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Prednisolone Bio-Transformation in the Culture of Filamentous Fungus *Acremonium strictum*

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Abstract: The whole cells of *Acremonium strictum* transformed prednisolone at its side chain to produce two steroid compounds. 21,21-Dimethoxy-11β-hydroxypregn-1,4-dien-3,20-dione was the main metabolite which its production has not been previously reported using microbiological means. This metabolite together with a hydroxylated derivative, 11β-hydroxyandrost-1,4-dien-3,17-dione, were purified with preparative TLC followed by their identification through ^{1}H, ^{13}C NMR and other spectroscopic data. Best fermentation condition was found to be 5 day incubation at 25°C and pH value of 6 according to TLC profiles. Optimum concentration of the substrate, which gave maximum bioconversion efficiency, was 1 mg mL^{-1} in one batch. Biotransformation was completely inhibited in a concentration above 5 mg mL^{-1}.

Key words: *Acremonium strictum*, biotransformation, prednisolone, steroid

INTRODUCTION

Microbial steroid transformations have been extensively investigated using various species of fungi during recent decades (Fernandes *et al*., 2003). These methods have been applied because of high regio and stereospecificity in steroid compounds modifications. Research in this area is of importance and often used as general mean to prepare steroid derivatives which those productions are difficult by means of chemical methods. Many attentions have been oriented to obtain new microorganisms and evaluated their abilities on various steroid biotransformations (Fernandes *et al*., 2003; Donova *et al*., 2005; Faramarzi *et al*., 2008).

Literature survey revealed that not much work has been done so far on the biotransformation of steroids using strains belonging to *Acremonium strictum* W. Gams 1971 in the family of Hypocreaceae (Yoshihama *et al*., 1989a, b).

Recently, we reported the conversions of hydrocortisone (Faramarzi *et al*., 2002), progesterone (Faramarzi *et al*., 2003), nandrolone decanoate (Tabatabaei Yazdi *et al*., 2006) and androst-1,4-dien-3,17-dione (Faramarzi *et al*., 2006) into compounds with alterations in the D ring and/or at the side chain. In this study, the same fungus was examined for biotransformation of prednisolone, a pharmaceutically steroid substance and the results are presented as follows.

MATERIALS AND METHODS

Chemicals and instruments: Prednisolone (pharmaceutical grade) was kindly donated by Aburaihan Pharmaceutical Co. (Tehran, Iran) Kieselgel 60 $HF_{254+366}$ for preparative TLC, Sabouraud-2%-dextrose broth and 4%-dextrose agar were purchased from Merck (Darmstadt, Germany). All other reagents and solvents were of analytical grade.

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. ^{1}H and ^{13}C nuclear magnetic resonance (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) were 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.

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Fungal strain and fermentation condition: The strain of *Acremonium strictum* PTCC 5282, which has been introduced in our previous report (Faramarzi *et al*., 2002), was maintained at 4°C on Sabouraud 4%-dextrose agar slant and freshly subcultured before using in transformation experiment. The organism was transferred to a fresh medium every two months.

The microbial spores, from the agar slant which incubated at 25°C, were inoculated into 1000 mL liquid medium of sabouraud-2%-dextrose broth equally divided into ten 500-mL conical flasks and cultured on a rotary shaker (150 rpm) at 25°C. After an incubation of 12 h, two milliliters of a 20 mL ethanol solution containing prednisolone (1 g) was directly added to each flask and the incubation was carried out for five days at the same conditions. The control was similarly processed without the microorganism.

Products isolation and analyses: After biotransformation procedures, media were collected by filtration and extraction with two equal volume of chloroform. The extract was washed with water and evaporated under reduced pressure resulting in a gummy material. The residue was dissolved in methanol and fractionated using preparative TLC with chloroform/acetone (20:80, v/v) as solvent system. After detection under uv light, the fractions were marked and cut from the plates. The metabolites were eluted from the adsorbent using a mixture of chloroform/methanol (80:20 and 20:80, v/v; three times) and then crystallized in suitable solvent.

