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Microbial hydroxylation of dydrogesterone by *Rhizopus stolonifer*

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Abstract

Microbial hydroxylation of dydrogesterone (9β,10α-pregna-4,6-diene-3,20-dione) (**1**) on fermentation with *Rhizopus stolonifer* afforded a new metabolite, 9β,12β-dihydroxy-9β,10α-pregna-4,6-diene-3,20-dione (**2**). Its structure was deduced on the basis of modern spectroscopic techniques.

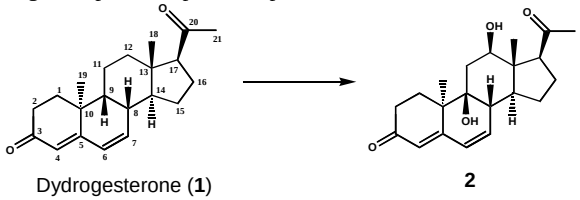
Keywords: Microbial hydroxylation; dydrogesterone; *Rhizopus stolonifer*

Introduction

Microbial transformation is one of the important tools for structural changes. The use of microorganisms for industrial processes is not new, although it has assumed renewed emphasis in recent years. Centuries ago, people in Asia and Africa learned to make wine, beer and vinegar with bacteria and yeast, without knowing the scientific basis of such productions. The technology related to microbial production of ethanol, lactic acid, butanol, riboflavin, etc. and enzymes such as protease, amylase and invertase was also developed as early as first few decades of the 20th century. Large scale production of the antibiotic penicillin, was perfected during World War II and the production of many other antibiotics, amino acids, nucleotides, enzymes, etc. had been successfully accomplished in the 1950's and later.

Dydrogesterone (**1**) is a synthetic hormone, similar to the naturally occurring sex hormone, progesterone (Choudhary et al. 2008). It is used to treat premenstrual syndrome, unusually heavy or long period in woman, period pains, irregular periods, infertility and endometriosis. It is also useful in helping prevent miscarriage in women who have suffered repeated miscarriages or who may miscarry.

In continuation of our studies on microbial transformation of bioactive natural and synthetic compounds (Choudhary et al. 2008; Choudhary et al. 2002; Azizuddin et al. 2008; Azizuddin et al. 2010), we have now investigated the fermentation of dydrogesterone (**1**) with *Rhizopus stolonifer* (ATCC 10404) yielded the dihydroxylated metabolite **2**. Structure of metabolite **2** was deduced through comparative spectroscopic studies with the substrate **1**.



Scheme 1. Microbial hydroxylation of dydrogesterone (**1**) by *R. stolonifer*.

Materials and Methods

General methods

The ^{1}H-NMR spectrum was recorded in $CDCl_3$ on Bruker AM-400 NMR spectrometer with TMS as an internal standard using UNIX operating system at 400 MHz. The ^{13}C-NMR spectra were recorded in $CDCl_3$ at 100 MHz on Bruker AM-400 NMR spectrometer. The HREIMS was recorded on Jeol JMS 600 and HX 110 mass spectrometers with the data system DA 5000. The IR spectra were recorded on a Jasco A-302 spectrophotometer. The UV spectra were recorded on a Hitachi U-3200 spectrophotometer. The optical rotations were measured on JASCO DIP-360 digital polarimeter. The melting point was determined on a Buchi 510 apparatus. Column chromatography (CC) was carried on silica gel column (70-230 Mesh). Purity of the samples was checked by TLC on pre-coated silica gel GF-254 preparative plates (20 × 20 cm, 0.25 mm thick, Merck) and were detected under the UV light (254 and 366 nm), while ceric sulphate was used as spraying reagent. Dydrogesterone (**1**) was purified from the tablets of a medicinal product, duphaston® (10 mg each, manufactured by Solvay Pharmaceuticals).

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Preparation of fermentation media

Three liter media for *Rhizopus stolonifer* (ATCC 10404) was prepared by mixing glucose (60 g), peptone (15 g), KH_2PO_4 (15 g), and yeast extract (9 g) into distilled water (3 L). The pH was maintained to 5.6 by adding a few drops of 0.04N aqueous NaOH solution. The fermentation medium thus obtained was distributed equally among 30 flasks of 250 mL capacity (100 mL in each) and autoclaved.

Cultivation of the microbes

Two-day-old spores of the microbe were transferred into the broth medium flasks (250 mL) of their respective media containing freshly prepared and autoclaved media (100 mL). The seed flasks of the fungi were incubated on a shake table at 30° C for two days.

Inoculation of the cultures

Broth culture (100 mL) from 2-day-old seed flasks of the fungi were equally distributed to 27 media flasks (250 mL) containing the respective media (100 mL). The incubation was continued for a further 2 days.

