*Biocatalysis and Biotransformation*, 2013; 31(4): 168–174

informa
healthcare

**ORIGINAL ARTICLE**

# Biotransformation of 3β-hydroxy-5-en-steroids by *Mucor silvaticus*

YANJIE WANG[1], DONGMEI SUN[1], ZHIBAO CHEN[2], HONGSHENG RUAN[2] & WENZHONG GE[2]

[1]*Department of Biotechnology and* [2]*Department of Pharmaceutical Engineering, College of Life Science and Technology, Heilongjiang Bayi Agricultural University, Daqing, P. R. China*



**Abstract**

The biotransformation of four 3β-hydroxy-5-en-steroids with varying substituents at C-16 or/and C-17 by *Mucor silvaticus* was investigated. The characterization of the metabolites was performed by IR, MS, $^{1}$H NMR, $^{13}$C NMR, and 2-D NMR. All the examined substrates were transformed, mainly by 7α-hydroxylation. Studies carried out with *M. silvaticus* demonstrated the versatility of this organism in introducing hydroxyl groups at the 7α-, 9α-, 11α-, and 14α-positions in 3-ol-5-ene steroids. The relationships between the substrate structures and hydroxylated positions are also discussed.

**Keywords:** *Mucor silvaticus, biotransformation, steroids, hydroxylation*

## Introduction

Microbial hydroxylations are among the most extensively studied and useful transformations, since they can be achieved under mild conditions with high chemo-, regio-, and stereoselectivity. Biotransformation of steroids allows for the production of important pharmaceutical intermediates which are difficult to synthesize by chemical methods (Donova & Egorova 2012, Bhatti & Khera 2012). Many microorganisms such as *Mucor racemosus* (Ge et al. 2008, Li et al. 2005), *Mucor piriformis* (Madyastha & Joseph 1995), *Mucor plumbeus* (Lamm et al. 2007), *Fusarium culmorum* (Kolek 1999), *Fusarium oxysporum* var. *cubense* (Peart et al. 2011, Wilson et al. 1999), *Cunninghamella elegans* (Choudhary et al. 2005), *Gibberella fujikuroi* (Choudhary et al. 2005), *Colletotrichum lini* (Romano et al. 2006), *Rhizopus stolonifer* (Choudhary et al. 2003), *Bacillus* strains (Schaaf & Dettner 2000), *Cephalosporium aphidicola* (Bensasson et al. 1998), *Circinella* sp. (Voishvillo et al. 1994), and *Mycobacterium* sp. (Sripalakit et al. 2006) have been used for the transformation of 3β-hydroxy-5-en-steroids. *M. racemosus* (Ge et al. 2008, Li et al. 2005) and *M. piriformis* (Madyastha & Joseph 1995) have generally shown the ability to carry out 7α-hydroxylation of 5-en-3β-ol steroids, while *M. plumbeus* could transform steroid substrates such as dehydroepiandrosterone, pregnenolone (Lamm et al. 2007), bufalin (Ye et al. 2005), and cinobufagin (He et al. 2006) by hydroxylation at different positions of the steroid core.

The fungus *Mucor silvaticus* was found to form ethylene in the presence of methionine (Lindberg et al. 1979), and more frequently was detected on carrots (Lugauskas et al. 2005). There is no report on the biotransformation of steroids by this organism.

During our screening procedure for isolation of microorganisms capable of converting various steroid compounds, it was found that *M. silvaticus* converted a series of 3β-hydroxy-5-en-steroids into hydroxylated products.

## Materials and methods

### *Instrumental methods*

Melting points (mp) were determined on a TX5 melting point apparatus and were uncorrected. Infrared (IR) spectra were recorded using KBr discs on a Bruker Vector-22 spectrometer. Mass spectra (MS) were obtained on an Esquire 3000 mass spectrometer by electrospray ionization (ESI). The $^{1}$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were obtained using a Bruker Avance

Correspondence: Wenzhong Ge, Department of Pharmaceutical Engineering, College of Life Science and Technology, Heilongjiang Bayi Agricultural University, Daqing, 163319, P. R. China. Tel: +86 459 6818606. Fax: +86 459 6819876. E-mail: wenzhongg@126.com

*(Received 6 February 2013; revised 14 April 2013; accepted 6 June 2013)*

ISSN 1024-2422 print/ISSN 1029-2446 online © 2013 Informa UK, Ltd.
DOI: 10.3109/10242422.2013.813490

DPX-400 spectrometer at 400 and 100 MHz, respectively, with Tetramethylsilane (TMS) as internal standard in DMSO-$d_6$. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS. Coupling constants ($J$) are given in hertz (Hz). Thin layer chromatography (TLC) was performed on 0.25 mm thick layer of silica gel G. Chromatography was performed with ethyl acetate/petroleum ether (bp 60–90°C) (3:1) and visualized by spraying the plates with 50% sulfuric acid solution and heating in an oven at 100°C for 3 min until the color developed.

### *Materials*

The 3β-hydroxy-5-en-steroids (dehydroepiandrosterone, pregnenolone, 16-dehydro-pregnenolone, and 16α, 17α-epoxy-3β-hydroxy-pregn-5-en-20-one) were of chemical grade and obtained from Hunan Steroid Chemicals Co., Ltd, China. Silica gel G (100–200 mesh) was purchased from Qingdao Marine Chemical Factory, China. All chemicals and solvents were of analytical grade and obtained from Shenyang Chemical Company, China.

### *Maintenance and growth of microorganism*

The strain of *M. silvaticus* AF 93204 was obtained from the China Center for Type Culture Collection. The strain was maintained on potato-2%-dextrose agar slope, grown at 28°C, stored at 4°C, and freshly subcultured before use in the transformation experiment.

