Research Journal of

# Microbiology

ISSN 1816-4935

Academic
Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 5 (6): 576-580, 2010
ISSN 1816-4935 / DOI: 10.3923/jm.2006.289.293
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# Microalgal Transformation of Androst-4-en-3,17-dione by *Nostoc ellipsosporum**

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**Abstract:** The ability of *Nostoc ellipsosporum* PTCC 1659, a cyanobacterium strain, for biotransformation of androst-4-en-3,17-dione (AD) was studied. Fermentation was performed in BG-11 medium supplemented with 0.05% AD at 25°C for seven days incubation. The single metabolite obtained was purified using chromatographically methods and characterized as testosterone on the basis of its spectroscopic features. Bioreaction characteristic observed was 17-carbonyl reduction. Time course study showed the accumulation of the product from the third day of the fermentation and reached to the maximum in the seventh day. Production of testosterone was not affected by aeration. Continuously illumination or 16 h light/8 h dark has no effect on the transformation yield. Optimum concentration of the substrate, which gave maximum bioconversion efficiency, was 0.5 mg $mL^{-1}$ in one batch. Biotransformation was completely inhibited in a concentration above 2.0 mg $mL^{-1}$.

**Key words:** *Nostoc ellipsosporum*, cyanobacterium, biotransformation, androst-4-en-3, 17-dione

## INTRODUCTION

The use of microorganisms for transformation of complex chemicals resulting in single and specific transformations has been effectively exploited in recent decades. They are useful biologically tools for production of organic compound either to improve the yields of already known substances or in preparation of novel substances. From the first patent in 1937, bacteria and fungi have been widely employed in steroid biotransformation studies (Smith, 1984). Among the various kinds of microorganisms, microalgae have been less investigated in conversion of steroid compounds. Earlier studies have already demonstrated the potential of microalgae for steroid modifications. In 1986, Abul-Hajj and Quian showed the ability of different strains of microalgae such as *Anabaena cylindrical*, *Scenedesmus quadricauda* and *Coelastrum proboscideum* in conversion of androstendione to testosterone (Abul -Hajj and Quian, 1986). Other researches proved that some algal strains induced regio- and stereoselective reduction of carbonyl groups, regioselective hydroxylations of the various carbons in the steroid substances and oxidation the molecule to give conjugated enones and dienones (Tabatabaei *et al*., 2004). Very recently, we also reported the bioconversion of hydrocortisone into some androstane and pregnane derivatives using two isolated strains of cyanobacteria from soil, *Nostoc muscorum* (Tabatabaei *et al*., 2004) and *Fischerella ambigua* (Tabatabaei *et al*., 2005).

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**Originally Published in Research Journal of Microbiology, 2006*

*Nostoc ellipsosporum* (Desm.) Rabenh. 1888 belongs to Nostocaceae family found in both terrestrial and aquatic habitats (Desikachary, 1958). It was recently studied for cyanovirin-N (CV-N) an 11 kDa protein that displays virucidal activity against several viruses, including human immunodeficiency virus. Until now, *N. ellipsosporum* is not known to apply in steroid bioconversion studies.

Androst-4-en-3,17-dione (AD) is one of the most useful intermediates for production of some valuable pharmaceutically steroid compounds and has been used in many studies as a substrate in biotransformation experiments (Smith, 1984). In this study the ability of an isolated strain of *Nostoc ellipsosporum* PTCC 1659 (Ghasemi *et al*., 2006) was investigated for transformation of androstendione as an exogenous steroid. This potential has not been previously examined. However, the effects of aeration, continuously illumination or 16 h light/8 h dark and the substrate concentration were also studied.

## MATERIALS AND METHODS

### Chemicals

Androst-4-en-3,17-dione was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents and solvents were from Merck and Fluka unless otherwise mentioned.

