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ALGAL TRANSFORMATION OF HYDROCORTISONE BY THE CYANOBACTERIUM *Nostoc ellipsosporum*

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The potential of Nostoc ellipsosporum *for biotransformation of hydrocortisone was studied. The microorganism was isolated during a screening program from soil samples collected from the paddy fields in the north of Iran and had not been previously examined for this purpose. The biotransformation yielded 11β,17α,20β,21-tetrahydroxypregn-4-en-3-one and 11β-hydroxyandrost-4-ene-3,17-dione. Both of the metabolites were purified chromatographically and characterized using instrumental analyses.*

Key words: Biotransformation, hydrocortisone, microalgae, *Nostoc ellipsosporum*.

Bacteria and fungi are generally employed in studies of steroid biotransformation, while cyanobacteria have been less investigated [1]. Cyanobacteria are photoautotrophic prokaryotes and do not require an organic substrate for energy. Consequently, their culture is simpler and cheaper than bacteria, yeasts, and fungi. They are more useful for biotransformation than cell culture of higher plants as they grow much faster in simple media and have a higher genetic diversity [2]. The potential of cyanobacteria for steroid biotransformation was firstly reported by Abul-Hajj and Qian [3]. They showed the ability of different strains of cyanobacteria such as *Anabaena cylindrica*, *Scenedesmus quadricauda*, and *Coelastrum proboscideum* to convert androstenedione to testosterone. More recently the ability of green algae for transformation of progesterone, prednisolone and some other steroids has been reported [4–6].

In a screening program for the isolation and identification of antimicrobial-producing cyanobacteria from paddy-fields in the north of Iran, we isolated some strains of *Nostoc* spp and *Fischerella* spp [7]. Previous observation showed that the isolated cyanobacteria *Nostoc muscorum* PTCC 1636 and *Fischerella ambigua* PTCC 1635 transformed hydrocortisone into some androstane and pregnane derivatives [8, 9]. However, no literature report has been found on the biotransformation of hydrocortisone using *Nostoc ellipsosporum*. The aim of this study is to study the ability of a locally isolated strain of *Nostoc ellipsosporum* to convert hydrocortisone as an exogenous substrate.

High specific reactions are required to produce functionalized steroids with therapeutic use and commercial value. Although some of the bioconversions on steroid compounds are well established, efforts are ongoing to identify new microorganisms capable of performing useful bioconversions. Earlier studies have clearly demonstrated that bacteria and fungi metabolize hydrocortisone. 1,2-Double bond formation using *Cylindrocarpon radicicola*, *Streptomyces laverdula*, *Fusarium causicam*, *Fasarium solari*, and *Septomyxa offinis* [10] and 1-dehydrogenation of hydrocortisone to prednisolone by *Arthrobacter (Corynobacterium) simplex*, *Bacillus sphaericus*, and *Bacterium cyclooxydans* have already been applied in industrial production [11]. Reduction of the 4, 5-double bond of hydrocortisone was also well studied by the Herbert L. Holand group [12]. In our previous works on biotransformation of hydrocortisone, it was found that *Acromonium strictum* PTCC 5282, *Nostoc muscorum* PTCC 1636, and *Fischerella ambigua* PTCC 1635 converted hydrocortisone into some pregnane and androstane derived products [8, 9, 13].

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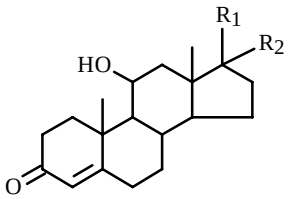
In the present study, we have shown the ability of *N. ellipsosporum* to transform hydrocortisone into two pregnane and androstane derivatives with the following characterizations. 11β-Hydroxyandrost-4-ene-3,17-dione (**1**) was less polar (R_f 0.8) than the substrate (**3**) (R_f 0.5) and the other metabolite, 11β,17α,20β,21-tetrahydroxypregn-4-en-3-one (**2**), was more polar (R_f 0.15).

