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14α-Hydroxylation of Androst-4-en-3, 17-dione by the Whole Cells of Cyanobacterium *Nostoc piscinale*

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Abstract: The potential of microalga *Nostoc piscinale* was evaluated in bioconversion of androst-4-en-3, 17-dione (I). Bio-reaction was performed in BG-11 liquid medium supplemented with 0.05% steroid substrate under continuous light photo-regime of 3000 lux at 25°C for 5 days. Three major metabolites were purified chromatographically and identified on the bases of their spectral data and physical constants as 17β-hydroxyandrost-4-en-3-one (II), 14α-hydroxyandrost-4-en-3,17-dione (III) and 14α,17β-dihydroxyandrost-4-en-3-one (IV). Bioconversion characteristics observed were 14α-hydroxylation and 17-keto reduction. Present results showed that the green biocatalyst is suitable for some specific alterations on androst-4-en-3,17-dione including 14α-hydroxylation.

Key words: *Nostoc piscinale*, microalgae, bioconversion, steroid, androst-4-en-3,17-dione

INTRODUCTION

Biocatalysts as both whole-cells and isolated enzymes are applied for commercial synthesis of complex and chiral compounds especially for industrial use (Pollard and Woodley, 2007; Woodley, 2008). Compared to isolated enzymes, application of whole cell catalysts has several advantages, including the much more ready and inexpensive preparation, more stability in the long-term reactions, diversity and ease of handling (Ishige *et al*., 2005). Steroids can be ranked among the most important classes of pharmaceutical substances. They are used for treating and prevention of many diseases in endocrinology, oncology, rheumatology and gynecology. So number of efficient pharmaceutical steroids continues to grow (Donova, 2007). Combination of techniques such as biotransformation and chemical process provides an effective synthetic strategy for manufacturing the steroids due to high regio- and stereo-selectivity of bio-reactions (Fernandes *et al*., 2003).

In recent years, biotransformation of a wide range of steroid substrates has been subjected in numerous researches (Donova, 2007; Faramarzi *et al*., 2008c; Fernandes *et al*., 2003). The produced derivatives can be used as new pharmaceutical agents or intermediates for synthesis of other steroidal compounds (Janeczko *et al*., 2009; Xiong *et al*., 2006). Although, many of whole cell biocatalysts including bacteria, fungi and algae have been extensively studied, however, only a small fraction of thousands of microorganism species and steroid substrates studied to date can actually be applied in the large scale manufacture of pharmaceutical steroids (Fernandes *et al*., 2003).

Screening and identifying of new microorganisms combined with recent developments in molecular biology, enzyme evolution, microbial genomics, strain engineering, bioinformatics (Donova, 2007) and process engineering (Huisman and Gray, 2002) have helped researchers to find out efficient biocatalysts with improved properties.

In the present study, the potential of an isolate strain of cyanobacterium *Nostoc piscinale* for biotransformation of androst-4-en-3,17-dione, a useful steroid intermediate, was investigated. To our knowledge, this ability has not been examined so far.

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MATERIAL AND METHODS

This study was conducted during May 15, 2008 and May 15, 2009 at Tehran University of Medical Sciences, in Tehran, Iran.

Chemicals and instruments: Androst-4-en-3,17-dione was purchased from Sigma chemical Co. (St. Louis, MO, USA). All other reagents and solvents were of analytical grade and from Merck (Darmstadt, Germany) unless otherwise mentioned.

