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Bioconversion of Hydrocortisone by Unicellular Microalga *Oocystis pusilla*

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Abstract: A unicellular microalga, *Oocystis pusilla*, was isolated from paddy-field and applied in the biotransformation experiment of hydrocortisone (1). This strain has not been previously tested for steroid bioconversion. Fermentation was carried out in BG-11 medium supplemented with 0.05% substrate at 25°C for 14 days incubation. The products obtained were chromatographically purified followed by their characterization using spectroscopic methods. 11β, 17α, 20β, 21-tetrahydroxypregn-4-en-3-one (2), 11β, 17β-dihydroxyandrost-4-en-3-one (3) and 11β-hydroxyandrost-4-en-3, 17-dione (4) were the main byproducts in the hydrocortisone bioconversion. Bioreaction characteristics observed were 20-ketone reduction for accumulation of compound 2 and side chain degradation of the substrate to prepare compounds 3 and 4. Time course study showed the accumulation of the product 2 from the second day of the fermentation and 3 as well as 4 from the third day. All the metabolites reached their maximum concentration in seven days. Optimum concentration of the substrate, which gave maximum bioconversion efficiency, was 0.5 mg mL^{-1} in one batch. Growth was not influenced by the addition of steroid substrate. Biotransformation was completely inhibited in a concentration above 2.0 mg mL^{-1}.

Key words: *Oocystis pusilla*, bioconversion, hydrocortisone

INTRODUCTION

Microalgae are a diverse group of photosynthetic microorganisms found in the soil and fresh water environments (Metting and Pyne, 1986). The ability of microalgae to modify exogenous steroids has been supported in several of our publications (Ghasemi *et al*., 2004, 2006; Tabatabaei Yazdi *et al*., 2004, 2005). Consequently their culture is simpler and cheaper than that of bacteria, yeasts or fungi. They are easily and rapidly cultured in an inexpensive medium containing simple salts which decreases the probability of contamination by other microorganisms. The use of microalgae for steroid bioconversion was firstly reported by Abul-Hajj and Qian (1986). They examined the conversion of 4-androsten-3, 17-dione to some hydroxylated derivatives with different strains of microalgae such as *Anabaena cylindrica*, *Scenedesmus quadricauda* and *Coelastrum proboscideum*. More recently, the ability of green algae for transformation of progesterone, prednisolone and some other steroids has been reported (Pollio *et al*., 1994, 1996). Only a few studies have so far been done to use microalgal strains isolated from local habitats for organic compounds bioconversion (Tabatabaei Yazdi *et al*., 2005).

Oocystis pusilla Hansgirg, belongs to chlorophytean *Oocystis* species, is distributed over the world and occurs in the quiet water and also in soils (Bold and Wynne, 1985). Preliminary taxonomical studies show that this strain seems common in the paddy fields of Fars province located in South of Iran, beside microalgae like *Chlorella*, *Scenedesmus* and some unicellular and filamentous cyanobacteria. There is only little work regarding the biological effects of *Oocystis* sp. In one study, the *Oocystis* sp. was screened for its ability to transformation of 4-androstene-3, 17-dione.

The algal culture did transform 4-androstene-3, 17-dione to testosterone and 6β-hydroxy-4-androstendione. In pursing present works on the bioconversion of steroids by *Nostoc muscorum* (Faramarzi *et al*., 2002; Tabatabaei Yazdi *et al*., 2004), *Fischerella ambigua*

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(Moradpour *et al*., 2006; Tabatabaei Yazdi *et al*., 2005) and *Nostoc ellipsosporum* (Ghasemi *et al*., 2006), the biotransformation of hydrocortisone as an exogenous steroid was carried out by a locally isolated strain of a unicellular microalgae, *Oocystis pusilla*. Until today, *Oocystis pusilla* has not been examined in transformation of hydrocortisone. The aim of this study is to identify the ability of locally isolated *Oocystis pusilla* to convert hydrocortisone as an exogenous substrate.

