Steroids 78 (2013) 1152–1158

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

# Biocatalyst mediated production of 6β,11α-dihydroxy derivatives of 4-ene-3-one steroids

Swati P. Kolet, Siddiqui Niloferjahan [1], Saikat Haldar [1], Rajesh Gonnade, Hirekodathakallu V. Thulasiram *

*Chemical Biology Unit, Department of Organic Chemistry, CSIR-National Chemical Laboratory, Pune 411008, India*

ARTICLE INFO

*Article history:*
Received 17 May 2013
Received in revised form 20 July 2013
Accepted 9 August 2013
Available online 25 August 2013

*Keywords:*
Biotransformation
4-Ene-3-one steroids
*Mucor* sp.

ABSTRACT

Biotransformation of steroids with 4-ene-3-one functionality such as progesterone (**I**), testosterone (**II**), 17α-methyltestosterone (**III**), 4-androstene-3,17-dione (**IV**) and 19-nortestosterone (**V**) were studied by using a fungal system belonging to the genera of *Mucor* (*M881*). The fungal system efficiently and quantitatively converted these steroids in regio- and stereo-selective manner into corresponding 6β,11α-dihydroxy compounds. Time course experiments suggested that the transformation was initiated by hydroxylation at 6β- or 11α-(10β-hydroxy in case of **V**) to form monohydroxy derivatives which upon prolonged incubation were converted into corresponding 6β,11α-dihydroxy derivatives. The fermentation studies carried out using 5 L table-top fermentor with substrates (**I** and **II**) clearly indicates that 6β,11α-dihydroxy derivatives of steroids with 4-ene-3-one functionality can be produced in large scale by using *M881*.



## 1. Introduction

Steroids are biologically and pharmaceutically important compounds and are involved in many physiological activities. Over 300 approved steroid based drugs are known and are widely used as anti-inflammatory, anti microbial, anti-tumor, anti-estrogenic, anti-allergic, anti-diabetic, anti-HIV and anti-convulsant agents [1–7]. Although many attempts have been made to produce steroids and their derivatives through conventional synthetic routes, they involve several steps and are usually time consuming [8,9]. On the other hand, biocatalyst mediated steroid transformation seems to be a powerful tool for the production of novel steroidal derivatives as well as their active pharmaceutical ingredients (APIs) in an efficient and regio- and stereo-selective manner. Biocatalysts are known to carry out the functionalization at remote and inactive carbons on steroids, which would be tedious or impossible through conventional chemical synthesis. Hydroxylation is one of the most important reactions of steroid functionalization as most of the hydroxylated steroids often prove to be more potent biologically active molecules than their non-hydroxylated precursors [1,4,10–12]. For last few decades, biocatalyst mediated hydroxylation on steroids has been well studied and documented. Although fungi belonging to the genera *Mucor* and *Rhizopus* have been shown to carryout hydroxylation at 6β- or/and 11α-positions, these are not found to be major transformations [13–16].

Earlier, fungal system belonging to the genera of *Mucor* (abbreviated as *M881*) [from National Collection of Industrial Microorganisms (NCIM), Pune catalogue No. 881] could able to carry out novel and preparatively useful transformations of limonoids [17]. This fungal strain *M881* showed the ability to carry out hydroxylation at 6β- and 11α-positions of 4-ene-3-one steroids. The present study focuses on the ability of the fungal system *Mucor* 881 (*M881*) to convert 4-ene-3-one steroids such as progesterone (**I**), testosterone (**II**), 17α-methyltestosterone (**III**) and 4-androstene-3,17-dione (**IV**) into their corresponding 6β,11α-dihydroxy derivatives. Further the large-scale fermentation studies have been carried out using **I** and **II** as substrates.

## 2. Experimental methods

### 2.1. Chemicals

Media ingredients, salts and acids were purchased from Merck (India) and Himedia, Mumbai. Analytical grade solvents were procured from Merck (India). Steroidal substrates such as progesterone (**I**), testosterone (**II**), 17α-methyltestosterone (**III**), 4-androstene-3,17-dione (**IV**) and 19-nortestosterone (**V**) were obtained from Sigma–Aldrich.

### 2.2. Microorganism

Microorganism used in present study, *Mucor* 881 (*M881*) was obtained from National Collection of Industrial Microorganisms

* Corresponding author. Tel.: +91 20 25902478; fax: +91 20 25902636.
*E-mail address:* hv.thulasiram@ncl.res.in (H.V. Thulasiram).
[1] These authors contributed equally.

0039-128X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.steroids.2013.08.004

(NCIM), Pune (catalogue No. 881). It was maintained and propagated as reported earlier [18].

### 2.3. Conditions for biotransformation

Fermentation procedures were carried out as reported earlier [17] in modified CzapekDox medium. Flasks (500 mL) containing 100 mL sterile medium were inoculated with 1 mL spore suspension from well grown (3–5 days) culture on a potato dextrose agar slants and incubated at 30 °C on a rotary shaker at 200 rpm for 36 h. After this growth period, pH was adjusted to 7.0 using sterile 1 M $K_2HPO_4$ and 70 mg substrate (**I**/**III**/**IV**/**V**) (100 mg in case of **II**) in 0.2 mL tetrahydrofuran (THF) was added to each flask and the incubation was continued for 6 days. Control experiments were also performed with the substrate but without microorganism and with the organism but without substrate. Substrate concentration studies were carried using varied concentration of substrates (0.3–1.2 g/L). In time course experiments, incubations were carried out for 1–12 days and the level of transformation at the end of each incubation were monitored by HPLC, and LCMS analyses. Quantitation and the levels of different metabolites formed were determined on the basis of area under the respective peak and compared with the standard graphs obtained for each metabolite by injecting the known amounts of these metabolites.

Resting cell experiments were carried out by incubating 3 g of well-washed mycelial pellet (36 h grown) with substrate (20 mg) in 50 mL of phosphate buffer (0.1 M, pH 7.0) on a rotary shaker (200 rpm). At the end of incubation period (36 h), the contents were extracted and analyzed by HPLC and LCMS.

### 2.4. Scale-up and fermentor studies

To standardize the scale-up fermentation conditions for large-scale production of hydroxylated steroids using *M881*, we carried out transformation in three different volumes (100 mL, 600 mL, 5000 mL) of modified CzapekDox medium. Time course experiments were performed in shake flasks with 100 mL and 600 mL medium and in 7 L capacity tabletop fermentor (New Brunswick Scientific) with 5 L fermentation media. Shake flask experiments were carried out as mentioned above. The fermentor containing 5 L medium was inoculated using 100 mL of 24 h grown culture of *M881* and incubated at 30 °C for 24 h with the following controlled parameters: rotary agitation- 200 rpm; dissolved oxygen-(DO) 60–80% (of saturation), airflow- 2 SLPM (Standard L/min). After this incubation period, substrate (3.5 g of **I** or 5 g of **II**) in 20 mL of THF was added to the fermentation media and incubation was continued. Aliquots were drawn aseptically at every 48 h, extracted with ethyl acetate and the level of transformation was monitored by HPLC and LCMS analyses.