Thin Layer Chromatography (TLC) and preparative TLC were performed, respectively, on 0.25 (DC-Fertigfolien, Schleicher and Schuell, Germany) and 0.5 mm layers of silica gel G (Kieselgel 60 $HF_{254+366}$, Merck, Germany). Layers were prepared on glass plates and activated at 105°C 1 h before use. Thin layer Chromatography was performed with acetone/chloroform/ethyl acetate (1:8:1, v/v/v) and visualized by spraying the plates with a mixture of phosphoric acid (85%)/distilled water (1:1, v/v) and heating in an oven at 110°C for 10 min until the colors developed. Mass Spectra (MS) were obtained with a Finnigan MAT TSQ-70 instrument by Electron Impact (EI) at 70 eV. Infrared (IR) spectra were recorded using KBr disks on Magna-IR 550 Nicolet FTIR spectrometer. The ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were obtained using a Bruker DRX (Avance 500) spectrometer at 500 and 125 MHz, respectively, with tetramethylsilane (TMS) as internal standard in $CDCl_3$. Chemical shifts (δ) are given in part per million (ppm) relative to TMS. The coupling constant (J) is given in hertz (Hz). Optical rotations were measured in the solvents specified for each product in 10 cm cells on Perkin-Elmer 142 automatic spectropolarimeter. Melting points (mp) were determined on a melting point apparatus, Gallenkamp, UK and were uncorrected. The compounds were also visualized under a UV lamp (Strstedt-Gruppe HP-UVIS) at 254 nm.

Algal strain, culture medium and maintenance: An axenic culture of *Nostoc piscinale* (Gharaei-Fathabad *et al*., 2007, 2008) was grown in BG-11 medium (Borowitzka and Borowitzka, 1988) and maintained at 4°C on BG-11 agar slants. The alga was transferred to fresh medium every 2 months.

BG-11 medium consisted of (g m^{-3}) $NaNO_3$, 1.5; K_2HPO_4, 0.04; $MgSO_4.7H_2O$, 0.075; $CaCl_2.2H_2O$, 0.036; ferric ammonium citrate, 0.006; citric acid, 0.006; Na_2EDTA, 0.001; Na_2CO_3, 0.02; trace element solution, 0.001 m^3; distilled water up to 1 m^3. Trace element solution contained (g m^{-3}) H_3BO_3, 2.86; $MnCl_2.4H_2O$, 1.81; $ZnSO_4.7H_2O$, 0.222; $Na_2MoO_4.2H_2O$, 0.3; $CuSO_4.5H_2O$, 0.079; $Co(NO_3)_2.6H_2O$, 0.494; distilled water up to 1 m^3 (Borowitzka and Borowitzka, 1988).

Bioconversion condition: The cyanobacterial cells of *N. piscinale* were inoculated into twenty 0.1 m^3 Erlenmeyer flasks containing 0.5 m^3 of sterile BG-11 liquid medium enclosed with cotton plugs. pH was adjusted to 7.0 with 1 M HCl and/or NaOH prior to autoclaving at 121°C for 20 min. Flasks were illuminated at 3000 lux supplied by fluorescent tubes under continuous light photoregime from all sides at 25°C and bubbled with sterile air without shaking for five days. After this period, AD (dissolved in 0.001 m^3 of dioxan) was added at a final concentration of 0.05% under sterile conditions and incubation was prolonged for another 5 days.

Purification of the bioproducts: After 5 days incubation, cultures were extracted three times with equal volume of chloroform. Organic phase was separated and filtered. The extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was spotted on preparative thin layer chromatography using the solvent system of acetone/chloroform/ethyl acetate (1:8:1, v/v/v). The purified metabolites were crystallized in chloroform and then identified using spectral data (^{13}C NMR, ^{1}H NMR, FTIR and MS) and physical constants (melting points and optical rotations).

Time course experiment and the effects of temperature, pH, aeration, photoregime and substrate concentration on the bioconversion: For a time course study, a 5 days growth cell of *N. piscinale* was transferred into a 0.5 m^3 Erlenmeyer flask containing 0.1 m^3 of BG-11 medium supplemented with 0.05 g of AD and then the incubation continued for 10 days at the same condition described above. Sampling was carried out every 24 h. Control was similarly processed except that no microalga was added.

Studies were performed to determine the optimum temperature and the maximum amount of substrate that could be transformed to the products. The temperature was varied from 20 to 40°C with an interval of 5°C. The effect of pH on the biotransformation procedure was studied in non-buffered media by adjusting the pH from 5 to 10 with NaOH and HCl. The optimum substrate concentration was determined using concentrations of 0.01, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 g m^{-3}. The effect of aeration on the biotransformation procedure was studied on a rotary shaker at 150 rpm and without shaking. In order to evaluate the impact of the photoregime on the biotransformation yield, two programs including continuous illumination at 3000 lux and 16/8 h light/dark periods were used.