MATERIALS AND METHODS

Instrumental analyses: Melting points (mp) were determined on a Reichert-Jung hot stage melting point apparatus. Optical rotations were measured in 1 dm cells on a Perkin-Elmer 142 automatic spectropolarimeter. ^{1}H and ^{13}C Nuclear Magnetic Resonances (NMR) spectra were recorded on Bruker (DRX-500 Avance) NMR spectrometer, in $CDCl_3$ with Tetramethylsilane (TMS) as internal standard. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS. Coupling constants (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 Hewlett-Packard 6890 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.

Chemicals: Hydrocortisone was kindly donated by Aburaihan Pharmaceutical Co. (Tehran, Iran), which had been purchased from Pharmacia and Upjohn S.A. (Guyancourt, USA). Other reagents and solvents were from Merck.

Collection, preservation and identification of the alga: The microalga was isolated during a screening program from soil samples collected from paddy fields of Shiraz located in the southern part of Iran (Fars province) from April to December 2004. Primary culturing was done in BG-11 medium (Ghasemi *et al*., 2004). After colonization, pure cultures of living specimens were prepared using subculturing with agar plate method in BG-11 medium (Allen, 1968). Preserved specimens were prepared and the living specimens were incubated in 50 mL-conical flasks, under unlimited carbon dioxide condition (using CO_2 enrichment condition). Constant illumination was used at 60 uE m^{-1} sec^{-1} intensity with white fluorescent lamps. Temperature was 25±2°C. The identification was done using famous manuals (Prescott, 1962; John *et al*., 2003).

Incubation conditions: The fermentation experiments were conducted in twenty 500 mL conical flasks, each containing 100 mL of BG-11 liquid medium. Inocula from the fresh culture of *Oocystis pusilla* was used at a final cell density of approximately 2.7-3×10^6 cells mL^{-1} and illuminated continuously with fluorescent lamps at 60 uE m^{-1} sec^{-1} intensity and incubated at a temperature of 25±2°C with shaking at 80 rpm for seven days. Hydrocortisone (1 g) was dissolved separately in 20 mL of ethanol. 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 14 days at the same conditions and the control was similarly processed without the microorganism. We also examined the optimum substrate concentration. The amount of the substrate varied from 0.025 to 0.2 g 100 mL^{-1} with a stepwise of 0.25. The results were obtained according to TLC analyses.

Cell density (number of cell mL^{-1}) was determined by both turbidity (optical density) and direct counting, using a light microscope with a 0.1 mm deep counting chamber (Neubauer haemocytomer). Correlation between these two methods was analyzed reaching to certainty (Andersen, 2005).

Products isolation and analyses: At the end of incubation, the content of the flasks was 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 (1:1 v/v) 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).

RESULTS

Identification of the algal strain: The strain was recognized by a colony of 4 ovate cells in closed by the enlarged mother cell wall; poles of the cells broadly rounded, without nodular thickenings; chloroplasts 1 or 2 parietal plates, pyrenoides present, but sometimes not obviously clear; cells 4-8.2 μ in diameter, 7-10 μ long, collected from paddy-fields and temporary waters of Fars Province. It seems that some little variations may be

occurred in this strain especially from biometrical point of view. It may be related to artificial environment too. According to these characters and as compared with the keys of green algae genera (John *et al*., 2003; Prescott, 1962), the selected strain was identified as a genus of *Oocystis*. The classification of the isolate alga was performed by Micoalgal Culture Collection of Shiraz University of Medical Sciences, Faculty of Pharmacy, Shiraz, Iran, as a strain of *Oocystis pusilla* Hansgirg MCCS 007.

