Bio-transformation of FXR antagonist CDRI 80/574[1]

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Abstract

The bio-transformation of 3-β-hydroxy-5, 16-dien-pregnane-20-one (CDRI 80/574, **1**) a novel antagonist of farnesoid X receptor (FXR) on hepatic membranes, with *Aspergillus niger* produced the oxidation products at C_3 and / or C_{11} (compound **2** and **3**) while the *Aspergillus ochraeus* promoted the oxidation at C_{11} and / or C_{15} (compound **4** and **5**). The products were characterized using 2D-NMR spectroscopy.

Keywords: 3β-Hydroxy-5, 16-dien-pregnane-20-one, bio-transformation, FXR receptor, *Aspergillus niger*

Introduction

Bile consists of bile acids (BAs), cholesterol, phosphatidylcholine, and bilirubin, and is secreted from the hepatocytes into the bile canaliculi. The amphipathic properties of bile acids promote the solubilization and subsequent absorption of dietary lipids in the digestive tract.[1] Besides their well-established roles in dietary lipid absorption and cholesterol homeostasis, BAs also act as signaling molecules with systemic endocrine functions. BAs activate mitogen-activated protein kinase (MAPK) pathways[2], nuclear hormone receptors such as farnesoid X receptor-α[3] (FXR) and are also ligands for the G-protein-coupled receptor TGR5[4]. Through activation of these diverse signaling pathways, BAs can regulate their own enterohepatic circulation and also triglyceride, cholesterol, energy, and glucose homeostasis. A major physiological role of FXR is to protect liver cells from the deleterious effects of BA overload by decreasing the endogenous production of BAs and by accelerating BA bio-transformation and excretion, thereby acting as an

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intracellular BA sensor.[5] 3β-Hydroxy-5, 16-dien-pregnane-20-one (CDRI/80-574), a close analogue of Guggulsterone has been demonstrated to be a novel FXR antagonist being devoid of the pharmacophoric groups of cholic, deoxycholic or chenodeoxycholic acids. The molecule has exhibited significant triglycerides, cholesterol, phospholipids and LDL cholesterol lowering activities in various animal models[6]. Guggulsterone and CDRI/80-574 are the only compounds of pregnane class which lower lipid profile through antagonism of FXR receptor and have generated interest world over[7]. CDRI/80-574 has successfully completed phase III human trial.
In the process of drug development, there comes a stage of assessing the oral bio-availability of the candidate drug after confirmation of its biological efficacy through screening in various animal models, to modulate its safe dose for optimum activity. Oral bio-availability of a drug candidate can be measured either through the analysis of parent molecule or its metabolites. The metabolites of the candidate drug are also required to establish its efficacy and safety. The bio-transformation of a drug is considered as a detoxification reaction, leading to the formation of more polar substances which are more easily eliminated from the biological system. However in some cases, this metabolism can lead to activation, producing either pharmacologically more active substances or toxic reactive metabolites.

