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Microbial transformation of oxandrolone with *Macrophomina phaseolina* and *Cunninghamella blakesleeana*

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ABSTRACT

Microbial transformation of oxandrolone (**1**) was carried out by using *Cunninghamella blakesleeana* and *Macrophomina phaseolina*. Biotransformation of **1** with *M. phaseolina* yielded four new metabolites, 11β,17β-dihydroxy-17α-(hydroxymethyl)-2-oxa-5α-androstan-3-one (**2**), 5α,11β,17β-trihydroxy-17α-methyl-2-oxa-androstan-3-one (**3**), 17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3,11-dione (**4**), and 11β,17β-dihydroxy-17α-methyl-2-oxa-5α-androstan-3-one (**5**). Whereas a new metabolite, 12β,17β-dihydroxy-17α-methyl-2-oxa-5α-androstan-3-one (**6**), was obtained through the microbial transformation of oxandrolone (**1**) with *C. blakesleeana*. The structures of isolated metabolites were characterized on the basis of MS and NMR spectroscopic data.

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1. Introduction

Biocatalysis has been extensively applied to the stereoselective synthesis of chiral molecules. The reactions catalyzed through biocatalysis are regio- and stereo-selective, and such reactions are rarely feasible through chemical methods [1–6]. Microorganisms contain factories of enzymes, which can catalyze a number of chemical reactions, such as oxidation, reduction, hydroxylation, and Michael addition. Microorganisms, particularly fungi, contains cytochrome P450 monooxygenase system, which is responsible for the stereoselective hydroxylation at various sites of steroidal skeletons. Stereoselective hydroxylation of steroids at non activated positions can be achieved with fungi. Fungi are also used as microbial models of mammalian steroid drug metabolism [7–9].

The anabolic–androgenic steroids are derivatives of testosterone. They increase the muscles tissues, and body mass [10]. Oxandrolone (17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one), (**1**) is a synthetic dehydrotestosterone derivative in which C-2 is replaced with an oxygen atom. It has a low androgenic and anabolic effects. Oxandrolone (**1**) does not aromatize and does not affect the production of testosterone in body in low dose (10 mg). In addition, oxandrolone is also used a for the treatment of number of ailments, such as HIV-related weight and muscle loss, trauma, severe burn injury, alcoholic hepatitis, and neuromuscular disorders, for more than 30 years [11–14].

In continuation to our study on the synthesis of bioactive compounds [15–18], and investigation on the metabolism of anabolic steroids through microbial transformation [19–21], we incubated oxandrolone (**1**) with *Macrophomina phaseolina* and *Cunninghamella blakesleeana*. This yielded five new metabolites. Four new metabolites **2**–**5** were obtained from the microbial transformation of **1** with *M. phaseolina* (Fig. 1), and a new metabolite **6** was obtained from the biotransformation of **1** with *C. blakesleeana* (Fig. 2).

2. Experimental

2.1. General experimental conditions

Oxandrolone (**1**) was procured from Hangzhou DayangChem (China). Sabouraud dextrose agar was obtained from Merck KGaA (Cat. No. 146392, Germany). Thin layer chromatography (TLC) was carried out on saturated plates (Merck KGaA, PF_{254}, Germany). Flash silica gel (E. Merck, Germany) was used for

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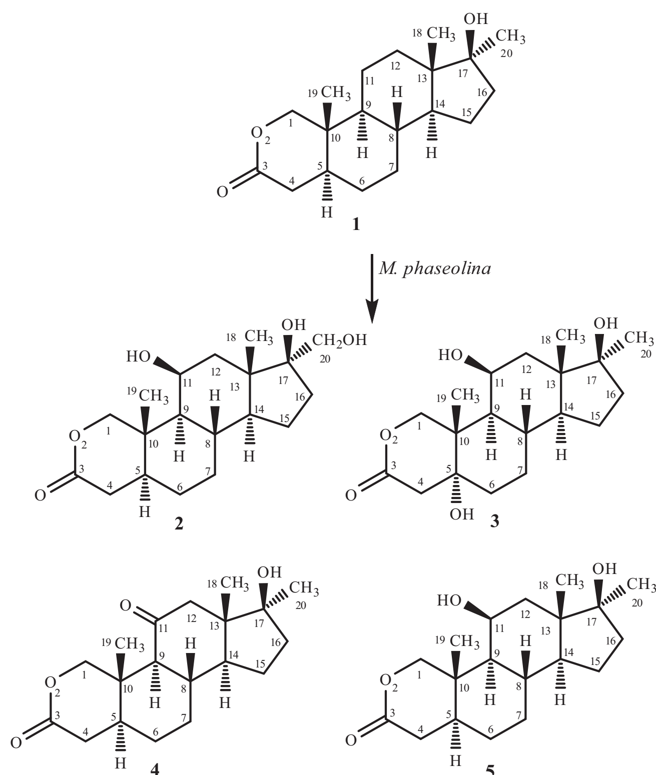

