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Seven new metabolites of drostanolone heptanoate by using *Beauveria bassiana*, and *Macrophomina phaseolina* cell suspension cultures†

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The present study reports the biotransformation of an anabolic-androgenic steroid (AAS) drostanolone heptanoate (**1**) by using two microbial cultures, *Beauveria bassiana*, and *Macrophomina phaseolina*. Fermentation of **1** with *B. bassiana* yielded five new transformed products **2–6**, while with *M. phaseolina* it afforded two new **7–8**, and two known **9–10** metabolites. The main sites of hydroxylation in the steroidal skeleton of **1** were at C-5, C-7, C-11, C-14, C-15, and C-20, hydrolysis of the ester moiety at C-17, and reduction of the carbonyl group at C-3. The structures of the transformed products were determined by using mass, NMR, and other spectroscopic techniques.

Introduction

Steroids constitute an important class of biologically active compounds due to their anabolic, anti-inflammatory, anti-microbial, anti-leishmanial, and anti-cancer properties. Therefore, there have been sustained efforts for the synthesis of new, and derivatizations of existing steroidal drugs with desired pharmacodynamic profiles. Currently a bio-catalytic approach has largely been employed for the structural modifications of steroids, particularly due to the ability of microorganisms to catalyze functionalization at almost every site of the inert steroidal skeleton.[1–3] Moreover, microorganisms can grow, and replicate quickly, and produce a variety of enzymes under ambient conditions. Biotransformations have been successfully employed where chemical methods were found to be difficult and expensive. In addition, the products obtained are highly regio-, enantio-, and stereo-selective, and the procedures are eco-friendly, and economical.[4,5]

Drostanolone heptanoate (**1**) ($C_{27}H_{44}O_3$) is a dihydrotestosterone (DHT)-derived anabolic androgenic steroid (AAS). Athletes, and body builders use it to increase muscles strength, and body mass without gaining fat.[6] However, due to its adverse effects on liver, serum lipid, and reproductive system, the use of drostanolone heptanoate (**1**), and other anabolic androgenic steroids have now been discontinued by the International Olympic Committee (IOC).[7] In contrary, many research groups have studied the treatment of estrogen-dependent breast carcinoma using **1** as an aromatase inhibitor.[8–11]

Several bio-transformed products of drostanolone from the urine of drostanolone-dosed rabbits have been reported in literature, including hydroxylations at C-15, C-16, and C-17, reduction at C-3, and oxidation at C-17.[12] Similarly, several metabolites of drostanolone on incubation with cryopreserved human hepatocytes are also reported.[13]

In continuation of our structural transformation studies on steroidal drugs,[14–20] biotransformation of drostanolone heptanoate (**1**) was carried out. Based on small scale screening results, compound **1** was subjected to biotransformation by using two microbial cell cultures, *Beauveria bassiana*, and *Macrophomina phaseolina*, for the first time, yielding seven new, and two known compounds. The purpose of this study was to synthesize structurally diverse analogues of drostanolone heptanoate (**1**) for their potential use in the biomedical research, by employing mild, and low-cost biotransformation procedures.

Experimental

General

All the chemicals used for microbial transformation were purchased from the Sigma Aldrich (Germany), and Oxoid

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Limited (UK), whereas drostanolone heptanoate (**1**) was purchased from the Shanghai Bettersyn Biotech Co., Ltd., Shanghai (China). Silica gel pre-coated thin layer chromatography (TLC) plates (E. Merck, Germany), and silica gel column chromatography (Germany) were used for analysis, and fractionations (initial purification), while preparative recycling reverse phase HPLC-LC-908, fitted with JAIGEL-ODS-L-80 columns (Japan), was used for the final purification of transformed products. The FAB-, and HRFAB-MS (JEOL HX110) (Japan), and ESI-, and HRESI-MS (Bruker, Maxis II) (Germany) techniques were used to deduce the molecular formulae of all compounds. The ^{1}H-, and the ^{13}C-NMR spectra were recorded on Bruker Avance spectrometers (300–600 MHz) (Switzerland). The melting points were recorded using Stuart SMP-10 instrument (Switzerland). The JASCO P-200 polarimeter was used to measure the optical rotations, while the IR data were recorded using KBr disks in $CHCl_3$ on the Bruker Vector 22 FT-IR spectrometer (Japan). Molibdophosphoric acid (MPA) was used as a staining reagent to detect the transformed products on TLCs.

Microbial cultures, and media preparation

Beauveria bassiana (ATCC-7159) was purchased from the American Type Culture Collection, while *Macrophomina phaseolina* (KUCC-730) was obtained from the Karachi University Culture Collection. Both cultures were grown on Sabouraud dextrose agar slants, and maintained at 4 °C.

The culture media for the growth of fungi was prepared by adding 50 g glucose, 25 g peptone, 25 g yeast extract, 25 g KH_2PO_4, 25 g NaCl, and 50 mL glycerol, in distilled water. The quantity, and recipe for both cultures were the same.

General fermentation, extraction, and purification protocol

The media for the growth of microorganisms was prepared by adding the specific amounts of media ingredients in distilled water. Media was then transferred to 100 mL Erlenmeyer flasks, cotton plugged, and autoclaved at 121 °C, followed by inoculation of spores of fungi from mycelia on SDA slants under sterilized conditions. Flasks were placed on a rotary shaker at 26 °C for the maximum growth of fungi. After suitable growth, substrate **1** was dissolved in acetone, and equally distributed in each fungus-containing flasks, and again placed on a rotary shaker at 26 °C with 125 rpm for the fermentation process. A positive control (only substrate in media), and a negative control (microbial culture in media) were prepared to identify the degradation of substrate in media, and fungal metabolites, respectively. The fermentation reaction was stopped by adding dichloromethane to each flask, followed by filtration of fungal biomass. The filtrate was then extracted with dichloromethane, dried by adding anhydrous sodium sulfate, filtered, and evaporated under high vacuum to afford a solid crude material. This crude material was then fractionated through silica gel column chromatography with gradient hexanes, and acetone elution. After TLC analysis, the fractions of similar polarities were combined together into ten main fractions. These combined fractions were then subjected to preparative recycling RP-HPLC, using an isocratic methanol and water solvent systems. A constant flow rate of 4 mL min^{-1} was maintained throughout the analysis to isolate pure metabolites **2–10**.