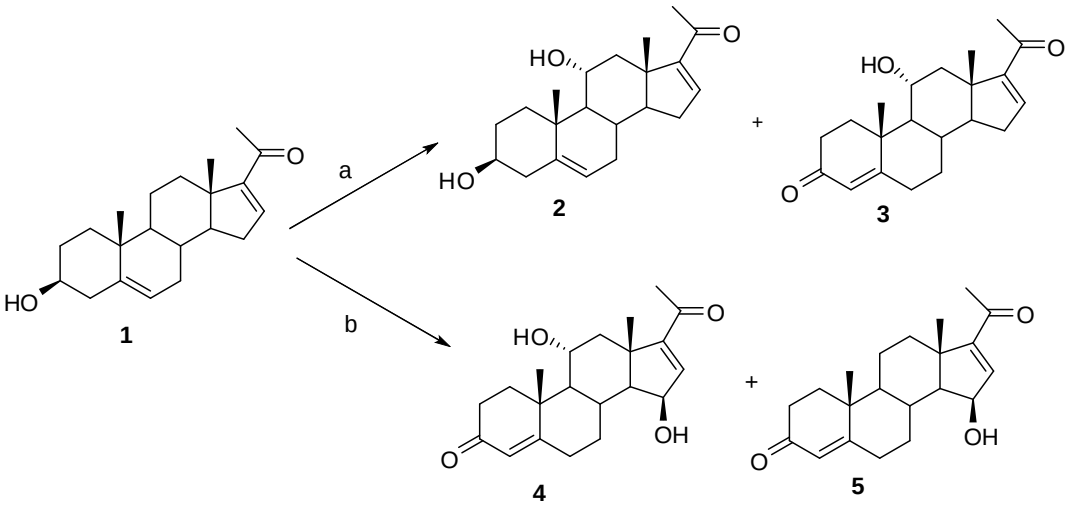
One way of finding the drug in plasma is through radio-tracer tagged onto the intact molecule or through the identification of possible metabolites by comparison with the standard samples. These possible standard metabolites are to be prepared either *via* microbial route or chemical synthesis. The oxidative reactions in animals involve various types of reactions essentially catalyzed by monoamine oxidases and flavine or cytochrome P-450 mono-oxygenases. The microbial hydroxylation of steroids and subsequently preparation of corticosteroids on industrial scale demonstrated that mono-oxygenase enzymes were present in microorganisms and proved to be mechanistically similar to mammalian hepatic mono-oxygenases. The systematic examination of microbial hydroxylation on a variety of model aromatic compounds and O- and N-dealkylation reactions, led Smith and Rosazza[8] to propose that the microbial transformation system could closely mimic most of transformation reactions of drugs observed in mammals. This therefore led us to study microbial bio-transformation of 3β-hydroxy-5, 16-dien-pregnane-20-one (CDRI/80-574, **1**) and report here the isolation and characterization of the bio-transformation products **2-5** by two fungal strains of *Aspergillus* viz. *Aspergillus ochraeus* and *Aspergillus niger* (Figure 1).

Results and Discussion

The two fungal species *Aspergillus ochraeus* and *Aspergillus niger*, were utilized for regioselective oxidation of 3β-hydroxy-5, 16-dien-pregnane-20-one (**1**). The C_{11} hydroxylation was found to be the most prevalent with both the organisms. Double bond at C_{16} seems to play a decisive role in bringing above regioselectivity. In pregnenolone[9] where C_{16} double is absent, no

hydroxylation products at C_{11} were obtained while with progesterone[10] hydroxylation occurred at C_{12} position instead of C_{11} on incubation with fungal strains.

The metabolites **2-5** were characterized using FT-IR, mass and ^{1}H, ^{13}C & 2D-NMR spectral analysis.



a=*Aspergillus ochraeus*, *b*= *Aspergillus niger*

Figure 1. Bio-transformations of the compound 80/574, **1**.

3β-Hydroxy-5, 16-dien-pregnane-20-one (CDRI/80-574, 1)

16-Dehydro-pregnenolone obtained from diosgenin is commonly utilized for the synthesis of steroidal hormones. Its spectral characteristics are well established.[11] In order to ascertain the structure of the metabolites, it was required to assign the chemical shifts of the parent molecule **1** itself by 1D and 2D NMR spectroscopy. In ^{1}H NMR, the two olefinic protons appeared at $C_{6,}$ C_{16} and one methine proton appeared distinctly at C_3 along with three methyl signals of C_{18}, C_{19} and C_{21}. The proton at C_3 appeared as multiplet at δ 3.52. The two olefinic protons at C_6 and C_{16} appear at δ 5.35 and 6.71 respectively. The most deshielded proton at δ 6.71 corresponds to C_{16}. In DEPT-90, the six methine carbons appeared at δ 144.5 (C_{16}), 121 (C_6), 71.7 (C_3), 56.4 (C_{14}), 50.4 (C_9) and 30.2 (C_8) while seven methylene carbons appear at δ 42.2 (C_4), 37.1(C_1), 34.6 (C_{12}), 31.5 (C_2 & C_7) and 20.7 (C_{11}). The chemical shifts of various protons and carbons were established using COSY, DEPT, HSQC and HMBC experiments and are given in tables 1 and 2.