For each experiment only one parameter was changed at a time. The procedure was carried out in triplicate for each analytical determination. Qualitative studies were performed using TLC and detection was done by UV at 254 nm.

RESULTS

Nostoc piscinale transformation of AD in 5 days led to the formation of three steroid derivatives (II to IV) (Fig. 1). No transformation occurred in the control. Steroid products were characterized on the bases of their analytical data. ^{13}C NMR assignments of the substrate as well as the bioproducts are listed in Table 1.

17β-Hydroxyandrost-4-en-3-one (II): Crystallized from chloroform; yield: % 36; mp 165-168°C, lit (Faramarzi *et al*., 2004) mp 165-168°C; $[\alpha]_D$ +97° (EtOH), lit (Faramarzi *et al*., 2004) $[\alpha]_D$ +97° (EtOH); IR ν_{max} 3414, 2936, 1661, 1607 cm^{-1}; MS (EI) m/z (%) 288 (60) (M^+, $C_{19}H_{28}O_2$), 246 (35), 228 (15), 203 (21), 164 (30), 147 (38), 124 (100), 109 (25), 81 (19); ^1H NMR ($CDCl_3$) δ 0.80 (3H, s, H-18), 1.19 (3H, s, H-19), 3.63 (1H, t, J = 8.4 Hz, H-17), 5.71 (1H, s, H-4); R_f in acetone/chloroform/ethyl acetate (1:8:1, v/v/v): 0.73.

14α-Hydroxyandrost-4-en-3,17-dione (III): Crystallized from chloroform; yield: % 17; mp 257-262°C, lit (Hill *et al*., 1991; Faramarzi *et al*., 2008a) mp 261-263°C; $[\alpha]_D$ +160° ($CHCl_3$), lit (Hill *et al*., 1991) $[\alpha]_D$ +162° ($CHCl_3$); IR ν_{max} 3457, 1734, 1662 and 1614 cm^{-1}; MS (EI) m/z (%) 302 (M+, C19H26O3), 227 (8), 133 (15), 119 (29), 79 (35), 69 (45), 57 (100); 1H NMR (CDCl3) δ 1.07 (3H, s, H-18), 1.25 (3H, s, H-19), 5.77 (1H, s, H-4); R_f in acetone/chloroform/ethyl acetate (1:8:1, v/v/v): 0.66.

14α,17β-Dihydroxyandrost-4-en-3-one (IV): Crystallized from chloroform; yield % 12; mp 181-184°C, lit (Hill *et al*., 1991; Faramarzi *et al*., 2008b) mp 183-186°C; $[\alpha]_D$ +121° ($CHCl_3$), lit (Hill *et al*., 1991) $[\alpha]_D$ +124° ($CHCl_3$); IR ν_{max} 3434, 1738, 1656 and 1612 cm^{-1}; MS (EI) m/z (%) 304 (M^+, $C_{19}H_{28}O_3$), 286 (35), 284 (56), 240 (14), 173 (15), 161

Table 1: ^{13}C NMR signals of the substrate and the metabolites (δ in ppm downfield from TMS, in $CDCl_3$)

Carbon atom No.	I	II	III	IV
1	35.6	35.7	36.1	36.2
2	33.8	32.7	33.5	34.4
3	199.0	199.4	200.0	200.2
4	124.0	123.8	124.5	124.3
5	170.1	171.2	170.6	171.3
6	32.4	33.8	32.8	33.0
7	31.2	31.5	26.0	29.0
8	35.0	35.6	38.4	39.3
9	53.7	53.9	47.3	47.2
10	35.8	38.6	39.1	39.2
11	20.2	20.6	19.5	20.1
12	30.6	36.4	24.9	33.1
13	47.3	42.7	53.0	47.4
14	50.7	50.4	81.1	83.8
15	21.6	23.3	30.7	30.0
16	35.6	30.3	34.3	26.5
17	220.0	81.5	218.9	79.0
18	13.5	11.0	18.3	15.3
19	17.0	17.4	17.7	17.7