Two metabolites (II, 253 mg; III, 195 mg) and the unconverted starting material (I) were identified by melting points, optical rotations and spectral data (^{13}C NMR, ^{1}H NMR, FTIR and MS).

Effect of temperature, pH and substrate concentration: Spores of *A. strictum* was transferred into a 500 mL Erlenmeyer flask containing 100 mL of SDB medium supplemented with 100 mg of prednisolone dissolved in 2 mL of absolute ethanol and then the incubation continued for 5 days at the same condition described above (Fungal strain and fermentation condition). Sampling was carried out every 24 h and the control was similarly processed without the microorganism.

Studies were performed to find out the optimum pH and temperature as well as the maximum amount of substrate that could be transformed to the products. The temperature was checked between 20 and 40°C with a stepwise of 5°C. The effect of pH on biotransformation procedure was studied in non-buffered media by adjusting the pH from 3 to 11 with NaOH and HCl. The stepwise of pH value was 0.5. The amount of the substrate varied from 0.5 to 5 mg mL^{-1} with a stepwise of 0.5. In each case, one parameter was studied and the others kept constant. TLC was applied to qualitatively studies of the influence of temperature, pH and the substrate concentration. Detection was done by UV at 254 nm.

RESULTS AND DISCUSSION

Acremonium strictum was found to transform prednisolone (I) into two steroid derivatives (II and III) (Fig. 1). The steroid products were isolated from a semisolid residue obtained of the fermentation broth and characterized by spectroscopic methods as follows:

21,21-Dimethoxy-11β-hydroxypregn-1,4-dien-3,20-dione (II): Crystallized from methanol; mp: 183-185°C, $[\alpha]^{25}_{D}$ +138° (MeOH), lit (Zoltan *et al*., 1963) m.p. 183-184°C; IR υ_{max} 3461, 2930, 1716, 1658, 1615, 1607 cm^{-1}; MS (EI) m/z (%) 388 (M+, $C_{23}H_{32}O_5$, 34), 370 (11), 315 (30), 297 (4), 269 (85), 242 (54), 227 (32), 213 (8), 173 (5), 161 (20), 145 (35), 91 (15), 58 (100); ^{1}H NMR ($CDCl_3$) δ 0.96 (3H, s, H-18), 1.48 (3H, s, H-19), 3.40 (6H, s, $CH(OC\underline{H}_3)_2$), 4.41 (1H, m, H-11), 4.46 (1H, s, H-21), 6.02 (1H, s, H-4), 6.28 (1H, d, J = 9.6 Hz, H-2), 6.26 (1H, d, J = 9.6 Hz, H-1); R*f* in chloroform/acetone (2:8): 0.6.

11β-Hydroxyandrost-1,4-dien-3,17-dione (III): Crystallized from methanol; mp: 210-214°C, $[\alpha]^{25}_{D}$ +90° ($CHCl_3$), lit (Hill *et al*., 1991) m.p. 212-214°C, $[\alpha]_{D}$ +86°; IR υ_{max} 3418, 2923, 1730, 1650, 1607 cm^{-1}; MS (EI) m/z (%) 300 (M+, $C_{19}H_{24}O_3$, 15), 299 (29), 282 (7), 250 (7), 240 (7), 225 (11), 178 (12), 160 (23), 145 (18), 132 (20), 121 (100), 90 (22); ^{1}H NMR ($CDCl_3$) δ 1.18 (3H, s, H-18), 1.48 (3H, s, H-19), 4.48 (1H, m, H-11), 6.03 (1H, m, H-4), 6.28 (1H, d, J = 10 Hz, H-2), 7.28 (1H, d, J = 10 Hz, H-1); R*f* in chloroform/acetone (2:8): 0.5.