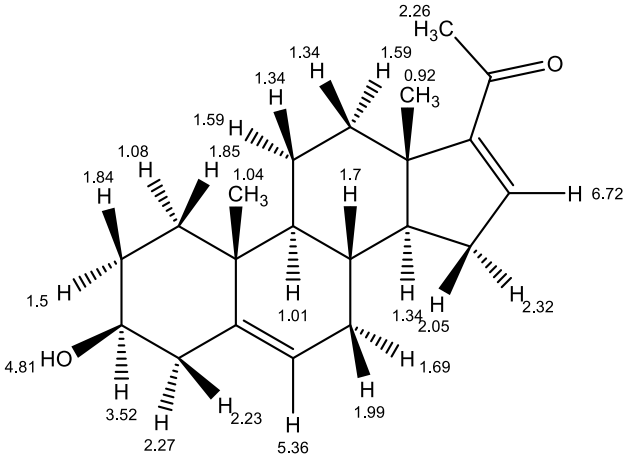
Table 1. ^{1}H-NMR chemical shifts of compound CDRI 80/574 **1** and metabolites **2-5**

^{1}H	**1**		**2**		**3**		**4**		**5**	
	δ	J (Hz)	δ	J (Hz)	δ	J (Hz)	δ	J (Hz)	δ	J (Hz)
1	1.08	m^{e}	2.54	td (3.5, 13.6)	2.59	td (4.5, 14.0)	2.52	m^{e}	1.7	m^{e}
1’	1.85	m^{e}	1.19	m^{e}	2.09	m^{e}	2.09	m^{e}	2.07	m^{e}
2	1.84	m^{e}	1.82	m^{e}	2.43	m^{e}	2.44	m^{e}	2.49	m^{e}
2’	1.5	m^{e}	1.54	m^{e}	2.34	m^{e}	2.27	m^{e}	2.29	m^{e}
3	3.52	m^{e}	3.53	m^{e}	-		-	-	-	-
4	2.27	m^{e}	2.29	m^{e}	5.74	brs	5.73	brs	5.72	brs
5	-	-	-	-	-	-	-	-	-	-
6	5.36	d (5.6)	5.43	d (5.6)	2.44	m^{e}	2.56	m^{e}	2.58	ddd (1.8,5.3, 14.7)
6’	-	-	-	-	2.34	m^{e}	2.36	ddd (14.6,2.5,3.9)	2.35	ddd (2.6,4.1,14.7)
7	1.99	m^{e}	2.02	m^{e}	1.88	m^{e}	2.24	m^{e}	2.21	m^{e}
7’	1.6	m^{e}	1.7	m^{e}	1.18	m^{e}	1.21	m^{e}	1.19	m^{e}
8	1.7	m^{e}	1.65	m^{e}	1.78	qd (3.1, 11.5)	2.05	m^{e}	2.05	m^{e}
9	1.01	m^{e}	1.08	t (9.8)	1.19	-	1.20	m^{e}	1.08	m^{e}
10	-	-	-	-	-	-	-		-	-
11	1.59	m^{e}	4.18	td (5.7, 10.3)	4.14	td (5.4, 10.3)	4.09	td(5.5, 10.0)	1.63	m^{e}
11’	1.34	m^{e}	-	-	-	-	2.59	m^{e}	-	-
12	2.4	td(3.1, 12.8)	2.78	dd (5.5, 11.9)	2.8	dd (5.5, 12.1)	1.29	m^{e}	2.27	m^{e}
12’	1.34	m^{e}	1.38	t (11.3)	1.40	m^{e}	-		1.27	m^{e}
13	-	-	-	-	-	-	-	-	-	-
14	1.42	m^{e}	1.55	m^{e}	1.57	td (6.4, 11.7)	1.39	-	1.28	m^{e}
15	2.32	m^{e}	2.33	m^{e}	2.35	dd (2.9, 5.3)	4.62	dd (2.9,5.3)	4.61	dd (2.9,5.3)
15’	2.05	m^{e}	2.05	m^{e}	2.08	m^{e}	-	-	-	-
16	6.72	dd (1.8,3.2)	6.72	dd (1.8,3.2)	6.72	dd (1.8, 3.2)	6.86	d (2.9)	6.8	d (2.9)
17	-	-	-	-	-	-	-	-	-	-
18	0.92	s	0.93	s	0.95	s	1.24	s	1.25	s
19	1.04	s	1.19	s	1.34	s	1.39	s	1.29	s
20	-	-	-	-	-	-	-	-	-	-
21	2.26	s	2.26	s	2.27	s	2.29	s	2.30	s

m^{e}: multiplet overlapped with other signals, s: singlet, brs: broad singlet, d: doublet, td: triplet of a doublet, dd: doublet of a doublet, qd=quartet of a doublet, ddd=doublet of a doublet of a doublet.

