformation of hydrocortisone was investigated. The microorganism produced two major metabolites after incubation with the substrate for seven days. Each microbial product was purified chromatographically and identified on the basis of spectral data. The products were identified as 11β,17α,20β,21-tetrahydroxypregn-4-en-3-one (II) and 11β-hydroxyandrost-4-en-3,17-dione (III) in the yields of 25% and 18.8%, respectively. Time course study showed the accumulation of both products from the second day of the fermentation and reached to the maximum in the seventh day. Optimum concentration of the substrate, which gave maximum bioconversion efficiency, was 1 mg ml^{-1} in one batch. Biotransformation was completely inhibited in a concentration above 2.5 mg ml^{-1}.

Keywords: *Neurospora crassa*; Hydrocortisone; Biotransformation; Fungi

Introduction

Microbial transformation is one of the most attractive approaches for introducing functional groups into various positions of organic compounds [1]. Using microorganisms in the biotransformation procedures has been investigated for decades with the advantages in production of the molecules with regio- and stereo-specificity [2,3]. One of the major biotechnological aspects in this area is the application of a wide range of the microorganisms including bacteria, fungi and microalgae in converting steroid substances into the pharmacoligically active compounds or other useful intermediates [4-6]. For instance, microorganisms are widely used in conducting of specific reactions such as side chain cleavage or hydroxylation in specific positions on steroids to obtain pharmacologically active substances [1].

The fungus *Neurospora crassa*, the common pink bread mold, is a filamentous ascomycete which produces many different enzymes [7] and has gained widespread usages [8]. It grows fast in high yields in simple synthetic media. Many phenotypes are readily registered and genetically accessible [7,8]. The potential of this fungus in the biotransformation of steroids has been rarely exploited. In this work, the ability of *Neurospora crassa* FGSC 4335 for bioconversion of hydrocortisone is studied and the results are presented.

[*] *E-mail: mtabataba@sina.tums.ac.ir*

Materials and Methods

Chemicals, Instruments and the Microorganism

Hydrocortisone was kindly donated by Aburaihan Pharmaceutical Co. (Tehran, Iran), which had been purchased from Pharmacia & Upjohn S.A. (Guyancourt, USA). Other reagents and solvents were from Merck (Germany) and of analytical grade unless otherwise mentioned. The instrumental analysis was conducted as previously described [9]. *Neurospora crassa* FGSC 4335, was obtained from the Fungal Genetics Stock Center, University of Kansas. It was maintained on Sabouraud-4%-dextrose agar slope and freshly subcultured before using in biotransformation experiments.

Incubation Conditions

Freshly obtained spores from agar slope cultures were inoculated in a 500-ml conical flask containing 100 ml of liquid medium of Sabouraud 2% dextrose broth (SDB) and incubated for 12 h at 25°C in a rotary shaker (150 rpm). Spores were collected with sterile normal saline solution containing 0.1% Tween 80. Ten 500-ml Erlenmeyer flasks, each containing 100 ml of SDB plus 2 ml of a 20 ml ethanol solution containing hydrocortisone (1 g) were inoculated with the spore solution and the incubation was carried out for seven days at 25°C on a rotary shaker (150 rpm).

Time Course Study and the Effect of Substrate Concentration

For a time course study, freshly spore solution obtained from a 12 h old culture of *N. crassa* was transferred into a 500-ml Erlenmeyer flask containing 100 ml of SDB supplemented with 100 mg hydrocortisone and then the incubation continued for seven days at the condition described above (see Incubation conditions). Sampling was carried out every 24 h. The effect of optimum substrate concentration was also examined. The amount of the substrate was varied from 0.5 to 2.5 mg ml^{-1} with a stepwise of 0.5. Results were obtained according to TLC analyses.

Biotransformations, Product Isolation and Analysis

The fermentation medium was separated from mycelium by filtration and the broth was extracted three times with chloroform. The extract was washed with water and evaporated under reduced pressure. The same procedure was done on the mycelia. Both of the extracts were checked for steroid metabolites using thin layer chromatography with chloroform/acetone (6:4 v/v). There was no steroid metabolite in the extract obtained from the mycelia. Then, the chloroform extract which prepared from the broth was loaded on silica gel plates (20×20 cm, 0.5 mm thickness) for preparative TLC; and fractionated with the same solvent system as described above. The metabolites were eluted first with chloroform/methanol (80:20 v/v), then with (20:80 v/v) from silicagel and after evaporation were crystallized from methanol. The metabolites were also analyzed on a Waters HPLC at 254 nm using a C18 column (4.6×150 mm). A mixture of methanol/water (45:55 v/v) was used as the mobile phase. The flow rate was adjusted to produce 1 ml/min.