*Fermentation of dydrogesterone (**1**)*

Dydrogesterone (**1**) (300 mg) dissolved in acetone (20 mL) and the resulting solution was evenly distributed among 28 conical flasks containing shake cultures of microbes, the fermentation was continued for 12 days.

Extraction and isolation

The culture media was filtered, mycelium was washed with EtOAc (300 mL) and the broth thus obtained was extracted with EtOAc (4 L). The ethyl extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to afford a brown gummy material (approximately, 1.12 g), analyzed by thin layer chromatography (TLC). A negative control flask containing fungi (for checking endogenous metabolites) and positive control flask containing compound **1** (for checking substrate stability) were also prepared in order to check the chemical changes due to media and for the presence of fungal metabolites. Control flask was also harvested and compared with the test sample by TLC to confirm the presence of bio-transformed products. The resulting brown gum was subjected to repeated column chromatography (silica gel) using the combination of petroleum ether and EtOAcwith increasing polarity. This yielded metabolite **2** (12.4 mg, petroleum ether: EtOAc, 5.2:4.8).

*9β,12β-Dihydroxy-9β,10α-pregna-4,6-diene-3,20-dione (**2**)*

Colorless crystalline solid; m.p. 190-192 °C; $[\alpha]_D^{25}$ 144.4° (*c* 0.018, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 284 nm (2.85); IR ($CHCl_3$) ν_{max} : 3411 (OH), 1718 (C=O), 1659, 1618 (C=C), 1234 cm^{-1} (C-O); EIMS *m/z* (rel. int. %): 344 [M^+] (21), 326 [M^+-H_2O] (07), 284 (07), 266 (06), 227 (10), 197 (6), 173 (11); HREIMS *m/z* 344.0226 (M^+, calcd 344.0136 for $C_{21}H_{28}O_4$); ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data listed in table 1.

Results and Discussion

In the present study, microbial transformation of dydrogesterone (**1**) by using *Rhizopus stolonifer* after 12 days yielded compound **2** (Scheme-1). Its structure was found to be 9*β*,12*β*-dihydroxy-9*β*,10*α*-pregna-4,6-diene-3,20-dione (**2**), which was identified on the basis of modern spectroscopic methods as a new compound. We were also interested to examine the biological activities of dydrogesterone (**1**) and its transformed product **2,** but it was difficult due to insufficient its quantity.

Table 1: ^{1}H-NMR (500 MHz, $CDCl_3$)[a] and ^{13}C-NMR (100 MHz, $CDCl_3$)[b][c] chemical shifts of compound **1** and new metabolite **2**. δ in ppm and J in Hz.

C. No	1		2	
	^{1}H	^{13}C	^{1}H	^{13}C
1	2.41-2.43 (m)[d]) 2.53-2.56 (m)[e])	34.0 *(t)*	2.38-2.40 (m) 2.53-2.56 (m)[d])	28.2 *(t)*
2	2.25-2.27 (m) 1.65-1.68 (m)	35.6 *(t)*	2.52-2.55 (m)[d]) 1.82-1.86 (m)	33.7 *(t)*
3	-	199.3 *(s)*	-	199.4 *(s)*
4	5.67 (br.s)	123.9 *(d)*	5.83 (s)	127.1 *(d)*
5	-	162.9 *(s)*	-	159.6 *(s)*
6	6.15 (d, *J* = 9.8)	127.1 *(d)*	6.23 (d, *J* = 9.7)	127.2 *(d)*
7	6.15 (dd, *J* = 9.8, 5.0)	140.3 *(d)*	6.03 (dd, *J* = 9.7, 4.6)	135.9 *(d)*
8	2.39-2.42 (m)[d])	38.6 *(d)*	2.62-2.64 (m)	48.4 *(d)*
9	1.82-1.85 (m)[f])	39.7 *(d)*	-	73.5 *(s)*
10	-	37.2 *(s)*	-	42.9 *(s)*
11	1.96-1.99 (m) 1.80-1.85 (m)[f])	20.6 *(t)*	2.34-2.36 (m) 2.12-2.15 (m)	37.1 *(t)*
12	1.93-1.96 (m) 1.61-1.64 (m)	37.8 *(t)*	4.65 (dd, *J* = 11.0, 7.0)	72.4 *(d)*
13	-	44.2 *(s)*	-	49.1 *(s)*
14α	1.75-1.78 (m)[g])	49.9 *(d)*	1.98-2.00 (m)	48.3 *(d)*
15	1.68-1.70 (m) 1.78-1.82 (m)	22.6 *(t)*	1.80-1.82 (m) 1.64-1.66 (m)[e])	21.6 *(t)*
16	1.75-1.77 (m)[g]) 1.88-1.90 (m)	25.2 *(t)*	1.72-1.75 (m) 1.63-1.66 (m)[e])	29.5 *(t)*
17α	2.52-2.54 (m)[e])	63.4 *(d)*	2.28-2.30 (m)	65.4 *(d)*
18	0.69 (s)	12.1 *(q)*	1.15 (s)	13.8 *(q)*
19	1.29 (s)	22.3 *(q)*	1.34 (s)	22.2 *(q)*
20	-	208.8 *(s)*	-	211.8 *(s)*
21	2.12 (s)	31.4 *(q)*	2.21 (s)	32.2 *(q)*

