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Biotransformation of hydrocortisone by a natural isolate of *Nostoc muscorum*

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Abstract

Hydrocortisone was converted in the culture of an isolated strain of the cyanobacterium *Nostoc muscorum* PTCC 1636 into some androstane and pregnane derivatives. The microorganism was isolated during a screening program from soil samples collected from paddy fields of north of Iran. The bioproducts obtained were purified using chromatographic methods and identified as 11β-hydroxytestosterone, 11β-hydroxyandrost-4-en-3,17-dione and 11β,17α,20β,21-tetrahydroxypregn-4-en-3-one on the basis of their spectroscopic features.

Keywords: Bioconversion; Hydrocortisone; Microalgae; Cyanobacteria; *Nostoc muscorum*

1. Introduction

The algal transformation of exogenous substrates has been investigated less than the higher plants (Giri et al., 2001; Stohs and Rosenberg, 1975; Suga and Hirata, 1990), fungi and bacteria (Mahato and Majumdar, 1993; Mahato and Garai, 1997). The potential of microalgae in compound alteration has been highly focused on organic pollutants degradation (Hook et al., 1999; Pavlostathis and Jackson, 2002; Semple et al., 1999) and steroid modification (Abul-Hajj and Qian, 1986; Fiorentino et al., 1991; Greca et al., 1996a; Greca et al., 1996b; Greca et al., 1996c; Greca and Previtera, 1996; Greca et al., 1997; Pollio et al., 1994; Pollio et al., 1996) because of their importance in environmental safety and therapeutic roles, respectively.

The use of microalgae in biotransformation of steroids has been mentioned in some limited articles during the past 20 years. Abul-Hajj and Qian (1986) indicated the conversion of androstendione to testosterone with 11 different strains of microalgae. Previtera's group showed the transformations of progesterone (Greca et al., 1996a; Pollio et al., 1994, 1996) and prednisolone (Greca et al., 1997) in several microalgal cultures within four studies. In other investigations, steroid substrates were 5α-androstane-3,17-dione (Fiorentino et al., 1991), adrenosterone (Greca et al., 1996c), androsta-1,4-diene-3,17-dione (Greca and Previtera, 1996) and 17-hydoxy-17α-methylandrosta-1,4-dien-3-one (Greca et al., 1996b). Bioreactions observed in the mentioned researches were reduction of carbonyl group (Abul-Hajj and Qian, 1986; Fiorentino et al., 1991; Pollio et al., 1994), oxidation (Greca et al., 1996b), hydration of C4/C5 double bond (Greca and Previtera, 1996; Greca et al., 1997; Pollio et al., 1996), C9/C10 bond cleavage (Pollio et al., 1994), side chain degradation (Pollio et al.,

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1996), hydroxylation in different positions (Abul-Hajj and Qian, 1986; Fiorentino et al., 1991; Greca et al., 1996a,1997; Greca and Previtera, 1996; Pollio et al., 1994, 1996), Bayer-Villigar (Greca et al., 1996a), and some other kinds of rearrangement (Greca et al., 1996b, 1997; Greca and Previtera, 1996). However, no literature report has been found in hydrocortisone biotransformation by the strains belonging to *Nostoc* sp.

Nostoc muscorum, a freshwater blue-green alga, is well known in producing a numerous important enzymes, including peroxidase (Tupyk and Los, 1978), hydrogenase (Tamagnini et al., 2002), nitrate reductase (Kumar et al., 1985), nitrogenase (Verma et al., 1990), glutamine synthetase and aminoligase (Srivastava and Alma, 1997), malate dehydrogenase (Sallal and Nimer, 1990), ascorbate peroxidase (Tel-OR and Huflejt, 1986) and alkalin phosphatase (Subramanian et al., 1994).

This study is focused on the ability of an isolated strain of the cyanobacterium *Nostoc muscorum* PTCC 1636 to convert hydrocortisone as an exogenous substrate.