Results

The crude extract obtained from seven days incubation of *Neurospora crassa* in the presence of hydrocortisone produced two major compounds (**II**, **III**), in addition to the substrate (**I**) as follows (Fig. 1).

Metabolite **II** was crystallized from methanol; mp 128-130°C, $[\alpha]_D$ +91°(MeOH); lit [9]: mp 133-135°C, $[\alpha]_D$ +85°; IR $_{\upsilon\ max}$ (KBr, cm^{-1}) 3462, 2924, 1649; MS (EI) m/z (%) 364 (18) (M^+,$C_{21}H_{32}O_5$), 346 (18), 331 (7), 315 (56), 303 (46), 285 (100), 267 (31), 227 (64), 148 (38), 124 (40), 91 (82), 79 (55); ^{1}H NMR ($CDCl_3$) δ 1.08 (3H, s, H-18), 1.46 (3H, s, H-19), 3.66 (2H, dd, J=18.4 Hz, J=4.8 Hz, H-21), 3.78 (1H, m, H-20), 4.36 (1H, s, H-11), 5.62 (1H, s, H-4); ^{13}C NMR ($CDCl_3$) δ 199.8 (C_3), 172.8 (C_5), 122.0 (C_4), 84.3 (C_{17}), 74.2 (C_{20}), 68.1 (C_{11}), 64.1 (C_{21}), 55.1 (C_9), 50.6 (C_{14}), 46.7 (C_{13}), 41.5 (C_{10}), 39.2 (C_{12}), 33.8 (C_1), 33.1 (C_2), 32.8 (C_{16}), 32.1 (C_6), 29.7 (C_7), 29.3 (C_8), 23.6 (C_{15}), 20.9 (C_{19}), 17.8 (C_{18}); R_f 0.1 ($CHCl_3$/Me_2CO; 6:4 v/v).

Metabolite **III** was also crystalized from methanol; mp196-197°C, $[\alpha]_D$ +226°(MeOH); lit [10]: mp 197-199°C, $[\alpha]_D$ +225°($CHCl_3$); IR $_{\upsilon\ max}$ (KBr, cm^{-1}) 3418, 1713, 1650; MS (EI) m/z (%) 302 (70) (M^+,$C_{19}H_{26}O_3$), 286 (20), 227 (24), 189 (37), 163 (100), 149 (46), 91 (24), 83 (18); ^{1}H NMR ($CDCl_3$) δ 1.17 (3H, s, H-18), 1.47 (3H, s, H-19) , 4.47 (1H, s, H-11), 5.70 (1H, m, H-4); ^{13}C NMR ($CDCl_3$) δ 218.9 (C_{17}), 199.3 (C_3), 171 (C_5), 122.6 (C_4), 67.9 (C_{11}), 56.7 (C_9), 52.4 (C_{14}), 46.7 (C_{13}), 41.0 (C_{10}), 39.3 (C_{12}), 36.9 (C_8), 35.3 (C_1), 35.0 (C_{16}), 33.8 (C_2), 31.8 (C_6), 31.5 (C_7), 21.4 (C_{15}), 21.1 (C_{19}), 15.8 (C_{18}); R_f 0.8 ($CHCl_3$/Me_2CO; 6:4 v/v).

HPLC analysis was performed to achieve the biotransformation yield of each metabolite (chromatogram not shown). Two major peaks of bioconverted products were obtained with retention times of 7.29 and 8.38 min, which related to the metabolites **II** and **III**, respectively. In this system, the retention time of