Metabolite II, $C_{23}H_{32}O_5$, showed in its mass spectrum a molecular ion peak at m/z 388, which indicated the addition of 28 units as compared to that of the substrate (m/z 360). However, the IR spectrum revealed absorption bands for hydroxyl group (3461 cm^{-1}) and two carbonyl groups (1716 and 1657 cm^{-1}). The ^{1}H NMR spectrum of metabolite II contained signals for two methyl groups (δ 0.96, H-18 and δ 1.48, H-19), as well as signal for H-4 at δ 6.02. The multiplet at δ 4.60 for two protons of H-21 in prednisolone was replaced by a singlet at δ 4.46 with integral of 1 H. The presence of a sharp singlet at δ 3.40 with integral of 6H indicated two methoxyl groups in produced metabolite. The rest significant chemical shifts were the same as in the substrate including two dd at δ 6.28 and δ 6.26 (J=9.6 Hz) for H-2 and H-1, respectively.

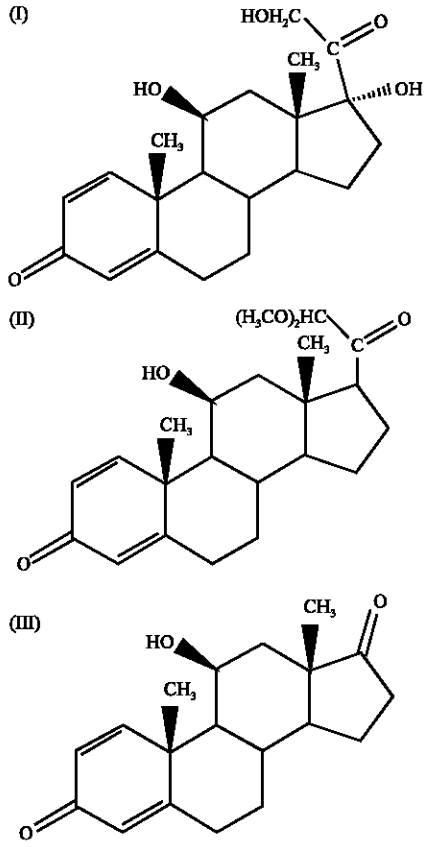

Fig. 1: The structures of prednisolone and its byproducts: prednisolone (I), 21,21-dimethoxy-11β-hydroxypregn-1,4-dien-3,20-dione (II), 11β-hydroxyandrost-1,4-dien-3,17-dione (III)

^{13}C NMR spectrum of the metabolite II, which its values and the assignment of its carbons with distortionless enhancement by polarization transfer experiment (DEPT) are shown in Table 1, was particularly interesting and deduced 23 carbon signals. However, the chemical shifts at δ 44.3 (C-13), 56.9 (C-14), 22.9 (C-16), 57.5 (C-17), 15.9 (C-18), 206.0 (C-20) and 103.3 (C-21) were all different. The main alterations were at C-17, C-20 and C-21. Furthermore, the spectrum contained two additional carbon signals at δ 53.9 and 54.3, which defined by DEPT experiment as two methyl groups. It also indicated the replacements of C-17, CH_2-21 with CH-17 and CH-21, respectively.

Metabolite III, $C_{19}H_{24}O_3$, exhibited in its mass spectrum the molecular ion peak at m/z 300, which easily conducted to imagine the substrate with losing its side chain. The IR spectrum displayed bands for hydroxyl group (3418 cm^{-1}) and two carbonyl groups (1730 and 1650 cm^{-1}). ^1H NMR spectrum edified the structure of metabolite III through two carbonyl signals at δ 186.0 for C-3, δ 218.0 for C-17, four sp^2 carbon signals at δ 155.5, 128.0 for C1-C2 double bond, δ 122.0, 169.0 for C4-C5 double bond, a hydroxylated carbon signal at δ 69.8 for C-11 and twelve other aliphatic carbon signals.