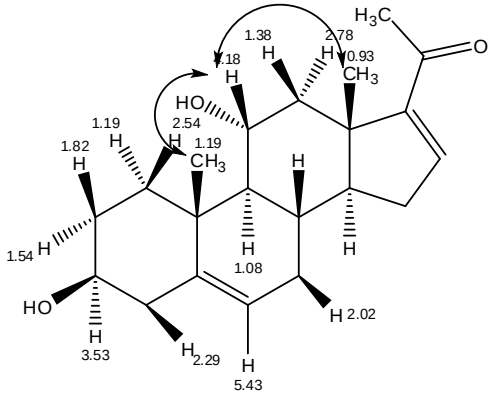
Table 2. ^{13}C-NMR chemical shifts of compound CDRI 80/574 and metabolites

^{13}C	1		2		3		4		5	
	δ	Type of carbon	δ	Type of carbon	δ	Type of carbon	δ	Type of carbon	δ	Type of carbon
1	37.1	CH_2	38.9	CH_2	37.1	CH_2	38.2	CH_2	36.7	CH_2
2	31.5	CH_2	31.7	CH_2	34.1	CH_2	35	CH_2	34.8	CH_2
3	71.7	CH	71.8	CH	200.3	C	202.9	CH	202.4	CH
4	42.2	CH_2	42.7	CH_2	124.5	CH	124.7	CH_2	124.3	CH_2
5	141.4	C	141.8	C	170.9	C	175.2	C	175.2	C
6	121	CH	120.9	CH	33.3	CH_2	34.6	CH	33.9	CH_2
7	31.5	CH_2	31.7	CH_2	31.6	CH_2	32.1	CH_2	32.3	CH_2
8	30.2	CH	30.3	CH	33.4	CH	32.2	CH	32.4	CH
9	50.4	CH	57.3	CH	59.3	CH	61.0	CH	56.1	C
10	36.6	C	38.3	C	40.0	C	41.8	C	40.3	CH_2
11	20.7	CH_2	68.7	CH_2	68.4	CH	68.7	CH_2	21.7	CH_2
12	34.6	CH_2	46.1	CH_2	46.3	CH_2	47.3	CH_2	36.0	CH_2
13	46.0	C	46.3	C	46.2	C	47.9	C	47.9	C
14	56.4	CH	55.5	CH	54.9	CH	59.0	CH	60.0	CH
15	32.2	CH_2	32.1	CH_2	31.9	CH_2	73.0	CH_2	73.2	CH_2
16	144.5	CH	144.6	CH	144.5	CH	145.3	CH	144.9	CH
17	155.3	C	154.6	C	154.4	C	157.3	C	157.9	C
18	15.7	CH_3	16.9	CH_3	17.1	CH_3	23.5	CH_3	22.4	CH_3
19	19.3	CH_3	19.2	CH_3	18.3	CH_3	18.8	CH_3	17.5	CH_3
20	196.9	C	196.6	C	196.5	C	199.7	C	200.0	C
21	27.1	CH_3	26.9	CH_3	26.9	CH_3	27.5	CH_3	27.6	CH_3