2α-Methyl-7α,11α,17β-trihydroxy-5α-androstan-3-one (2). White solid; melting point: 230–233 °C; $[\alpha]_D^{25} = -46.2°$ (*c* 0.017, MeOH); IR ($CHCl_3$) λ_{max} (cm^{-1}): 3475, 3330, 3326 (3× O–H), 1687 (C=O); retention time (min): 17 (MeOH : H_2O; 75 : 25); HRFAB-MS *m*/*z* 337.2356 ([M + H]$^+$; calcd. 336.2301); FAB-MS *m*/*z* 337 [M + H]$^+$; ^{1}H-NMR (CD_3OD, 300 MHz): Table 1; ^{13}C-NMR (CD_3OD, 100 MHz): Table 2.

2α-Methyl-7α,15α,17β-trihydroxy-5α-androstan-3-one (3). Colourless solid; melting point: 236–238 °C; $[\alpha]_D^{25} = +25°$ (*c* 0.028, MeOH); IR ($CHCl_3$) λ_{max} (cm^{-1}): 3733, 3375, 3278 (3× O–H), 1687 (C=O); retention time (min): 19 (MeOH : H_2O; 75 : 25); HRFAB-MS *m*/*z* 337.2372 ([M + H]$^+$; calcd. 336.2301); FAB-MS *m*/*z* 337 [M + H]$^+$; ^{1}H-NMR (CD_3OD, 300 MHz): Table 1; ^{13}C-NMR (CD_3OD, 150 MHz): Table 2.

2α-Hydroxymethyl-11α,17β-dihydroxy-5α-androstan-3-one (4). White solid; melting point: 145–147 °C; $[\alpha]_D^{25} = +43.8°$ (*c* 0.021, MeOH); IR ($CHCl_3$) λ_{max} (cm^{-1}): 3737, 3410, 3369 (3 × O–H), 1703 (C=O); retention time (min): 18 (MeOH : H_2O; 70 : 30); HRFAB-MS *m*/*z* 337.2366 ([M + H]$^+$; calcd. 336.2301); FAB-MS *m*/*z* 337 [M + H]$^+$; ^{1}H-NMR (CD3OD, 300 MHz): Table 1; ^{13}C-NMR (CD_3OD, 100 MHz): Table 2.

2α-Methyl-7β,11α,17β-trihydroxy-5α-androstan-3-one (5). Colourless solid; melting point: 202–205 °C; $[\alpha]_D^{25} = +78.7°$ (*c* 0.024, MeOH); IR ($CHCl_3$) λ_{max} (cm^{-1}): 3743, 3429, 3375 (3 × O–H), 1699 (C=O); retention time (min): 26 (MeOH : H_2O; 70 : 30); HRFAB-MS *m*/*z* 337.2365 ([M + H]$^+$; calcd. 336.2301); FAB-MS *m*/*z* 337 [M + H]$^+$; ^{1}H-NMR (CD_3OD, 300 MHz): Table 1; ^{13}C-NMR (CD_3OD, 100 MHz): Table 2.

2α-Methyl-11β,15α,17β-trihydroxy-5α-androstan-3-one (6). Colourless solid; melting point: 244–246 °C; $[\alpha]_D^{25} = +27.4°$ (*c* 0.028, MeOH); IR ($CHCl_3$) λ_{max} (cm^{-1}): 3733, 3421, 3305 (3 × O–H), 1689 (C=O); retention time (min): 24 (MeOH : H_2O; 70 : 30); HRFAB-MS *m*/*z* 337.2372 ([M + H]$^+$; calcd. 336.2301); FAB-MS *m*/*z* 337 [M + H]$^+$; ^{1}H-NMR (CD_3OD, 300 MHz): Table 1; ^{13}C-NMR (CD_3OD, 100 MHz): Table 2.

2α-Methyl-3β,5α,17β-trihydroxy-5α-androstane (7). White solid; melting point: 248–251 °C; $[\alpha]_D^{25} = -113°$ (*c* 0.013, MeOH); IR (CH_3Cl) λ_{max} (cm^{-1}): 3373 (broad O–H); retention time (min): 21 (MeOH : H_2O; 75 : 25); HRESI-MS *m*/*z* 340.2851 ([M + NH_4]$^+$; calcd. 322.2508); ESI-MS *m*/*z* 340.3 ([M + NH_4]$^+$; 304 [M − H_2-O]$^+$); ^{1}H-NMR (CD_3OD, 300 MHz): Table 1; ^{13}C-NMR (CD_3OD, 150 MHz): Table 2.

2α-Methyl-3β,14α,17β-trihydroxy-5α-androstane (8). White solid; melting point: 272–275 °C; $[\alpha]_D^{25} = +21.8°$ (*c* 0.02, MeOH); IR ($CHCl_3$) λ_{max} (cm^{-1}): 3377 (O–H); retention time (min): 27 (MeOH : H_2O; 75 : 25); HRESI-MS *m*/*z* 323.2586 ([M + H]$^+$; calcd. 322.2508); ESI-MS *m*/*z* 323.3 [M + H]$^+$; ^{1}H-NMR (CD_3OD, 400 MHz): Table 1; ^{13}C-NMR (CD_3OD, 100 MHz): Table 2.

2α-Methyl-17β-hydroxy-5α-androstan-3-one (9). White solid; melting point: 150–152 °C; $[\alpha]_D^{25} = +31.9°$ (*c* 0.007, MeOH); IR ($CHCl_3$) λ_{max} (cm^{-1}): 3425 (O–H), 1706 (C=O); HRESI-MS *m*/*z* 305.2480 ([M + H]$^+$; calcd. 304.2402); ESI-MS *m*/*z* 305.2 [M + H]$^+$; 287.2 ([M + H]$^+$–H_2O); ^{1}H-NMR (CD_3OD, 300 MHz): Table 1; ^{13}C-NMR (CD_3OD, 100 MHz): Table 2.