The mass spectrum of metabolite **1** showed the molecular ion at *m/z* 302, which suggested the reduction of 60 units as compared to hydrocortisone. Features of note in the IR spectrum of this compound include peaks at 1735 cm^{-1} (C=O) and 1657 cm^{-1} (C=O), which confirmed the existence of two carboxyl groups in compound **1**. In addition, the IR spectra indicated the existence of at least one hydroxyl group in metabolite **1** and **2**. The IR data has also been supported by the related ^{13}C NMR spectra. Two signals at δ 198.3 ppm and 218.6 ppm in compound **1** have been imputed to C-3 and C-17, respectively. The chemical shift of H-11 was reported for hydrocortisone and other 11-hydroxysteroids in δ 4.3–4.4 [14]. The mass spectrum of metabolite **2** 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 supposed that one carboxyl group or double bond in the substrate has been reduced. The IR spectrum showed only one carbonyl group at 1649 cm^{-1}, which indicated that the conjugated ketone in C-3 position has not been altered. The elimination of 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 ppm in the ^1H NMR spectrum as compared to the substrate confirmed the metabolite **2**.



1 - 3

1: $R_1 = R_2 = O$
2: $R_1 = OH$, $R_2 = CHOH\text{-}CH_2OH$
3: $R_1 = OH$, $R_2 = CO\text{-}CH_2OH$

None of the metabolites were altered in its 4-en-3-oxo system. They differed in substitution in the C-17 position or side-chain. The conversion included ketone reduction and side-chain cleavage. Cleavage of the steroid side chain is an important research subject because of the potential application of this reaction for the production of high value therapeutic pharmaceuticals from abundantly available and inexpensive steroidal substrates. The steroids used for side-chain cleavage have mainly been cholestane-based compounds, and little work has been reported on shorter side chain substrates, e.g., pregnanes [15]. In this study we have reported that *N. ellipsosporum* is capable of achieving steroidal side-chain cleavage in a pregnane-based steroid. Also the nature of the side-chain cleavage enzyme in this microorganism is not clearly understood, but it might be similar to the enzyme which catalyzes the conversion of cholesterol to pregnenolone in mammals.

As these results show, the isolated cyanobacterium may be considered a useful biocatalyst for some kinds of biotransformation. It has a potential for site- and regioselective bioconversion of hydrocortisone and probably other pregnane – like steroids. Among all the cyanobacteria studies for assessment of steroids biotransformation, it seems *N. ellipsosporum* is being reported for the first time. The biotransformation observed in this work may be a research subject for further studies in using this microorganism and the other related cyanobacteria.

EXPERIMENTAL

Chemicals. Hydrocortisone was kindly donated by Aburaihan Pharmaceutical Co. (Tehran, Iran), which had been purchased from Pharmacia and Upjohn S. A. (Guyancourt, USA). Reagents and solvents were of analytical grade from Merck (Germany).

Instrumental Analyses. All the chemicals and reagents were purchased from Merck (Darmstadt, Germany). Hydrocortisone was kindly donated by Aburaihan Pharmaceutical Co. (Tehran, Iran), which had been purchased from Pharmacia

and Upjohn S. A. (Guyancourt, USA). Thin layer chromatography (TLC) and preparative TLC were carried out, respectively, on 0.25 mm and 0.5 mm thick layers of silica gel G (Kieselgel 60 $HF_{254+366}$, Merck). Chromatography was performed with chloroform/acetone (6:4) and visualized under UV spectrometer. HPLC analyses were performed on a Shimadzu HPLC equipment at 254 nm using a μ Bondapak C18 column (25×4.6 cm). A mixture of methanol/water (45:55) was used as mobile phase at a flow rate of 1 mL/min. The EI-MS spectra were obtained with a Finnigan MAT TSQ-70 instrument. The ^1H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, with a FTNMR Varian Unity Plus spectrometer in $CDCl_3$. The IR spectra were determined on a Magna-IR 550 Nicolet FTIR spectrometer. Melting points (mp) were determined on a Reichart-Jung hot stage melting point apparatus and were uncorrected.

The Biocatalyst. The blue-green alga was isolated during a screening program from soil samples collected from paddy fields north of Iran (Mazandaran and Golestan provinces) from May to November 2001. Primary culturing was done in BG-11 and modified Allen and Arnon media [2, 16] After colonization, pure cultures of living specimens were prepared using subculturing with the agar plate method in BG-11 medium [16]. Preserved specimens were prepared and the living specimens were incubated in 50 ml-tubes bubbling in the presence of 1% carbon dioxide. Constant illumination was used at 40 $\mu Em^{-2}S^{-1}$ intensity with white fluorescent lamps. Temperature was 25±2°C. Identification was done using semi-permanent slides (glycerin mount) and living specimen according to the cyanobacter genera [17].