### *Incubation and biotransformation conditions*

Ten 500 ml Erlenmeyer flasks, each containing 100 ml of sterilized potato-2%-dextrose broth, were inoculated with freshly obtained spores from agar slope cultures and incubated for 2 days at 28°C in a rotary shaker (150 rpm). The 3β-hydroxy-5-en-steroid substrates (1.0 g) were each dissolved in 20 ml acetone. To each 500 ml Erlenmeyer flasks, 2 ml of the acetone solution was added. Incubation was continued for 4 days under the same conditions.

### *Product isolation and analysis*

All of the fermentation media were exhaustively extracted with ethyl acetate and filtered to separate the broth from the mycelium. After the extract had been evaporated under reduced pressure, the residue was separated by silica gel column chromatography using ethyl acetate/petroleum ether (3:1) as eluant to obtain the metabolites, which were identified by melting points and a combination of IR, MS, and two-dimensional NMR.

### *Transformation of 3β-hydroxy-5-androsten-17-one (1)*

Elution with ethyl acetate/petroleum ether (2:1) gave three metabolites.

3β, 7α-dihydroxy-androst-5-en-17-one **(5)**, mp: 179.4–181.7°C; IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3364, 3301, 2930, 2855, 1728, 1660, 1629, 1460, 1380, 1289, 1202, 1036; $^1$H NMR (DMSO-$d_6$) δ (ppm): 3.56 (m, H-3), 5.64 (dd, $J$ = 3.6, 4.2 Hz, H-6), 3.97 (dd, $J$ = 3.6, 7.6 Hz, H-7), 0.90 (s, H-18), 1.08 (s, H-19); MS m/z: 327 $[M+Na]^+$, 343 $[M+K]^+$; and Yield: 38.4%.

3β, 7α, 17β-trihydroxy-androst-5-en **(6)**, mp: 271.6–273.1°C; IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3432, 2934, 2920, 2855, 1663, 1462, 1435, 1026; $^1$H NMR (DMSO-$d_6$) δ (ppm): 3.31 (m, H-3), 5.41 (d, $J$ = 4.4 Hz, H-6), 3.58 (br. s, H-7), 3.44 (t, $J$ = 8.8 Hz, H-17), 0.63 (3H, s, H-18), 0.90 (3H, s, H-19); MS m/z: 329 $[M+Na]^+$, 345 $[M+K]^+$; and Yield: 14.7%.

3β, 7β, 17β-trihydroxy-androst-5-en **(7)**, mp: 251.2~252.4°C; IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3291, 2959, 2936, 2904, 2884, 2849, 1472, 1457; $^1$H NMR (DMSO-$d_6$) δ (ppm): 3.26 (m, H-3), 5.13 (s, H-6), 3.55 (t, $J$ = 7.6 Hz, H-7), 3.40 (m, H-17), 0.62 (3H, s, H-18), 0.96 (3H, s, H-19); MS m/z: 329 $[M+Na]^+$, 345 $[M+K]^+$; and Yield: 6.2%.

### *Transformation of 3β-hydroxy-pregn-5-en-20-one (2)*

Elution with ethyl acetate/petroleum ether (3:2) gave three metabolites. The original spectra of compound **9** are shown in Supplementary Figures.

3β, 7α-dihydroxy-pregn-5-en-20-one **(8)**, mp: 183.8–184.3°C; IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3420, 2935, 1698, 1661, 1458, 1433, 1358, 1229, 1186, 1054; $^1$H NMR (DMSO-$d_6$) δ (ppm): 3.59 (m, H-3), 5.62 (dd, $J$ = 1.2, 5.2 Hz, H-6), 3.87 (m, H-7), 2.61 (t, $J$ = 9.2 Hz, H-17), 0.64 (3H, s, H-18), 1.00 (3H, s, H-19), 2.14 (3H, s, H-21); MS m/z: 355 $[M+Na]^+$, 371 $[M+K]^+$; and Yield: 40.3%.

3β, 7α, 11α-trihydroxy-pregn-5-en-20-one **(9)**, mp: 268.1–269.1°C; IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3328, 2969, 2936, 1700, 1463, 1357, 1234, 1191, 1157, 1046, 1024; $^1$H NMR (DMSO-$d_6$) δ (ppm): 3.33 (m, H-3), 5.44 (d, $J$ = 5.4 Hz, H-6), 3.56 (d, $J$ = 8.5 Hz, H-7), 3.81 (dd, $J$ = 5.6, 10.3 Hz, H-11), 2.59 (t, $J$ = 9.1 Hz, H-17), 0.50 (3H, s, H-18), 1.00 (3H, s, H-19), 2.07 (3H, s, H-21); MS m/z: 371 $[M+Na]^+$; and Yield: 15.6%.

3β, 7α, 9α-trihydroxy-pregn-5-en-20-one **(10)**, mp: 239.6–241.2°C; IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3511, 3291, 2960, 2933, 2897, 1693, 1635, 1381, 1364, 1064, 1025; $^1$H NMR (DMSO-$d_6$) δ (ppm): 3.32 (m, H-3), 5.47 (d, $J$ = 5.2 Hz, H-6), 3.74 (dd, $J$ = 4.4, 9.2 Hz, H-7), 2.66 (t, $J$ = 8.8 Hz, H-17), 0.52 (3H, s, H-18), 0.99 (3H, s, H-19), 2.08 (3H, s, H-21);

MS m/z: 371 $[M+Na]^+$, 387 $[M+K]^+$; and Yield: 8.1%.

*Transformation of 3β-hydroxy-pregn-5, 16 (17)-dien-20-one (3)*

Elution with ethyl acetate/petroleum ether (3:1) gave four metabolites.