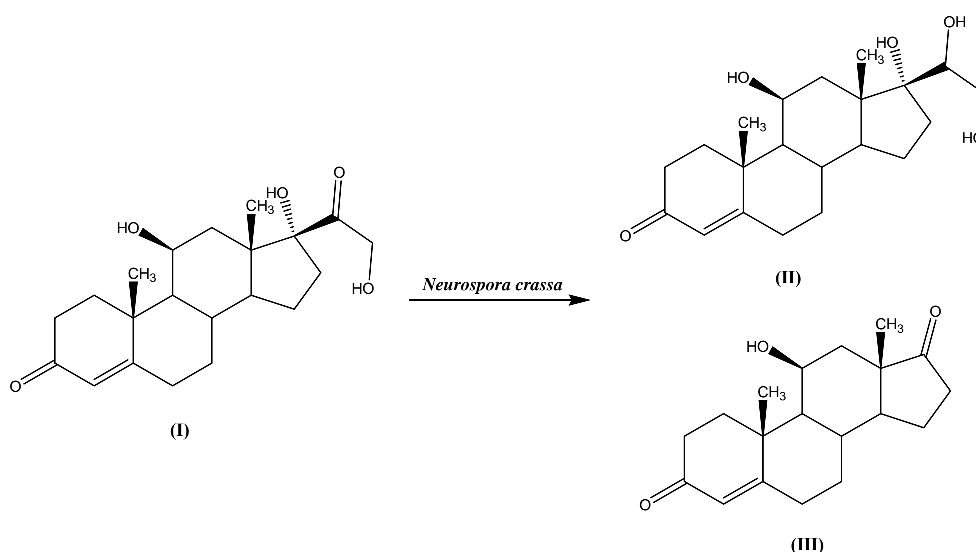

Figure 1. The structures of hydrocortisone and the biotransformed products: hydrocortisone (**I**), 11β,17α,20β,21-tetrahydroxypregn-4-en-3-one (**II**), 11β-hydroxyandrost-4-en-3,17-dione (**III**).

hydrocortisone was 10.46 min. The yield of each product expressed as percentage of the sum of the total transformed products and the remaining substrate by direct computational integration of the individual peaks was found to be 18.75 and 25% for metabolites **II** and **III**, respectively. The HPLC analysis revealed trace quantities of other metabolites in the transformed mixture, which were not purified for characterization.

For a time course study, production of **II** and **III**, as a function of incubation time, was detected by thin layer chromatography. The starting material, **I**; 1 mg ml^{-1}, was transformed to the products within seven days. According to TLC profile, metabolites **II** and **III** were accumulated in the broth from the second day of incubation and reached to the maximum concentrations within seven days.

N. crassa was also examined to convert hydrocortisone at different concentrations between 0.5 to 2.5 mg ml^{-1}. Based on TLC profile, best substrate concentration was 1 mg ml^{-1}, and in a concentration above 2.5 mg ml^{-1}, hydrocortisone was not converted to any metabolite.

Discussion

From the results of the experiment, it appears that *Neurospora crassa* transformation of hydrocortisone (**I**) led to the formation of two major bioproducts (**II** and **III**) with the following characterizations.

Metabolite **II** was less polar (R_f: 0.1) than the substrate (R_f: 0.5) in a solvent system as $CHCl_3/Me_2CO$; 6:4 v/v and the other metabolite, **III**, was much polar (R_f: 0.8) than hydrocortisone.

The mass spectrum of metabolite **II** showed the molecular ion at m/z 364, which indicated the addition of two units as compared to that of hydrocortisone (m/z 362). It can be imagined that one carboxyl group or double bond in substrate have been reduced. The IR spectrum showed only one carbonyl group at 1649 cm^{-1} that indicated the conjugated ketone in C-3 position has not been altered. The elimination of C-20 carbonyl group absorption in the IR spectrum showed that the reduction has taken place at C-20. Additional multiplet resonance at δ 3.66 in ^{1}H-NMR spectrum as compared to the substrate confirmed the metabolite **II.** In addition, the other notable observation obtained was stereospecific reduction at the C-20 ketone group. The configuration of the C-20 hydroxyl group was recognized mainly with comparison of its melting point with the compounds having α-hydroxyl and β-hydroxyl groups at C-20 [11]. Melting point value of the metabolite **II** was similar to the compound with β-hydroxyl group at C-20 position.

The mass spectrum of metabolite **III** showed the molecular ion at m/z 302, which suggested the reduction of 60 units as compared to hydrocortisone. Feature of note in the IR spectrum of this compound included peaks at 1713 cm^{-1} (C=O) 1650 cm^{-1} (C=O), which confirmed the existence of two carboxyl groups in this compound. In addition, the IR spectra indicated the existence of at least one hydroxyl group in metabolite **III**. The IR data has been also supported by the related

^{13}C NMR spectra. Two signals at δ 199.3 and 218.9 in compound **III** have been imputed to C-3 and C-17, respectively. The chemical shift of H-11 was reported for hydrocortisone and other 11-hydroxy steroids in δ 4.3-4.4 [12].

Thus, metabolites **II** and **III** were identified as 11β,17α,20β,21-tetrahydroxypregn-4-en-3-one and 11β-hydroxyandrost-4-en-3,17-dione, respectively.