Biotransformation of hydrocortisone: The crude extract obtained from 14 days incubation of *Oocystis pusilla* in the presence of hydrocortisone produced three steroid compounds 11β,17α,20β,21-tetrahydroxypregn-4-en-3-one (2), 11β,17β-dihydroxyandrost-4-en-3-one (3) and 11β-hydroxyandrost-4-en-3,17-dione (4), in addition to the substrate (1) as (Fig. 1):

Metabolite (2) was crystallized from methanol; m.p.: 132-134°C, $[\alpha]_D$ +91° (MeOH); lit. (Carvajal *et al*., 1959): m.p.: 133-135°C, $[\alpha]_D$ +85°; IR υ_{max} (KBr, cm^{-1}) 3536, 2910, 1661; MS (EI) m/z (%) 364 (18) (M^+,$C_{21}H_{32}O_5$), 346 (19), 331 (8), 315 (56), 303 (46), 285 (100), 267 (31), 227 (64), 148 (38), 124 (40), 91 (82), 79 (55); ^{1}H NMR ($CDCl_3$) δ 1.1 (3H, s, H-18), 1.43 (3H, s, H-19), 3.63 (2H, dd, J = 18.2 Hz, J = 4.9 Hz, H-21), 3.70 (1H, m, H-20), 4.37 (1 H, s, H-11), 5.66 (1H, s, H-4); ^{13}C NMR ($CDCl_3$) δ 199.8 (C_3), 172.7 (C_5), 122.1 (C_4), 84.2 (C_{17}), 74.3 (C_{20}), 68.2 (C_{11}), 64.2 (C_{21}), 55.3 (C_9), 50.5 (C_{14}), 46.6 (C_{13}), 41.3 (C_{10}), 39.4 (C_{12}), 33.7 (C_1), 33.2 (C_2), 32.8 (C_{16}), 32.1 (C_6), 29.7 (C_7), 29.3 (C_8), 23.6 (C_{15}), 20.8 (C_{19}), 17.7 (C_{18}); R_f: 0.18 (chloroform/acetone; 1:1 v/v).

Compound (3): Crystallized from methanol; mp: 240-242°C, $[\alpha]_D$ +164° (MeOH); lit. (Brannon *et al*., 1965): m.p.: 241-243°C, $[\alpha]_D$ +142°; IR ν_{max} (KBr, cm^{-1}) 3433, 2976, 1657; MS (EI) m/z (%) 304 (78) (M^+, $C_{19}H_{28}O_3$), 303 (20), 261 (100), 235 (55), 188 (20), 123 (60), 109 (50), 82 (85); ^{1}H NMR ($CDCl_3$) δ 1.11 (3H, s, H-18), 1.47 (3H, s, H-19), 3.82 (1H, m, H-17), 4.37 (1H, m, H-11), 5.77 (1H, s, H-4); ^{13}C NMR ($CDCl_3$) δ 199.9 (C_3), 171.4 (C_5), 122.7 (C_4), 84.6 (C_{17}), 67.1 (C_{11}), 55.4 (C_9), 49.4 (C_{14}), 46.2 (C_{12}), 43.2 (C_{13}), 38.4 (C_1), 35.6 (C_{10}), 35.5 (C_{16}), 34.3 (C_2), 32.6 (C_6), 32.2 (C_7), 32.6 (C_6), 31.1 (C_8), 25.8 (C_{15}), 21.3 (C_{19}), 15.3 (C_{18}); R_f: 0.6 (chloroform/acetone; 1:1 v/v).

Metabolite (4) was also crystallized from methanol; m.p.: 196-199°C, $[\alpha]_D$ +226°(MeOH); lit. (Rao, 1961): m.p.: 197-198°C, $[\alpha]_D$ +225°($CHCl_3$); IR υ_{max} (KBr, cm^{-1}) 3522, 1734, 1663; MS (EI) m/z (%) 302 (100) (M^+,$C_{19}H_{26}O_3$), 286 (32), 227 (41), 189 (64), 149 (40), 123 (80); 91 (80), 75 (60); ^{1}H NMR ($CDCl_3$) δ 1.20 (3H, s, H-18), 1.52 (3H, s, H-19), 4.52 (1H, s, H-11), 5.74 (1H, m, H-4); ^{13}C NMR ($CDCl_3$) δ 219.8 (C_{17}), 199.9 (C_3), 171.7 (C_5), 122.8 (C_4), 68.6 (C_{11}), 57.1 (C_9), 52.8 (C_{14}), 47.1 (C_{13}), 39.7 (C_{10}), 41.4 (C_{12}), 31.4 (C_8), 35.7 (C_1), 35.4 (C_{16}), 34.2 (C_2), 32.2 (C_6), 31.9(C_7), 22.1 (C_{15}), 21.5 (C_{19}), 16.3 (C_{18}); R_f: 0.7 (chloroform/acetone; 1:1 v/v).