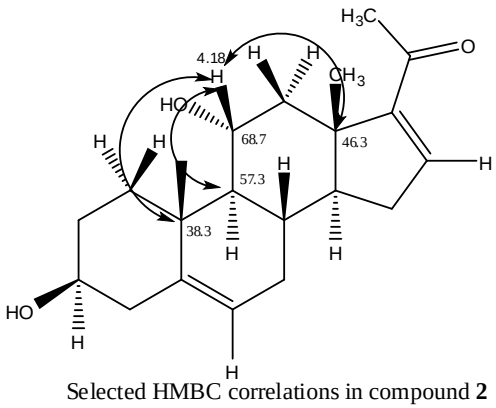


Compound CDRI 80/574

3β, 11α-Dihydroxy-5, 16-dien-pregnane-20-one (2). Compound **2** had a molecular weight of 330 m/e an increment of 16 m/e unit from parent molecule. This clearly indicates an introduction of hydroxyl functionality in the molecule. In ^{1}H NMR, there appeared an additional multiplet at 4.18 ppm corresponding to the proton attached with carbon having oxygen functionality. The two olefinic protons appeared at δ 5.43 and 6.72 as in compound **1**, which indicates that the hydroxylation has not occurred at the allylic positions. The assignments of hydrogen and carbon signals are given in table I and II respectively. As compared with parent compound **1** there is a significant difference in chemical shift of protons at C_1, C_{11} and C_{12} and also of the carbons at C_9, C_{10}, C_{11} and C_{12} indicating the substitution at C_{11}. One proton at C_1 showed significant difference in the chemical shift because of its proximity to 11-hydroxyl functional group as suggested by the three dimensional structure of the compound. The two oxymethine protons (at C_3 and C_{11}) appeared at δ 3.52 and 4.18 respectively showing HSQC correlations with oxymethine carbons at δ 71.6 and 68.7 respectively. The oxymethine proton at 4.18 ppm showed HMBC correlations with the signals of C_{10} at 38.3, C_{13} at 46.3 and C_9 at 57.3 ppm. The relative stereochemistry of C_{11} hydroxyl group is found to be α, which is evident from NOE correlations.



Selected NOESY correlations in compound **2**

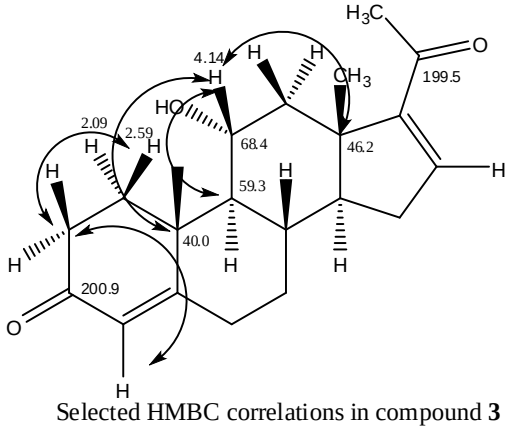


Selected HMBC correlations in compound **2**

In NOESY, the oxymethine proton (C_{11}H) signal at δ 4.18 showed correlations with β-methyl protons at C_{18} and C_{19} as evident from their proximity in three-dimensional structure.

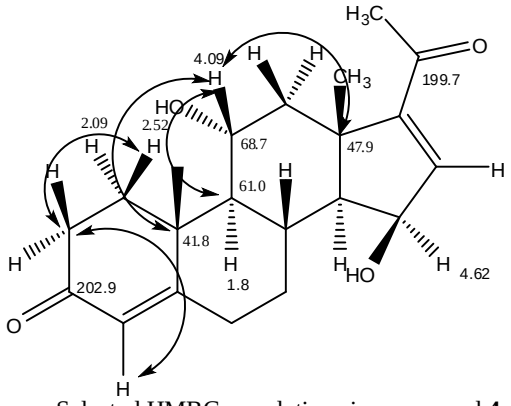
11α-Hydroxy-4, 16-dien-pregnan-3, 20-dione (3). The ^{13}C NMR spectrum of compound **3** provided twenty one signals which include three methyls, six methylenes, six methines and six quaternary carbons. Among quaternary carbons an additional carbonyl carbon at δ 200.3 was observed instead of an aliphatic methylene carbon as evaluated from the DEPT edited spectra. The olefinic proton of compound **1** at δ 5.36 showed a downfield shift and appeared at δ 5.74 for compound **3**. On analysis of the ^{1}H NMR spectrum it was observed that the olefinic proton at δ 5.74 appeared as a broad singlet and showed COSY correlation with the proton at δ 2.43. One of the methylene protons showed correlation with the carbon at δ 34.1 in the HSQC spectrum. This olefinic proton provided a long range correlation with the same carbon (at δ 34.1) in the HMBC spectrum. Moreover, the C_{19} methyl proton provided long range correlation with the C_1