Fig. 1. Biotransformation of oxandrolone (**1**) with *Macrophomina phaseolina*.

column chromatography. Isolated compounds were purified on a reverse phase recycling preparative HPLC–LC–908, equipped with JAIGEL–ODS–L–80 column (L = 250 mm, I.D. = 20 mm). Melting points were determined on a Buchi M-560 melting point apparatus. Optical rotations were measured with a JASCO P-2000 polarimeter. ^{1}H-NMR spectra were recorded in CD_3OD on Bruker Avance-NMR (600 MHz) spectrometer, whereas ^{13}C-NMR spectra were recorded in CD_3OD on Bruker Avance-NMR (75, 100 and 150 MHz) spectrometers. Jeol JMS-600H (Japan) mass spectrometer was used to record EI-MS and HREI-MS of isolated compounds. HRESI-MS were measured on QSTAR XL mass spectrometer. JEOL TMS-HX110 (Japan) mass spectrometer was used for recording HRFAB-MS. IR Spectra were recorded on a Vector 22 spectrophotometer (Bruker). All the reagents, solvents, and media were used in this experiment were of analytical grade.

2.2. Fungal culture and medium

The fungal culture of *C. blakesleeana* (ATCC 8688A) and *M. phaseolina* (KUCC 730) were purchased from the American Type Culture Collection (ATCC), and Karachi University Culture Collections (KUCC). The fungal strains were grown on Sabouraud dextrose agar, and stored at 4 °C.

The culture medium for *C. blakesleeana* (ATCC 8688A) and *M. phaseolina* (KUCC 730) was prepared in distilled water by mixing the following ingredients, glucose (10.0 g), peptone (5.0 g), KH_2PO_4 (5.0 g), NaCl (5.0 g), and glycerol (10.0 mL) for each liter.

2.3. Fermentation and extraction conditions of oxandrolone (1) with M. phaseolina

The microbial culture medium (4 L) was prepared by mixing aforementioned ingredients, dispensed equally into 40 Erlenmeyer flasks of 250 mL (each flask containing 100 mL of the medium), and autoclaved at 121 °C. Flasks were inoculated with fungal culture and placed on a shaker (121 rpm) at 26 ± 2 °C for incubation. When suitable growth was observed, 1 g of compound **1** was dissolved in the 40 mL of MeOH, and distributed equally among all flasks. The flasks were then placed on shaker (121 rpm) at 26 ± 2 °C for 19 days. Two control experiments were run simultaneously. In one experiment only the microbial culture was used, whereas second experiment was carried out by adding substrate **1** to a flask that is containing all ingredients of medium without fungal culture. These control experiments were used to evaluate the transformation of oxandrolone (**1**).

The degree of transformation was analyzed on TLC. In 19 days most of the substrate **1** was consumed, therefore the fermentation process was stopped. After the completion of cultivation process, the microbial mass was separated by filtration, and washed with dichloromethane (DCM). The collective organic and aqueous material was extracted with DCM three times. The DCM extract was dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to obtain 1.9 g of the brown gum. The crude extract was separated over silica gel column. The mobile phase comprises of gradient of dichloromethane–methanol mixture. The polarity of solvent system was increased gradually by adding methanol (1%) in CH_2Cl_2. The fractions of similar polarity were pooled together. As a result, four major fractions (1–4) were obtained. These fractions were subjected to reversed-phase recycling HPLC. Compound **2** (25 mg, R_T = 12 min.) was obtained from fraction 1 by reverse phase recycling HPLC, with methanol–water (70:30) as mobile phase. Compound **3** (10 mg, R_T = 20 min.) was purified from fraction 2, using methanol–water (75:25) as the mobile phase. Similarly, fraction 3 yielded compound **4** (15 mg, R_T = 26 min) on elution with methanol–water (75:25). Compound **5** (30 mg R_T = 22 min) was obtained from fraction 4 using 60% methanol–water as the mobile phase. During purification process, we also recovered some unused oxandrolone (**1**) along with several minor transformed products, which we could not purify due to their very low quantities.