Table 1 ^{1}H-NMR chemical shift assignments of compounds **1–10** (δ_H, multiplicity [J = Hz])

Positions	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	2.08 dd (J = 12.6, 5.7); 1.05 m	2.44 dd (J = 13.8, 5.8); 1.98 m	2.10 m; 1.00 m	2.91 dd (J = 13.8, 6.0); 1.32 m	2.54 br. t (J = 13.8); 1.88 dd (J = 13.8, 3.0)	2.44 br. t (J = 13.8); 1.16 m	1.61 m; 1.04 m	1.35 m; 1.00 br. t (J = 12.8)	2.08 dd (J = 13.2, 6.0); 1.04 m	1.34 m; 1.05 m
2	2.55 m	2.58 m	2.54 m	2.63 m	2.58 m	2.56 m	1.42 overlap	1.41 m	2.55 m	1.51 m
3	—	—	—	—	—	—	3.67 m	3.70 m	—	3.69 m
4	2.42 br. t (J = 13.8); 2.00 dd (J = 14.1, 3.3)	2.93 d (J = 6.0); 1.19 m	2.46 m; 2.08 m	2.44 br. t (J = 14.1); 2.00 overlap	2.82 dd (J = 3.8, 4.8); 1.25 m	2.87 dd (J = 13.8, 5.7); 1.20 m	1.50 overlap; 1.45 m	1.51 m; 1.39 m	2.87 br. t (J = 13.5); 1.99 m	1.82 m; 1.54 m
5	1.54 m	1.60 m	1.57 m	1.57 m	1.49 m	1.55 m	—	1.33 m	0.91 m	0.75 m
6	1.75 m; 1.31 overlap	1.56 m; 1.42 m	1.63 m; 1.44 m	1.40 m; 1.37 m	1.57 overlap; 1.43 m	1.37 m; 1.33 m	1.21 m; 1.18 m	1.41 m; 1.25 m	1.33 m; 1.30 m	1.61 m; 1.28 m
7	1.58 m; 1.32 overlap	3.24 m	3.30 m	1.55 m; 1.26 m	3.77 m	2.06 m; 1.13 m	1.63 m; 1.53 m	1.53 m; 1.36 m	1.74 m; 0.88 m	1.21 m; 1.16 m
8	1.50 m	1.66 m	1.60 m	1.52 m	1.52 m	1.67 m	1.43 m	1.71 m	1.46 overlap	1.42 m
9	0.77 m	0.88 br. t (J = 10.2)	0.82 m	0.86 br. t (J = 10.2)	1.42 m	0.86 br. t (J = 10.2)	1.73 m	1.48 m	1.47 overlap	1.66 m
10	—	—	—	—	—	—	—	—	—	—
11	1.40 m; 1.33 overlap	3.92 dt (J = 10.5, 5.1)	1.67 m; 1.66 m	3.93 dt (J = 10.8, 5.0)	3.88 dt (J = 10.8, 5.1)	3.87 m	1.51 overlap; 1.41 overlap	1.56 m; 1.32 m	1.56 m; 1.40 m	1.67 m; 0.89 m
12	1.18 dt (J = 12.9, 3.9); 1.71 m	2.08 dd (J = 12.0, 4.8); 1.10 m	1.82 m; 1.10 m	2.11 dd (J = 12.0, 4.8); 1.10 m	2.06 overlap; 1.07 br. t (J = 11.7)	2.07 overlap; 1.16 m	1.57 m; 1.44 m	1.43 m; 1.25 m	1.85 m; 1.01 m	1.42 m; 1.00 m
13	—	—	—	—	—	—	—	—	—	—
14	1.08 m	1.20 m	1.03 m	1.08 m	1.40 m	1.05 m	1.55 m	—	0.91 m	0.95 m
15	2.12 m; 1.47 m	1.88 m; 1.67 m	4.06 dt (J = 9.0, 4.8)	1.73 m; 1.70 m	1.68 m; 1.26 m	3.90 m	1.37 m; 1.33 m	1.64 m; 1.51 m	3.90 m	1.57 m; 1.24 m
16	1.52 m; 1.47 m	1.95 m; 1.44 m	2.04 m; 1.93 m	2.02 overlap; 1.48 m	2.01 m; 1.56 overlap	2.05 overlap; 1.84 m	1.98 m; 1.46 m	2.14 m; 1.52 m	1.53 m; 1.26 m	1.94 m; 1.46 m
17	4.58 t (J = 8.7)	3.55 t (J = 8.4)	3.74 t (J = 9.0)	3.59 t (J = 8.7)	3.60 t (J = 8.4)	3.78 t (J = 9.0)	3.61 t (J = 8.4)	4.20 t (J = 7.2)	3.55 t (J = 8.7)	3.54 t (J = 8.4)
18	0.83 s	0.77 s	0.78 s	0.76 s	0.75 s	0.76 s	0.72 s	0.81 s	0.74 s	0.71 s
19	1.12 s	1.24 s	1.14 s	1.22 s	1.20 s	1.23 s	0.94 s	0.83 s	1.11 s	0.82 s
20	0.96 d (J = 6.3)	0.94 d (J = 6.6)	0.97 d (J = 6.3)	3.75 dd (J = 10.8, 5.1); 3.47 dd (J = 11.1, 5.7)	0.93 d (J = 6.3)	0.93 d (J = 6.3)	0.91 d (J = 5.4)	0.91 d (J = 6.8)	0.94 d (J = 6.6)	0.91 d (J = 6.9)
21	—	—	—	—	—					
22	2.29 t (J = 7.2)	—	—	—	—					
23	1.59 m; 1.32 overlap	—	—	—	—					
24	1.38 overlap; 1.33 overlap	—	—	—	—					
25	1.30 overlap; 1.29 overlap	—	—	—	—					
26	1.34 overlap; 1.30 overlap	—	—	—	—					
27	0.90 t (J = 6.9)	—	—	—	—					

Table 2 ^{13}C-NMR chemical shift assignments of compounds **1–10**

Carbons	1	2	3	4	5	6	7	8	9	10
1	49.8	45.6	49.3	45.7	45.6	46.1	35.1	42.3	49.9	42.2
2	42.1	42.2	42.0	49.9	42.8	42.3	31.3	33.1	42.1	40.0
3	215.4	215.5	215.0	214.3	215.5	215.9	71.3	71.4	215.6	71.4
4	45.5	51.2	45.0	46.3	51.4	51.7	38.0	37.8	45.6	38.1
5	36.3	47.4	46.6	50.2	42.8	50.5	77.0	48.8	55.2	56.1
6	32.4	40.2	37.8	30.3	38.0	30.4	29.3	29.2	29.8	21.5
7	26.2	75.0	74.8	24.2	67.4	33.0	27.5	27.0	32.5	29.4
8	49.4	43.3	44.8	36.1	40.2	35.9	33.2	40.2	36.6	36.6
9	55.2	59.1	58.5	60.9	52.9	61.0	49.6	39.7	49.6	33.1
10	37.3	38.8	45.8	39.0	44.1	39.3	42.4	37.6	37.7	37.4
11	29.8	69.6	22.1	69.5	69.7	69.2	24.1	20.6	22.2	32.9
12	38.2	49.4	38.2	49.0	48.5	49.6	33.4	30.0	38.0	37.8
13	43.9	44.9	58.4	44.4	49.6	45.6	44.1	49.0	44.1	44.1
14	51.9	51.1	52.1	51.3	45.9	58.5	44.8	84.8	52.2	52.4
15	28.5	27.2	73.1	32.4	23.4	72.5	26.2	32.7	72.5	24.3
16	23.5	31.1	40.5	30.7	30.6	43.0	30.6	30.1	24.3	30.6
17	84.0	81.8	79.5	81.9	82.0	79.2	82.3	79.6	79.2	82.5
18	12.6	12.6	13.3	12.5	12.3	13.9	10.8	15.7	11.6	11.7
19	14.2	12.9	12.7	12.9	11.9	13.0	14.7	12.3	12.6	12.6
20	14.9	15.0	14.9	62.5	15.0	15.0	19.2	19.0	15.0	19.0
21	175.5	—	—	—	—	—	—	—	—	—
22	35.3	—	—	—	—	—	—	—	—	—
23	26.2	—	—	—	—	—	—	—	—	—
24	29.8	—	—	—	—	—	—	—	—	—
25	32.6	—	—	—	—	—	—	—	—	—
26	23.5	—	—	—	—	—	—	—	—	—
27	14.3	—	—	—	—	—	—	—	—	—