3β, 7α-dihydroxy-pregn-5, 16 (17)-dien-20-one **(11)**, mp: 112.1–112.8°C; IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3480, 3415, 3366, 3309, 2934, 1660, 1588, 1460, 1432, 1372, 1233, 1055, 1019; $^1$H NMR (DMSO-$d_6$) δ (ppm): 3.32 (m, H-3), 5.43 (d, $J$ = 4.8 Hz, H-6), 3.69 (m, H-7), 6.90 (m, H-16), 0.82 (3H, s, H-18), 0.93 (3H, s, H-19), 2.21 (s, H-21); MS m/z: 353 $[M+Na]^+$, 369 $[M+K]^+$; and Yield: 17.6%.

3β, 7α, 11α-trihydroxy-pregn-5, 16-dien-20-one **(12)**, mp: 274.3–275.6°C; IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3493, 3368, 2973, 2936, 2874, 1646, 1457, 1436, 1376, 1051, 1024; $^1$H NMR (DMSO-$d_6$) δ (ppm): 3.30 (m, H-3), 5.45 (d, $J$ = 5.2 Hz, H-6), 3.67 (dd, $J$ = 5.6, 8.8 Hz, H-7), 1.36 (H-9), 3.93 (m, H-11), 1.16 (H-12), 2.52 (H-12), 6.90 (t, $J$ = 1.2 Hz, H-16), 0.80 (3H, s, H-18), 1.03 (3H, s, H-19), 2.21 (3H, s, H-21); MS m/z: 369 $[M+Na]^+$, 385 $[M+K]^+$; and Yield: 8.9%.

3β, 7α, 9α-trihydroxy-pregn-5, 16-dien-20-one **(13)**, mp: 261.4–263.1°C; IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3347, 2938, 1662, 1648, 1457, 1437, 1373, 1053, 1019; $^1$H NMR (DMSO-$d_6$) δ (ppm): 3.28 (m, H-3), 5.48 (dd, $J$ = 1.2, 4.8 Hz, H-6), 3.82 (br. s, H-7), 6.93 (t, $J$ = 1.2 Hz, H-16), 0.84 (3H, s, H-18), 1.02 (3H, s, H-19), 2.23 (3H, s, H-21); MS m/z: 369 $[M+Na]^+$, 385 $[M+K]^+$; and Yield: 15.4%.

3β, 14α-dihydroxy-pregn-5, 16-dien-7, 20-dione **(14)**, mp: 196.3–197.8°C; IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3361, 3230, 2946, 2755, 1750, 1640, 1619, 1467, 1371, 1275, 1202, 1024; $^1$H NMR (DMSO-$d_6$) δ (ppm): 3.41 (m, H-3), 5.62 (d, $J$ = 2.0 Hz, H-6), 6.83 (t, $J$ = 1.6 Hz, H-16), 0.93 (3H, s, H-18), 1.13 (3H, s, H-19), 2.22 (3H, s, H-21); MS m/z: 367 $[M+Na]^+$, 383 $[M+K]^+$; and Yield: 13.2%.

*Transformation of 16α, 17α -epoxy-3β-hydroxy-pregn-5-en-20-one (4)*

Elution with ethyl acetate/petroleum ether (1:1) gave two metabolites.

3β, 7α, 11α-trihydroxy-16α, 17α-epoxy-pregn-5-en-20-one **(15)**, mp: 251.2–253.7°C; IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3484, 3292, 2973, 2934, 2891, 1693, 1458, 1437, 1419, 1375, 1362, 1062, 1044; $^1$H NMR (DMSO-$d_6$) δ (ppm): 3.30 (m, H-3), 5.43 (d, $J$ = 5.6 Hz, H-6), 3.55 (br. s, H-7), 1.29 (H-9), 3.87 (m, H-11), 1.18 (H-12), 2.14 (H-12), 3.94 (s, H-16), 0.93 (3H, s, H-18), 1.01 (3H, s, H-19), 1.97 (3H, s, H-21); MS m/z: 385 $[M+Na]^+$, 401 $[M+K]^+$; and Yield: 22.6%.

3β, 11α-dihydroxy-16α, 17α-epoxy-pregn-5-en-7, 20-dione **(16)**, mp: 280.5–282.1°C; IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3528, 3409, 2966, 2941, 1703, 1459, 1377, 1361, 1299, 1078, 1059; $^1$H NMR (DMSO-$d_6$) δ (ppm): 3.38 (m, H-3), 5.65 (s, H-6), 3.98 (m, H-11), 3.93 (t, $J$ = 4.8 Hz, H-16), 0.95 (3H, s, H-18), 1.25 (3H, s, H-19), 1.97 (3H, s, H-21); MS m/z: 383 $[M+Na]^+$, 399 $[M+K]^+$; and Yield: 8.3%.

The $^{13}$C NMR signals of the compounds are presented in Table I.

Table I. $^{13}$C NMR signals for the substrates and the metabolites of 3β-hydroxy-5-ene steroids (δ ppm).