### 2.5. Extraction of metabolites

At the end of incubation period, the contents from all flasks were pooled and filtered. Broth and mycelia were extracted four times with ethyl acetate separately. As TLC and HPLC analyses indicated that the metabolites present in both the extracts were same, the extracts were pooled, dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure on a rotary evaporator. The crude residue obtained was subjected to column chromatography to isolate the metabolites.

### 2.6. Chromatographic procedure

Metabolites formed were purified by subjecting the crude extract to column chromatography over silica gel (230–400 mesh) and eluting with methanol/dichloromethane ($CH_2Cl_2$) gradient (1–10% of methanol in $CH_2Cl_2$) mixture. Compounds were analyzed by thin-layer chromatography (TLC) using solvent system I (5% methanol in $CH_2Cl_2$) and system II (10% methanol in $CH_2Cl_2$) and compounds were visualized by spraying with a solution of 3.2% anisaldehyde, 2.8% sulfuric acid, 2% acetic acid in ethanol followed by heating. TLC was performed on pre-coated silica gel 60-F254 plates (0.25 mm). High-performance liquid chromatography (HPLC) analyses of metabolites were carried out on Waters 600A HPLC instrument, with X-bridge $C_{18}$ column (4.6 × 100 mm) and methanol/water gradient elution at 1 mL/min flow rate and 245 nm wavelength. For preparative HPLC purification of metabolites, X-bridge $C_{18}$ column (19 × 100 mm) was used with methanol/water isocratic elution (30–50% of methanol in water) at flow rate of 15 mL/min. $^1H$ and $^{13}C$ NMR spectra were recorded in $CD_3OD$ on Bruker AC-400 at 400.13 and 100.03 MHz or on a Bruker DRX-500 spectrometer at 500.13 and 125.78 MHz respectively. Chemical shifts are reported in parts per million with respect to TMS (tetramethylsilane) as internal standard. IR spectra were recorded on Schimadzu 8400 series FTIR instrument and values are reported in $cm^{-1}$. Optical rotations ($[\alpha]_D$) were recorded using Jasco, P-1020 polarimeter and are reported in deg/dm and the concentration (c) is given in g per 100 mL in the specified solvent. Mass spectra were recorded on Waters make QTOF (SYNAPT-HDMS).

### 2.7. X-ray crystallographic data

X-ray intensity data measurements of the compounds **Ib** and **IIc** were carried out on a Bruker SMART APEX I CCD diffractometer with graphite-monochromatized ($MoK_\alpha$ = 0.71073 Å) radiation at room temperature. The X-ray generator was operated at 50 kV and 30 mA. Data were collected with $\omega$ scan width of 0.3° at different settings of $\varphi$ (0°, 90°, 180° and 270°) keeping the sample-to-detector distance fixed at 6.145 cm and the detector position ($2\theta$) fixed at −28°. The X-ray data collection was monitored by SMART program[19]. All the data were corrected for Lorentzian, polarization and absorption effects using SAINT and SADABS programs[19]. SHELX-97 was used for structure solution and full matrix least-squares refinement on $F^2$ [20]. ORTEPs were generated using Mercury-3.0 program [21].

## 3. Results and discussion

### 3.1. Transformation of progesterone (*I*)

A batch of 30 flasks was inoculated with *M881* and the substrate **I** (0.7 g/L) was added. At the end of 6 days of incubation period, the contents of all flasks were pooled and processed as described in experimental section. The crude extract (1.87 g) upon TLC and HPLC analyses indicated the presence of one major and two minor metabolites along with small amount of substrate **I**. The crude extract was subjected to column chromatography on silica gel (230–400 mesh, 50 g) and elution of metabolites was carried out with methanol/dichloromethane ($CH_2Cl_2$) gradient mixture. Eluting the column with 2% methanol in $CH_2Cl_2$ yielded unmetabolized substrate **I** (38 mg, $R_f$: 0.83 system: I; $R_t$: 41.0 min,). Further elution of the column with 3% methanol in dichloromethane yielded a fraction (1.01 g) containing three metabolites ($R_f$: 0.41, 0.40, 0.29 system: I; $R_t$: 26.3 min, 16.8 min, 19.5 min respectively), which were purified by preparative HPLC (117 mg, 54 mg, 741 mg respectively).

The HRMS spectrum of compound with $R_f$: 0.41 (system: **I**) showed $[M+Na]^+$ peak at *m/z* 353.2065 ($C_{21}H_{30}O_3Na$) indicating the introduction of a hydroxy group to the parent structure (**I**). Signal at δ 3.97 (1H, dt, *J* = 4.6, 10.7 Hz,) in $^1H$ NMR and shift of signal

**Table 1**
$^1$H-NMR spectral data of progesterone (**I**), testosterone (**II**), 17α-methyltestosterone (**III**), 4-androstene-3,17-dione (**IV**), 19-nortestosterone (**V**) and their metabolites in $CD_3OD$.