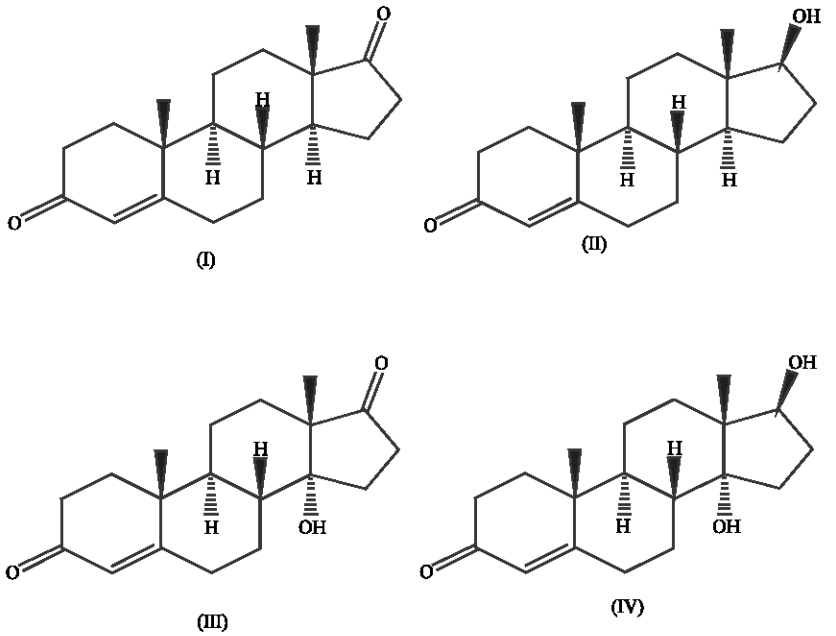

Fig. 1: Chemical structures of AD (I) and its metabolites produced by *Nostoc piscinale*. 17β-hydroxyandrost-4-en-3-one (II), 14α-hydroxyandrost-4-en-3,17-dione, (III) and 14α,17β-dihydroxyandrost-4-en-3-one (IV)

(20), 148 (29), 123 (50), 91 (47), 56 (100); ^{1}H NMR ($CDCl_3$) δ 0.94 (3H, s, H-18), 1.24 (3H, s, H-19), 4.34 (1H, t, J =7.7, H-17), 5.76 (1H, s, H-4); R_f in acetone/chloroform/ethyl acetate (1:8:1, v/v/v): 0.57.

For a time course study, production of II to IV, as a function of incubation time, was detected by thin layer chromatography. The starting material, AD (I) 0.5 g m^{-3}, was transformed into three metabolites within 5 days. According to TLC profile, compounds III and IV appeared in the broth from the second day of fermentation and the amount of all bioproducts reached to the maximum level in fifth day. Extended incubation time had no positive effect on the biotransformation process.

The optimum substrate concentration was 0.05% (w/v) and in concentrations between 0.2 to 0.4% (w/v), substrate was converted in low amounts only to II and III. At the concentration of 0.5% (w/v) compound III was the sole product in very low amount and no biotransformed metabolite was accumulated in the broth at 0.6% (w/v) of AD.

Optimum temperature was recorded at 25°C. Transformation experiments at 20 and 30°C was resulted in the production of the same metabolites but in lower concentrations. The highest bioconversion rates of AD were obtained within the pH range 6.5-7.5. The transformation process was markedly retarded at pH values below 6.5 or above 7.5 for all products (II, III and IV). The optimal pH for the production of all metabolites was seven. Production of the metabolites was not affected by aeration. At continuous illumination or 16 h light/ 8 h dark, the same results were observed.

DISCUSSION

Cyanobacteria are known as one of the oldest forms of life on the earth and have been used as food and medicine in various parts of the world for centuries. In the last 20 years due to their remarkable degree of secondary metabolites and bioactivities diversity, these ancient phototrophic organisms are of interest for pharmaceutical researches (Svircev *et al*., 2008; Mehner *et al*., 2008).

