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Synthesis of 7α-hydroxy-dehydroepiandrosterone and 7β-hydroxy-dehydroepiandrosterone

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Abstract

The fermentation of dehydroepiandrosterone synthesized from the starting material diosgenin using *Mucor racemosus* produced 7α-hydroxy-dehydroepiandrosterone and 7β-hydroxy-dehydroepiandrosterone. The bioactivity of the microbial metabolites is also discussed. The species *M. racemosus* was isolated by screening among stains from soil samples collected from various parts of China.

Keywords: Microbial transformation; DHEA; Hydroxylation; *Mucor racemosus*; 7-Hydroxy

1. Indroduction

The microbiological hydroxylation of steroids has been described in terms of a triangular relationship between two binding sites and the site of hydroxylation [1,2]. The oxidoreduction of steroids governs the biological activity and metabolic fate. 7-Oxygenated steroids are widespread in mammals, birds, fish, and plants [3]. The major 3β-hydroxysteroids, including dehydroepiandrosterone (DHEA, **4**), pregnenolone, cholesterol and androstane-3β,17β-diol are efficiently 7α-hydroxylated in diverse tissues including the brain[4–11]. In recent years, The significant bioactivity of DHEA and 7-OH-DHEA has been noted. The metabolism of DHEA is also of some interest. DHEA and its metabolites has been shown to promote the immune response in experimental animals [12–17]. The major metabolic pathway for DHEA in extra-hepatic tissues is via 7-hydroxylation [18–20]. Some scientists have observed that 7α-OH-DHEA is more active than DHEA in preventing hypoxic cell death of neurons in vitro. Therefore, 7-oxygenation seems to be associated with the activation of DHEA [21]. There is still ongoing debate about the stereoconfiguration of the active metabolite. In peripheral tissues, 7-OH-DHEA has been found to up-regulate immunity and to counteract immunosuppression [22–24], Moreover, in both Alzheimer's patients and a control group, the conversion of DHEA to Δ5-androstene-3β,17β-diol and to 7α-OH-DHEA occurred in the frontal cortex, hippocampus, amygdala, cerebellum. The formation of these metabolites within distinct brain regions negatively correlated with the density of β-amyloid deposits [24].

With the aim of synthesizing 7-hydroxy-DHEA, we first synthesized **4** from diosgenin (**1**) which is a readily available commercal product. Our next objective was to screen microorganisms capable of producing 7-hydroxy steroids. In our search for various physiologically important steroid derivatives by microbial transformations, we were able to isolat a common strain of *Mucor racemosus* (A.C.C.C. 0401) from soil using **4** as the substrate.

In this study, *M. racemosus* was used to convert **4** into 7α-hydroxy-dehydroepiandrosterone (**5**) and 7β-hydroxy-dehydroepiandrosterone (**6**). The metabolites were characternized by various spectroscopic methods. It was reported that membrane-bound *M. racemosus* lipases could be entrapped in cryogel beads obtained from polyvinyl alcohol

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(PVA) by a freezing–thawing method in two-phase system, and due to the porosity of PVA-cryogels, high molecular-weight substances can penetrate beads of the biocatalyst. The biocatalyst could be applied for various hydrolysis and synthesis reactions [25]. But we believe this to be the first reported instance of the microbial hydroxylation of steroids using *M. racemosus*. Moreover, we produced **5** and **6** from the readily available material diosgenin, and with a considerable yield.

2. Experimental

2.1. Instrumental methods

Melting points were determined on a XT5 melting point apparatus and are uncorrected. Infrared spectra were recorded using KBr discs on a Bruker Vectror-22 spectrometer. Mass spectra were obtained on an Esquire3000 mass spectrometer by electrospray ionization. Optical rotations were measured in 1-dm cells at 20 °C on a Perkin-Elmer 341 automatic spectropolarimeter in methanol solutions. The ^{1}H and ^{13}C nuclear magnetic resonance spectra were obtained using a Bruker Avance DPX-400 spectrometer at 400 and 100 MHz, respectively, with tetramethylsilane as internal standard in DMSO-d_6,and chemical shifts are given as δ values. Coupling constants (*J*) are given in hertz (Hz). Thin layer chromatography (TLC) was performed on a 0.25 mm thick layer of silica gel G (Qingdao Marine Chemical Factory, China). Chromatography was performed with petroleum ether (bp 60–90 °C)/acetone (7:3) or chloroform/methanol (8:1) and visualized by spraying the plates with 50% sulfuric acid solution and heating in an oven at 100 °C for 3 min until the colors developed.