| Metabolites | 18-$CH_3$ | 19-$CH_3$ | 4-H | 6α-H | 11β-H | Other significant signals |
|---|---|---|---|---|---|---|
| **I** | δ 0.67 (3H, s, H-18) | 1.23 (3H, s, H-19) | 5.71 (1H, s, H-4) | | | 2.12 (3H, s, H-21) |
| **Ia** | 0.69 (3H, s, H-18) | 1.35 (3H, s, H-19) | 5.72 (1H, s, H-4) | | 3.96 (1H, dt, *J* = 4.6, 10.7 Hz, H-11β) | 2.14 (3H, s, H-21) |
| **Ib** | 0.67 (3H, s, H-18) | 1.46 (3H, s, H-19) | 5.74 (1H, s, H-4) | 4.22 (1H, t, H-6α) | 3.97 (1H, dt, *J* = 4.6, 10.7 Hz, H-11β) | 2.10 (3H, s, H-21) |
| **Ic** | 0.64 (3H, s, H-18) | 1.58 (3H, s, H-19) | 5.79 (1H, s, H-4) | 4.28 (1H, t, H-6α) | | 2.11 (3H, s, H-21) |
| **II** | 0.82 (3H, s, H-18) | 1.27 (3H, s, H-19) | 5.74 (1H, s, H-4) | | | 3.61 (1H, t, H-17α) |
| **IIa** | 0.81 (3H, s, H-18) | 1.39 (3H, s, H-19) | 5.78 (1H, s, H-4) | 4.26 (1H, t, H-6α) | | 3.59 (1H, t, H-17α) |
| **IIb** | 0.81 (3H, s, H-18) | 1.36 (3H, s, H-19) | 5.71 (1H, s, H-4) | | 3.99 (1H, dt, *J* = 4.6, 10.7 Hz, H-11β) | 3.62 (1H, t, H-17α) |
| **IIc** | 0.83 (3H, s, H-18) | 1.51 (3H, s, H-19) | 5.77 (1H, s, H-4) | 4.25 (1H, t, H-6α) | 4.00 (1H, dt, *J* = 4.6, 10.7 Hz, H-11β) | 3.62 (1H, t, H-17α) |
| **III** | 0.86 (3H, s, H-18) | 1.14 (3H, s, H-19) | 5.67 (1H, s, H-4) | | | 1.21 (3H, s, 17α-CH3) |
| **IIIa** | 0.92 (3H, s, H-18) | 1.36 (3H, s, H-19) | 5.71 (1H, s, H-4) | | 4.01 (1H, dt, *J* = 4.6, 10.7 Hz, H-11β) | 1.21(3H, s, 17α-CH3) |
| **IIIb** | 0.92 (3H, s, H-18) | 1.40 (3H, s, H-19) | 5.78 (1H, s, H-4) | 4.26 (1H, t, H-6α) | | 1.19(3H, s, 17α-CH3) |
| **IIIc** | 0.94 (3H, s, H-18) | 1.52 (3H, s, H-19) | 5.78 (1H, s, H-4) | 4.25 (1H, t, H-6α) | 4.04 (1H, dt, *J* = 4.6, 10.7 Hz, H-11β) | 1.22 (3H, s, 17α-CH3) |
| **IV** | 0.93 (3H, s, H-18) | 1.26 (3H, s, H-19) | 5.73 (1H, s, H-4) | | | |
| **IVa** | 0.97 (3H, s, H-18) | 1.44 (3H, s, H-19) | 5.83 (1H, s, H-4) | 4.34 (1H, t, H-6α) | | |
| **IVb** | 0.95 (3H, s, H-18) | 1.38 (3H, s, H-19) | 5.73 (1H, s, H-4) | | 4.01 (1H, dt, *J* = 4.6, 10.7 Hz, H-11β) | |
| **IVc** | 0.97 (3H, s, H-18) | 1.53 (3H, s, H-19) | 5.81 (1H, s, H-4) | 4.31 (1H, t, H-6α) | 4.05 (1H, dt, *J* = 4.6, 10.7 Hz, H-11β) | |
| **IVd** | 0.94 (3H, s, H-18) | 1.27 (3H, s, H-19) | 5.75 (1H, s, H-4) | | | 3.45 (1H, dt, H-7α) |
| **V** | 0.81 (3H, s, H-18) | | 5.80 (1H, d, H-4, *J* = 1.83 Hz) | | | 3.59 (1H, t, H-17α) |
| **Va** | 0.82 (3H, s, H-18) | | 5.86 (1H, d, H-4, *J* = 1.83 Hz) | 4.29 (1H, t, H-6α) | | 3.59 (1H, t, H-17α) |
| **Vb** | 0.80 (3H, s, H-18) | | 5.73 (1H, d, H-4, *J* = 1.83 Hz) | | | 3.58 (1H, t, H-17α) |

[a]Progesterone (**I**), 11α-hydroxyprogesterone (**Ia**), 6β,11α-dihydroxyprogesterone (**Ib**), 6β-hydroxypregn-4-ene-3,11,20-trione (**Ic**), Testosterone (**II**), 6β-hydroxytestosterone (**IIa**), 11α-hydroxytestosterone (**IIb**), 6β,11α-dihydroxytestosterone (**IIc**), 17α-methyltestosterone (**III**), 11α-hydroxy-17α-methyltestosterone (**IIIa**), 6β-hydroxy-17α-methyltestosterone (**IIIb**), 6β,11α-dihydroxy-17α-methyltestosterone (**IIIc**), 4-androstene-3,17-dione (**IV**), 6β-hydroxy-4-androstene-3,17-dione (**IVa**), 11α-hydroxy-4-androstene-3,17-dione (**IVb**), 6β,11α-dihydroxy-4-androstene-3,17-dione (**IVc**), 7β-hydroxy-4-androstene-3,17-dione (**IVd**), 19-nortestosterone (**V**), 6β-hydroxy-19-nortestosterone (**Va**), 10β-hydroxy-19-nortestosterone (**Vb**).

from δ 22.1 to δ 69.3 in $^{13}$C NMR spectra (Tables 1 and 2) confirmed the insertion of a hydroxy group in progesterone at C-11 position. The stereochemistry of the newly introduced hydroxyl group at C-11 was deduced to be α on the basis of coupling constants and NOESY correlations of H-18β (δ = 0.69) and H-19β (δ = 1.35) with H-11 (δ = 3.97) (Supplement S12). From the spectral data, the metabolite was identified as 11α-hydroxyprogesterone (**Ia**) and the spectral data (Tables 1 and 2 and Supplement ST1) for **Ia** matched well with the earlier report [22].

The molecular formula for compound with $R_f$: 0.29 (system: I) was deduced as $C_{21}H_{30}O_4$ from HRMS analysis which showed $[M+Na]^+$ ion peak at *m/z* 369.2038, indicating introduction of two hydroxy group in the substrate **I**. $^1$H NMR spectrum of compound showed signal at δ 3.97 (1H, dt, *J* = 4.6, 10.7 Hz,) and at δ 4.22 (1H, t). Chemical shift for C-6 and C-11 carbons from δ 34.7 and δ 22.1 to δ 73.8 and δ 69.3 respectively (Tables 1 and 2 and Supplement ST1 and S16) revealed the presence of 6- and 11-hydroxyl groups. The NOESY correlations of C11 with C18 methyl and C19 methyl further confirmed the hydroxyl group to be α in nature. The stereochemistry of the C-6 hydroxyl group was deduced to be β on the basis of the NOESY interaction between H-6 and H-14α (δ = 1.35). The position and stereochemistry of newly introduced hydroxyl groups were further confirmed from X-ray diffraction data of metabolite (**Ib**) (CCDC reference number = CCDC 922747, supplement) and it was identified as 6β,11α-dihydroxyprogesterone (Fig. 1). The spectral data (Tables 1 and 2 and Supplement ST1) for the compound was in good agreement with the earlier report [23].