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

	Compounds			
Carbon atom	I	II	DEPT of II	III
1	156.8	156.8	CH	155.5
2	127.1	127.3	CH	128.0
3	185.8	186.6	C	186.0
4	121.8	121.9	CH	122.0
5	170.5	170.7	C	169.0
6	31.8	31.8	CH_2	30.9
7	31.2	33.7	CH_2	31.8
8	34.0	31.1	CH	32.7
9	55.4	55.5	CH	55.9
10	38.9	44.1	C	40.9
11	68.9	69.6	CH	69.8
12	47.1	47.3	CH_2	44.0
13	51.1	44.3	C	46.9
14	44.1	56.9	CH	51.8
15	23.8	23.8	CH_2	21.9
16	33.5	22.9	CH_2	35.2
17	88.6	57.5	CH	218.0
18	16.9	15.9	CH_3	15.8
19	20.9	20.7	CH_3	21.2
20	212.1	206.0	C	-
21	66.5	103.3	CH	-
$CH(OCH_3)_2$	-	53.9, 54.3	CH_3	-

Prednisolone (I), 21,21-dimethoxy-11β-hydroxypregn-1,4-dien-3,20-dione (II) and 11β-Hydroxyandrost-1,4-dien-3,17-dione (III)

The highest bioconversion rate of prednisolone was obtained at pH values of 6.5. The bioconversion reaction proceeded well at 25°C for production of all the metabolites. In 40°C, the substrate remained unconverted in the broth. Increasing concentration of prednisolone from 0.5 to 5 mg mL^{-1} showed that higher concentration of the substrate (≥5 mg mL^{-1}) decrease microbial conversion. The optimum substrate concentration, which gave the maximum efficiency in microbial conversion of prednisolone by *A. strictum*, was 1 mg mL^{-1}.

As data showed, fungal transformation of prednisolone using *A. strictum* led to accumulation of two steroid compounds. Dimethoxyl derivative steroid (II) was the major product and there has so far been no literature report on its production using microorganisms except that of Zoltan and co-workers, which introduced compound II in a synthetic procedure (Zoltan *et al*., 1963). We also reported the purification of an 11-hydroxy derived compound (III) in the fermentation broth. Both metabolites were produced from prednisolone within the microbial alterations in the side chain of the substrate (I).

Prednisolone is an important glucocorticosteroid which used to treat inflammatory conditions. A number of bacteria and fungi have been so far applied for the production of prednisolone from hydrocortisone through dehydrogenation via microbial dehydrogenase enzymes (El-Hadi, 2003; Fernandes *et al*., 2003). The microbial

1-dehydrogenation of hydrocortisone using *Arthrobacter simplex* ATCC 6946 led to formation of prednisolone. Hydroxylations on C-9α, C-16β, C-16α and C-16α and C-16β methylation on prednisolone are necessary in the chemical and microbial pathways towards production of some valuable pharmaceutical steroids, e.g., dexamethasone, fluocinolone, betamethasone, etc. (Smith, 1984). Attempts have been done to produce this highly active inflammatory commercial product from cortexolone using immobilized mixed cultures of fungi (Fernandes *et al*., 2003). Beside bacteria and fungi, the green alga T76 *Scenedesmus quadricauda* was also examined for algal transformation of prednisolone. Hydration of the Δ^4 double bond and some rarely rearrangements were the characteristic observed through this biotransformation (DellaGreca *et al*., 1997).

In our previous studies, *A. strictum* was applied as a biocatalyst with the ability of converting esterane-, androstane-and pregnane-like steroids at D-ring and/or side chain. The observed characteristics for those biotransformations were 21-hydroxyl acetylating, C-20 carbonyl reduction and side chain degradation on hydrocortisone (Faramarzi *et al*., 2002); 15α- and 21-hydroxylations on progesterone (Faramarzi *et al*., 2003); 15α-hydroxylation and side chain degradation on nandrolone decanoate (Tabatabaei Yazdi *et al*., 2006) and 15α-hydroxylation, 1,2-hydrogenation and C-17 carbonyl reduction on androst-1,4-dien-3,17-dione (Faramarzi *et al*., 2006), respectively. In nearly all cases, the fungal strain selectively attacked D-ring and the side chain of steroid substrates. The same result was obtained on prednisolone biotransformation by the same strain. It seems that the strain is a suitable biocatalyst for some alterations on D-ring and side chain of steroid compounds.

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