2.3.1. 11β,17β-Dihydroxy-17α-(hydroxymethyl)-2-oxa-5α-androstan-3-one (2)

White solid; m. p. 279–281 °C; $[\alpha]_D^{25}$ = −126.2 (*c* 0.018, MeOH); IR (KBr): υ_{max} 3550 (O–H stretching), 1617 (C=O stretching); HRFAB-MS m/z 339.2195 $[M+H]^+$ (mol. formula, $C_{19}H_{31}O_5$, calcd.

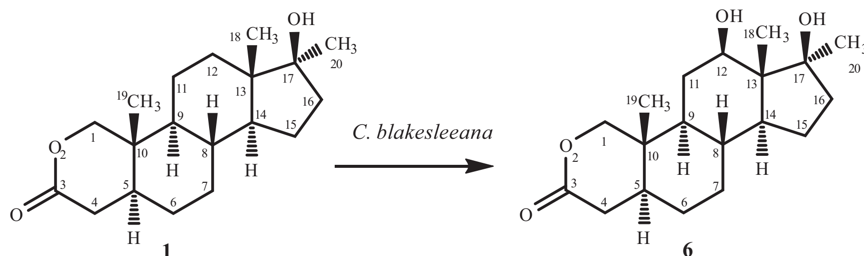

Fig. 2. Biotransformation of oxandrolone (**1**) with *Cunninghamella blakesleeana*.

339.2171); ^{1}H-NMR (CD_3OD, 600 MHz); Table 1; ^{13}C-NMR (CD_3OD, 150 MHz); Table 2.

*2.3.2. 5α,11β,17β-Trihydroxy-17α-methyl-2-oxa-androstan-3-one (**3**)*

White solid; m. p. 287–288 °C; $[\alpha]_D^{25}$ = 62.5 (*c* 0.01, MeOH); IR ($CHCl_3$): υ_{max} 3550 (O–H stretching), 1617 (C=O stretching); HRESI-MS *m/z* 339.2165 [M+H]$^+$ (mol. formula, $C_{19}H_{31}O_5$, calcd. 339.2171); ^{1}H-NMR (CD_3OD, 600 MHz); Table 1; ^{13}C-NMR (CD_3OD, 150 MHz); Table 2.

*2.3.3. 17β-Hydroxy-17α-methyl-2-oxa-5α-androstan-3,11-dione (**4**)*

White solid; m. p. 243–245 °C; $[\alpha]_D^{25}$ = −257.5 (*c* 0.02, MeOH); IR ($CHCl_3$): υ_{max} 3512 (O–H stretching), 1708 (C=O stretching); HREI-MS *m/z* 320.1889 [M]$^+$ (mol. Formula $C_{19}H_{29}O_4$, calcd. 320.1988); ^{1}H-NMR (CD_3OD, 600 MHz) Table 1; ^{13}C-NMR (CD_3OD, 75 MHz) Table 2.

*2.3.4. 11β,17β-Dihydroxy-17α-methyl-2-oxa-5α-androstan-3-one (**5**)*

White solid; m. p. 287–289 °C; $[\alpha]_D^{25}$ = −25 (c 0.01, $CHCl_3$), IR ($CHCl_3$); υ_{max} 3783 (O–H stretching), 1722 (C=O stretching); HREI-MS *m/z* 322.2158 [M]$^+$ (mol. formula, $C_{19}H_{30}O_4$, calcd. 322.2144); ^{1}H-NMR (CD_3OD, 600 MHz) Table 1; ^{13}C-NMR (CD_3OD, 150 MHz) Table 2.