2. Results and discussion

Hydrocortisone (**III**) was undergone the transformation reactions in the cultures of the blue-green alga *Nostoc moscurum* PTCC 1636. At the end of incubation procedure, the cultures were analyzed for metabolites by TLC. Thus, four products with the following characterization were identified (see Fig. 1). 11β-Hydroxyandrost-4-en-3,17-dione (**I**) and 11β,17β-dihydroxyandrost-4-en-3-one (**II**), both were less polar than the substrate and the third metabolite (**III**) had the same R_f, melting point and spectral data with hydrocortisone, so it was concluded that it is unconverted substrate and the last metabolite, 11β,17α,20β,21-tetrahydroxypregn-4-en-3-one (**IV**), had a R_f value less than hydrocortisone. The mass spectra of compound **I** and **II** showed the molecular ions at m/z 302 and 304, respectively, which suggested the reduction of 60 and 58 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 **I** and **II**. Furthermore, in compound **I**, IR spectra showed two absorptions at1661 and 1735 cm^{-1}, which confirmed the existence of two carbonyl groups in C-3 and C-17, respectively. These IR data have been also supported by the related ^{13}C NMR spectra (Table 1). Two signals at δ 199.3 and 218.9 in ^{13}C NMR spectra have been imputed to C3 and C17, respectively. In compound **II**, the IR spectra showed only one carbonyl group at 1662 cm^{-1}, which was conjugated to C4/C5 double bond. The resonances at δ 3.8 and 4.38 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 3.8 has been attributed to CH–OH in C-17. These data were supported by ^{13}C NMR, which showed a downfield resonance at δ 84.0 for CH–OH in C-17. The mass spectra of compound **IV** showed the molecular ion at m/z 364 which indicated the addition of two units of m/z as compared to that of hydrocortisone (m/z 362). It

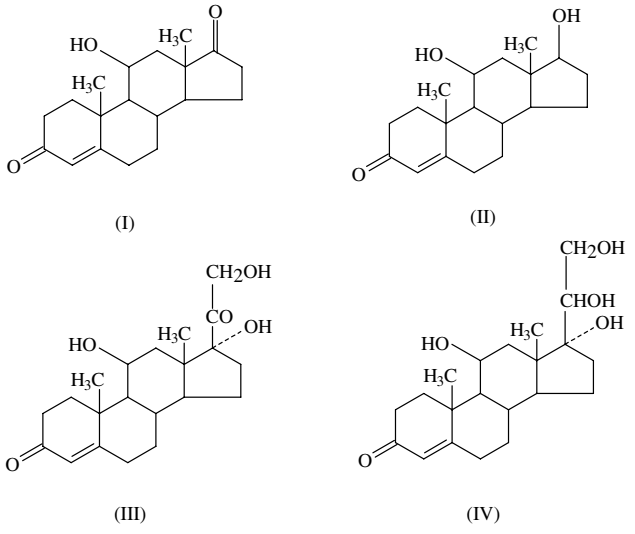

Fig. 1. The structures of hydrocortisone and the biotransformed products. 11β-Hydroxyandrost-4-en-3,17-dione (**I**), 11β,17β-dihydroxyandrost-4-en-3-one (**II**), hydrocortisone (**III**), 11β,17α,20β,21-tetrahydroxypregn-4-en-3-one (**IV**).

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

Carbon atom	Compounds				
	I	**II**	**III**	**IV**	**V**
1	35.3	37.9	34.1	33.8	34.1
2	33.8	33.7	33.8	33.1	33.8
3	199.3	198.2	199.1	199.8	199.1
4	122.6	122.2	121.5	122.2	121.5
5	171.5	171.1	172.7	172.7	172.7
6	31.8	29.3	32.8	32.1	32.8
7	31.5	32.1	31.5	29.7	31.5
8	36.9	31.9	31.6	29.3	31.6
9	56.7	55.1	55.9	55.1	55.9
10	41.0	35.0	38.5	41.5	38.5
11	67.9	66.7	66.1	68.5	66.1
12	39.3	45.6	39.3	39.2	39.3
13	46.7	42.7	46.0	46.7	46.0
14	52.4	48.9	51.5	50.6	51.5
15	21.4	22.6	23.2	23.6	23.2
16	35.0	30.6	32.5	32.8	32.5
17	218.9	84.0	88.5	85.2	88.5
18	15.8	14.1	161	17.8	161.1
19	21.1	20.8	20.4	20.9	20.4
20	–	–	212.3	73.2	212.3
21	–	–	65.8	64.6	65.8