Microbial transformation of steroid has been studied by almost every type of microorganisms including fungi. They are able to convert the substrates to their derivatives in a high level of stereospecificity [1]. Fungi that grow in simple conditions and having no harmful effects on human health are of importance in this field. Among the fungi, *N. crassa* has the potential of industrial applications [4,13]. In the present study, *N. crassa* was applied to biotransformation of hydrocortisone. The microorganism converted the substrate into a pregnane and an androstane derivative. The obtained results indicated that the main reaction was side chain cleavage. Microbial scission of carbon-carbon bonds is important in cleavage of sterol side chains to produce useful C-19 intermediates [1]. Another bioreaction was the reduction of carbonyl group at C-20 position. Reduction of carbonyl group has been widely occurred [1]. In 1999, Wilson *et al.* [4] reported that this kind of reduction in the biotransformation of cortisone by *Fusarium oxysporum*. The characteristic features were C-20 carbonyl reduction and side chain cleavage at C-17 with the formation of a carbonyl group. In another report, Faramarzi *et al.* [9] showed the bioconversion of hydrocortisone using *Acremonium strictum*. The bioreactions were side chain cleavage, C-20 carbonyl reduction and hydroxylation at C-17 position. Porter *et al.* [14] performed the steroid microbial biotransformation by *Exophiala jeanselmei* and reported C-20 ketone reduction, C-17 side chain cleavage which produced C-17 hydroxyl group and further transformation resulted in conversion of hydroxyl function into a carbonyl group at C-17 position. The application of *Neurospora crassa* for biotransformation of hydrocortisone had not been previously studied. However, Luke and Burton [7] reported a novel application for *Neurospora crassa* in bioremediation of phenol compounds. They concluded that *Neurospora crassa* produced intra- and extracellular oxidases at high levels of activity which were able to convert efficiently those substances. In this work, it can be concluded that *Neurospora crassa* acts as a biocatalyst in the transformation of steroidal compounds specially for the purpose of side chain cleavage and C-20 ketone reduction on pregnane-like substances.

Acknowledgement

This work was supported by a grant from the research council of Tehran University of Medical Sciences, Tehran, Iran.

References

1. Smith L.L. Steroids. In: Rehm H.J. and Reed G. (Eds.), *Biotechnology*, Vol. 6a, Chrmie GmbH, Weinheim, pp. 31-78 (1984).
2. Fraga B.M., Hernandez M.G., Gonzalez P., Charmy M.C., and Garbarino J.A. The biotransformation of 18-hydroxy-9-epi-ent-pimara-7,15-diene by *Gibberella fujikuroi*. *Phytochemistry*, **53**: 395-399 (2000).
3. Hu S., Tian X., and Han G. Novel microbial hydroxylation of 13-ethyl-17β-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one. *Steroids*, **63**: 88-92 (1988).
4. Wilson M.R., Gallimore W.A., and Reese P.D. Steroid transformations with *Fusarium oxysporum* var. cubense and *Colletotrichum musae*. *Steroids*, **64**: 834-843 (1999).
5. Farooq A. and Tahara S. Biotransformation of testosterone and pregnenolone catalyzed by the fungus *Botrytis cinerea*. *J. Nat. Prod.*, **63**: 489-491 (2000).
6. Lisowska K. and Dfugonski J. Concurrent corticosteroid and phenantherene transformation by filamentous fungus *Cunninghamella elegans*. *J. Steroid Biochem. Molec. Biol.*, **85**: 63-69 (2003).
7. Luke A.K. and Burton S.G. Novel application for *Neurospora crassa*: progress from batch culture to a membrane bioreactor for the bioremediation of phenols. *Enzyme Microb. Technol.*, **99**: 348-356 (2001).
8. Bankel L., Holm E., Lindstedt G., and Lindstedt S. Oxygenases involved in thymine and thymidine metabolism in *Neurospora crassa*. *FEBS Lett.*, **21**: 135-138 (1972).
9. Faramarzi M.A., Tabatabaei Yazdi M., Shafiee A., and Zarrini G. Microbial transformation of hydrocortisone by *Acremonium strictum* PTCC 5282. *Steroids*, **67**: 869-872 (2002).
10. Rao P.N. Manganese dioxide oxidation: the optional introduction of Δ6-double bond with simultaneous cleavage of dihydroxyacetone or 17,20-glycol side chains in Δ4-3-ketosteroids. *J. Org. Chem.*, **26**: 2149-2152 (1961).
11. Carvajal F., Vitale O.F., Gentles M.J., Herzog H.L., and Hershberg E.B. Microbial transformation of steroids. VI. Stereospecific reductions of the 20-carbonyl group. *J. Org. Chem.*, **24**: 695-698 (1959).
12. Kirk D.N., Toms H.C., Douglas C., and White K.A. A survey of the high-field 1H NMR spectra of the steroid hormones, their hydroxylated derivatives, and related compounds. *J. Chem. Soc. Perkin. Trans. II*, **9**: 1567-1594 (1990).
13. Watanaba K. Microorganisms relevant to bioremediation. *Curr. Opin. Biotechnol.*, **12**: 237-241 (2001).
14. Porter R.B.R., Gallimore W.A., and Reese P.B. Steroid transformations with *Exophiala jeanselmei* var lecanii-corni and *Ceratocystis paradoxa*. *Steroids*, **64**: 770-779 (1999).