*2.4. Biotransformation oxandrolone (**1**) with C. blakesleeana*

Same ingredients were used for culture medium of *C. blakesleeana*, as described for *M. phaseolina*. *C. blakesleeana* was grown in 40 flasks (each flask contain 100 mL of culture medium). Oxandrolone (**1**) 1.0 g was dissolved in 40 mL of methanol, and evenly distributed among 40 flasks of 4-day old cultures of *C. blakesleeana*. Fermentation was continued for 18 days on a rotatory shaker (121 rpm) at 26 ± 2 °C. The biotransformation reaction was compared through control experiments that were run simultaneously. Only a limited quantity of substrate **1** was transformed by this fungus, as monitored by periodic TLC analysis.

After fermentation, fungal mass was separated by filtration and washed with dichloromethane (DCM). The collective organic and aqueous layers were extracted with DCM (three times). The organic layer of DCM extract was dried over anhydrous Na_2SO_4, and concentrated under reduced pressure on a rotary evaporator. The concentrated extract was subjected to silica gel column chromatography using gradient hexanes-acetone as solvent system. Fractions of similar polarity were combined to a major fraction, which yielded compound **6** (8 mg, R_T = 15 min.) on purification with reverse phase recycling HPLC, using methanol–water (70:30) as solvent system. Most of the substrate **1** recovered while purifying the metabolite **6**. Several minor transformed products were also observed on TLC. We could not isolate the minor products due to their very low quantities.

*2.4.1. 12β,17β-Dihydroxy-17α-methyl-2-oxa-5α-androstan-3-one (**6**)*

White solid; m. p. 195–196 °C; $[\alpha]_D^{25}$ = −55 (*c* 0.01, MeOH), IR ($CHCl_3$); υ_{max} 3389.2 (O–H stretching), 1666 (C=O stretching); HREI-MS *m/z* 322.2147 [M]$^+$ (mol. formula, $C_{19}H_{30}O_4$, calcd. 322.2144); ^{1}H-NMR ($CDCl_3$, 600 MHz) Table 1; ^{13}C-NMR (CD_3OD, 100 MHz) Table 2.

2.5. Results and discussion

Fermentation of oxandrolone (**1**, $C_{19}H_{30}O_3$) with *M. phaseolina* afforded four new metabolites **2**–**5**, whereas the biotransformation of **1** with *C. blakesleeana* yielded new metabolite **6**.

Compound **2** was isolated as a white solid. The molecular ion peak [M+H]$^+$ appeared in HRFAB-MS at *m/z* 339.2195 (calcd. 339.2171), consistent with the formula $C_{19}H_{31}O_5$. This showed the addition of two oxygen atoms in substrate **1**. The IR absorbances at 3474, 1735, 1699, 1637, and 1617 cm^{-1} were due to the hydroxyl and lactone, respectively. A new methine proton signal at δ 4.05 (d, $J_{11a,12e}$ = 3 Hz), and methylene proton signals at δ 3.55 (d, *J* = 11.1 Hz), and 3.36 (d, *J* = 11.1 Hz) appeared in the ^{1}H-NMR spectrum of compound **2**, indicated hydroxylation. Two new downfield carbon signals, methine (δ 68.2) and methylene (δ 67.6), in the ^{13}C-NMR spectrum, supported dihydroxylation. The COSY-45° spectrum showed cross-peaks between the new methine proton (δ 4.05) and H-9 (δ 0.89) and H_2-12 (δ 1.73, 1.45). This indicated the presence of an OH at C-11. The H_2-12 (δ 1.73, 1.45) showed HMBC correlations with newly formed methine carbon (δ 68.2), further suggested an OH at C-11 (Fig. 3). The C-20 methylene protons (δ 4.45, 4.10) showed HMBC correlations with C-17 (δ 84.7), and C-16 (δ 32.7). This indicated the second OH at C-20. The H-11 (δ 4.05) showed the NOESY correlation with H-9 (δ 0.89). As H-9 is α-oriented in substrate **1**, its correlations with H-11 suggested that H-11 is also α-oriented (Fig. 4). Therefore a β-hydroxylation was inferred at C-11. Thus the structure of new metabolite **2** was deduced as 11β,17β-dihydroxy-17α-(hydroxymethyl)-2-oxa -5α-androstan-3-one.