Although, bacteria and fungi have usually used as biocatalyst for the biotransformation of various substrates (Mahato and Garai, 1997; Fernandes *et al*., 2003), genetic and biochemical diversity and simple nutritional requirements of microalgae are made them as a good substitute to bacterial and fungal systems. Despite the attractive properties of these microorganisms, researchers had not given them much importance, until 1986, when the potential of some algal strains to convert steroids was investigated by Abul-Hajj and Qian (1986). Although, the ability of few species for bioconversion of steroids has been studied up to now, wide variety modifications performed by microalgae have been reported (Faramarzi *et al*., 2008c).

Nostoc piscinale GT-319, a soil isolate cyanobacterium from paddy-fields of Iran, was examined for its cytotoxic activity in our previous study. The results showed that it is a potent source of cytotoxic compounds against five different cell lines including HeLa, Vero, Caco-2, HepG and CHO more than vincristine, 5-fluorouracil and methotrexate (Gharaei-Fathabad *et al*., 2007). Further study indicated that the cytotoxic effects may rise via an action on cellular DNA (Gharaei-Fathabad *et al*., 2008). Antifungal activity of extracellular products of *Nostoc piscinale* (Zulpa *et al*., 2003) and the ability of the algae for removing Pb in aqueous solutions (Inthorn *et al*., 2002) has been also studied. There is no report on the ability of *N. piscinale* in biotransformation experiments.

In the present study, we applied the isolate strain of *N. piscinale* GT-319 for bioconversion of androst-4-en-3,17-dione, a useful intermediate compound in the production of some pharmaceutical steroids. The results from present experiments showed that the bioconversion characteristics were as C-14á hydroxylation and 17-keto reduction.

14α-Hydroxyandrost-4-en-3,17-dione is used as an important intermediate in manufacturing of steroid hormones (Mahato and Garai, 1997). In some previous studies, algal production of 14α-hydroxylated derivative of AD by *Anacystis nidulans*, *Scenedesmus quadricauda* and *Coelastrum proboscideum* var *grucile* was reported. It was also observed the accumulation of 14α-hydroxytestosterone in the culture media of *Anabaena cylindrical*, *Anacystis nidulans*, *Ankistrodesmus angustus* and *Scenedesmus quadricauda* (Faramarzi *et al*., 2008c).

Microbial reduction of 17-keto- to 17α-hydroxysteroids was firstly reported in 1937, in which androst-4-ene-3,17-dione was transformed to testosterone by *Saccharomyces cerevisiae*. So far, conversion of 17-oxo to 17β -hydroxysteroids have been observed for a wide range of microorganisms and substrates. The biological importance of 17β-reduction for microorganisms is indicated by its wide natural occurrence. In some cases, 17-ketoreduction was observed along with other reactions such as hydroxylations. For example, 17β-reduction of AD by *Bacillus* sp., accompanied monohydroxylation at C-6, C-7, C-11 and C-14. In *Phycomyces blakesleeanus* and *Absidia coerulea*, 17β-reduction was occurred together with 14α-hydroxylation of substrates. In the culture medium of *Botryosphaerica obtuse*, 6-hydroxylated and 6,7-dihydroxylated derivatives was accumulated along with 17β-reductive metabolite (Donova *et al*., 2005).

In conclusion, we report here 14α-hydroxylation and 17-keto reduction of androst-4-en-3,17-dione as two important biotransformation products using the soil isolate cyanobacterium *Nostoc piscinale*.

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REFERENCES

Abul-Hajj, Y.J. and X. Qian, 1986. Transformation of steroids by algae. J. Nat. Prod., 48: 244-248.

Borowitzka, M.A. and J.L. Borowitzka, 1988. Microalgal Biotechnology. Cambridge University Press, Cambridge, pp: 456-465.

Donova, M.V., O.V. Egorova and V.M. Nikolayeva, 2005. Steroid 17β-reduction by microorganisms a review. Biochemistry, 40: 2253-2262.

Donova, M.V., 2007. Transformation of steroids by actinobacteria a review. App. Biotechol. Microb., 43: 1-14.

Faramarzi, M.A., M. Tabatabaei Yazdi, M. Amini, F. Azizmohseni, G. Zarrini, A. Amani and A. Shafiee, 2004. Microbial production of testosterone and testololactone in the culture of *Aspergillus terreus*. World J. Microbiol. Biotechnol., 20: 657-660.