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 imagined that one carboxyl group or double

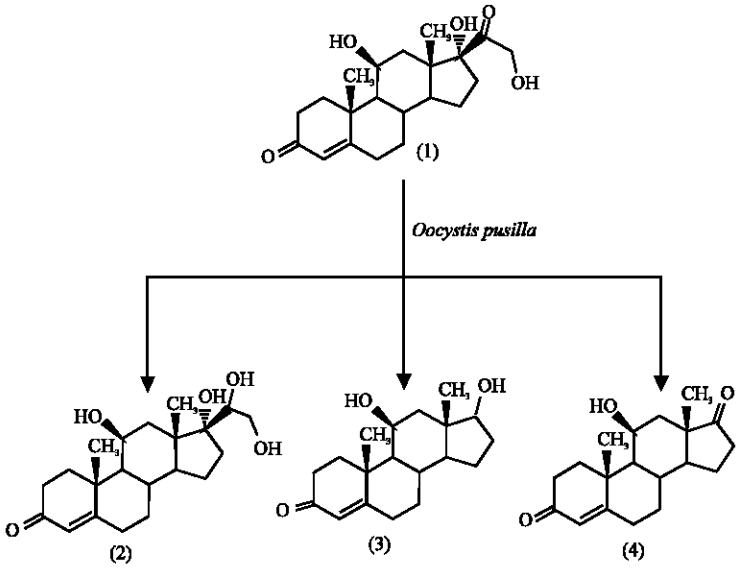

Fig. 1: Chemical structure of hydrocortisone (1) and the biotransformed products: 11β,17α,20β,21-tetrahydroxypregn-4-en-3-one (2), 11β,17β-dihydroxyandrost-4-en-3-one (3) and 11β-hydroxyandrost-4-en-3,17-dione (4)

bond in substrate have been reduced. The IR spectrum showed only one carbonyl group at 1661 cm^{-1} indicating that 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 had taken place at C-20. Additional multiplet resonance at δ 3.70 in ^{1}H-NMR spectrum as compared to the substrate confirmed the metabolite (2). 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 (Faramarzi *et al*., 2002). Melting point value of the metabolite (2) was similar to the compound with β-hydroxyl group at C-20 position.

The mass spectra of compound (3) and (4) showed the molecular ions at m/z 304 and 302, respectively, suggesting the reduction of 58 and 60 units of m/z as compared to hydrocortisone (m/z 362). The IR spectra indicated the existence of at least one hydroxyl group in compounds (3) and (4). Furthermore, in compound (3), the IR spectra showed only one carbonyl group at 1657 cm^{-1}, which was conjugated to C4-C5 double bond. The resonances at δ 3.82 and 4.37 in ^{1}H NMR spectra clearly showed the existence of two hydroxyl groups. The chemical shift of H-11 was reported for hydrocortisone and other 11-hydroxylated steroids in δ 4.3-4.4 (Kirk *et al*., 1990), so the resonance in δ 3.82 has been attributed to CH–OH in C-17. These data were supported by ^{13}C NMR, which showed a downfield resonance at δ 84.6 for CH-OH in C-17. In compound (4), IR spectra showed two absorptions at 1663 and 1734 cm^{-1}, which confirmed the existence of two carbonyl groups in C-3 and C-17, respectively. These IR data have also been supported by the related ^{13}C NMR spectra. Two signals at δ 199.9 and 219.8 in ^{13}C NMR spectra have been imputed to C-3 and C-17, respectively. As these results show, the isolated alga may be considered useful biocatalysts for some kinds of biotransformation. It has a potential for site- and regioselective bioconversion of hydrocortisone and probably other pregnane like steroids.