### Instrumental Analyses

Melting points (mp) were determined on a Reichert-Jung hot stage melting point apparatus and are uncorrected. Optical rotations were measured in 1 dm cells on a Perkin-Elmer 142 automatic spectropolarimeter. $^1H$ and $^{13}C$ nuclear magnetic resonances (NMR) spectra were recorded using FTNMR Varian Unity plus spectrometer at 400 and 100 MHZ, respectively, in $CDCl_3$ with tetramethylsilane (TMS) as internal standard. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS. Coupling constant (J) were given in hertz (Hz). Infrared (IR) spectra were recorded on a Magna-IR 550 Nicolet FTIR spectrometer. Mass spectra (MS) were obtained with a Finnigan MAT TSQ-70 instrument by electron impact (EI) at 70 eV. Thin layer chromatography (TLC) and preparative TLC were performed, respectively, on 0.25 and 0.5 mm thick layers of silica gel G (Kieselgel 60 $HF_{254+366}$, Merck). Layers were prepared on glass plates and activated at 105°C 1 h before use. Chromatography was performed with acetone/hexane (1:1, v/v) and visualized by spraying the plates with a mixture of methanol/sulfuric acid (6:1, v/v) and heating in an oven at 100°C for 3 min until the colors developed. The compounds were also visualized under UV lamp (Strstedt-Gruppe HP-UVIS) at 254 nm.

The chromatographic apparatus consisted of a Knauer model K-1001 pump, a K-2600 UV variable-wavelength detector and an online degasser, all from Knauer (Berlin, Germany). Samples were injected to a Knauer D-14163 injector system with a 20 μL sample loop. The data were acquired and processed by means of Eurochrom chromatography software (Knauer, Berlin, Germany). Chromatographic separation was achieved on a Finepak SIL C18-10 reverse-phase column (C18, 25 × 0.46 cm i.d., 10 μm particle size) from Jasco Corporation (Japan).

### Algal Strain

Nostoc ellipsosporum PTCC 1659 (Ghasemi *et al*., 2006) was maintained at 4°C on BG-11 agar slant and freshly subcultured before use in transformation experiments. The organism was transferred to fresh medium every two months.

### Incubation Conditions

The fermentation experiments was conducted in twenty 500 mL conical flasks, each containing 100 mL of BG-11 liquid media, 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. AD (1 g) was dissolved separately in 20 mL of 1,4-dioxan. One milliliter 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 seven days at the same conditions and the control was similarly processed without the microorganism.

**Product Isolation and Analyses**

At the end of incubation, the content 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 acetone/hexane (1:1) solvent system and then the metabolite was crystallized in ethanol. Purified metabolite was identified by melting point and spectral data ($^{13}$C NMR, $^{1}$H NMR, FTIR and MS). The purity as well as the amount of metabolite was checked with HPLC analyses (Faramarzi *et al*., 2006). A mobile phase consisting methanol/acetonitrile/water (20:25:55, v/v/v) was used. Flow rate was 1.5 mL min$^{-1}$. The chromatogram at 254 nm showed a complete resolution for the peaks (Fig. 1).

**Time Course Study And the Effect of Aeration, Illumination and Substrate Concentration**

For a time course study, *N. ellipsosporum* was transferred into a 500 mL Erlenmeyer flask containing 100 mL of BG-11 broth supplemented with 50 mg AD and then the incubation continued for seven days at the condition as described above. Sampling was carried out every 24 h. We also examined the effect of aeration, continuously illumination or 16 h light/8 h dark and optimum substrate concentration. Fermentation procedure was checked on a rotary shaker at 150 rpm and without shaking. The photoperiod was 16 h light-8 h dark in comparison with illuminated continuously provided by day light fluorescence lamps at 40 $\mu$Em$^{-2}$S$^{-1}$. The amount of the substrate was varied from 0.025 to 0.2 g 100 mL$^{-1}$ with a stepwise of 0.25. In each case, one parameter was studied and the others kept constant. Results were obtained according to TLC analyses.

## RESULTS AND DISCUSSION

The single metabolite was isolated and purified from androstendione biotransformation by *N. ellipsosporum* (Fig. 1). HPLC profile of the fermentation extract presented well-resolved peaks (Fig. 2). Retention times for the metabolite and the substrate were 8.6 (II) and 24.3 (I) min, respectively. The yield of the product (II) was found to be 32.6% according to HPLC analysis. The percentage of the unconverted substrate (I) was 55.6% at the end of the fermentation.

The analytical data of compound II is as below:

Compound II: mp 165-168°C, $[\alpha]_D$ +97° (EtOH), lit (Faramarzi *et al*., 2004) mp 165-168°C, $[\alpha]_D$ +97° (EtOH); IR $v_{max}$ 3413, 2936, 1661, 1607 cm$^{-1}$; MS (EI) m/z (%) 288 (60) ($M^+$, $C_{19}H_{28}O_2$), 246