| *C* | *1* | *5* | *6* | *7* | *2* | *8* | *9* | *10* |
|---|---|---|---|---|---|---|---|---|
| 1 | 37.2 | 35.4 | 36.7 | 36.5 | 37.3 | 37.0 | 38.4 | 29.0 |
| 2 | 31.5 | 31.4 | 31.4 | 31.6 | 31.6 | 31.6 | 31.6 | 31.3 |
| 3 | 71.4 | 69.9 | 69.9 | 69.9 | 71.7 | 71.3 | 70.2 | 69.3 |
| 4 | 42.2 | 42.2 | 42.2 | 41.8 | 42.3 | 42.2 | 42.8 | 42.3 |
| 5 | 141.3 | 144.0 | 143.8 | 141.4 | 140.9 | 146.3 | 144.6 | 141.2 |
| 6 | 120.8 | 124.4 | 124.6 | 127.3 | 121.4 | 123.7 | 124.3 | 123.5 |
| 7 | 31.5 | 62.6 | 63.1 | 71.8 | 31.8 | 65.2 | 63.5 | 64.4 |
| 8 | 31.5 | 37.1 | 37.6 | 39.9 | 31.9 | 37.4 | 37.1 | 38.5 |
| 9 | 50.3 | 41.9 | 41.8 | 48.3 | 50.1 | 41.9 | 47.8 | 74.7 |
| 10 | 36.7 | 37.1 | 36.9 | 36.2 | 36.6 | 37.4 | 38.5 | 42.4 |
| 11 | 20.4 | 19.8 | 20.1 | 20.5 | 21.1 | 20.7 | 67.2 | 26.4 |
| 12 | 30.8 | 31.2 | 36.3 | 36.8 | 38.9 | 38.2 | 49.1 | 34.0 |
| 13 | 47.5 | 46.6 | 42.0 | 42.8 | 44.0 | 43.8 | 43.6 | 43.4 |
| 14 | 51.8 | 44.7 | 43.8 | 50.8 | 57.0 | 49.7 | 49.5 | 45.0 |
| 15 | 21.8 | 21.5 | 23.0 | 30.2 | 24.5 | 24.4 | 23.7 | 23.7 |
| 16 | 35.8 | 36.7 | 30.0 | 25.7 | 22.9 | 22.9 | 22.6 | 22.4 |
| 17 | 221.3 | 220.2 | 80.3 | 80.0 | 63.8 | 63.5 | 62.8 | 62.6 |
| 18 | 13.2 | 13.1 | 11.1 | 11.4 | 13.2 | 13.0 | 14.1 | 12.3 |
| 19 | 19.4 | 18.1 | 18.0 | 18.9 | 19.4 | 18.2 | 17.7 | 21.4 |
| 20 | – | – | – | – | 209.4 | 209.6 | 208.7 | 208.7 |
| 21 | – | – | – | – | 31.5 | 31.3 | 31.3 | 31.4 |

| *C* | *3* | *11* | *12* | *13* | *14* | *4* | *15* | *16* |
|---|---|---|---|---|---|---|---|---|
| 1 | 36.7 | 36.3 | 38.1 | 28.9 | 36.0 | 37.1 | 38.1 | 38.0 |
| 2 | 29.6 | 31.2 | 31.5 | 31.2 | 31.2 | 31.5 | 31.5 | 31.3 |
| 3 | 69.9 | 69.6 | 70.0 | 69.2 | 69.0 | 71.6 | 70.0 | 69.6 |
| 4 | 42.1 | 42.0 | 42.8 | 42.3 | 41.9 | 42.2 | 42.8 | 42.4 |
| 5 | 141.6 | 143.9 | 144.8 | 141.4 | 168.2 | 141.1 | 144.7 | 167.2 |
| 6 | 120.0 | 124.2 | 124.0 | 123.5 | 125.6 | 120.9 | 124.0 | 124.1 |
| 7 | 31.3 | 63.4 | 63.6 | 64.7 | 199.2 | 31.3 | 63.1 | 200.4 |
| 8 | 30.9 | 35.4 | 35.6 | 37.0 | 46.9 | 29.7 | 35.0 | 38.9 |
| 9 | 49.9 | 41.5 | 48.3 | 75.2 | 43.2 | 50.3 | 48.3 | 55.2 |
| 10 | 36.2 | 36.8 | 38.5 | 42.5 | 37.9 | 36.6 | 40.9 | 40.2 |
| 11 | 20.1 | 19.8 | 67.0 | 26.5 | 19.3 | 30.4 | 66.9 | 66.6 |
| 12 | 34.3 | 34.1 | 45.6 | 30.6 | 25.4 | 31.4 | 42.3 | 41.9 |
| 13 | 45.4 | 45.2 | 45.7 | 45.8 | 52.1 | 41.5 | 38.6 | 41.6 |
| 14 | 55.8 | 49.5 | 49.7 | 45.1 | 80.7 | 45.5 | 39.0 | 42.2 |
| 15 | 31.7 | 31.7 | 31.5 | 31.6 | 43.0 | 27.5 | 26.4 | 28.7 |
| 16 | 145.1 | 145.7 | 146.0 | 146.0 | 144.4 | 60.5 | 60.8 | 60.9 |
| 17 | 154.2 | 154.2 | 154.2 | 154.4 | 150.6 | 71.0 | 70.2 | 69.3 |
| 18 | 15.5 | 15.4 | 16.8 | 15.0 | 20.3 | 15.1 | 15.9 | 16.0 |
| 19 | 18.9 | 17.7 | 17.7 | 21.4 | 17.0 | 19.3 | 17.7 | 16.8 |
| 20 | 196.3 | 196.1 | 196.2 | 196.3 | 196.6 | 204.9 | 205.0 | 205.0 |
| 21 | 26.9 | 26.9 | 27.1 | 27.1 | 27.2 | 25.9 | 25.9 | 25.9 |

## Results

The following substrates were examined: 3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone; DHEA) **(1)**, 3β-hydroxy-pregn-5-en-20-one (PRG) **(2)**, 3β-hydroxy-pregn-5, 16 (17)-dien-20-one (16-dehydro-pregnenolone) **(3)**, and 16α, 17α-epoxy-3β-hydroxy-pregn-5-en-20-one **(4)**. The courses of transformation of the substrates are shown in Figure 1.

