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Biotransformation of Dydrogesterone by Cell Suspension Cultures of *Azadirachta indica*

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Biotransformation of dydrogesterone (**1**) by using cell suspension cultures of *Azadirachta indica* yielded a metabolite 20*R*-hydroxy-9β,10α-pregna-4,6-diene-3-one (**2**). The structure of this compound was deduced on the basis of various spectroscopic techniques.

Key Words: Biotransformation, dydrogesterone, cell suspension cultures, *Azadirachta indica*.

Introduction

Plant cell suspension cultures exhibit a vast biochemical potential for the production of specific secondary metabolites. They can serve as tools for the in vivo production of secondary metabolites[1,2] as well as for the biotransformation of foreign substrates.[3–5] These cultures are considered useful biocatalysts for reactions such as hydroxylation at allylic positions, oxidation–reduction between alcohols and ketones, and the reduction of carbon–carbon double bonds.[6,7] Plant cell culture mediated biotransformations are now increasingly employed by synthetic chemists for the structural modifications of various organic compounds. In continuation of our studies on the biotransformation of bioactive compounds,[8,9] dydrogesterone (**1**) was incubated with *Azadirachta indica* cell suspension cultures, which are employed for the first time for structural modifications of dydrogesterone (**1**). This afforded a metabolite, **2**, resulting from the reduction of the ketonic group, which was reported for the first time in this way. The structure of this compound was deduced by various spectroscopic methods.

Dydrogesterone (**1**) is a synthetic hormone, similar to the naturally occurring sex hormone progesterone. It is a common medicine, used to treat premenstrual syndrome, infertility, and endometriosis, which are due to the deficiency of progesterone in women.

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1. Experimental

General Methods: The ^{1}H-NMR spectrum was recorded in $CDCl_3$ on a Bruker AM-400 NMR spectrometer with TMS as an internal standard using the UNIX operating system at 400 MHz. The ^{13}C-NMR spectra were recorded in $CDCl_3$ at 100 MHz on a 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 a JASCO DIP-360 digital polarimeter. The melting point was determined on a Buchi 510 apparatus. Column chromatography (CC) was carried out on a 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 UV light (254 and 366 nm), while ceric sulfate was used as the spraying reagent. Dydrogesterone (**1**) was purified from the tablets Duphaston® (Solvay Pharmaceuticals).

Callus Culture: The callus cultures of the plant were derived from young leaves cultivated in 300 mL jars, each having 25 mL of Murashige and Skoog media,[10] supplemented with sucrose (30 g/L), 3-indole butyricacid (IBA) (4 mg/L), and 6-benzyl aminopurine (BA) (1 mg/L), and solidified by agar (6 g/L) at 25 ± 1 °C in complete darkness.

Biotransformation Protocol: Cell suspension cultures were derived from static cultured calli in Erlenmeyer flasks (1000 mL), each containing 400 mL of the Murashige and Skoog media, supplemented with ingredients as mentioned above, except BA and agar. After 15 days of preculturing on a gyratory platform shaker at 100 rpm and with a 16 h photoperiod at 25 ± 1 °C, a solution of substrate (100 mg in 1 mL of acetone) was added to each flask through a 0.2 μM membrane filter and the flasks were placed on a shaker for 20 days. The time course study was carried out by taking aliquots from the culture on a daily basis and the content of transformation was analyzed by TLC. A negative control containing only plant cell suspension cultures and a positive control containing compound **1** in the media were also prepared in order to check for the presence of plant metabolites in the cell culture and chemical changes as a result of a chemical reaction (if any) due to media components, respectively.

Extraction and Isolation Procedure: After 20 days of incubation, the cells and the media were separated by filtration. The filtrate was extracted with CH_2Cl_2 (3 × 1.5 L) and the cells were extracted in an ultrasonic bath with CH_2Cl_2 (3 × 500 mL) at room temperature. The combined extract was dried over anhydrous Na_2SO_4, and concentrated under reduced pressure, which afforded a brown residue (1.32 g). The transformed metabolites were isolated from this gummy crude by repeated column chromatography (silica gel) with petroleum ether/EtOAc gradient, which afforded metabolites 2 (58.6 mg, petroleum ether:EtOAc, 7.2:2.8, 14.6% yield).