Oocystis pusilla 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

The unicellular microalgae may be considered useful bioreactor for biotransformation because of the simple nutritional requests of these microorganisms. To sum up, as far as we know *Oocystis pusilla* transformation of hydrocortisone and other steroids has never been reported before. A few studies have been done to apply unicellular microalgae isolated from local habitats for biotransformation of organic compounds. In one study, the unicellular cyanobacterium *Synechococcus* sp. PCC 7942 could enantioselectively reduce several aryl methyl ketone to the corresponding (S)-alcohols (Nakamura *et al*., 2000). In another study, *Synechococcus* sp. PCC 7002 has been investigated for its ability to transform aniline, naphthalene and phenol (Wurster *et al*., 2003). Bacteria and fungi are the microorganisms usually employed in steroid biotransformation, while microalgae have been less investigated up to now (Tabatabaei Yazdi *et al*., 2005). There are also some limited reports on the conversion of steroid substances using strains belonging to the microalgae (Abul-Hajj and Qian, 1986; Pollio *et al*., 1994, 1996). The use of microalgae to produce biologically active compounds is an interesting research subject because of the simple nutritional requests of these microorganisms.

Hydrocortisone 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 and Steroids, 1984).

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 lavendulae*, *Fusarium causicam*, *Fusarium solani* and *Septomyxa affinis* and 1-dehydrogenation of hydrocortisone to prednisolone by *Anthrobacter* (*Corynebacterium*) *simplex*, *Bacillus sphaericus* and *Bacterium cyclooxydans* has already been applied in industrial productions (Tabatabaei Yazdi *et al*., 2005).

In our earlier study on hydrocortisone bioconversion using microalgal strains, it was reported that *Nostoc muscorum* (Tabatabaei Yazdi *et al*., 2004), *Fischerella ambigua* (Tabatabaei Yazdi *et al*., 2005) and *Nostoc ellipsosporum* (Ghasemi *et al*., 2006), converted the substrate into some pregnane- and androstane-derived products. The main characteristics observed were hydrocortisone side chain cleavage and C-20 ketone reduction. Here, we also found that the isolate strain of *Oocystis pusilla* transformed hydrocortisone at the same characteristics to obtain some androstane and pregnane like steroids.

11β, 17β-dihydroxyandrost-4-en-3-one (3) and 11β-hydroxyandrost-4-en-3, 17-dione (4) both were less polar (R_f:0.6 and 0.7, respectively) than the substrate (R_f : 0.45)

and the other metabolite, 11β, 17α, 20β, 21-tetrahydroxypregn-4-en-3-one (2), was much polar (R_f: 0.18). The mass spectrum of metabolite (2) showed the molecular ion at m/z 364. It can be imagined that one carboxyl group or double bond in substrate have been reduced. The IR spectrum showed that the reduction had taken place at C-20. Additional multiplet resonance at δ 3.70 in ^{1}H-NMR spectrum as compared to the substrate confirmed the metabolite (2), (Tabatabaei Yazdi *et al*., 2004, 2005).

The mass spectra of compound (3) and (4) showed the molecular ions at m/z 304 and 302, respectively. The IR spectra of compound (3) showed only one carbonyl group at 1657 cm^{-1}, which was conjugated to C4-C5 double bond. The resonances at δ 3.82 and 4.37 in ^{1}H NMR spectra clearly showed the existence of two hydroxyl groups. These data were supported by ^{13}C NMR, which showed a downfield resonance at δ 84.6 for CH–OH in C-17. In compound (4), IR spectra confirmed the existence of two carbonyl groups in C-3 and C-17. These IR data have also been supported by the related ^{13}C NMR spectra. Two signals at δ 199.9 and 219.8 in ^{13}C NMR spectra have been imputed to C-3 and C-17, respectively (Tabatabaei Yazdi *et al*., 2005).

Among all of the microalgae studied for assessment of steroids biotransformation, it seems that *Oocystis pusilla* is being reported for the first time. Biotransformation observed in this research may be a research subject for further studies in using this microorganism and other related unicellular microalgae.

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