HRMS for the metabolite with $R_f$: 0.40 (system: I) showed a peak at *m/z* 367.1885 ($[M+Na]^+$) indicating the molecular formula was $C_{21}H_{28}O_4Na$ (cal: 367.1886). The $^1$H NMR spectrum showed appearance of narrow triplet at δ 4.28 indicating the presence of hydroxyl functionality at 6β- position. In $^{13}$C NMR spectrum of the compound, two new signals at δ 211.0, δ 73.8 and disappearance of signals at δ 22.1, δ 34.7 were observed along with downfield shift of C-12 carbon from δ 39.7 to δ 57.3 (Tables 1 and 2 and Supplement ST1 and S20), indicating introduction of hydroxyl at C-6 position and oxo group at C-11 position. The stereochemistry of the hydroxyl group was confirmed from NOESY correlation of C-6 proton with H-14α (Supplement S20). From these observations the metabolite was identified as 6β-hydroxypregn-4-ene-3,11,20-trione (**Ic**).

**Table 2**
$^{13}$C-NMR spectral data of progesterone, testosterone, 17α-methyltestosterone, 19-nortestosterone, 4-androstene-3,17-dione and their metabolites in $CD_3OD$.

| Carbon atoms | Compounds | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **I** | **Ia** | **Ib** | **Ic** | **II** | **IIa** | **IIb** | **IIc** | **III** | **IIIa** | **IIIb** | **IIIc** | **IV** | **IVa** | **IVb** | **IVc** | **IVd** | **V** | **Va** | **Vb** |
| 1 | 36.8 | 38.6 | 38.9 | 37.1 | 36.8 | 37.7 | 38.6 | 38.5 | 36.8 | 38.6 | 38.4 | 38.7 | 36.3 | 38.3 | 36.6 | 36.7 | 36.6 | 27.2 | 27.2 | 32.7 |
| 2 | 34.7 | 34.9 | 35.2 | 34.8 | 34.7 | 35.0 | 34.9 | 35.2 | 34.7 | 34.9 | 35.1 | 35.2 | 34.7 | 35.0 | 34.9 | 35.2 | 34.7 | 37.3 | 37.1 | 33.2 |
| 3 | 202.3 | 203.0 | 203.6 | 203.6 | 202.3 | 203.2 | 203.0 | 203.6 | 202.3 | 203.0 | 203.2 | 203.7 | 202.2 | 203.0 | 203.0 | 203.7 | 202.1 | 202.9 | 203.3 | 202.3 |
| 4 | 124.2 | 124.7 | 127.2 | 127.2 | 124.1 | 126.6 | 124.6 | 127.2 | 124.1 | 124.6 | 126.7 | 127.5 | 124.3 | 126.9 | 124.8 | 127.3 | 125.0 | 124.7 | 125.6 | 124.7 |
| 5 | 175.0 | 175.3 | 171.8 | 171.8 | 175.2 | 171.6 | 175.6 | 171.9 | 175.2 | 175.6 | 171.6 | 172.0 | 174.5 | 171.1 | 175.1 | 171.6 | 171.2 | 170.8 | 168.8 | 168.7 |
| 6 | 33.9 | 34.7 | 73.8 | 73.8 | 33.9 | 73.6 | 34.8 | 73.8 | 33.0 | 34.8 | 73.6 | 73.8 | 32.5 | 73.4 | 34.6 | 73.6 | 43.5 | 36.5 | 72.5 | 34.5 |
| 7 | 33.2 | 33.0 | 40.6 | 40.0 | 32.9 | 38.3 | 32.6 | 40.3 | 32.7 | 32.8 | 39.2 | 40.3 | 32.0 | 36.6 | 31.7 | 40.2 | 75.1 | 32.0 | 39.1 | 34.5 |
| 8 | 36.8 | 36.2 | 29.7 | 31.3 | 36.9 | 30.6 | 36.6 | 30.2 | 37.7 | 37.5 | 31.9 | 30.8 | 36.6 | 30.7 | 35.7 | 29.4 | 43.8 | 41.6 | 34.9 | 36.5 |
| 9 | 55.1 | 60.0 | 60.0 | 62.8 | 51.8 | 51.7 | 60.3 | 60.3 | 51.5 | 60.2 | 51.5 | 60.3 | 52.0 | 55.1 | 60.2 | 60.12 | 52.1 | 51.0 | 50.9 | 54.5 |
| 10 | 40.0 | 41.3 | 40.3 | 39.1 | 40.0 | 39.3 | 41.5 | 40.7 | 40.0 | 41.5 | 39.4 | 40.8 | 40.0 | 39.4 | 41.5 | 40.7 | 39.4 | 43.8 | 39.5 | 70.9 |
| 11 | 22.1 | 69.3 | 69.3 | 210.8 | 21.7 | 21.7 | 69.4 | 69.4 | 21.8 | 69.8 | 21.8 | 69.8 | 21.4 | 21.3 | 69.1 | 69.1 | 21.4 | 27.7 | 27.2 | 21.2 |
| 12 | 39.7 | 50.9 | 50.8 | 57.3 | 37.7 | 39.4 | 51.1 | 51.0 | 34.0 | 44.4 | 32.7 | 44.3 | 33.7 | 32.5 | 43.5 | 43.4 | 32.4 | 37.7 | 37.7 | 37.5 |
| 13 | 45.1 | 45.2 | 45.3 | 47.9 | 43.9 | 44.0 | 44.6 | 44.7 | 46.6 | 47.3 | 46.8 | 47.5 | 48.8 | 49.1 | 49.1 | 49.0 | 49.1 | 44.1 | 44.4 | 44.0 |
| 14 | 57.2 | 56.5 | 56.4 | 55.1 | 55.5 | 55.3 | 49.1 | 49.0 | 55.4 | 50.9 | 55.2 | 50.8 | 55.3 | 51.1 | 51.3 | 51.1 | 51.8 | 51.1 | 50.9 | 51.4 |
| 15 | 25.3 | 25.2 | 25.2 | 24.9 | 24.2 | 24.2 | 24.1 | 24.1 | 24.2 | 24.2 | 24.2 | 24.1 | 22.6 | 22.7 | 22.6 | 22.6 | 25.9 | 24.1 | 24.0 | 24.3 |
| 16 | 23.8 | 23.8 | 23.8 | 24.4 | 30.6 | 31.1 | 30.7 | 30.0 | 39.2 | 39.2 | 39.5 | 39.2 | 36.7 | 38.5 | 38.5 | 37.5 | 36.8 | 30.6 | 30.6 | 30.6 |
| 17 | 64.5 | 64.3 | 64.3 | 63.2 | 82.2 | 81.3 | 81.3 | 81.9 | 82.1 | 81.7 | 82.2 | 81.7 | 221.8 | 223.5 | 221.8 | 222.1 | 223.5 | 82.2 | 82.3 | 82.3 |
| 18 | 13.7 | 14.7 | 14.7 | 14.7 | 11.6 | 11.6 | 12.7 | 12.7 | 14.6 | 15.7 | 14.6 | 15.7 | 14.0 | 14.1 | 14.9 | 14.9 | 14.3 | 11.6 | 11.6 | 11.5 |
| 19 | 17.7 | 18.6 | 20.3 | 19.3 | 17.7 | 19.7 | 18.7 | 20.4 | 17.7 | 18.7 | 19.7 | 20.4 | 17.6 | 19.7 | 18.7 | 20.4 | 17.6 | – | – | _ |
| 20 | 212.1 | 211.6 | 211.7 | 211.0 | – | – | – | – | 26.1 | 26.1 | 26.0 | 26.1 | – | – | – | – | – | – | – | _ |
| 21 | 31.6 | 31.4 | 31.4 | 32.3 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | _ |