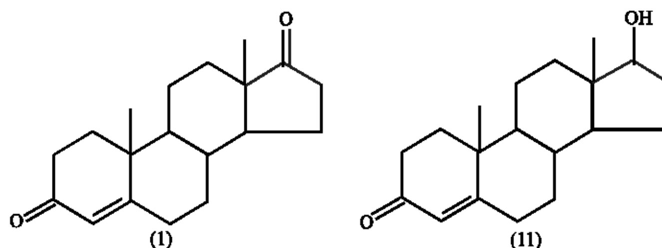


Fig. 1: The structures of androst-4-en-3,17-dione (I) and testosterone (II)

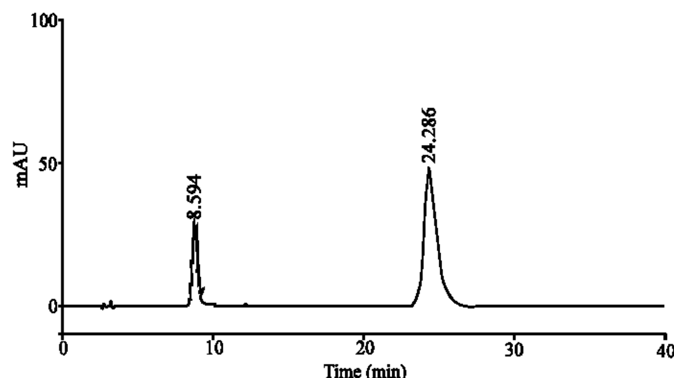


Fig. 2: HPLC profile of androstendione transformation by *Nostoc ellipsosporum*: androst-4-en-3,17-dione (I), 24.3 min; testosterone (II), 8.6 min

(42), 228 (15), 203 (21), 164 (35), 147 (38), 124 (100), 109 (25), 81 (19); $^{1}$H NMR ($CDCl_3$) δ0.80 (3H, s, H-18), 1.19 (3H, s, H-19), 3.65 (1H, t, J = 8.4 Hz, H-17), 5.73 (1H, s, H-4) ; $^{13}$C NMR ($CDCl_3$) δ 199.6 ($C_3$), 171.3 ($C_5$), 123.9 ($C_4$), 81.6 ($C_{17}$); $R_f$ in acetone/hexane (1:1, v/v): 0.61.

Compound II was found as a mono-hydroxy derived of androstendione according to its mass (m/z 288, $C_{19}H_{28}O_2$) and infrared spectrum (presence of a peak at 3413 cm$^{-1}$). The IR spectrum also revealed absorption bands for 3-ketone (1661 cm$^{-1}$) conjugated with double bond at C-4 position (1607 cm$^{-1}$). The $^{1}$H NMR spectrum contained signals for two methyl groups (δ0.8, H-18 and δ1.19, H-19; each as singlet) and a singlet at δ5.73 for H-4. The 17β-hydroxyl group was confirmed through the absence of chemical shift for carbonyl group at δ220.4 in $^{13}$C NMR spectrum which was replaced by a signal at δ81.5 and also by the appearance of a distinctly visible triplet at δ3.65 (J = 8.4 Hz) (Kirk *et al*., 1990). It was characterized as testosterone.

For a time course study, production of II, as a function of incubation time, was detected by thin layer chromatography. The starting material, AD 0.05 g 100 mL$^{-1}$, was transformed to a single steroid metabolite within seven days. According to TLC profile, testosterone was accumulated in the broth from the third day of incubation and reached to its maximum concentration within seven days.

*N. ellipsosporum* was also examined to convert ADD at different concentrations between 0.025 to 0.2 g 100 mL$^{-1}$. Based on TLC profile, best substrate concentration was 0.05 g 100 mL$^{-1}$ and in a concentration above 0.15 g 100 mL$^{-1}$, AD was not converted to any metabolite. Production of testosterone was not affected by aeration. Continuously illumination or 16 h light/8 h dark has no effect on the fermentation.

From the results of the experiment, it appears that *Nostoc ellipsosporum* transformation of AD for seven days at 25°C was led to formation of testosterone. The bioconversion characteristic observed was 17-ketone reduction. Spectroscopic analysis approved that the stereochemistry of 17-hydroxyl group was as β-form. It seems that 17-keto reduction is common bioreactions on AD by some microalgal strains (Abul-Hajj and Qian, 1986). This study showed 17-ketone of AD is a suitable site for bioconversion using *Nostoc ellipsosporum*. The tested cyanobacterium conducted carbonyl reduction to C-17 on the substrates whereas the other carbonyl group (C-3) as well as the other carbon atoms remained intact.

## ACKNOWLEDGEMENT

This study was supported by a grant from Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran.

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