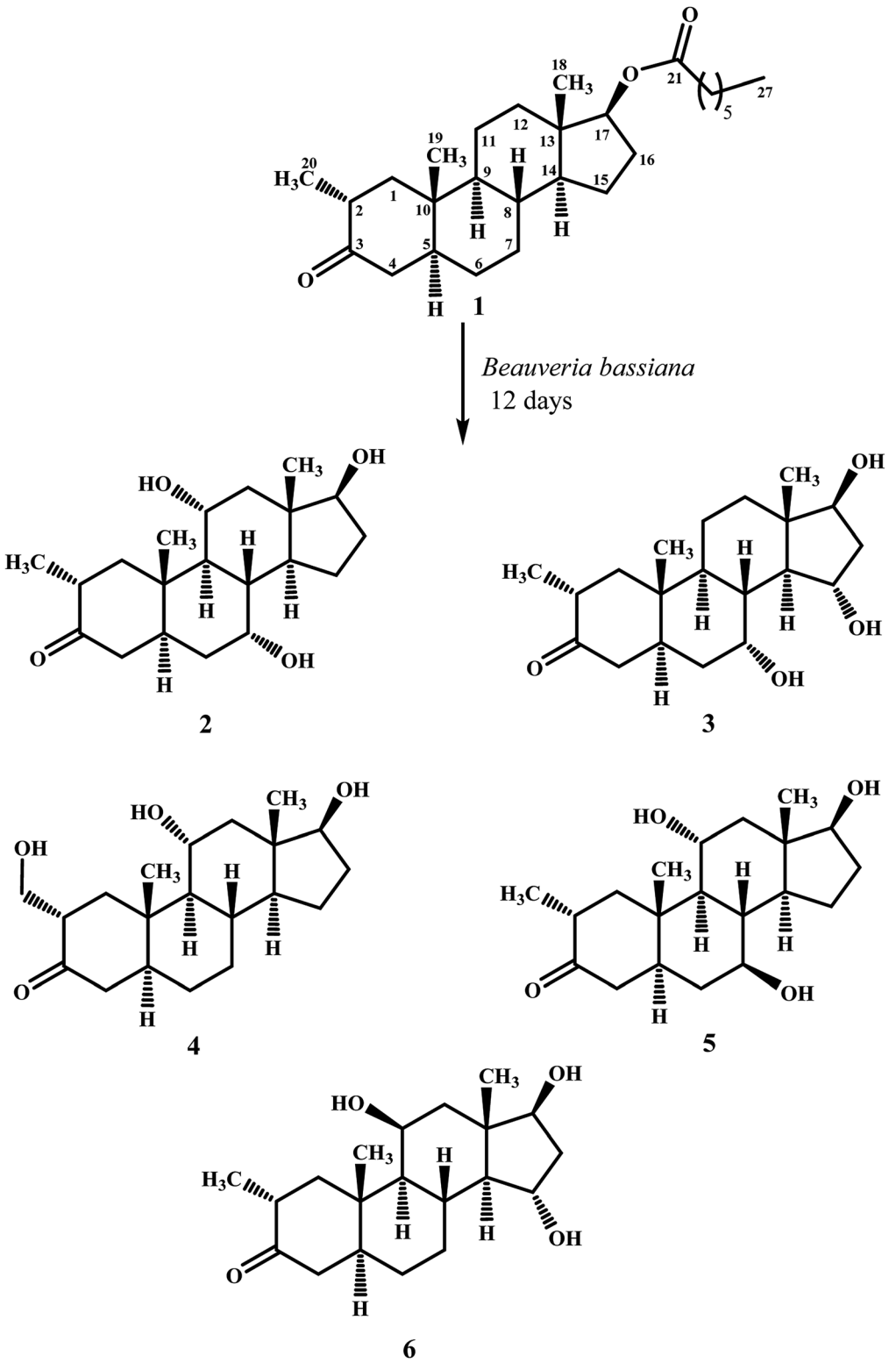
2α-Methyl-3β,17β-dihydroxy-5α-androstane (10). White solid; melting point: 240–242 °C; $[\alpha]_D^{25} = +19.6°$ (*c* 0.026, MeOH); IR ($CHCl_3$) λ_{max} (cm^{-1}): 3425 (broad O–H); HRESI-MS *m*/*z* 324.2902 ($[M + NH_4]^+$; calcd. 306.2599); ESI-MS *m*/*z* 324.3 $[M + NH_4]^+$; ^{1}H-NMR (CD_3OD, 300 MHz): Table 1; ^{13}C-NMR (CD_3OD, 125 MHz): Table 2.

Results and discussion

Biotransformation of drostanolone heptanoate (**1**) with cell suspension cultures of *B. bassiana*, and *M. phaseolina* produced nine transformed products **2–10**. *B. bassiana* yielded five new metabolites **2–6** (Scheme 1), whereas *M. phaseolina* afforded two new **7** and **8**, and two known **9** and **10** metabolites (Scheme 2). The structures of new compounds were deduced by using different spectroscopic techniques, and by comparing their spectral data with substrate **1**. The structures of known metabolites were also determined by comparing their spectroscopic data with the reported data.