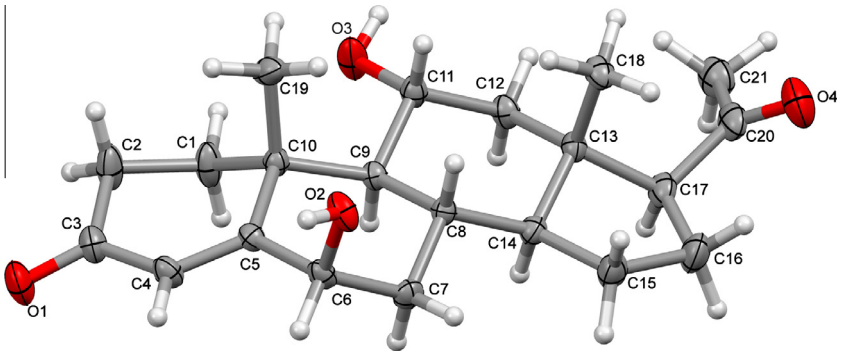



**Fig. 1.** X-ray structure of **Ib**.

Substrate concentration studies indicated that the organism could transform progesterone (**I**) into **Ia**, **Ib** and **Ic** in efficient manner at the concentration of 0.7 g/L. Increase in the substrate concentration decreased the level of metabolites formation. Time course experiment carried out using **I** revealed that during early stages of incubation (two days) nearly 35% of **I** was transformed into metabolites **Ia**, **Ib** and **Ic**. When the incubation period was extended to 12 days, the transformation was increased to 98% with formation of 6β,11α-dihydroxyprogesterone (**Ib**) as a major metabolite (96%) (Figs. 2 and 3a).

*M881* has initiated the transformation of **I** by hydroxylation at 11α-position to form **Ia** which further undergoes one more hydroxylation at 6β-position to form **Ib**. This was further confirmed by incubating **Ia** with *M881*, which transformed into **Ib**. Moreover, the formation of 6β-hydroxyprogesterone was not observed by HPLC and LCMS analyses. Small amount of **Ib** was converted into **Ic** as the microorganism oxidizes 11α-hydroxyl group on **Ib**. Based on these results, the biotransformation pathway of progesterone by *M881* was deduced (Scheme 1).

### 3.2. Biotransformation of testosterone (*II*)

Transformation of **II** (1.0 g/L) was carried out using 30 flasks as described in experimental section. The crude extract (2.3 g) obtained after 6 days of incubation was subjected to HPLC and LC–MS analyses, which indicated the presence of three metabolites along with trace amount of unmetabolised substrate **II**. The metabolites were purified over silica gel column (230–400 mesh, 38 g) chromatography by eluting with gradient mixture of methanol in dichloromethane. Elution with 2% methanol in $CH_2Cl_2$ yielded a fraction containing substrate **II** (27 mg $R_f$: 0.79 system: II; $R_t$: 30.2 min). Other three metabolites were subsequently eluted with 2.5, 2.8 and 3.1% methanol in $CH_2Cl_2$ (369 mg, 251 mg, 546 mg, $R_f$: 0.60, 0.44, 0.32 system II; $R_t$: 16.0 min, 19.0 min, 10.5 min, respectively). From HRMS, IR, $^1$H, $^{13}$C NMR spectral data analyses (Tables 1 and 2 and Supplement ST1) and X-ray diffraction data (Supplement Fig. S1) (CCDC reference number = CCDC 922257), the compounds were characterized as 6β-hydroxytestosterone (**IIa**), 11α-hydroxytestosterone (**IIb**) and 6β,11α-dihydroxytestosterone (**IIc**). The spectral data for these compounds agreed well with the earlier reports [24,25].

The optimum concentration for the transformation was determined from substrate concentration studies and it was found to be 1.0 g/L. Time-course experiments indicate that *M881* could transform 66% of testosterone (**II**) into 6β-hydroxytestosterone (**IIa**), 11α-hydroxy testosterone (**IIb**) 6β,11α-dihydroxytestosterone (**IIc**) at the end of 48 h incubation. At the end of twelfth day of incubation, **IIc** was found to be major metabolite (84%) (Supplement Figs. S2 and S3a). The biotransformation pathway for **II** (Scheme 2) was confirmed by incubating 6β-hydroxytestosterone