Determination of Stereochemistry by Horeau's Method: A mixture of 20*R*-hydroxy-9β,10α-pregna-4,6-diene-3-one (**2**) (6.1 mg), C_5H_5N (0.5 mL), and racemic 2-phenylbutanoic anhydride (0.1 mL) was stirred overnight at room temperature. After the completion of the reaction (TLC analysis), distilled water (5.0 mL) was added and the reaction mixture was allowed to stand for 30 min. Then, 0.01 M NaOH was added dropwise until the pH became 9 and the solution was then extracted with $CHCl_3$. The remaining aqueous layer was acidified to pH 3 using 1 M HCl. The acidic layer was then extracted with benzene (10 mL). The benzene extract was evaporated to adjust the volume to 1.0 mL. This work-up process afforded 2-phenylbutanoic acid (after hydrolysis of the left-over anhydride), which showed positive optical rotation, thereby establishing the "*R*" configuration at C-20 in compound **2**.

Results

20*R*-Hydroxy-9β,10α-pregna-4,6-diene-3-one (2): Colorless solid, mp 165-166 °C, $[\alpha]_D^{20}$ –118.7° (*c* 0.032, $CHCl_3$). UV (MeOH) λ_{max} (log ε): 286 nm (4.99). IR ($CHCl_3$) ν_{max}: 3408 (OH), 1725 (C=O), 1659, 1620 (C=C), 1386 cm^{-1} (C-O). EIMS*m/z* (rel. int. %): 314 [M^+] (8), 296 [M^+-H_2O] (100), 281 [M^+-H_2O-CH_3] (56), 267 (21), 227 (18), 201 (7), 161 (44). HREIMS *m/z*: 314.2358 (M^+, $C_{21}H_{30}O_2$; calcd 314.2352). ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) data listed in Tables 1 and 2, respectively.

Table 1. ^{1}H-NMR (400 MHz, $CDCl_3$)[a]) chemical shifts of compound **1** and its metabolite **2**. δ in ppm and *J* in Hz.

C. NO.	1	2
1	2.42 (m)[b])	2.44 (m)
	2.55 (m)	2.56 (m)
2	2.26 (m)	2.26 (m)
	1.67 (m)	1.68 (m)[b])
3	-	-
4	5.67 (br.s)	5.65 (br. s)
5	-	-
6	6.15 (d, J = 9.8)	6.15 (d, J = 9.8)
7	6.15 (d, J = 9.8)	6.16 (d, J = 9.8)
8β	2.40 (m)[b])	2.40 (m)
9β	1.84 (m)[c])	1.81 (m)
10	-	-
11	1.98 (m)	1.94 (m)
	1.83 (m)[c])	1.81 (m)
12	1.95 (m)	1.78 (m)[c])
	1.62 (m)	1.36 (m)
13	-	-
14α	1.76 (m)	1.64 (m)
15	1.69 (m)	1.67 (m)[b])
	1.80 (m)	1.79 (m)[c])
16	1.76 (m)	1.60 (m)
	1.89 (m)	1.75 (m)
17	2.53 (m)	1.35 (m)
18	0.69 (s)	0.74 (s)
19	1.29 (s)	1.25 (s)
20	-	3.73 (quintet, J= 6.2)
21	2.12 (s)	1.22 (d, J = 6.3)

[a]Assignments based on COSY and HMQC.

[b,c]Signals may be interchanged.

Table 2. ^{13}C-NMR (100 MHz, $CDCl_3$)[a])[b]) chemical shifts of compound **1** and its metabolite **2**.

C. NO.	**1**	**2**
1	34.0 (*t*)	34.0 (*t*)
2	35.6 (*t*)	35.6 (*t*)
3	199.3 (*s*)	199.5 (*s*)
4	123.9 (*d*)	123.7 (*d*)
5	162.9 (*s*)	163.3 (*s*)
6	127.1 (*d*)	126.9 (*d*)
7	140.3 (*d*)	141.0 (*d*)
8	38.6 (*d*)	38.4 (*d*)
9	39.7 (*d*)	39.8 (*d*)
10	37.2 (*s*)	37.2 (*s*)
11	20.6 (*t*)	20.3 (*t*)
12	37.8 (*t*)	37.7 (*t*)
13	44.2 (*s*)	42.0 (*s*)
14	49.9 (*d*)	49.5 (*d*)
15	22.6 (*t*)	24.9 (*t*)
16	25.2 (*t*)	25.2 (*t*)
17	63.4 (*d*)	58.3 (*d*)
18	12.1 (*q*)	11.3 (*q*)
19	22.3 (*q*)	22.2 (*q*)
20	208.8 (*s*)	70.0 (*d*)
21	31.4 (*q*)	23.6 (*q*)

[a]Multiplicities were determined by DEPT experiments.

[b]Assignment based on HMQC and HMBC.

Discussion

Biotransformation of dydrogesterone (**1**) by cell suspension cultures of *Azadirachta indica* yielded compound **2**.