Compound **3** was isolated as a white solid. The [M+H]$^+$ appeared in HRESI-MS (+ve) at *m/z* 339.2165 ($C_{19}H_{31}O_5$, calcd. 339.2171), which indicated an addition of two oxygens in substrate **1**. The absorptions in IR spectrum indicated the presence hydroxyl groups (3550 cm^{-1}), lactone (1735 cm^{-1}), and ketonic carbonyl (1637 cm^{-1}). A new downfield methine proton appeared at δ 3.98 (q, $J_{11a,9a}$ = $J_{11a,12a}$ = 6 Hz, $J_{11a,12e}$ = 3.5 Hz) in the ^{1}H-NMR spectrum. The ^{13}C-NMR spectrum also supported the presence of two hydroxyl groups (δ 68.5 and δ 72.6). The new methine proton (δ 3.98) showed cross-peaks with H_2-12 (δ 1.69, 1.42) in the COSY-DFQF spectrum, suggested the hydroxylation at C-11. The H_2-12 (δ 1.69, 1.42) and H-9 (δ 1.61) showed HMBC correlations with methine carbon (δ 68.5) (Fig. 3). This suggested a hydroxylation at C-11. The H-19 (δ 1.40), and H-4 (δ 2.73, 2.36), showed HMBC correlations with C-5 (δ 72.6). Therefore, second OH was placed at C-5. The β stereochemistry of OH at C-11 was inferred on the basis of NOESY correlations of αH-9 (δ 1.61) with H-11 (δ 3.98) (Fig. 4). The OH (δ 4.81) at C-5 showed NOESY correlation with α-oriented H-9 (δ 1.61) suggesting an α OH at C-5. The new metabolite **3** was identified as 5α,11β,17β-trihydroxy-17α-methyl-2-oxa-androstan-3-one.

Compound **4** was isolated as a white amorphous solid. The HREI-MS exhibited the [M]$^+$ at *m/z* 320.1889 (calcd. 320.1988), corresponding to the formula $C_{19}H_{29}O_4$. The increase of 14 amu suggested oxidation of substrate **1**. The IR spectrum showed the absorbance for hydroxyl (3512 cm^{-1}) and lactone carbonyl (1708 cm^{-1}). The ^{1}H-NMR spectrum showed downfield methylene protons resonating at δ 2.30 d ($J_{12a,12e}$ = 12.6 Hz) and 2.10 ($J_{12e,12a}$ = 12.6 Hz). The ^{13}C-NMR spectrum showed a new downfield carbonyl carbon signal at δ 212.8, which indicated the oxidation of compound **1**. H-9 (δ 1.94) and H_2-12 (δ 2.30, 2.10) showed HMBC correlations with newly formed carbonyl carbon (δ 212.8), which suggested oxidation at C-11 (Fig. 3). The stereochemistry of compound **4** was found similar to substrate **1**. The new compound **4** was thus identified as 17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3,11-dione.

Compound **5** was isolated in a white crystalline form. The HREI-MS showed the [M]$^+$ at *m/z* 322.2158 (calcd. 322.2144), in agreement with $C_{19}H_{30}O_4$. The IR spectrum showed the presence of OH group (3783 cm^{-1}) and carbonyl functionality (1722 cm^{-1}). The ^{1}H-NMR spectrum showed a new methine proton signal at δ 4.08 (d, $J_{11a,12e}$ = 3 Hz), suggested hydroxylation of substrate **1**. The ^{13}C-NMR spectrum showed a new downfield carbon signal (δ 68.2), which further supported the presence of an OH in compound

Table 1
^{1}H-NMR (CD_3OD, 600 MHz) chemical shift assignments (δ in ppm, J in Hz) of compounds **1–6**.