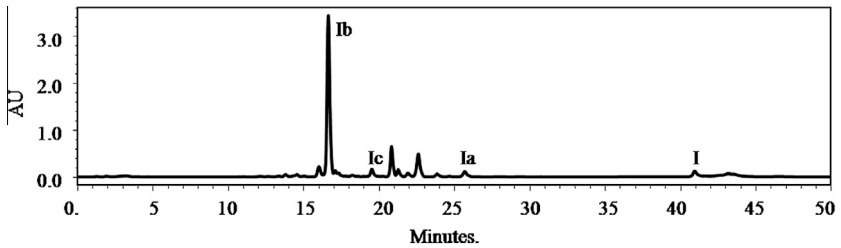



**Fig. 2.** The HPLC analysis of the broth extract of progesterone (**I**) after 8 days of incubation.

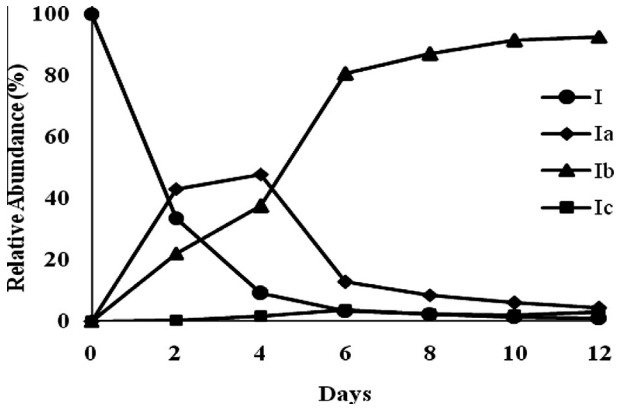
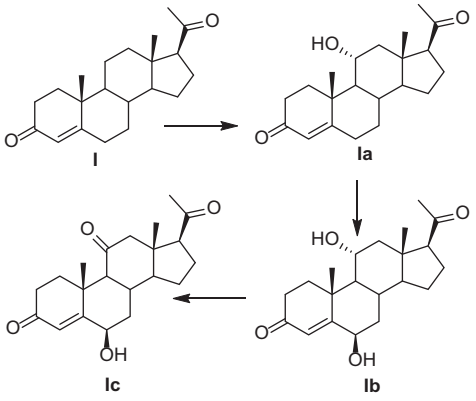



**Fig. 3a.** Time course study of progesterone (**I**) transformation in shake flask fermentation: (●) progesterone (**I**), (♦) 11α-hydroxyprogesterone (**Ia**), (▲) 6β,11α-dihydroxyprogesterone (**Ib**), (■) 6β-hydroxypregn-4-ene-3,11,20-trione (**Ic**).




**Scheme 1.** Transformation of progesterone (**I**) into corresponding hydroxy derivatives by fungal strain *M881*.

(**IIa**), 11α-hydroxytestosterone (**IIb**) with *M881*. It was observed that both metabolites **IIa** and **IIb** were transformed into dihydroxy metabolite **IIc**.

### 3.3. Transformation of 17α-methyltestosterone (*III*)

For the purification of metabolites, transformation of **III** (0.7 g/L) was carried using 30 flasks inoculated with *M881* as described in experimental section. The TLC, HPLC and LC–MS analyses of crude extract (1.79 g) obtained after 6 days of incubation showed the presence of three metabolites along with trace amount of unmetabolised substrate **III**. The metabolites were purified over silica gel column (230–400 mesh, 44 g) chromatography with gradient mixture of methanol in $CH_2Cl_2$ as mobile phase. Elution of the column with different % of methanol in $CH_2Cl_2$ yielded fractions containing pure compounds with $R_f$ values 0.81, 0.65, 0.41, 0.35 (system: II) respectively. Based on the spectral data analyses [HRMS, IR, $^1$H and $^{13}$C NMR (Tables 1 and 2 and Supplement ST1)] the metabolites were identified as 11α-hydroxy-17α-methyltestosterone (**IIIa**) (539 mg, $R_f$: 0.41, $R_t$: 18.8 min), 6β-hydroxy-17α-methyltestosterone (**IIIb**) (417 mg, $R_f$: 0.65, $R_t$: 17.6 min) and 6β,11α-dihydroxy-17α-methyltestosterone (**IIIc**) (365 mg, $R_f$: 0.35, $R_t$: 12.5 min). The spectral data for purified compounds (Tables 1 and 2 and Supplement ST1) was completely agreed with that of earlier report [26,27].

From time course experiments (concentration 0.7 g/L), it was observed that the fungal system can convert 17α-methyltestosterone (**III**) into 11α-hydroxy-17α-methyltestosterone (**IIIa**), 6β-hydroxy-17α-methyltestosterone (**IIIb**) and 6β,11α-dihydroxy-17α-methyltestosterone (**IIIc**) (Scheme 3) (Supplement Figs. S4 and S5). However, with prolonged incubation, increase in the concentration of **IIIc** was observed. At the end of 12 days of incubation, 90% of **III** was converted into **IIIc** as a major metabolite.

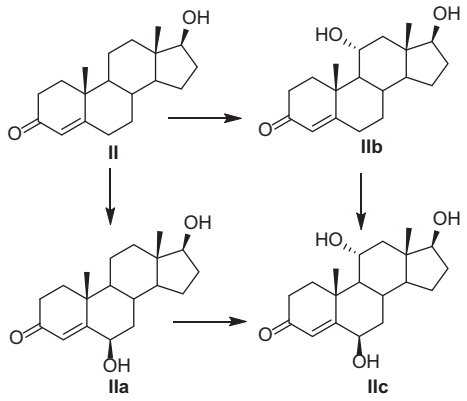
### 3.4. Transformation of 4-androstene-3,17-dione (*IV*)

TLC and HPLC analyses of the crude extract (1.63 g) obtained after processing a batch of 30 flasks for the transformation of 4-androstene-3,17-dione (**IV**), inoculated with *M881*, indicated the presence of four metabolites along with small amount of substrate **IV**. The metabolites [57 mg (**IV**), 338 mg, 472 mg, 198 mg, and 391 mg, $R_f$: 0.82 (**IV**), 0.64, 0.58, 0.56, and 0.32 system: I, $R_t$: 46.2 (**IV**), 26.1 min, 24.8 min, 21.0 min, and 12.5 min respectively] were purified over silica gel (230–400 mesh, 44 g) column chromatography by eluting with methanol/dichloromethane ($CH_2Cl_2$) gradient mixture. From the spectral data (Tables 1 and 2 and Supplement ST1) analyses and by comparing the data with earlier reports [1,25,28] the metabolites were identified as 6β-hydroxy-4-androstene-3,17-dione (**IVa**), 11α-hydroxy-4-androstene-3,17-dione (**IVb**), 7β-hydroxy-4-androstene-3,17-dione (**IVd**) and 6β,11α-dihydroxy-4-androstene-3,17-dione (**IVc**).

Time-course for the transformation of 4-androstene-3,17-dione (**IV**) revealed that in the early stages of incubation nearly 50% of **IV** was transformed into four metabolites (**IVa–d**) with 11α-hydroxy-4-androstene-3,17-dione (**IVb**) as major metabolite (Supplement Figs. S6 and S7). It was observed that on prolong incubation percentage of 6β,11α-dihydroxy metabolite was increased with decrease in the percentage of both 6β- and 11α-hydroxy metabolites (**IVa** and **IVb**) (Scheme 4).