Compound **2** was obtained as a colorless solid. The molecular formula was deduced from the HREIMS as $C_{21}H_{30}O_2$, corresponding to the M^+ at m/z 314.2358 (Calcd 314.2352) and consistent with 7 degrees of unsaturation. The IR spectrum of compound **2** showed hydroxyl absorption at 3408 cm^{-1}, indicating the reduction of the C-20 ketonic group in the compound. The spectrum also showed ketonic and olefinic absorptions at 1725 and 1620 cm^{-1}, respectively. The ^{1}H-NMR spectrum of **2** also indicated the reduction of the C-20 ketonic carbonyl group by the presence of an additional oxymethine proton at δ_H 3.73 (quintet, J = 6.2 Hz, H-20). The ^{1}H-NMR spectrum of **2** closely resembled that of substrate **1**. The 2 main differences were that the C-17 methine proton was shifted upfield at δ_H 1.35 (m) due to reduction of carbonyl group, and the C-21 methyl protons resonated upfield as a doublet at δ_H 1.22 (J = 6.3 Hz) due to the same reason. The ^{13}C-NMR spectra also indicated the reduction of the C-20 carbonyl group by the lack of the C-20 ketonic carbon signal and the presence of an upfield methine carbon signal at δ_C70.0 (C-20). The observed upfield

shifts in C-21 methyl carbon at δ_C 23.6 and the C-17 methine carbon at δ_C 58.3 were due to the reduction of a C-20 carbonyl group. In the HMBC spectrum, the C-20 proton (δ_H 3.73) showed ^2J correlations with C-17 (δ_C 58.3) and C-21 (δ_C 23.6) and ^3J correlations with C-13 (δ_C 42.0). Similarly, the C-21 methyl protons (δ_H 1.22) showed HMBC correlations with C-17 (δ_C 58.3) and C-20 (δ_C 70.0). The COSY-45 $^\circ$ spectrum showed correlations between the 2 vicinal protons at δ_H 1.35 (H-17α) and 3.73 (H-20). The latter proton was further coupled with the C-21 methyl protons. The *R*-configuration at C-20 was deduced by Horeau's method.[11]

Compound **2** was reacted with racemic 2-phenyl-butanoic anhydride and the unreacted (*S*)-2-phenyl-butanoic acid was recovered from the water layer after hydrolysis of the left-over anhydride. This indicated that only the '*R*' form of the racemic 2-phenyl-butanoic anhydride was consumed in the ester formation. Compound **2** was identified as 20*R*-hydroxy-9β,10α-pregna-4,6-diene-3-one by spectral comparison with the literature values. This compound was previously isolated during several metabolic studies on dydrogesterone (**1**).[12,13]

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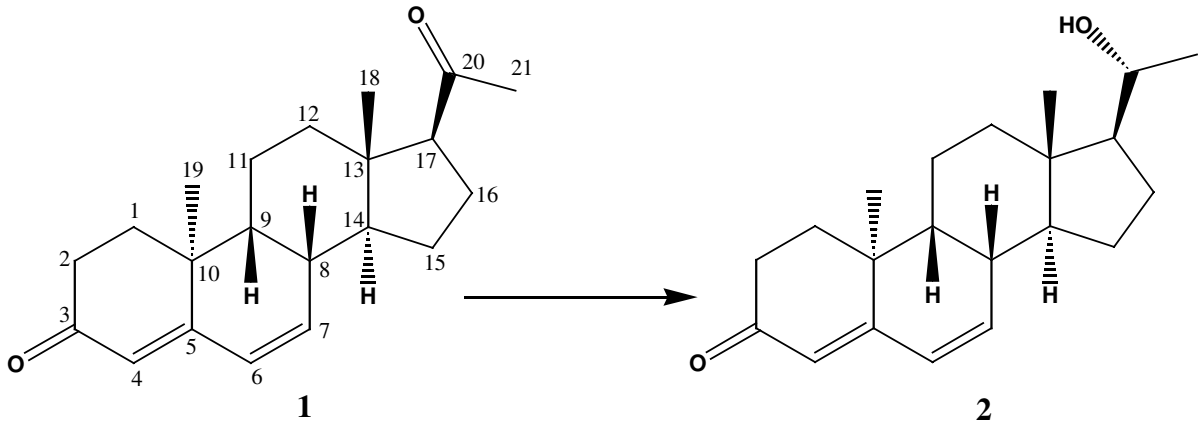

Scheme. Biotransformation of dydrogesterone (1) by cell suspension cultures of Azadirachta indica.

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