[a]) Assignments based on COSY and HMQC.
[b]) Multiplicities were determined by DEPT experiments.
[c]) Assignment based on HMQC and HMBC.
[d,e,f,g]) Signals may be interchanged.

Compound **2** was found to be a dihydroxylated product of substrate **1**. The IR spectrum ($CHCl_3$) showed hydroxyls absorption at 3411 cm^{-1}. The spectrum also showed ketonic and olefinic absorptions at 1718 and 1659 cm^{-1}, respectively. The HR-EI-MS of metabolite **2** showed the M^+ at *m/z* 344.0226, corresponding to the formula $C_{21}H_{28}O_4$ (calcd 344.0136), indicating an addition of two oxygen functions in substrate **1**. The ^{1}H- and ^{13}C-NMR spectra (Table-1) of compound **2** showed the additional methine proton signal at δ_H 4.65 (dd, *J* = 7.0, 11.0 Hz, H-12*α*), which indicating the presence of an OH group at δ 72.4 (C-12) in HMQC. The C-18 methyl and C-8 methine protons were seem to experience a downfield shift to δ_H 1.15 and 2.62, respectively, as compared to the substrate **1** due to the deshielding effect of the neighboring hydroxyl groups. The ^{13}C-NMR spectrum also showed an additional quaternary carbon signal at δ 73.5 (C-9), indicating the presence of OH group (Choudhary et al. 2008). Hydroxylation at a quaternary C-9 was also supported by the downfield shifts of adjacent methine and methylene carbons at δ 48.4 (C-8), and 42.9 (C-10), respectively. Both hydroxylation was also confirmed by the downfield shift of central methylene carbon at δ 37.1 (C-11). These observations were further confirmed through the HMBC spectrum, in which the C-8 proton (δ_H 2.62) showed ^2J correlations with C-9 (δ_C 73.5) and C-7 (δ_C 135.9) (Fig. 1). The C-19 methyl protons (δ_H 1.34) showed ^2J correlation with C-10 (δ_C 42.9) and ^3J correlation with C-9 (δ_C 73.5). The C-12 proton (δ_H 4.65) showed ^2J correlations with C-11 (δ_C 37.1) and C-13 (δ_C 49.1), and ^3J correlation with C-14 (δ_C 48.3). The C-18 methyl protons (δ_H 1.15) showed ^2J correlation with C-13 (δ_C 49.1) and ^3J correlation with C-12 (δ_C 72.4).

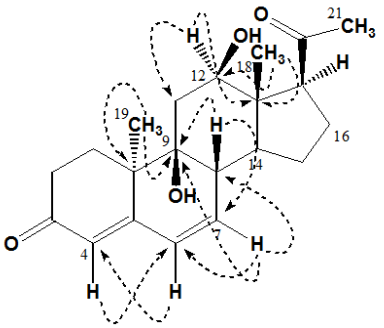

Figure 1: Key HMBC correlations for compound 2

The β-stereochemistry of C-12 hydroxyl group was deduced on the basis of NOESY interaction of H-12 with α-oriented C-19 methyl protons (Fig. 2). These spectral observations supported the structure as 9*β*,12*β*-dihydroxy-9*β*,10*α*-pregna-4,6-diene-3,20-dione for compound **2**.

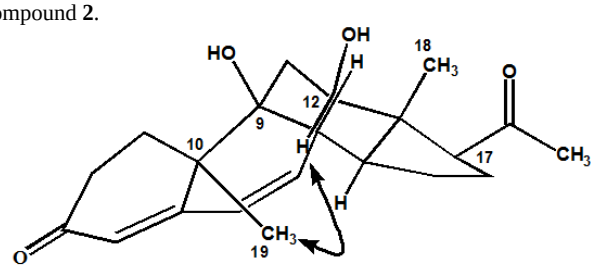

Figure 2: Key NOESY interaction for compound 2

Acknowledgements

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