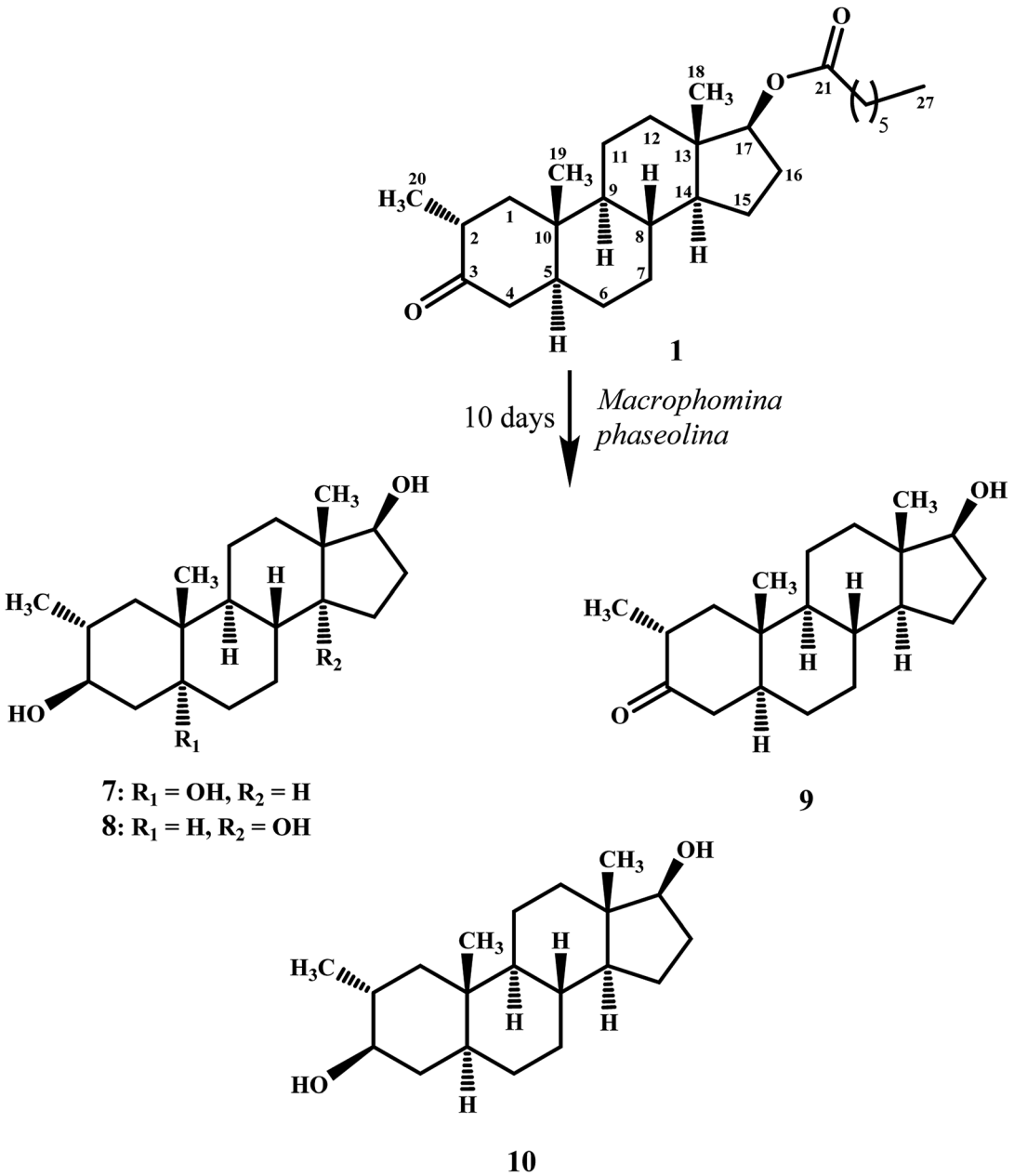
Metabolite **2** was purified as optically active, colorless solid through recycling RP-HPLC. HRFAB-MS showed the $[M + H]^+$ at *m*/*z* 337.2356 (calcd. 336.2301, $C_{20}H_{32}O_4$), 24 amu lesser than substrate **1**. This suggested hydrolysis of ester group, and addition of two oxygen atoms. Absorption bands at ν_{max} (cm^{-1}) 3475, 3330, 3326, and 1687 were observed in the IR spectra for three OH, and a carbonyl groups. In the ^{1}H-NMR spectrum, three downfield methine signals at δ 3.92 (dt, $J_{11a,9a/12a} = 10.5$ Hz, $J_{11a,12e} = 5.1$ Hz), 3.55 (t, $J_{17\alpha,16\alpha/\beta} = 8.4$ Hz), and 3.24 (m) were appeared, indicating presence of hydroxyl groups. Similarly, downfield signals for oxymethine carbons were observed in the ^{13}C-NMR spectrum at δ 81.8, 75.0, and 69.6. In the HMBC spectrum of compound **2** (Fig. 1), H-12 showed HMBC interactions with C-9, C-11, C-14, C-17, and C-18, supporting the positions of both OH groups at C-11, and C-17. Similarly, H-9 showed cross peaks with C-7, C-8, C-10, C-11, and C-14, suggested OH groups at C-7, and C-11. The configuration of H-7, H-11, and H-17 were deduced as β, β, and α (Fig. 2), based on NOESY correlations of H-7 with β-oriented H-8, and H-11 with β-oriented H_3-19, and H_3-18 (Fig. 2). Thus the structure of new metabolite was determined as 2α-methyl-7α,11α,17β-trihydroxy-5α-androstan-3-one (**2**).

Compound **3** was obtained as optically active, colorless solid by using recycling RP-HPLC. HRFAB-MS showed the $[M + H]^+$ at *m*/*z* 337.2372 (calcd. 336.2301, $C_{20}H_{32}O_4$), suggesting hydrolysis of ester group, and addition of two oxygen atoms. This was 24 amu lesser than substrate **1**. IR showed absorption bands at ν_{max} (cm^{-1}) 3733, 3375, 3278, and 1687 for three OH, and a carbonyl groups. The ^{1}H-, and the ^{13}C-NMR spectra of compounds **2**, and **3** were found to be distinctly similar. In the ^{1}H-NMR spectrum, three downfield methine signals at δ 4.06 (dt, $J_{15\beta,16\alpha/\beta} = 9.0$ Hz, $J_{15\beta,14\alpha} = 4.8$ Hz), 3.74 (t, $J_{17\alpha,16\alpha/\beta} = 9.0$ Hz), and 3.30 (m) were assigned to the protons geminal to the OH groups. Consistent with this observations, the ^{13}C-NMR spectrum showed methine carbons resonances at δ 79.5, 74.8, and 73.1. However, the HMBC, COSY, and NOESY spectra of both compounds were found different. Compound **3** displayed HMBC interactions (Fig. 1) of H-9 with C-5, C-7, C-10, and C-19, suggesting one of the OH groups at C-7. Similarly, the other two



Scheme 1 Biotransformation of drostanolone heptanoate (1) with *B. bassiana*.