2.2. 3β-Acetoxy-5,16-pregnadien-20-one (*2*)

Compound **2** was synthesized as described in the literature [26] with a few variations noted in the Results and discussion. mp 172–174 °C. $[\alpha]_D = -30°$ (c, 0.11, CH_3OH). IR (KBr) ν: 2944, 2905, 2865, 1730, 1661, 1583, 1438, 1370, 1248, 1307 cm^{-1}. ^{1}H NMR (DMSO-d_6, 400 MHz) see Table 1. ^{13}C NMR (DMSO-d_6, 400 MHz) δ: 196.3 (C20), 169.8 (CH_3COO), 154.4 (C17), 145.2 (C16), 140.1 (C5), 121.9 (C6), 73.3 (C3), 56.0 (C14), 50.0 (C9), 45.6 (C13) ppm. MS (ESI) *m/z* $[M + Na]^+$ 379.

Table 1
^{1}H NMR ($CDCl_3$, 400 MHz) data of compounds **2**, **3**, **4**, **5** and **6**

	3-H	6-H	7-H	18-H	19-H
2	4.45, m	5.36, d *J* = 4.4		0.85, s	1.01, s
3	4.61, m	5.38		0.63, s	1.02, s
4	3.54, m	5.40, d *J* = 5.20		0.90, s	1.04, s
5	3.58, m	5.64, d *J* = 2.0	3.97	0.89, s	1.02, s
6	3.57, m	5.32	3.95, d *J* = 8.0	0.90, s	1.08, s

2.3. 3β-Acetoxy-5,16-pregnadien-20-oxime (*3*)

A mixture of **2** (2.5 g, 6.7 mmol), hydroxylamine hydrochloric acid (0.85 g, 12.2 mmol), pyridine (3 ml) and 95% ethanol (13 ml) was refluxed for 30 min, the progress of the reaction was monitored by TLC (acetone/ether (bp 60–90 °C) (3:7). Then the mixture was cooled with ice, washed with hot water, dried under a vacuum. A white solid was obtained, 2.27 g; yield 87%. mp 227–229 °C (literature 228–230 °C [27]), IR (KBr) ν: 3476, 2939, 1721, 1373, 1269, 1034 cm^{-1}. ^{1}H NMR (see Table 1).

2.4. Dehydroepiandrosterone(*4*)

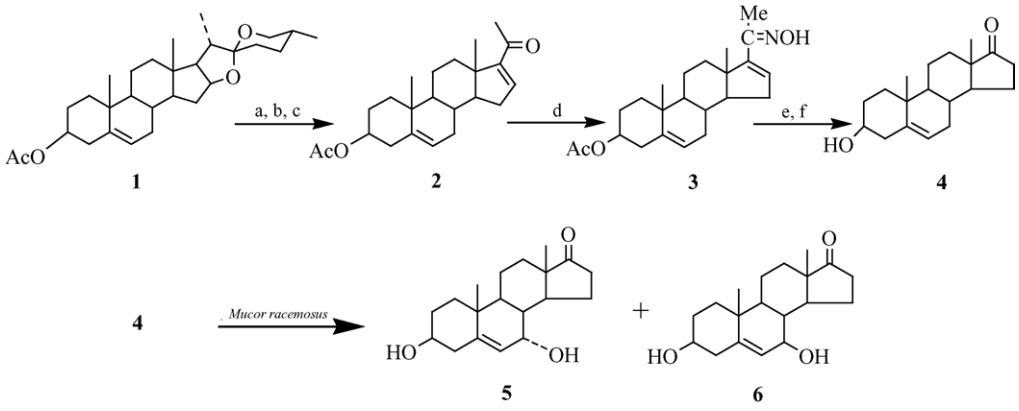
A mixture of **3** (0.70 g, 1.9 mmol) and unhydrous pyridine (70 ml) was first stirred for 2 h at 10–15 °C, then for an additional 2 h at room temperature. The mixture was then poured into ice water and put into a refrigerator for one night. The next day, the mixture was extracted with ethyl acetate (3 × 20 ml). The organic layer was evaporated under reduced pressure to obtain a solid which was dissolved in methanol and 2.5% potassium hydroxide (30 ml) and refluxed. The mixture was then extracted with ethyl acetate (3 × 20 ml), and the organic layer was evaporated under reduced pressure to obtain product **4**, which was recrystallized with methanol to afford 0.476 g; yield: 51.8%. mp 150.6–151.7 °C (literature 150–152 °C). IR (KBr) ν: 3462, 2935, 2854, 1732, 1460, 1371, 1298, 1219, 1136, 1061, 839, 802, 592 cm^{-1}. ^{1}H and ^{13}C NMR data (see Tables 1 and 2).