methylene carbon at δ 37.1. The corresponding C_1 protons at δ 2.59 and 2.09 showed long range correlations with the carbonyl carbon at δ 200.3. Other than the two long range correlations of C_1 protons with the carbonyl carbon at δ 200.3, two more long range correlations of protons at δ 2.43 and 2.34 were observed with of the methylene carbon at δ 34.1. This pattern of correlations can only be obtained when the carbonyl is at C_3 and the double bond is between C_4 and C_5. It was further reinstated by the down field shift (~ 29.5 ppm) of C_5 at δ 170.9 with respect to the compound **1** due to the conjugation in **3**. The confirmation of C_5 chemical shift was carried out on the basis of long range correlations of C_{19} methyl with C_5 respectively. The position of the hydroxyl group at C_{11} was confirmed on the basis of distinct multiplicity, correlations in the COSY spectrum as well as the long-range correlations in the HMBC spectrum. The NOESY correlations of C_{11} with C_{18} methyl and C_{19} methyl further confirmed the hydroxyl group to be α in nature. The detailed ^1H and ^{13}C assignments are presented in tables I and II.



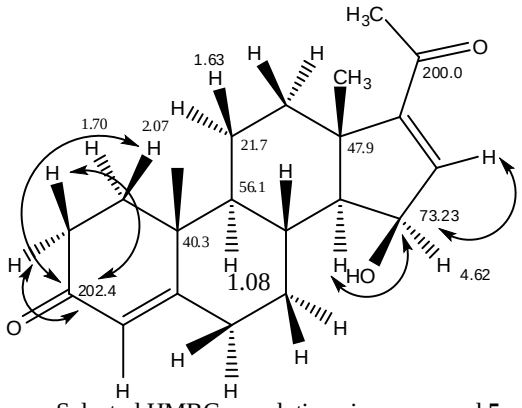
Selected HMBC correlations in compound **3**

11α, 15β-Dihydroxy-4, 16-dien-pregnan-3, 20-dione (4). The compound **4** showed similar spectral features as of compound **3** with an additional hydroxyl group. In ^{13}C spectrum, there appeared two signals of carbonyl carbons at δ 202.9 and 199.7 and the signal at δ 202.9 showed HMBC correlations similar to compound **3**, this suggest that the carbonyl is at C_3. The two methine signals attached with oxygen appeared at δ 4.09 and 4.62. The down field shift of one of these protons indicates its allylic position. The position of the hydroxyl group at C_{15} was confirmed on the basis of the COSY spectrum as it showed correlations with the protons at δ 6.86 (olefinic proton, H_{16}) and δ 1.39 ppm (H_{14}). It was further reconfirmed by the HMBC correlations. The relative stereochemistry of the hydroxyl group was confirmed by the coupling constant of 5.3 Hz between H_{14} and H_{15}. The detailed assignments are given in tables I and II.



Selected HMBC correlations in compound **4**

15β-Hydroxy-4, 16-dien-pregnan-3, 20-dione (5). The spectral pattern of compound **5** shows similar profile in comparison to compound **4** except the absence of C_{11} hydroxyl group. Carbonyl carbon at δ 202.4 showed HMBC correlations with protons at δ 1.7, 2.07, 2.49 and 2.29 corresponding to C_1, $C_{1'}$ and $C_{2'}$ protons. Similarly carbon at δ 73.23 showed HSQC correlation with C_{15} proton (δ 4.62) and HMBC correlations with C_{14} and C_{16} protons (δ1.28 and 6.80). The relative stereochemistry of the hydroxyl group was confirmed by the coupling constant of 5.3 Hz between H_{14} and H_{15}.



Selected HMBC correlations in compound **5**

Compound **2** and **3** have earlier been prepared either from 11-hydroxy-progesterone, 11-oxo-16-dehydropregnenolone or 11-hydroxy-diosgenin following chemical route[12,13] or *via* bio-transformation with *Aspergillus ochraeus*[14]. In above methods, 11-hydroxy-progesterone has been prepared from 11-hydroxy-diosgenin. The compound **4** is novel and has not been reported so far whereas compound **5** has been reported as microbial biotransformation product of 16-dehydroprogesterone by a fungus *Absidia orchidis*[15].