I—11β-hydroxyandrost-4-en-3,17-dione; **II**—11β,17β-dihydroxyandrost-4-en-3-one; **III**—unconverted substrate; **IV**—11β,17α,20β,21-tetrahydroxypregn-4-en-3-one; **V**—hydrocortisone standard.

can be imagined that one carbonyl group or double bond in the structure of hydrocortisone has been reduced. The IR spectra showed only one carbonyl group at 1649 cm^{-1} that indicated that the conjugated ketone in C-3 position has not been altered. The elimination of 20-carbonyl group absorption in IR spectra showed that the reduction has been done in C-20. Additional multiplet resonance at 3.78 in ^1H NMR spectra as compared to the substrate confirmed the structure of **IV**. The stereochemistry of C-20 in the product **IV** was determined by comparison of its melting point with the compounds having a α-hydroxyl and β-hydroxyl groups at C-20 (for α-hydroxyl m.p. 253–257 °C literature (Gardi et al., 1965) and for β-hydroxyl m.p. 133–135 °C literature (Kirk et al., 1990)). Melting point value of the product **IV** was similar to the compound with β-hydroxyl group at C-20 position (Kirk et al., 1990).

Winter et al. (1984) reported the isolation of compound **IV** in 20α-hydroxyl form in the culture of *Clostridium scindens* containing hydrocortisone. In contrast, 20β-hydroxyl form of this product was obtained using *Arthrobacter globiformis* 193 (Arinbasarova et al., 1985), *Eubacterium desmolans* and *Clostridium cadavaris* (Bokkenheuser et al., 1986) with the same substrate.

As these results indicate, the isolated alga is able to effect on the cyclopentane ring and the side chain attached to the D-ring of the substrate. 20-Ketone reduction was mainly occurred to accumulate the compound **IV**. Side chain degradation to prepare two androstane derivatives (**I**, **II**) was also seen. However, no alteration was found in the rest of the molecule. Thus, the isolated strain of *N. muscorum* may be considered useful biocatalyst for some kinds of biotransformations. It has a potential for site- and regioselective bioconversion of hydrocortisone and probably other pregnane like steroids.

3. Experimental

3.1. Chemicals

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

3.2. General experimental procedures

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 with a FTNMR Varian Unity Plus spectrometer in $CDCl_3$. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS. Coupling constants (J) are given in hertz (Hz). The IR spectra were determined on a Magna-IR 550 Nicolet FTIR spectrometer. Optical rotations were measured on solutions of methanol in 1-dm cells on a Perkin–Elmer 142 automatic spectropolarimeter. Melting points (m.p.) were determined on a Reichart-Jung hot stage melting point apparatus and were uncorrected. 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 chloroform/acetone (6:4) and visualized by spraying the plates with a mixture of methanol–sulfuric acid (6:1) and heating in an oven at 100 °C for 3 min until the colors developed. HPLC analyses were also done using HPLC (JASCO, JAPAN) equipment on a C_{18} column (4.6×250 mm). An isocratic column elution was monitored by UV detector (JASCO 1570 UV/Visible detector). The wavelength was set on 254 nm and the mobile phase used was methanol–water (45:55 v/v) at a flow rate 1 ml/min.

3.3. Collection, preservation and identification of the alga

The blue-green alga was isolated during a screening program from soil samples collected from paddy fields of north of Iran (Mazandaran and Golestan provinces) from May to November 2001. Primary culturing was done in BG-11 and modified Allen and Arnon medium (Allen, 1968; Borowitzka, 1988). 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-tubes bubbling by one percent carbon dioxide. Constant illumination was used at 40 $\mu E\, m^{-2}\, S^{-1}$ intensity with white fluorescent lamps. Temperature was 25 ± 2 °C. The identification was done using semi-permanent slides (glycerin mount) and living specimen according to cyanobacter genera (Anagnostidis and Komarek, 1988).

3.4. Identification of the algae

The strain was recognized by colony with indefinite and irregular shape, black to dark brown; filaments compact and spirally arranged; cells 3–4 in diameter, oblong cylindric, 5–8 μm in length; heterocysts 5–7 μm in diameter, oblong; akinestes 5 μm broad, 7–10 μm long, several attached to each other. According to these characters and comparing with the keys of cyanobacteria genera (Anagnostidis and Komarek, 1988), the selected strain was identified as a genus of *Nostoc*. The classification of the isolate alga was performed by Persian Type Culture Collection (PTCC), Tehran, Iran, as a strain of *N. muscorum*, PTCC 1636. The strain was

maintained on BG-11 agar slope and freshly subcultured before using in transformation experiment.