Biotransformation Condition and Analytical Procedure. The experiment was conducted in twenty 500 mL conical flasks, each containing 100 mL of BG-11 liquid medium, illuminated continuously with fluorescent lamps at 40 $\mu Em^{-2}S^{-1}$, and incubated at a temperature of 25±2°C without shaking for seven days. Hydrocortisone (1 g) was dissolved in 40 ml of absolute ethanol. Two milliliters of the ethanol solution was added to each 500 mL conical flask (final concentration of the substrate was 0.05% in each flask). Incubation was continued for another ten days at the same conditions. All determinations were carried out in triplicate. In addition, two types of control were used. Control 1: the medium contained the cyanobacterium (steroid free) and control 2: the medium with 2 mL of the ethanol solution of the substrate (cyanobacterium free).

At the end of incubation, the contents of the flasks were extracted with three volumes of chloroform. The extract was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was loaded on preparative TLC and fractionated with chloroform/acetone (6:4). The purified metabolites were crystallized in methanol. Pure compounds were identified by melting points and spectral data (^{13}C NMR, ^1H NMR, FTIR, and MS). The purity as well as the amount of individual metabolites was checked with HPLC [9].

The strain was recognized as a colony with irregular shape, olive; cells subcylindrical to cylindrical and subspherical, 5–6 μ long, 3–4 μ broad; spores spherical to ovate, yellow to green, 5–7 μ × 4–6 μ, when mature to 10–12 μ. According to these characters and comparing with the keys of cyanobacteria genera [16], the selected strain was identified as a genus of *Nostoc*. The classification of the isolated alga was performed by the Persian Type Culture Collection (PTCC), Tehran, Iran, as a strain of *N. ellipsosporum* PTCC 1659. The strain was maintained on BG-11 agar slope freshly subcultured before use in the transformation experiment.

Three metabolites were isolated and purified from hydrocortisone biotransformation by *N. ellipsosporum* as followed. No biotransformation reaction occurred in the control media.

11β-Hydroxyandrost-4-ene-3,17-dione (1): mp 196–197°C (methanol), $[\alpha]_D^{25}$ +225° (*c* 1.5, MeOH); lit [18] mp 197–199°C, $[\alpha]_D$ +228°; IR ν_{max} 3463, 1735, 1657 cm^{-1}; MS (EI) *m/z* (%): 302 (70) (M^+, $C_{19}H_{26}O_3$), 286 (19), 269 (21), 227 (24), 189 (39), 163 (100), 149 (45), 123 (53), 91 (22), 83 (18); ^1H NMR ($CDCl_3$) δ 1.17 (3H, s, H-18), 1.46 (3H, s, H-19), 4.47 (1H, m, H-11), 5.70 (1H, s, H-4); ^{13}C NMR ($CDCl_3$) δ 218.6 (C_{17}), 198.3 (C_3), 171.6 (C_5), 121.4 (C_4), 65.8 (C_{11}); R_f in chloroform–acetone (6:4): 0.8.

11β,17α,20β,21-Terahydroxypregn-4-en-3-one (2): mp 128–131°C (methanol), $[\alpha]_D^{25}$ +91° (*c* 1.1, MeOH); lit [19] mp 133–135°C, $[\alpha]_D$ +85°; IR ν_{max} 3462, 2924, 1649 cm^{-1}; MS (EI) *m/z* (%): 364 (60) (M^+, $C_{21}H_{32}O_5$), 346 (25), 331 (7), 315 (56), 303 (46), 285 (100), 267 (31), 227 (64), 148 (38), 124 (40), 91 (82), 79 (55); ^1H NMR ($CDCl_3$) δ 1.12 (3H, s, H-18), 1.46 (3H, s, H-19), 3.66 (2H, m, H-21), 3.78 (1H, m , H-20), 4.36 (1H, m, H-11), 5.62 (1H, s, H-4); ^{13}C NMR ($CDCl_3$) δ 198.2 (C_3), 172.7 (C_5), 121.2 (C_4), 84.04 (C_{17}) 75.04 (C_{20}), 67.7 (C_{11}), 63.8 (C_{21}); R_f in chloroform–acetone (6:4): 0.15.

HPLC profile of the extract obtained from hydrocortisone biotransformation presented well-resolved peaks (data not shown), which correlate with the TLC profile (data not shown). The two major bioconverted products obtained from hydrocortisone had retention times of 16.5 and 20.9 min for metabolite **1** and **2**, respectively. In this system, the retention time of hydrocortisone was 14.25 min. The yield of each product expressed as a 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 60.1% and

21.2% for metabolites **1** and **2**, respectively. The percentage of the unconverted substrate was 18.7%. HPLC analysis revealed that few other metabolites were found in the transformed mixture, which was not purified for characterization due to its trace quantity.

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