### 3.5. Transformation of 19-nortestosterone (*V*)

Transformation of **V** (0.7 g/L) was carried out using 30 flasks inoculated with *M881* as described in experimental section. The crude extract (1.6 g) on TLC, HPLC and LCMS analyses indicated the presence of two metabolites with a trace amount of unmetabolized substrate **V**. The metabolites were purified over silica gel



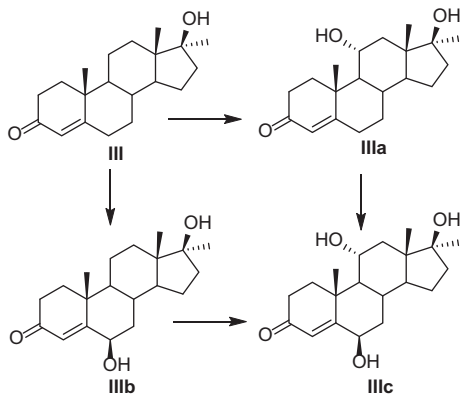

**Scheme 2.** Transformation of testosterone (**II**) into corresponding hydroxy derivatives by fungal strain *M881*.

column (230–400 mesh, 38 g) chromatography. Elution of the column with 1% of methanol in $CH_2Cl_2$ yielded a fraction containing substrate **V** (608 mg, $R_f$: 0.81, $R_t$: 22.8 min). Other two metabolites were then eluted as a mixture with 1.5% of methanol in $CH_2Cl_2$. Two metabolites were then purified using preparative HPLC (326 mg, 289 mg, $R_f$: 0.64, 0.64 system: II; $R_t$: 10.1 min, 11.8 min respectively) and were identified as 6β-hydroxy-19-nortestosterone (**Va**) and 10β-hydroxy-19-nortestosterone (**Vb**) from the spectral data analysis (Tables 1 and 2 and Supplement ST1). The spectral data for both the metabolites were in good agreement with the earlier report [26].

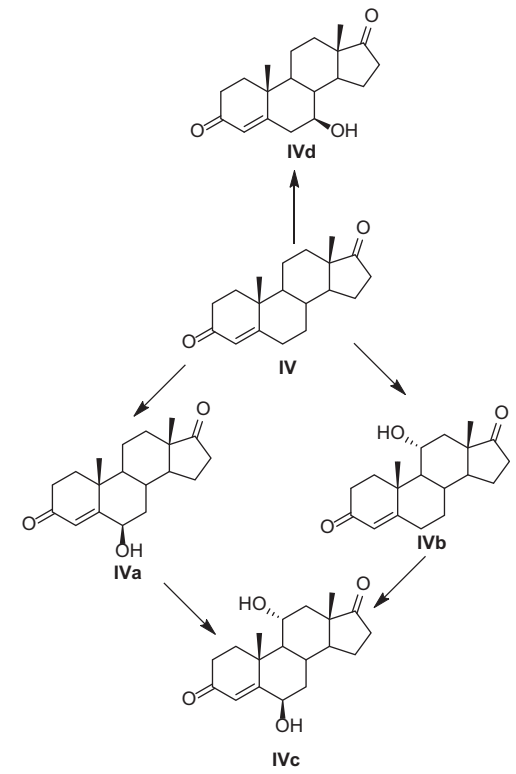
Time course experiment for the transformation of 19-nortestosterone (**V**) explained that fungal strain *M881* transformed 19-nortestosterone (**V**) into **Va** and **Vb** (Supplement Figs. S8 and S9). By prolonging the incubation period to 12 days, the transformation of **V** was increased to 97% with proportionate increase in the level of **Va** and **Vb** (Scheme 5). However, the formation of dihydroxy derivative (s) was not observed as seen with **I**–**IV**.

### 3.6. Studies on large-scale fermentation

Scale-up studies were carried out in shake flasks (600 mL media) containing progesterone (**I**) (0.7 g/L) or Testosterone (**II**) (1.0 g/L) as substrate. Flask was inoculated with *M881* and incubated on a rotary shaker. At the end of every 2 days the aliquots were drawn aseptically from flask, extracted and analyzed by HPLC and LC–MS. These results revealed that, the fungal system quantitatively converted **I** and **II** into corresponding dihydroxy



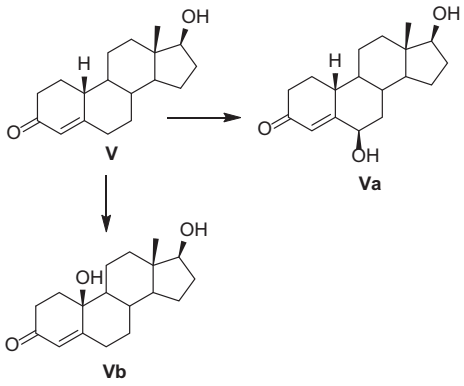

**Scheme 3.** Transformation of 17α-methyltestosterone (**III**) into corresponding hydroxyl derivatives by fungal strain *M881*.




**Scheme 4.** Transformation of 4-androstene-3,17-dione (**IV**) into corresponding hydroxy derivatives by fungal strain *M881*.

metabolites in 10 days of incubation. Similar results were obtained when fermentor was used with 5000 mL of CzapekDox media containing **I** (0.7 g/L) or **II** (1.0 g/L) (Fig. 3b and Supplement S3b). Thus, the fungal system, *M881* can be used for the large-scale production of 6β,11α-dihydroxy derivatives of **I**, **II**, **III**, and **IV** with fine tuning of the fermentation conditions.

In conclusion, the versatile fungal strain *Mucor* sp. (*M881*) could able to carry out the regio- and stereo- specific hydroxylation at 6β,11α-positions in steroids with 4-ene-3-one functionality. *M881* efficiently carried out hydroxylation at C-6β and C-11α position on progesterone (**I**), testosterone (**II**), 17α-methyltestosterone (**III**) and 4-androstene-3,17-dione (**IV**) to form corresponding 6β,11α-dihydroxy derivatives **Ib**, **IIc**, **IIIc** and **IVc** as a major metabolites. However the fungal system failed to carryout hydroxylation at 11α position when 19-nortestosterone (**V**) was used as a




**Scheme 5.** Transformation of 19-nortestosterone (**V**) into corresponding hydroxy derivatives by fungal strain *M881*.

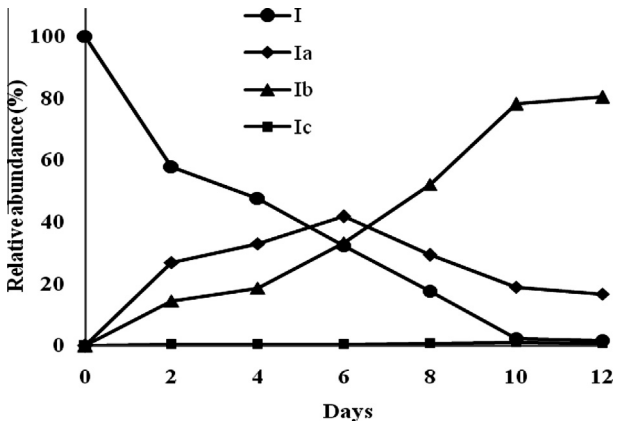



**Fig. 3b.** Time course study of progesterone (**I**) transformation in fermentor: (●) progesterone (**I**), (♦) 11α-hydroxyprogesterone (**Ia**), (▲) 6β,11α-dihydroxyprogesterone (**Ib**), (■) 6β-hydroxypregn-4-ene-3,11,20-trione (**Ic**).

substrate indicates that the methyl group at C10 position is necessary for the 11α-hydroxylation by *M881*. The large scale fermentation studies in 5 L fermentor with progesterone (**I**) and testosterone (**II**) clearly indicates that this fungal system can be used for large scale asymmetric synthesis of 6β,11α-dihydroxy derivatives of **I**, **II**, **III** and **IV**.