The mass spectrum of metabolite **5** showed a quasimolecular ion in $[M + Na]^+$ at m/z 327, which suggested that one oxygen atom was incorporated into the substrate. The presence of signals at δ 124.4 and δ 144.0 ppm in the $^{13}C$ NMR spectrum of Compound 5 and signal at δ 5.64 (dd, $\mathcal{J} = 3.6$, 4.2 Hz, H-6) ppm in the $^1H$ NMR spectrum showed that the C5–C6 double bond had been retained. The $^1H$ NMR spectrum showed a new downfield signal for the oxygen-bearing methine proton at δ 3.97 (dd, $\mathcal{J} = 3.6$, 7.6 Hz) ppm, which indicated introduction of a C-7 hydroxyl group in this compound. The hydroxyl group at C-7 was also confirmed by the connectivity between H-7 and the olefinic H-6 (δ 5.64 ppm) in the $^1H$-$^1H$ COSY spectrum of **5** and downfield shifts were observed for C-6 (δ 124.4 ppm) and C-8 (δ 37.1 ppm) and a γ–gauche upfield shift for C-9 (δ 41.9 ppm).

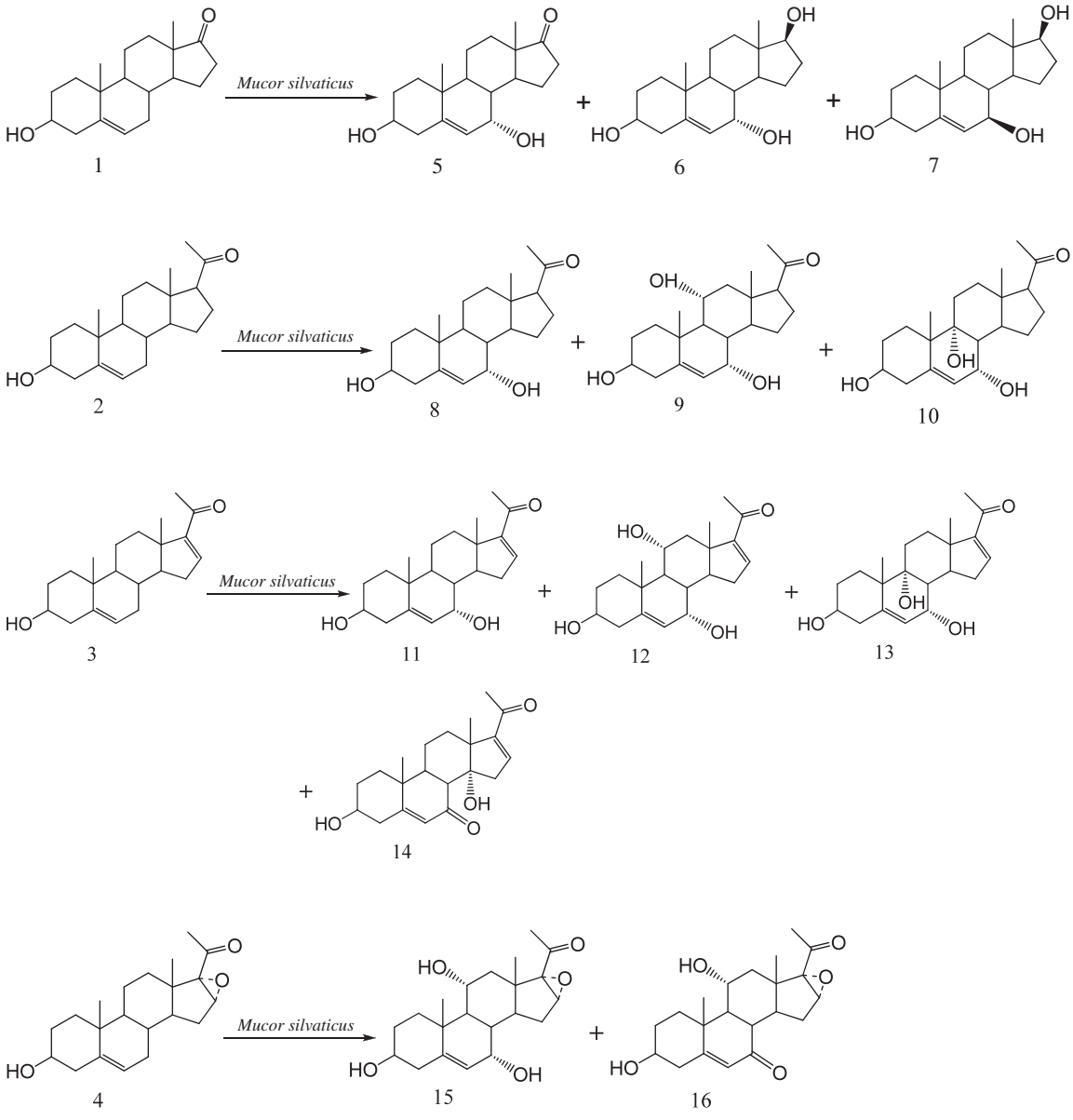


Figure 1. Transformation of 3β-hydroxysteroids by *Mucor silvaticu*.

The IR spectrum of **6** confirmed that the characteristic absorption for the 17-carbonyl had disappeared and the $^{13}$C NMR spectrum showed a new oxygen-bearing methine carbon signal at δ 80.3 ppm, which indicated that the 17-carbonyl group had been reduced. Interpretation of the HMBC spectrum revealed correlation between $H_3$-18 (δ 0.63 ppm) and the carbon at δ 80.3 ppm, which suggested that the signal at δ 80.3 ppm was assigned to C-17. The presence of 17β-hydroxyl group was confirmed by the presence of a characteristic signal for 17α-H at δ 3.44 (t, $\mathcal{J} = 8.8$ Hz) ppm which was easily deduced to be resonating peak of H-17 from the HSQC spectrum. The presence of a 7-hydroxyl group was deduced from the connectivity between H-7 (δ 3.58 ppm) and the olefinic H-6 (δ 5.41 ppm, d, $\mathcal{J} = 4.4$ Hz) in the $^1$H-$^1$H COSY spectrum, and was also confirmed by the HMBC correlations between H-7 and the carbons at δ 143.8 and δ 124.6 ppm, respectively.