OH groups were placed at C-15, and C-17, based on the HMBC correlations of H-16 with C-13, C-15, and C-17. The configuration of H-7, H-15, and H-17 were deduced as β, β, and, based on NOESY correlations of H-7 with β-oriented H-8, H-15 with axially-oriented H_3-18, and H-17 with α-oriented H-14 (Fig. 2). Thus the structure of new compound was deduced as 2α-methyl-7α,15α,17β-trihydroxy-5α-androstan-3-one (**3**).



Scheme 2 Biotransformation of drostanolone heptanoate (**1**) with *M. phaseolina*.

Metabolite **4** was obtained as a white solid using recycling RP-HPLC. The HRFAB-MS of **4** showed the $[M + H]^+$ at *m*/*z* 337.2366 (calcd. 336.2301, $C_{20}H_{32}O_4$) suggesting the addition of two oxygen atoms, along with hydrolytic loss of ester moiety. The IR spectrum revealed the presence of three OH (3737, 3410, and 3369 cm^{-1}), and a carbonyl (1703 cm^{-1}) groups. Signal for the C-20 methyl protons in the ^{1}H-NMR spectrum was also found missing. An additional downfield signal at δ 62.5 for C-20 in the ^{13}C-NMR spectrum also indicated the oxidation of the methyl group as CH_2–OH. New downfield signals for H-11 at δ 3.93 (dt, $J_{11a,9a/12a} = 10.8$ Hz, $J_{11a,12a} = 5.0$ Hz), H-17 at δ 3.59 (t, $J_{17\alpha,16\alpha/\beta} = 8.7$ Hz), and H_2-20 at δ 3.75 (dd, $J_{20a,20b} = 10.8$ Hz, $J_{20a,2\beta} = 5.1$ Hz), and δ 3.47 (dd, $J_{20b,20a} = 11.1$ Hz, $J_{20b,2\beta} = 5.7$ Hz) were appeared in the ^{1}H-NMR spectrum. This was further established through the HMBC correlations (Fig. 1). The HMBC correlations between H-1 and C-2, C-3, C-5, C-10, and C-20, and from H-12 to C-9, C-11, C-13, C-14, and C-17 were observed. These correlations supported the OH groups at C-11, C-17, and C-20. In the COSY spectrum (Fig. 2), the homonuclear ^{1}H–^{1}H couplings were observed between H-11, and H-9, and H-12, while H-2 showed the COSY cross peaks with H-1, and H_2-20. The coupling constant of H-11 (dt, $J_{11a,9a/12a} = 10.8$ Hz, $J_{11a,12a} = 5.0$ Hz), and its NOESY correlations with H_3-18 (δ 0.76 s), implied that OH group at C-11 was equatorial (α) (Fig. 2). Likewise, H-17 showed NOESY cross peaks with H-14, indicating a β-orientation of the OH group at C-17. This new

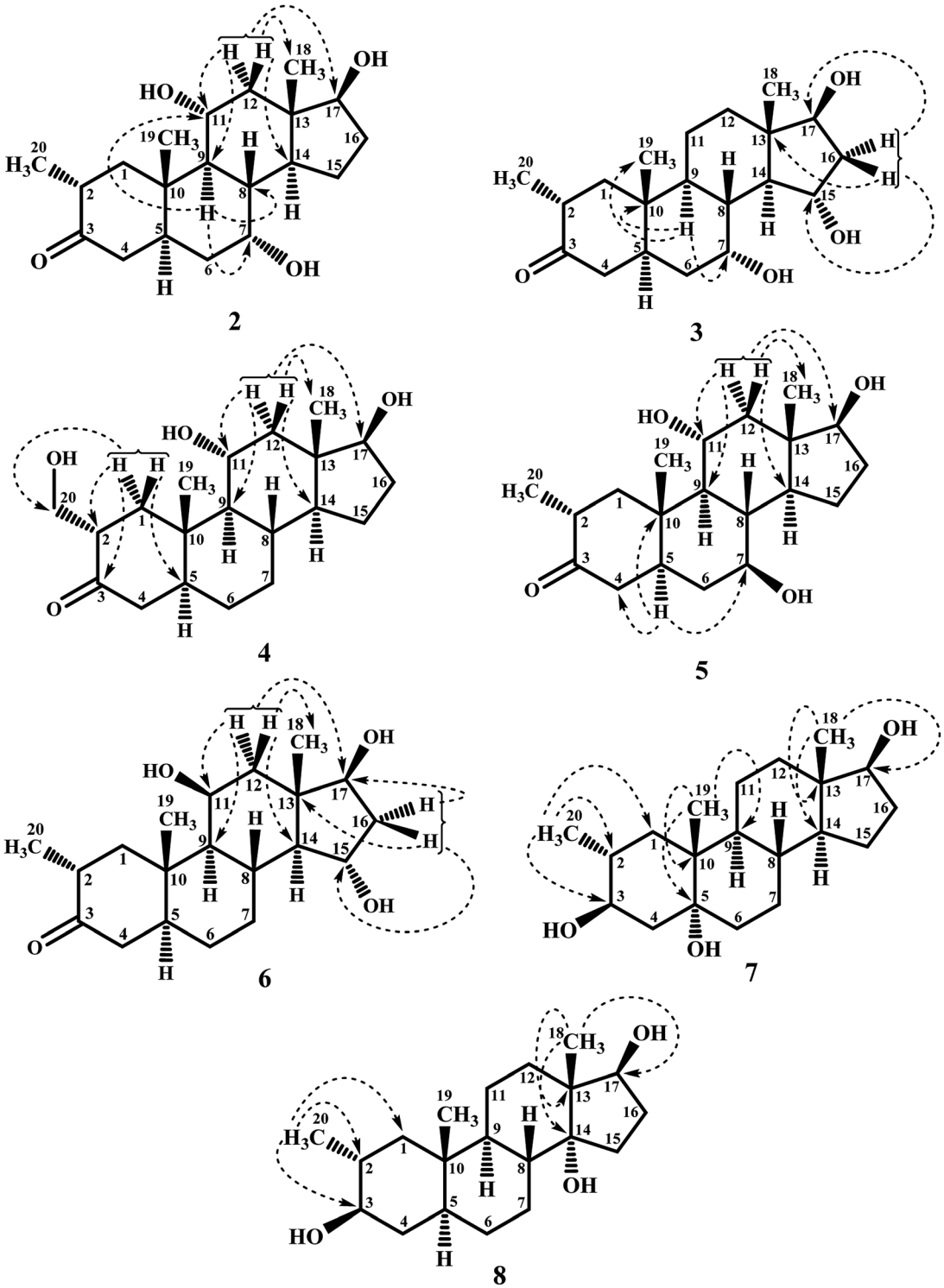


Fig. 1 Key HMBC interactions of metabolites **2**–**8**.

metabolite was thus identified as 2α-hydroxymethyl-11α,17β-dihydroxy-5α-androstan-3-one (**4**).