2.5. 7α-Hydroxy-dehydroepiandrosterone(*5*) and 7β-hydroxy –dehydroepiandrosterone(*6*)

Ten 500-ml Erlenmeyer flasks, each containing 100 ml sterilized (peptone dextrose agar) broth, were inoculated with

Table 2
^{13}C NMR ($CDCl_3$, 400 MHz) data of compounds **4**, **5** and **6**

Carbon	**4**	**5**	**6**
1	37.2	35.8	36.8
2	31.5	31.1	31.2
3	71.4	71.2	71.3
4	42.2	41.9	41.6
5	141.3	146.6	143.7
6	120.8	123.6	125.5
7	31.5	64.3	72.9
8	31.5	37.2	40.5
9	50.3	42.6	48.2
10	36.7	37.2	36.7
11	20.4	20.1	20.4
12	30.8	31.3	31.5
13	47.5	47.1	47.8
14	51.8	44.9	51.2
15	21.8	21.9	24.6
16	35.8	37.0	36.0
17	221.3	220.0	221.2
18	13.2	13.3	13.6
19	19.4	18.3	19.2



Scheme 1. Reagents and conditions: (a) $(CH_3CO)_2O$, NH_4Cl, Py, 140 °C; (b) CrO_3, CH_3CO_2H, $ClCH_2CH_2Cl$, 0 °C; (c) AcONα·$3H_2O$ Δ; (d) $NH_2OH{\cdot}HCl$, Py, C_2H_5OH, reflux; (e) $POCl_3$, Py, benzene, condensed HCl; (f) CH_3OH, KOH.

freshly obtained spores from agar slope cultures and incubated for 2 days at 27 °C in a rotary shaker (150 rpm). DHEA (1.5 g) was dissolved in 20 ml acetone, and 2 ml of this solution was added to each 500-ml Erlenmeyer flask. Incubation was continued for 4 days under the same conditions. The fermentation media were extracted exhaustively with ethyl acetate (5 × 200 ml) and filtered to separate the broth from the mycelium. The extract was evaporated under reduced pressure. The transformation products were separated by silica gel column chromatography by using $CHCl_3/CH_3OH$ (8:1) as eluent. Two metabolites, **5** (613 mg)and **6** (276 mg), were purified. Compound **5**: yield 40.9%. mp 179.4∼181.7 °C (literature 181.5 ∼ 183.5 °C [28]). IR (KBr) ν: 3364, 3301, 2930, 2855, 1730, 1660, 1629, 1460, 1380, 1297, 1132, 1056 cm^{-1}. ^1H and ^{13}C NMR data (see Tables 1 and 2). MS *m/z* 326.7, $[M + Na]^+$, 342.7 $[M+K]^+$. Analysis calculated for $C_{19}H_{28}O_3$: C, 74.96%; H, 9.27% O, 15.77%. Found: C, 74.83%; H, 9.34%; O, 15.70%.

Compound **6**, yield 18.0%. mp 216.2∼217.6 °C (literature 215 ∼ 216 °C [28]). IR (KBr) ν: 3431, 2933, 1730, 1657,1621, 1461, 1375, 1247, 1112, 1057, 1029 cm^{-1}. ^1H and ^{13}C NMR data (see Tables 1 and 2). MS *m/z*, 326.7 $[M + Na]^+$, 342.7 $[M + K]^+$. Analysis calculated for $C_{19}H_{28}O_3$: C, 74.96%; H, 9.27% O, 15.77% found C, 74.78%; H, 9.16%; O, 15.67%.

3. Results and discussion

3β-Acetoxy-5, 16-pregnadien-20-one (**2**) was prepared according to the literature [25] from diosgenin. However, we obtained better results by keeping the reaction at a higher temperature after the oxidant (CrO_3/CH_3CO_2H) was added, and by cooling the product after it was treated with hot water rather than by treating it with cold water directly. With **2** as the precursor of 3β-acetoxy-5,16-pregnadien-20-oxime (**3**) according to the literature [26], we produced **3**. Next, dehydroepiandrosterone (**4**) was prepared from **3** using Bechmanns' reaction and hydrolysis. In this step, toluene was used as a solvent instead of benzene because of its lower toxicity. Finally methanol and 2.5% potassium hydroxide was used in the hydrolysis step, which produced fairly quickly (Scheme 1.). The target compounds 7α-hydroxy-dehydroepiandrosterone (**5**) and 7β-hydroxy-dehydroepiandrosterone (**6**) were obtained via microbiological hydroxylation. The steroid-converting fungus was isolated by screening among 96 stains from samples collected from various parts of China. The strain was grown at 27 °C on a peptone dextrose agar slope, stored at 4 °C, and freshly subcultured before the transformation experiment. The selected strain was identified as the species *M. racemosus* by China General Microbiological Culture Collection Center.