The $^1$H NMR spectrum of **7** showed an olefinic proton signal at δ 5.13 ppm and two new signals at δ 3.55 (t, $\mathcal{J} = 7.6$ Hz, H-7) and 3.40 (m, H-17) ppm, which indicated the presence of hydroxyl substituents at C-7 and C-17. The $^{13}$C NMR spectrum also showed two new methine carbon signals at δ 71.8 (C-7) and δ 80.0 (C-17) ppm. The HMBC spectrum exhibited couplings of H-7 with C-6 (δ 127.3 ppm) and C-8 (δ 39.9 ppm). The vicinal coupling constant $\mathcal{J}_{6,7\alpha}$ was too small to be noted, which meant that the hydroxyl group attached at C-7 of Compound **7** has an equatorial orientation.

The IR spectrum of Compound **8** showed characteristic absorption at 3420, 1698, and 1661 cm$^{-1}$ for hydroxyl, saturated ketone, and double bond groups, respectively. The hydroxyl group at C-7 was deduced from HMBC correlations between H-7 (δ 3.87 ppm) and the carbons at δ 146.3 and δ 123.7 ppm, which was also supported by the connectivity between H-7 (δ 3.87 ppm) and the olefinic H-6 (δ 5.62 ppm, dd, $\mathcal{J} = 1.2$, 5.2 Hz) in the $^1$H-$^1$H COSY spectrum of **8**.

The mass spectrum of **9** showed a quasimolecular ion in $[M + Na]^+$ at m/z 371, suggesting the addition of 32 mass units to **2**. In the $^1$H NMR spectrum, two new downfield signals were observed for oxygen-bearing methine protons at δ 3.56 (d, $\mathcal{J} = 8.5$ Hz) and δ 3.81 ppm, which indicated the hydroxyl groups introduced in Compound **2** were at C-7α and C-11α. For hydroxylation at C-11, a new signal indicative of an oxygen-bearing methine carbon appeared at δ 67.2 ppm, and downfield shifts were observed for C-12 (δ 49.1 ppm) and a γ–gauche upfield shift for C-13 (δ 43.6 ppm).

The spectra of $^{13}$C NMR and DEPT of metabolite **10** showed a new oxygen-bearing methine carbon signal at δ 64.4 ppm (C-7) and a tertiary carbon at δ 74.7 ppm (C-9). The presence of 9-hydroxyl group was deduced from HMBC correlations between $H_3$-19 (δ 0.99 ppm) and the carbons at δ 141.2 (C-5) and δ 74.7 ppm, respectively. The HSQC spectrum of **10** showed no related signal was found in the $^1$H NMR spectrum for its proton related to C-9 which indicated the insertion of a hydroxyl group added to a tertiary carbon. The hydroxyl group at C-9 was also confirmed by the downfield shifts for C-8 (δ 38.5 ppm), C-10 (δ 42.4 ppm), C-11 (δ 26.4 ppm), and a γ–gauche upfield shift for C-12 (δ 34.0 ppm).

The presence of signals at δ 5.43 (d, $\mathcal{J} = 4.8$ Hz, H-6) and δ 6.90 (m, H-16) ppm in the $^1$H NMR spectrum of Compound **11** and signals at δ 143.9, 124.2, 145.7, and 154.2 ppm in the $^{13}$C NMR spectrum showed that the two double bonds C5–C6 and C16–C17 had been retained. The hydroxyl group at C-7 was deduced by the connectivity between H-7 (δ 3.69 ppm) and the olefinic H-6 (δ 5.43 ppm) in the $^1$H-$^1$H COSY spectrum of **11**.

In the $^1$H NMR spectrum of Compound **12**, two new downfield signals were observed for oxygen-bearing methine protons at δ 3.67 (dd, $\mathcal{J} = 5.6$, 8.8 Hz) and δ 3.93 ppm, which indicated the hydroxyl groups introduced in Compound **3** were at C-7α and C-11α. The HMQC spectrum showed connectivity of δ 3.93 ppm with C-11 (δ 67.0 ppm), while the HMBC spectrum showed cross peaks between C-11 and H-9 (δ 1.36 ppm), $H_2$-12 (δ 1.16, 2.52 ppm), and also correlation of H-11 (δ 3.93 ppm) with C-9 (δ 48.3 ppm).

The mass spectrum of **13** showed a quasimolecular ion in $[M + Na]^+$ at m/z 369, suggesting the addition of 32 mass units to **3**. The HMBC spectrum of **13** showed the correlations between $H_3$-19 (δ 1.02 ppm) and the carbons at δ 141.4 (C-5) and δ 75.2 ppm, respectively, which indicated a hydroxyl group introduced into Compound **3** at C-9α. Substitution at C-9 was further supported in the product $^{13}$C NMR spectrum by the downfield shifts for C-8 (δ 37.0 ppm) which shared with C-10 (δ 42.5 ppm), C-11 (δ 26.5 ppm) and a γ–gauche upfield shift for C-12 (δ 30.6 ppm).

The $^{13}$C NMR data coupled with the DEPT and HSQC spectra showed the presence of two carbonyls and two double bonds in Compound **14**. The presence of a 14α-hydroxyl group was deduced from HMBC correlations between $H_3$-18 (δ 0.93 ppm) and the carbons at δ 80.7 and δ 150.6 (C-17) ppm, respectively. The hydroxyl group at C-14 was also supported by the downfield shifts for C-8 (δ 46.9 ppm),

C-13 (δ 52.1 ppm) and C-15 (δ 43.0 ppm). The large shielding effects experienced by the olefinic carbons suggested that the double bond C5–C6 was in conjugation with a carbonyl. The 7-carbonyl group was supported by the observed HMBC correlations between H-6 and C-7 (δ 199.2 ppm) and C-5 (δ 168.2 ppm).