Faramarzi, M.A., M. Aghelnejad, M. Tabatabaei Yazdi, M. Amini and N. Hajarolasvadi, 2008a. Metabolism of androst-4-en-3 17-dione by the filamentous fungus *Neurospora crassa*. Steroids, 73: 13-18.

Faramarzi, M.A., M. Badiee, M. Tabatabaei Yazdi, M. Amini and M. Torshabi, 2008b. Formation of hydroxysteroid derivatives from androst-4-en-3,17-dione by the filamentous fungus *Mucor racemosus*. J. Mol. Cat. B Enzymatic, 50: 7-12.

Faramarzi, M.A., S. Aderangi and M. Tabatabaei-Yazdi, 2008c. Microalgal biotransformation of steroids. J. Phycol., 44: 27-37.

Fernandes, P., A. Cruz, B. Angelova, H.M. Pinheiro and J.M.S. Cabral, 2003. Microbial conversion of steroids compounds recent developments. Enzyme Microb. Technol., 32: 688-705.

Gharaei-Fathabad, E., M. Tabatabaei Yazdi, S.N. Ostad, S. Shokravi, Z. Sepehrizadeh, M.A. Faramarzi and M. Amini, 2007. *Nostoc piscinale* GT-319, a new cyanobacterial strain with cytotoxic activity. Biotechnology, 6: 505-512.

Gharaei-Fathabad, E., M. Tabatabaei Yazdi, S.N. Ostad and M.A. Faramarzi, 2008. *In vitro* genotoxicity of extracellular extract from *Nostoc piscinale*. Toxicol. Environ. Chem., 90: 795-799.

Hill, R.A., D.N. Kirk, H.L.J. Makin and G.M. Murphy, 1991. Dictionary of Steroids Chemical Data Structures and Bibliographies. 1st Ed., Chapma Hall Ltd., London, pp: 4-55, 510, 512.

Huisman, G.W. and D. Gray, 2002. Towards novel processes for the fine-chemical and pharmaceutical industries. Curr. Opin. Biotechnol., 13: 352-358.

Inthorn, D., N. Sidtitoon, S. Silapanuntakul and A. Incharoensakdi, 2002. Sorption of mercury cadmium and lead by microalgae. Sci. Asia, 28: 253-261.

Ishige, T., K. Honda and S. Shimizu, 2005. Whole organism biocatalysis. Curr. Opin. Chem. Biol., 9: 174-180.

Janeczko, T., G.J. Dmochowska, E.S. Kostrzewa, A. Bialonska and Z. Ciunik, 2009. Biotransformations of steroid compounds by *Chaetomium* sp.. KCH 6651. Steroids, 74: 657-661.

Mahato, S.B. and S. Garai, 1997. Advances in microbial steroid biotransformation. Steroids, 62: 332-345.

Mehner, C., D. Müller, S. Kehraus, S. Hautmann, M. Gütschow and G.M. König, 2008. New peptolides from the cyanobacterium *Nostoc insulare* as selective and potent inhibitors of human leukocyte elastase. Chem. Bio. Chem., 9: 2692-2703.

Pollard, D.J. and J.M. Woodley, 2007. Biocatalysis for pharmaceutical intermediates the future is now. Trends Biotechnol., 25: 66-73.

Svircev, Z., D. Cetojevic-Simin, J. Simeunovic, M. Karaman and D. Stojanovic, 2008. Antibacterial antifungal and cytotoxic activity of terrestrial cyanobacterial strains from Serbia. Sci. China Ser. C Life Sci., 51: 941-947.

Woodley, J.M., 2008. New opportunities for biocatalysis making pharmaceutical processes greener. Trends Biotechnol., 26: 321-327.

Xiong, Z., Wei, Q., H. Chen, S. Chen, W. Xu, G. Qiu, S. Liang and X. Hu, 2006. Microbial transformation of androst-4-ene-3,17-dione by *Beauveria bassiana*. Steroids, 71: 979-983.

Zulpa, G., M.C. Zaccaro, F. Boccazzi, J.L. Parada and M. Storni, 2003. Bioactivity of intra and extracellular substances from cyanobacteria and lactic acid bacteria on wood blue stain fungi. Biol. Control, 27: 345-348.