Two metabolites, **5**, **6**, and the unconverted substrate **4** were purified and identified by melting points and spectral data (IR, MS, ^1H NMR, ^{13}C NMR) (Scheme 1). The mass spectra of metabolites **5** and **6** showed the quasi-molecular ion $[M + Na]^+$ at *m/z* 326.7, which suggested that they incorporate one oxygen atom into the substrate. In the NMR spectra for compound **5**, the signal at δ 3.58 was assigned to H-3, which was similar to that of compound **6**. In the HMQC spectra for compound **5**, the signal at δ 5.64 correlated to C-6 and at 3.97 correlated to C-7,which were assigned to H-6 and H-7 respectively. Similarly, in compound **6**, there were corresponding signals at 5.32 and at 3.95, which were also assigned respectively to H-6 and H-7. Regarding the H-H COSY spectra for compound **5**, the resonance of H-7(δ = 3.97) correlated with H-6 (δ = 5.64) and H-8(δ = 1.69), while for compound **6**, the resonance of H-7(δ = 3.95) only correlated with H-8(δ = 1.83). These correlated peaks show that the hydroxyl group attached at C-7 of compound **5** has an was axial orientation, while the hydroxyl group attached at C-7 of compound **6** has an equatorial orientation.

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References

[1] Browne John w, Denny William A, Ewart Sir, Jones RH, Neakins Denis, Morisawa Yasu, et al. Microbiological Hydroxylation of steroids part VII. The pattern of dihydroxylation of mono-oxo-5α-androstanes and 5α-estranes with the fungus *Rhizopus nigricans*. Journal of the Chemical Society, Perkin Transactions I 1973:1493–9.

[2] Chambers Virginia EM, Denny William A, Evans John M, Jones Sir Ewart RH, Kasal Alexander, Meakins G Denis, et al. Microbiological hydroxylation of steroids part VIII. The pattern of monohydroxylation of diketones and keto-alcohols derived from 5α-androstane with cultures of the fungus, *Rhizopus nigricans*. J Chem Soc, Perkin Trans I 1973:1500–17.

[3] Lathe Richard. Steroid and sterol 7-hydroxylation: ancient pathways. Steroids 2002;67:967–77.

[4] Akwa Y, Morfin RF, Robel P, Baulieu EE. Neurosteroid metabolism: 7alpha-hydroxylation of dehydroepiandrosterone and pregnenolone by rat brain microsomes. Biochem J 1992;288:959–64.

[5] Khalil MW, Strutt B, Vachon D, Killinger DW. Metabolism of dehydroepiandrosterone by cultured human adipose stromal cells: identitication of 7alpha-hydroxydehydroepiandrosterone as a major metabolite using high performance liquid chromatography and mass spectrometry. J Sreroid Biochem Mol Biol 1993;46:585–95.

[6] Zhang J, Larsson O. 7alpha-Hydroxylation of 25-hydroxycholesterol and 27-hydroxycholesterol in human fibroblasts. Biochim Biophys Acta 1995;1256:353–9.

[7] Paync DW, Shackleton C, Toms H, Ben-Shlomo I, Kol S, deMoura M, et al. A novel nonhepatic hydroxycholesterol 7alpha-hydroxylase that is markedly stimulated by interleukin-1beta. Characterization in the immature rat ovary. J Biol Chem 1995;270:18888–96.

[8] Doostzadh J, Morfin R. Studies of the enzyme complex responsible for pregnenolonc and dehydrooepiandrosterone 7alpha-hydroxylation in mouse tissues. Steroids 1996;61:613–20.

[9] Doostzadh J, Morfin R. Effects of cytochrome P450 inhibitors and of steroid hormones on the formation of 7-hydroxylated metabolites of pregnenolone in mouse brain microsomes. J Endocrinol 1997;155:343–50.

[10] Doostzadh J, Cotillon AC, Benalycherif A, Morfin R. Inhibition studies of dehydroepiandrosterone 7alpha-and 7beta-hydroxylation in mouse liver microsomes. Steroids 1998;63:608–14.