Position	**1**	**2**	**3**	**4**	**5**	**6**
1	4.25 d ($J_{1a,1e}$ = 10.5), 4.01 d ($J_{1e,1a}$ = 10.5)	4.46 d ($J_{1a,1e}$ = 10.5), 4.10 d, ($J_{1e,1a}$ = 10.5)	4.55 d ($J_{1a,1e}$ = 10.0), 4.31 d ($J_{1e,1a}$ = 10.0)	4.81 d ($J_{1a,1e}$ = 10.8), 3.91 d, ($J_{1e,1a}$ = 10.8)	4.11 d ($J_{1a,1e}$ = 10.5), 4.46 d, ($J_{1e,1a}$ = 10.5)	4.22 d ($J_{1a,1e}$ = 10.5), 4.01 d ($J_{1e,1a}$ = 10.8)
2	–	–	–	–	–	–
3	–	–	–	–	–	–
4	2.49 dd ($J_{4a,4e}$ = 18.9, $J_{4a,5a}$ = 6.0) 2.23 dd ($J_{4e,4a}$ = 18.9, $J_{4e,5a}$ = 12.9)	2.49 dd ($J_{4a,4e}$ = 18.9, $J_{4a,5a}$ = 6.3), 2.25 dd ($J_{4e,4a}$ = 18.9, $J_{4e,5a}$ = 12.3)	2.73 d ($J_{4a,4e}$ = 18.5), 2.36 d ($J_{4e,4a}$ = 18.5)	2.40 dd ($J_{4a,4e}$ = 18.6, $J_{4a,5a}$ = 5.4), 2.20 dd ($J_{4e,4a}$ = 19.2, $J_{4e,5a}$ = 13.2)	2.22 dd ($J_{4a,4e}$ = 19.2, $J_{4a,5a}$ = 6.6), 2.48 dd ($J_{4e,4a}$ = 18.9, $J_{4e,5a}$ = 12.3)	2.50 dd ($J_{4a,4e}$ = 18.9, $J_{4a,5a}$ = 6.0), 2.26 dd, ($J_{4e,4a}$ = 18.9, $J_{4e,5a}$ = 12.9)
5	1.77 m	1.45 m	–	1.77 m	1.76 m	1.77 m
6	1.46 m, 1.23 m	1.42 m, 1.26 m	1.85 m, 1.62 m	1.48 dq ($J_{6a,5a}$ = $J_{6a,7a}$ = 13.2, $J_{6a,7e}$ = 3.6), 1.27 td ($J_{6e,7e}$ = 12.6, $J_{6e,7a}$ = 3.6)	1.81 m, 1.87 m	1.48, 1.21 m
7	1.74 m, 0.95 dq ($J_{7a,6a}$ = $J_{7a,8a}$ = 13.2, $J_{7a,6e}$ = 4.2)	1.92 ddd ($J_{7a,6a}$ = $J_{7a,8a}$ = 14.4, $J_{7a,7e}$ = 9.6, $J_{7a,6e}$ = 6.0) 1.88 m	1.61 m, 1.69 m	1.86 m, 1.18 dq ($J_{7a,6a}$ = $J_{7a,8a}$ = 12.6, $J_{7a,6e}$ = 3.6)	1.27 m, 1.29 m	1.75 m, 1.45 m
8	1.49 m	0.98 dq ($J_{8a,7a}$ = $J_{8a,9a}$ = $J_{8a,14a}$ = 13.2, $J_{8a,7e}$ = 4.2)	1.35 m	1.81 m	0.98 dq ($J_{8a,7a}$ = $J_{8a,9a}$ = $J_{8a,14a}$ = 13.2, $J_{8a,7e}$ = 4.2)	1.43 m
9	0.85 dd ($J_{9a,8a}$ = $J_{9a,11a}$ = 12.0, $J_{9a,11e}$ = 4.5)	0.89 dd ($J_{9a,8a}$ = 11.1, $J_{9a,11e}$ = 4.2)	1.61 m	1.94 m	0.89 dd ($J_{9a,8a}$ = 11.4, $J_{9a,11e}$ = 4.2)	0.97 td ($J_{9a,8a}$ = $J_{9a,11a}$ = 11.4, $J_{9a,11e}$ = 4.4)
10	–	–	–	–	–	–
11	1.47 m, 1.37 m	4.07 d ($J_{11a,12e}$ = 3.0)	3.99 q ($J_{11a,9a}$ = $J_{11a,12a}$ = 6.0, $J_{11a,12e}$ = 3.5)	–	4.08 d ($J_{11a,12e}$ = 3.0)	1.46 m, 0.92 dd ($J_{11a,9a}$ = $J_{11a,12a}$ = 12.6, $J_{11a,11e}$ = 4.2)
12	1.52 m, 1.32 m	1.73 m, 1.45 m	1.69 dd ($J_{12a,12e}$ = 13.5, $J_{12a,11a}$ = 6.0), 1.47 dd ($J_{12e,12a}$ = 13.5, $J_{12e,11a}$ = 3.5)	2.30 d ($J_{12a,12e}$ = 12.6), 2.10 d ($J_{12e,12a}$ = 12.6)	1.67 dd ($J_{12a,11a}$ = 13.8 $J_{12a,12e}$ = 2.4), 1.46 m	3.68 dd ($J_{12a,11a}$ = 12.6, $J_{12a,11e}$ = 6.9)
13	–	–	–	–	–	–
14	1.24 m	1.28 m	1.25 m	1.91 m	1.20 m	1.18 m
15	1.59 m, 1.25 m	1.61 m, 1.36 m	1.16 m, 1.18 m	1.73 m, 1.38 dq ($J_{15a,14a}$ = $J_{15a,16a}$ = 12.6, $J_{15a,16e}$ = 6.0)	1.62 m, 1.54 m	1.62 m, 1.37 m
16	1.83 m, 1.62 m	1.89 m, 1.59 m	1.81 m, 1.41 m	1.91 m, 1.80 m	1.63 m, 1.85 m	1.83 m, 1.64 m
17	–	–	–	–	–	–
18	0.85 s	1.09 s	1.07 s	0.77 s	1.07 s	0.89 s
19	1.01 s	1.23 s	1.40 s	1.14 s	1.24 s	1.01 s
20	1.15 s	3.55 d (J = 11.1), 3.36 d (J = 11.1)	1.09 s	1.24 s	1.13 s	1.31 s