Conclusion

3-β-Hydroxy-5, 16-dien-pregnane-20-one (**1**), when subjected to microbial conversion with two fungal species of *Aspergillus* leads to the formation of regioselective products being oxidized at C_3, C_{11} or C_{15} position. Our study demonstrates a simple method of obtaining compound **2** which is the precursor for the synthesis of the most of the hormone class of compounds. We have isolated four metabolites **2-5** and the novel metabolite **4** has been characterized.

Experimental Section

Cultures. A fungal strain *Aspergillus ochraeus* and a strain of *Aspergillus niger*, isolated from soil samples collected from Ambikapur, Chhattisgarh (India) were used in present study. Pure cultures were isolated on Sabouraud dextrose agar plates containing antibiotic Rifampicin (1µg/ml). The stock cultures were routinely maintained on Sabouraud dextrose agar slants in 6"x1" test tubes at 4 ^oC.

Media. Sabouraud-dextrose agar containing glucose-40 g/L, peptone-10 g/L and agar 20 g/L was used as culture maintenance medium. Czapek Dox broth containing cane sugar 30 g/l, sodium nitrate 3 g/L, di-potassium hydrogen phosphate-1 g/L, magnesium sulfate-0.5 g/L, potassium chloride 0.5 g/L, ferrous sulfate-10 mg at pH-7 in 100 ml/L Erlenmeyer flask autoclaved at 10 pounds /10 min was used as production medium.

Preparation of spores suspension. Spore suspension from 10-15 days old slant culture, was prepared in 200 ml of sterilized distilled water (Approximate cell count was 2-6 x10 ^{6}).

Inoculation of production medium. In order to achieve 2-6x10^5 spores / flask appropriate volume of spore suspension was used to inoculate each production flask and incubated for 48 hrs to achieve fine pellet at 28 ± 1 ^oC on rotatory shaker assembly revolving at 180 rpm with a throw of 1 cm. 5 ml of ethanol containing 25 mg of 3β-hydroxy-5, 16-dien-pregnane-20-one (**1**) was added in each flask to achieve 0.25 mg/ml final substrate concentration and continued the incubation for 48 hrs.

Recovery. Biomass was separated out by vacuum filtration. Filtrate was extracted twice by equal volume of chloroform and biomass (17-20 g/wet wt. and 3.4-5 g/L dry wt.) was extracted firstly by acetone followed by evaporation and re-extracted by chloroform. Combined extracts were evaporated to dryness and dried crude product was subjected to column chromatography.

General. The progress of the transformation was monitored by thin layer chromatography on Merck silica-gel 60-F_{254} coated plates. Spots were run in acetone and benzene mixture (20:80 v/v) and observed under UV light. Transformed products were separated by column chromatography on silica-gel (100-200 mesh) column using benzene and ethyl acetate mixture as eluent. IR spectra were recorded on Perkin–Elmer RXI FT-IR spectrometer using KBr pellets or

neat (expressed in cm^{-1}). ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) were recorded in CD_3OD or $CDCl_3$ (Aldrich) as solvent on a Bruker Avance DRX-400 calibrated with TMS as internal standard. ^1H and ^{13}C chemical shifts of all the five compounds are given in tables 1 and 2 respectively. ES-MS was recorded in CH_3OH on a Micromass Quattro II. The FAB-MS was recorded using a Jeol SX-120/DA6000 mass spectrometer using Argon as the FAB gas.

3β-Hydroxy-5, 16-dien-pregnane-20-one (1). M.p. 205-207ºC, M^++1=315, IR=1735cm^{-1}.

3β, 11α-Dihydroxy-5, 16-dien-pregnane-20-one (2). M.P. 162^{0}C, M^++1(328); IR (KBr): 1661 cm^{-1}.

11α-Hydroxy-4, 16-dien-pregnan-3, 20-dione (3). M.P. 205^{0}C; M^++1(330); IR (KBr): 1652 cm^{-1}.

11α, 15β-Dihydroxy-4, 16-dien-pregnan-3, 20-dione (4). M.P. 200 ^{0}C; M^++1 (345); IR (KBr): 1657 cm^{-1}.

15β-Hydroxy-4, 16-dien-pregnan-3, 20-dione (5). M.P. 210 ^{0}C; M^++1 (329); IR (KBr): 1596 cm^{-1}.

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