[11] Sonderfan AJ, Arlotto MP, Parkinson A. Identification of the cytochrome P-450 isozymes responsible for testosterone oxidation in rat lung. Kidney, and testis: evidence that cytochrome P-450a (P450 II Al) is the physiologically important testosterone 7alpha-hydroxylase in rat testis. Endocrinology 1989;125:857–66.

[12] Daynes RA, Dudley DJ, Araneo BA. Regulation of murine lymphokine production in vivo. II. Dehydroepiandrosterone is ma natural enhancer of in terleukin 2 synthesis by helper T cells. Eur J Immunol 1990;20:793–802.

[13] Danenberg HD, Alpert G, Lustig S, Ben-Nathan D. Dehydroepiandrosterone protects mice from endotoxin and reduces tumor necrosis factor production. Antimicrob Agents Chemother 1992;36:2275–9.

[14] Danenberg HD, Ben-Yehuda A, Zakay-Rones Zakay-Rones Z, Friedman G. Dehydroepiandrosterone (DHEA) treatment reverses the impaired immune response of old mice to influenza vaccination and protects from influenza infection. Vaccine 1995;13:1445–8.

[15] Padgctt DA, Loria RM. Endocrine regulation of murine macrophage function: effects of dehydroepiandrosterone, androstenediol, and androstenetriol. J Neuroimmunol 1998;84:61–8.

[16] Padgett DA, Loria RM, Sheridan JF. Endocrine regulation of the immune response to influenza virus infection with a metabolite of DHEA-androstenediol. J Neuroimmunol 1997;78:203–11.

[17] Solerte SB, Fioravanti M, Vignati G, Giustina A, Cravello L, Ferrari E. Dehydroepiandrosterone sulfate enhances natural killer cell cytotoxicity in humans via locally generated immunoreactive insulin-like growth factor I. J Clin Endocrinol Metab 1999;84:3260–7.

[18] Rose KA, Stapleton G, Dott K, Kieny MP, Best R, Schwarz M, et al. Cyp7b, a novel brain cytochrome P450, catalyzes the synthesis of neurosteroids 7alpha-hydroxy dehydroepiandrosterone and 7alpha-hydroxy pregnenolone. Proc Natl Sci USA 1997;94:4925–30.

[19] Martin KO, Reiss AB, Lathe R, Javitt NB. 7alpha-Hydroxylation of 27-hydroxycholesterol: biologic role in the regulation of cholesterol synthesis 7alpha-hydroxylation of 27-hydroxycholesterol: biologic role in the regulation of cholesterol synthesis. J Lipid Res 1997;38:1053–8.

[20] Rose K, Allan A, Gauldie S, Stapleton G, Dobbie L, Dott K, et al. Neurosteroid hydroxylase CYP7B: vivid reporter activity in dentate gyrus of gene-targeted mice and abolition of a widespread pathway of steroid and oxysterol hydroxylation. J Biol Chem 2001;276:23937–44.

[21] Lathe Richard. Steroid and sterol 7-hydroxylation: ancient pathways. Steroids 2002;67:967–77.

[22] Morfin R, Courchay G. Pregnenolone and dehydroepiandrosterone as precursors of native 7-hydroxylated metabolites which increase the immune response in mice. J Steroids Biochem Mol Biol 1994;50:91–100.

[23] Lafaye P, chmielewski V, Nato F, Mazie JC, Morfin R. The 7alpha-hydroxysteroids produced in human tonsils enhance the immune response to tetanus toxoid and *Bordetella pertussis antigens*. Biochim Biophys Acta 1999;1472:222–31.

[24] Schumacher M, Weill-Engerer S, Liere P, Robert F, Franklin RJM, Garcia-Segura LM, et al. Steroids hormones and neurosteroids in normal and pathological aging of the nervous system. Neurobiology 2003;71:3–29.

[25] Szczesna-Antczak M, Antczak T, Rzyska Malgorzata, Bielecki S. Catalytic properties of membrane-bound Mucor lipase immobilized in a hydrophilic carrier. J Mol Catal B: Enzymatic 2002;19–20:261–8.

[26] Micovic IV, Ivanovic MD, Piatak DM. Simplified preparation of 16-dehydropregnenolone acetate. Synthesis 1990;7:591–2.

[27] XueKong Qin, Nan Li. The progress of natural hormone DHEA. Chin Tradi Herb Drugs 2000;31:871–3.

[28] Crabb Trevor A, Dawson Philip J, Williams Roger O. Microbiological transformations. Part 3. [1,2] The oxidation of androstetene derivatives with the fungus *Cunninghamella Elegans*. J C S Perkin 1980;11:2535–41.