Compound **5** was isolated as colorless solid through recycling RP-HPLC with a retention time of 26 min. It showed the $[M + H]^+$ at *m*/*z* 337.2365 (calcd. 336.2301, $C_{20}H_{32}O_4$) in the HRFAB-MS. The IR spectrum showed absorption bands at υ_{max} (cm^{-1}) 3743, 3429, 3375, and 1699, for OHs, and a carbonyl, respectively. The ^{1}H-, and ^{13}C-NMR spectra of compound **5**, were found to be distinctly similar to compounds **2**, and **3**. Three new downfield signals for oxymethine protons were observed at δ 3.88 (dt, $J_{11a,9a/12a}$ = 10.8 Hz, $J_{11a,12e}$ =5.1 Hz), 3.77 (m), and 3.60 (t, $J_{17\alpha,16\alpha/\beta}$ = 8.4 Hz) in the ^{1}H-NMR spectrum. Similarly,

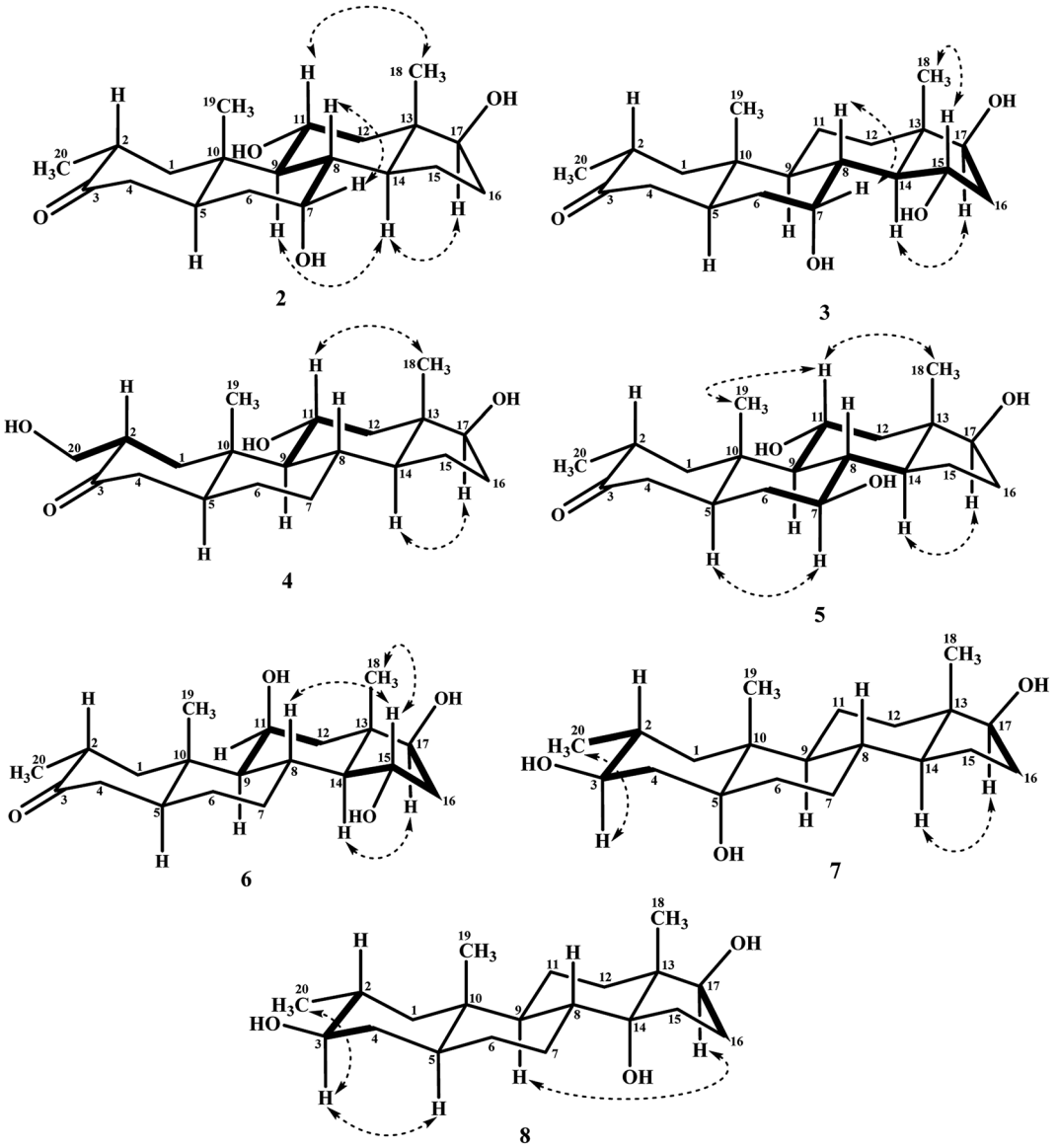


Fig. 2 Key COSY, and NOESY interactions in metabolites **2**–**8**.

^{13}C-NMR spectrum of **5** showed downfield signals for oxymethine carbons at δ 69.7, 67.4, and 82.0. The positions of OH groups at C-11, and C-17 were deduced through the HMBC correlations (Fig. 1) of H-12 (δ 2.06, overlap; δ 1.07 br. t; $J_{12a,12e/11a}$ = 11.7 Hz) with C-9 (δ 52.9), C-11 (δ 69.7), C-13 (δ 49.6), C-14 (δ 45.9), and C-17 (δ 82.0) indicated the OH groups at C-11, and C-17. Similarly, OH group at C-5 was determined through the HMBC correlations of H-5 (δ 1.52, m) with C-4 (δ 51.4), C-7 (δ 67.4), and C-10 (δ 44.1) (Fig. 1). An OH group at C-11 was deduced as α on the basis of NOESY interactions (Fig. 2) of H-11 (δ 3.88 dt, $J_{11a,9a/12a}$ = 10.8 Hz, $J_{11a,12e}$ = 5.1 Hz) with axially-oriented H_3-18 (δ 0.75 s), and H_3-19 (δ 1.20 s). Similarly, the β-orientations of OH groups at C-7, and C-17 in compound **5** were deduced through the key NOESY correlations of axially-oriented H-5 with H-7, and axially-oriented H-14 with H-17 (δ 3.60 t, $J_{17\alpha,16\alpha/\beta}$ = 8.4 Hz), respectively. The structure of a new metabolite was thus deduced as 2α-methyl-7β,11α,17β-trihydroxy-5α-androstan-3-one (**5**).