Table 2
^{13}C-NMR chemical shift assignments of (δ in ppm) compounds **1–6**.

Position	**1**[a]	**2**[b]	**3**[c]	**4**[d]	**5**[e]	**6**[f]
1	82.6	81.9	77.8	80.8	81.9	82.2
2	–	–	–	–	–	–
3	174.2	174.1	173.9	173.4	174	173.6
4	34.5	34.2	42.6	34.2	34.2	34.5
5	41.2	42.2	72.6	41.0	42.2	41.2
6	28.0	27.9	38.9	27.7	32.6	28.1
7	31.9	33.2	26.3	32.3	27.9	31.4
8	36.9	33.2	33.2	38.6	33.4	36.0
9	50.7	54.5	46.2	60.6	54.6	49.2
10	35.5	35.7	39.7	35.6	35.9	35.7
11	22.0	68.2	68.5	212.8	68.2	31.1
12	32.4	41.4	41.6	51.5	41.3	73.9
13	46.6	45.8	45.9	50.6	45.9	48.4
14	51.5	53.6	52.9	50.3	53.1	50.7
15	24.2	24.7	24.4	23.7	24.3	23.9
16	38.9	32.7	32.6	39.1	38.9	39.1
17	82.3	84.7	82.7	80.8	82.6	82.7
18	14.6	17.2	16.9	15.5	16.8	9.1
19	10.3	12.5	16.9	10.2	12.4	10.3
20	26.0	67.6	24.0	26.1	26.2	25.9

[a] 75 MHz.
[b] 150 MHz.
[c] 150 MHz.
[d] 75 MHz.
[e] 150 MHz.
[f] 100 MHz.