The mass spectrum of metabolite **15** showed a quasimolecular ion in $[M + Na]^+$ at m/z 385, which suggested that two oxygen atoms were incorporated into the substrate. The chemical shift values of two oxygenated carbons at δ 60.8 and 70.2 ppm indicated the presence of an epoxy group. The Compound **15** was identified by two new downfield signals in the $^1$H NMR spectrum of the metabolite at δ 3.55 and δ 3.87 ppm, indicating hydroxylation at C-7 and C-11. The $^{13}$C NMR spectrum, in comparison to **4**, demonstrated a downfield shift for C-12 (δ 42.3 ppm) and a γ–gauche upfield shift for C-13 (δ 38.6 ppm). The OH-bearing methine proton signal at δ 3.87 ppm was assigned to C-11, which showed homonuclear couplings ($^1$H-$^1$H COSY) with $H_2$-12 (δ 1.18, 2.14 ppm) and H-9 (δ 1.29 ppm), while long-range $^1$H-$^{13}$C correlations of C-11 (δ 66.9 ppm) with H-9 (δ 1.29 ppm) and H-12 (δ 1.18 ppm) were observed in HMBC spectrum of **15**.

A new signal indicative of an oxygen-bearing methine carbon appeared at δ 66.6 ppm, and downfield shifts were observed for C-9 (δ 55.2 ppm) and C-12 (δ 41.9 ppm) in the $^{13}$C NMR spectrum of Compound **16**, and a new downfield signal was also observed at δ 3.98 ppm in the $^1$H NMR spectrum, which indicated the hydroxylation at C-11. The large shielding effects experienced by the olefinic carbons suggested that the double bond C5–C6 was in conjugation with a carbonyl. The 7-carbonyl group was also supported by the HMBC correlations between H-6 (δ 5.65 ppm) and the carbons at δ 200.4 (C-7) and δ 167.2 (C-5) ppm.

## Discussion

The biotransformation of 3β-hydroxy-5-en-steroids with varying substituents at C-16 and/or C-17, that is, dehydroepiandrosterone (DHEA), pregnenolone, 16-dehydropregnenolone, and 16α, 17α-epoxy-pregnenolone in *M. silvaticus* culture showed that all substrates were mainly hydroxylated at the 7α position, depending on the structure of the substrate.

Studies carried out with *M. silvaticus* demonstrated the versatility of this organism in introducing hydroxyl groups at the 7α-, 9α-, 11α-, and 14α-positions in 3-ol-5-ene steroids and reduction of the carbonyl group of dehydroepiandrosterone occurred.

For the C-21 steroids, a second hydroxylation step may occur in a secondary position (11α- and/or 9α-), again producing polyhydroxylated derivatives; this may be related to the presence of C-17 acetyl side chain. The 9α-hydroxylated steroids are important intermediates in the synthesis of highly effective fluorinated anti-inflammatory remedies, which could be obtained by microbial oxidation of natural sterols (Sukhodolskaya et al. 2007, Donova et al. 2005) or by the transformation of steroids (Faramarzi et al. 2008, Angelova et al. 1996).

It is interesting to note that the minor 14α-hydroxylation, which occurs on a tertiary carbon atom, was observed in the transformation of **3**, which rarely occurs in microbial transformation of 3β-hydroxy-5-en-steroids (Hu et al. 1995). A possible bioconversion sequence from substrate **3** is of allylic hydroxylation at C-7, which was hydroxylated at C-14, followed by the oxidation of the 7α-hydroxyl group yielding 14.

Dehydroepiandrosterone **(1)** was transformed by *M. silvaticus* in a different way; apart from the hydroxylation at the 7-position giving rise to both α and β-epimers, reduction of the 17-keto group was also observed. The other substrates did not undergo carbonyl reduction. Mucor strains such as *M. piriformis* (Madyastha & Joseph 1995) and *M. plumbeus* (Lamm et al. 2007) have the ability to reduce the C-17 carbonyl group. It seems that the enzyme involved in 17-carbonyl reduction does not act on the 20-carbonyl group in the presence of C-17 acetyl side chain in the mentioned strain.

Fermentation of pregnenolone with *M. silvaticus* was shown to produce 3β, 7α-dihydroxy-pregn-5-en-20-one **(8)** and 3β, 7α, 11α-trihydroxy-pregn-5-en-20-one **(9)**. This is consistent with previous studies when the substrate was exposed to *M. racemosus* (Ge et al. 2008) and *M. piriformis* (Madyastha & Joseph 1995), respectively.

There are only a few reports describing biotransformation of 16α, 17α -epoxy-3β-hydroxy-pregn-5-en-20-one **(4)**. In our previous study on substrate **4** with *M. racemosus* (Ge et al. 2008), metabolic products of 3β, 7α-dihydroxy-16α-methyloxy-pregn-5-en-20-one and 3β, 7α-dihydroxy-16α, 17α-epoxy-pregn-5-en-20-one were observed. Another report showed the hydroxylation of Compound **4** by *Circinella sp.* (Voishvillo et al. 1994) at 7α, 9α-positions. In the present study, the monohydroxylated product was not available.

In conclusion, these results show that the structure of the investigated 3β-hydroxy-5-en-steroids plays an important role in the hydroxylation position, and that this strain of *M. silvaticus* could be employed for the regioselective hydroxylation of steroids of interest in the pharmaceutical industry.