Metabolites **6** was obtained as colorless solid by using RP-HPLC with a retention time of 24 min. The molecular formula of compound **6** was consistent with $C_{20}H_{32}O_4$, deduced from the HRFAB-MS at *m*/*z* 337.2365 (calcd. 336.2301; $[M + H]^+$). Absorption bands in the IR spectrum at υ_{max} (cm^{-1}) 3733, 3421, 3305, and 1689, were due to the presence of three OHs, and a carbonyl, respectively. The ^{1}H-, and ^{13}C-NMR spectra of compounds **5**, and **6** were found to be distinctly similar to compounds **2**, and **3**. The ^{1}H-NMR spectrum showed three new downfield signals for methine protons at δ 3.78 (t, $J_{17\alpha,16\alpha/\beta}$ = 9.0 Hz), 3.87 (m), and 3.90 (m). Signals for deshielded methine carbons at δ 69.2, 72.5, and 79.2 were appeared in the ^{13}C-NMR spectrum of **6**. This suggested the presence of three OH groups. OH Groups at C-11, C-17 were deduced through the HMBC

correlations (Fig. 1) of H-12 (δ 2.07 overlap; δ 1.16 m) with C-9 (δ 61.0), C-11 (δ 69.2), C-13 (δ 45.6), C-14 (δ 58.5), and C-17 (δ 79.2), whereas, OH group at C-15 was determined through the HMBC correlations of H-16 with C-13, C-14, C-15, and C-17. In the NOESY spectrum (Fig. 2) of compound **6**, cross peaks were observed between axially-oriented H-14, and H-17 suggesting β-orientation of OH group at C-17. Similarly, β-stereochemistry of OH groups at C-11, and C-15 were also deduced from the NOESY correlations of H-11 with axially-oriented H-9, and H-15 with axially-oriented H-8 and H_3-18. The structure of a new metabolite was thus deduced as 2α-methyl-11β,15α,17β-trihydroxy-5α-androstan-3-one (**6**).

Compound **7** was purified as a white solid through recycling RP-HPLC with a retention time of 21 min. The HRESI-MS of metabolite **7** showed the $[M + NH_4]^+$ at *m*/*z* 340.2851 (calcd. 322.2508, $C_{20}H_{32}O_3$). This suggested dihydroxylation, along with hydrolysis of side chain ester group. The IR spectrum showed broad absorbances at υ_{max} 3373 cm^{-1} for OH groups. Absence of the bridge methine signals in the ^{1}H-, and the ^{13}C-NMR spectra of **7** suggested the hydroxylation at tertiary carbon. An OH group at C-5 was deduced through the HMBC correlations of H-3, H_3-19, H_2-6, and H-9 with C-5 (Fig. 1). Stereochemistry of OH group at C-3 was assigned as β, based on NOESY correlations of axially-oriented H-3 with equatorially-oriented H_3-20 (Fig. 2). An OH group at C-5 (δ 77.0) was placed as α, based on the comparison of the reported chemical shifts for 5α-OH in steroids.[15,20] Thus the structure of a new metabolite was identified as 2α-methyl-3β,5α,17β-trihydroxy-androstane (**7**).

Metabolite **8** was obtained as a white solid by using RP-HPLC with a retention time of 27 min. The HRESI-MS of **8** showed the $[M + H]^+$ at *m*/*z* at 323.2586 (calcd. 322.2508), indicating hydrolysis of side chain ester group, along with dihydroxylation. Broad absorption bands at υ_{max} 3377 cm^{-1} in the IR spectrum were due to the OH groups. The ^{1}H-, and ^{13}C-NMR spectra of metabolite **7** were apparently identical to **8**. Absence of the bridge methine signals in the ^{1}H-, and ^{13}C-NMR spectra of **8** suggested the hydroxylation at tertiary carbon. An OH group at C-14 was deduced through the HMBC correlations of H-17, H-8, H_2-15, H_2-16, and H_3-18 with C-14 (Fig. 1). The relative stereochemistry at C-3 in compound **8** was deduced from the NOESY interactions between axially-oriented H-3, and equatorially-oriented H_3-20 (Fig. 2). Similarly, OH group at C-14 was deduced as α on the basis of reported chemical shift for 14α-OH in similar steroidal compound.[18] Thus the structure of a new compound was deduced as 2α-methyl-3β,14α,17β-trihydroxy-5α-androstane (**8**).

Metabolites **9**, and **10** were obtained as white solids by repeated column chromatography. HRESI-MS of compound **9** showed the $[M + H]^+$ at *m*/*z* 305.2480 (calcd. 304.2402; $C_{20}H_{30}O_2$) 47 amu less than the substrate **1**, suggesting hydrolysis of side chain ester group. Whereas the HRESI-MS of metabolite **10** at *m*/*z* 324.2902 (calcd. 306.2599; $[M + NH_4]^+$) suggested a molecular composition of $C_{20}H_{34}O_2$. This indicated the reduction of ketonic carbonyl group, along with hydrolysis of ester side chain of substrate **1**. Metabolites **9**, and **10** were previously reported by Templeton and Kim from the urine of drostanolone dosed rabbits.[12] The spectroscopic data of metabolites **9**, and **10** was consistent with the literature data. Their structures were identified as 2α-methyl-17β-hydroxy-5α-androstan-3-one (**9**), and 2α-methyl-3β,17β-dihydroxy-5α-androstane (**10**).

Conclusion

Microbial biotechnology has wide applications in the synthesis of libraries of structurally diverse analogues of bioactive natural products, and drugs. The present study afforded seven new, and two known metabolites of drostanolone heptanoate (**1**) by using two microbial cultures, *Beauveria bassiana*, and *Macrophomina phaseolina*. Incubation of **1** with *B. bassiana* led to the synthesis of five new metabolites **2–6**, whereas *M. phaseolina* afforded two new **7**, and **8**, and two known transformed products **9**, and **10**. Hydroxylation, reduction, and hydrolysis were the main reactions during structural transformation of **1**. The transformed products will be evaluated for potential biological activities in the future. In addition, metabolites **2–10** will also be studied at enzymatic level to understand the mechanism involved during biocatalysis.

Conflicts of interest

Authors has no conflicts of interest.

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