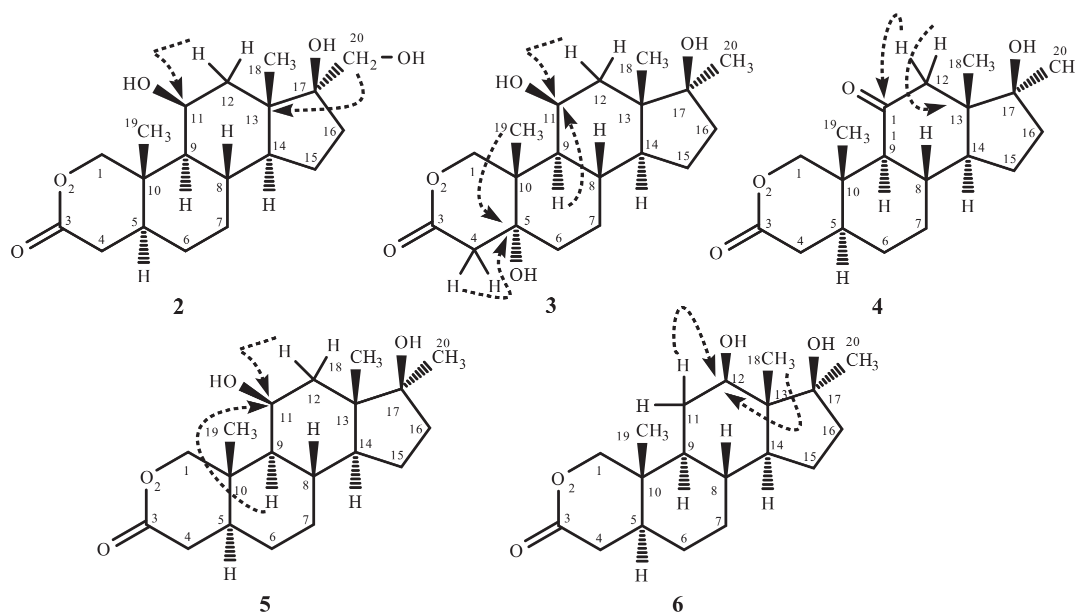

Fig. 3. Key HMBC correlations in compounds **2–6**.

5. The HMBC correlations of H_2-12 (δ 1.67, 1.46) and H-9 (δ 0.089) with C-11 (δ 68.2) supported hydroxylation at this site (Fig. 3). The stereochemistry of compound **5** was deduced by NOESY correlations of H-11 (δ 4.08) with α-oriented H-9 (δ 0.89), suggesting the α-orientation of H-11 (Fig. 4). Therefore, β-hydroxylation was inferred at C-11. Thus the structure of compound **5** was identified as 11β,17β-dihydroxy-17α-methyl-2-oxa-5α-androstan-3-one.

Compound **6** was isolated in a white crystalline form. The addition of an OH group in compound **6** was inferred from the $[M]^+$ at m/z 322.2147 ($C_{19}H_{30}O_4$, calcd. 322.2144). The IR spectrum showed hydroxyl (3784 cm^{-1}) and ketonic carbonyl (1703 cm^{-1}). The ^{1}H-NMR spectrum of compound **6** showed a new downfield methine proton signal at δ 3.70 (dd, $J_{12a,11a}$ = 12.6 Hz, $J_{12a,11e}$ = 6.9 Hz), geminal to an OH. A new downfield carbon signal (δ 73.9) in the ^{13}C-NMR spectrum supported the presence of an OH in compound **6**. The position of OH was deduced on the basis of HMBC correlations of H-18 (δ 0.89) and H-11 (δ 1.41, 0.92) with newly formed methine carbon (δ 73.9) (Fig. 3). Therefore, OH was placed at C-12 of steroidal skeleton. The stereochemistry of newly formed stereocenter (C-12) was deduced on the basis of NOESY correlations. H-12 (δ 3.70) showed NOESY correlations with α-oriented H-20 (δ 1.31) and αH-9 (δ 0.98) (Fig. 4). Therefore, β-OH was inferred at C-12. The structure of compound **6** was deduced as 12β,17β-dihydroxy-17α-methyl-2-oxa-5α-androstan-3-one.

Fig. 4. Key NOESY correlations in compounds **2**–**6**.

2.6. Conclusion

In conclusion, a number of new hydroxylated derivatives were synthesized through the microbial transformation of androgenic steroid, oxandrolone (**1**). Five new compounds **2**–**6** were obtained from the microbial transformation of oxandrolone (**1**) with *M. phaseolina* and *C. blakesleeana*. Stereoselective hydroxylation and oxidation at various positions of oxandrolone skeleton were observed in this study. *M. phaseolina* and *C. blakesleeana* provide a unique metabolic pattern which might be useful in the investigation of the mammalian drug metabolism, as well as in the synthesis of new analogs.

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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.2015.06.008.

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