3.5. Fermentation conditions

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 $\mu E\,m^{-2}\,S^{-1}$, and incubated at a temperature of 25 ± 2 °C without shaking for five 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 seven days at the same conditions and the control was similarly processed without the microorganism.

3.6. Analytical procedure

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) solvent system and then purified metabolites were crystallized in methanol. Purified metabolites were identified by melting points and spectral data (^{13}C NMR, ^{1}H NMR, FTIR and MS). The purity as well as the amount of individual metabolites was checked with HPLC analysis.

3.7. Bioconversion of hydrocortisone

Four metabolites were purified from hydrocortisone bioconversion with *N. muscorum* PTCC 1636 as follows:

3.7.1. (I) 11β-Hydroxyandrost-4-en-3,17-dione

This compound was crystallized from methanol; (170 mg), m.p. 196–197 °C, $[\alpha]_D$ +225° (MeOH), lit. (Rao, 1961) m.p. 197–199 °C, $[\alpha]_D$ +225°; IR ν_{max} 3473, 1735, 1661 cm^{-1}; MS (EI) *m/z* (%) 302 (70) (M^+, $C_{19}H_{28}O_3$), 286 (19), 269 (20), 227 (25), 189 (38), 163 (100), 149 (44), 123 (50), 91 (25), 83 (18); ^{1}H NMR ($CDCl_3$) δ 1.17 (3H, s, H-18), 1.47 (3H, s, H-19), 4.47 (1H, m, H-11), 5.70 (1H, s, H-4); R_f in chloroform/acetone (6:4): 0.8.

3.7.2. (II) 11β,17β-Dihydroxyandrost-4-en-3-one

This compound was crystallized from methanol; (126 mg), m.p. 237–240 °C, $[\alpha]_D$ +164° (MeOH), lit. (Brannon et al., 1965) m.p. 241–243 °C, $[\alpha]_D$ +142°; IR ν_{max} 3527, 3385, 1662 cm^{-1}; MS (EI) *m/z* (%) 304 (4) (M^+, $C_{19}H_{28}O_3$), 303 (20), 302 (53), 284 (11), 269 (11), 213 (21), 189 (34), 163 (100), 123 (52), 91 (41); ^{1}H NMR ($CDCl_3$) δ 1.17 (3H, s, H-18), 1.48 (3H, s, H-19), 3.80 (1H, m, H-17), 4.38 (1H, m, H-11), 5.65 (1H, s, H-4); R_f in chloroform/acetone (6:4): 0.66.

3.7.3. (III) Hydrocortisone

This compound was crystallized from methanol; (305 mg), m.p. 217 °C, $[\alpha]_D$ +163–164° (MeOH), lit. (Hill et al., 1991) IR ν_{max} 3431, 2931, 1710, 1639 cm^{-1}; MS (EI) *m/z* (%) 362 (38) (M^+, $C_{21}H_{30}O_5$), 344 (20), 329 (16), 303 (25), 285 (65), 242 (62), 227 (100), 173 (30), 161.1 (29), 123 (52), 91 (45), 74 (35), 55 (45); ^{1}H NMR ($CDCl_3$) δ 0.74 (3H, s, H-18),1.36 (3H, s, H-19), 4.62 (1H, m, H-11), 5.55 (1H, s, H-4); R_f in chloroform/acetone (6:4) 0.49.

3.7.4. (IV) 11β,17α,20β,21-Tetrahydroxypregn-4-en-3-one

This compound was crystallized from methanol; (215 mg), m.p. 128–130°C, $[\alpha]_D$ +91° (MeOH), lit. (Carvajal et al., 1959) m.p. 133–135°C, $[\alpha]_D$ +85°; IR ν_{max} 3462, 1649 cm^{-1}; 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, m, H-21), 3.78 (1H, m, H-20), 4.36 (1H, m, H-11), 5.62 (1H, s, H-4); R_f in chloroform/acetone (6:4): 0.1.

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