***Declaration of interest***: The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

Financial support was provided by Natural Science Foundation of Heilongjiang Province of China (B200812) and Program of Science and Technology Innovation Teams Building in Heilongjiang Province (2012TD006).

## References

Angelova B, Mutafov S, Avramova T, Dimova I, Boyadjieva L. 1996. 9α-Hydroxylation of 4-androstene-3,17-dione by resting *Rhodococcus* sp. cells. Process Biochem 31:179–184.

Bensasson CM, Hanson JR, Hunter AC. 1998. The hydroxylation of $\Delta^5$-androstenes by *Cephalosporium aphidicola*. Phytochemistry 49:2355–2358.

Bhatti HN, Khera RA. 2012. Biological transformations of steroidal compounds: a review. Steroids 77:1267–1290.

Choudhary MI, Batool I, Shah SAA, Nawaz SA, Atta-ur-Rahman. 2005. Microbial hydroxylation of pregnenolone derivatives. Chem Pharm Bull 53:1455–1459.

Choudhary MI, Shah SAA, Musharraf SG, Shaheen F, Atta-ur-Rahman. 2003. Microbial transformation of dehydroepiandrosterone. Nat Prod Res 17:215–220.

Donova MV, Egorova OV. 2012. Microbial steroid transformations: current state and prospects. Appl Microbiol Biotechnol 94:1423–1447.

Donova MV, Gulevskaya SA, Dovbnya DV, Puntus IF. 2005. *Mycobacterium sp.* Mutant strain producing 9α-hydroxyandrostenedione from sitosterol. Appl Microbiol Biotechnol 67: 671–678.

Faramarzi MA, Aghelnejad M, Tabatabaei Yazdi M, Amini M, Hajarolasvadi N. 2008. Metabolism of androst-4-en-3, 17-dione by the filamentous fungus *Neurospora crassa*. Steroids 73:13–18.

Ge WZ, Wang SM, Shan LH, Nan Li, Liu HM. 2008. Transformation of 3β-hydroxy-5-en-steroids by *Mucor racemosus*. J Mol Cat B Enzym 55:37–42.

He X, Tang J, Qiao A, Wang G, Jiang M, Liu R, Yiao X. 2006. Cytotoxic biotransformed products from cinobufagin by *Mucor spinosus* and *Aspergillus Niger*. Steroids 71: 392–402.

Hu SH, Genain G, Azerad R. 1995. Microbial transformation of steroids: contribution to 14α-hydroxylations. Steroids 60: 337–352.

Kolek T. 1999. Biotransformation XLVII: transformations of 5-ene steroids in *Fusarium culmorum* culture. J Steroid Biochem Mol Biol 71:83–90.

Lamm AS, Chen ARM, Reynolds WF, Reese PB. 2007. Steroid hydroxylation by *Whetzelinia sclerotiorum*, *Phanerochaete chrysosporium* and *Mucor plumbeus*. Steroids 72: 713–722.

Li HP, Liu HM, Ge WZ, Huang LH, Shan LH. 2005. Syntheses of 7α-hydroxy-dehydroepiandrosterone and 7β-hydroxy-dehydroepiandrosterone. Steroids 70:970–973.

Lindberg T, Granhall U, Berg B. 1979. Ethylene formation in some coniferous forest soils. Soil Biol Biochem 11:637–643.

Lugauskas A, Repeckiene J, Novosinskas H. 2005. Micromycetes, producers of toxins, detected on stored vegetables. Ann Agric Environ Med 12:253–260.

Madyastha KM, Joseph T. 1995. Transformation of dehydroepiandrosterone and pregnenolone by *Mucor piriformis*. Appl Microbiol Biotechnol 44:339–343.

Peart PC, McCook KP, Russell FA, Reynolds WF, Reese PB. 2011. Hydroxylation of steroids by *Fusarium oxysporum*, *Exophiala jeanselmei* and *Ceratocystis paradoxa*. Steroids 76: 1317–1330.

Romano A, Romano D, Ragg E, Costantino F, Lenna R, Gandolfi R, Molinari F. 2006. Steroid hydroxylations with *Botryodiplodia malorum* and *Colletotrichum lini*. Steroids 71: 429–434.

Schaaf O, Dettner K. 2000. Transformation of steroids by *Bacillus* strains isolated from the foregut of water beetles (Coleoptera: Dytiscidae): II. Metabolism of 3β-hydroxypregn-5-en-20-one (pregnenolone). J Steroid Biochem Mol Biol 75: 187–199.

Sripalakit P, Wichai U, Saraphanchotiwitthaya A. 2006. Biotransformation of various natural sterols to androstenones by *Mycobacterium* sp. and some steroid-converting microbial strains. J Mol Cat B Enzym 41:49–54.

Sukhodolskaya GV, Nikolayeva VM, Khomutov SM, Donova MV. 2007. Steroid-1-dehydrogenase of *Mycobacterium* sp. VKM Ac-1817D strain producing 9α-hydroxy-androst-4-ene-3, 17-dione from sitosterol. Appl Microbiol Biotechnol 74: 867–873.

Voishvillo NE, Istomina ZI, Kamernitsky AV. 1994. Transformed steroids 195. Introduction of the 9α-hydroxygroup into $\Delta^5$-3β-hydroxysteroids by *Circinella* sp. mold. Russ Chem Bull 43: 689–695.

Wilson MR, Gallimore WA, Reese PB. 1999. Steroid transformations with *Fusarium oxysporum* var. *cubens*e and *Colletotrichum musae*. Steroids 64:834–843.

Ye M, Han J, Tu G, An D, Guo D. 2005. Microbial hydroxylation of bufalin by *Cunninghamella blakesleana* and *Mucor spinosus*. J Nat Prod 68:626–628.

## Supplementary materials available online

Supplementary: Spectra of Compound **9**