## Acknowledgment

S.K. and S.H. thank CSIR-New Delhi for the fellowship. This work was supported by CSIR Network grant (NWP0006, BSC0124, and CSC0106).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2013.08.004.

## References

[1] Al-Awadi S, Afzal M, Oommen S. Studies on *Bacillus stearothermophilus*. Part III. transformation of testosterone. Appl Microbiol Biotechnol 2003;62:48–52.
[2] Mahato SB, Garai S. Advances in microbial steroid biotransformation. Steroids 1997;62:332–45.
[3] Koenig HL, Schumacher M, Ferzaz B, Thi AN, Ressouches A, Guennoun R, Jung-Testas I, Robel P, Akwa Y, Baulieu EE. Progesterone synthesis and myelin formation by Schwann cells. Science 1995;268:1500–3.
[4] Traish AM, Saad F, Guay A. The dark side of testosterone deficiency: II. Type 2 diabetes and insulin resistance. J Androl 2009;30:23–32.
[5] Powers ML, Florini JR. Direct effect of testosterone on muscle cells in tissue culture. Endocrinology 1975;97:1043–7.
[6] Mor G, Eliza M, Song J, Wiita B, Chen S, Naftolin F. 17 alpha-Methyl testosterone is a competitive inhibitor of aromatase activity in jar choriocarcinoma cells and macrophage-like THP-1 cells in culture. J Steroid Biochem 2001;79:239–46.
[7] Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T, Lehmann JM. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell 1998;92:73–82.
[8] Ye M, Guo D. Substrate specificity for the 12 beta-hydroxylation of bufadienolides by *Alternaria alternata*. J Biotech 2005;117:253–62.
[9] Ma E, Choi T. An efficient 4 beta-hydroxylation of steroidal 5-en-3 beta-ols and 1,4-conjugation of steroidal 4-en-3-ones using $SeO_2$ oxidation. Bull Korean Chem Soc 2009;30:245–8.
[10] Ashley RL, Arreguin-Arevalo JA, Nett TM. Binding characteristics of the ovine membrane progesterone receptor alpha and expression of the receptor during the estrous cycle. Reprod Biol Endocrinol 2009;7:42.
[11] Spratt DI. Altered gonadal steroidogenesis in critical illness: is treatment with anabolic steroids indicated? Best Pract Res Clin Endocrinol 2001;15:479–94.
[12] Jayyosi Z, Cooper KO, Thomas PE. Brain cytochrome P450 and testosterone metabolism by rat brain subcellular fractions: presence of cytochrome P450 3A immunoreactive protein in rat brain mitochondria. Arch Biochem Biophys 1992;298:265–70.
[13] Hu S, Genain G, Azerad R. Microbial transformation of steroids-contribution to 14-alpha-hydroxylations. Steroids 1995;60:337–52.
[14] Madyastha KM. Preparative useful transformation of steroids and morphine alkaloids by *Muco piriformis*. P Indian AS Chem Sci 1994;106:1203–12.
[15] Elmonem A, Elrefai H, Sallam LA, Elkady IA. Studies on mechanism of progesterone transformation with *Rhizopus nigricans*. J Gen Appl Microbiol 1970;16:137–44.
[16] Peterson DH, Murray HC, Eppstein SH, Reineke LM, Weintraub A, Meister PD, Leigh HM. Microbiological transformations of steroids I. introduction of oxygen at carbon-11 of progesterone. J Am Chem Soc 1952;74:5933–6.
[17] Haldar S, Kolet SP, Thulasiram HV. Biocatalysis: fungi mediated novel and selective 12β- or 17β- hydroxylation on the basic limonoid skeleton. Green chem 2013;15:1311–7.
[18] Thulasiram HV, Madyastha KM. Transformation of a monoterpene ketone, piperitenone, and related terpenoids using *Mucor piriformis*. Can J Microbiol 2005;51:447–54.
[19] Bruker (2003). SADABS (Version 2.05), S. V., SAINT (Version 6.45) and SHELXTL (Version 6.14). Bruker AXS Inc., Madison, Wisconsin, USA.
[20] Sheldrick GM. A short history of SHELX. Acta Crystallogr A 2008;64:112–22.
[21] Macrae CF, Edgington PR, McCabe P, Pidcock E, Shields GP, Taylo R, Towler M, van De Streek J. Mercury: visualization and analysis of crystal structures. J Appl Crystallogr 2006;39:453–7.
[22] Farooq A, Hanson JR, Iqbal Z. Hydroxylation of progesterone by *Cephalosporum aphidicola*. Phytochemistry 1994;37:723–6.
[23] Al-Awadi S, Afzal M, Oommen S. Studies on *Geobacillus stearothermophilus*-part V: transformation of 11 alpha-hydroxyprogesterone. Biocatal Biotransform 2005;23:323–8.
[24] Faramarzi MA, Badiee M, Yazdi MT, Amini M, Torshabi M. Formation of hydroxysteroid derivatives from androst-4-en-3,17-dione by the filamentous fungus *Mucor racemosus*. J Mol Catal B-Enzym 2008;50:7–12.
[25] Xiong ZG, Wei Q, Chen HM, Chen SW, Xu WJ, Qiu GF, Liang SC. Hu XM Microbial transformation of androst-4-ene-3,17-dione by *Beauveria bassiana*. Steroids 2006;71:979–83.
[26] Huszcza E, Dmochowska-Gladysz J. Transformations of testosterone and related steroids in *Absidia glauca* culture. J Basic Microbiol 2003;43:113–20.
[27] Hanson JR, Nasir H, Parvez A. The hydroxylation of testosterone and some relatives by *Cephalosporium aphidicola*. Phytochemistry 1996;42:411–5.
[28] Holland HL, Thomas EM. Microbial hydroxylation od steroids 8. Incubation of Cn halo- and other substituted steroids with Cn hydroxylating fungi. Can J Chem 1982;60:160–4.