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Microbial transformation of androst-4-ene-3,17-dione by *Beauveria bassiana*

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ABSTRACT

The microbial transformation of androst-4-ene-3,17-dione (**I**) by the fungus *Beauveria bassiana* CCTCC AF206001 has been investigated using pH 6.0 and 7.0 media. Two hydroxylated metabolites were obtained with the pH 6.0 medium. The major product was 11α-hydroxyandrost-4-ene-3,17-dione (**II**) whereas the minor product was 6β,11α-dihydroxyandrost-4-ene-3,17-dione (**III**). On the other hand, four hydroxylated and/or reduced metabolites were obtained with the pH 7.0 medium. The major product was 11α,17β-dihydroxyandrost-ene-3-one (**V**) and the minor products were 17β-hydroxyandrost-ene-3-one (**IV**), 6β,11α,17β-trihydroxyandrost-ene-3-one (**VI**) and 3α,11α,17β-trihydroxy-5α-androstane (**VII**). The products were purified by chromatographic methods, and were identified on the basis of spectroscopic methods. This fungus strain is clearly an efficient biocatalyst for 11α-hydroxylation and reduction of the 17-carbonyl group.

1. Introduction

Microbial transformation is an efficient and often key means for the preparation of many natural products and their derivatives. This technique has been widely applied for the conversion and synthesis of steroids. As example, many microorganisms have been used for the transformation of androst-4-ene-3,17-dione (**I**) [1–5] and the hydroxylated derivatives of **I** are valuable intermediates in the synthesis of steroidal drugs.

It was reported some strains of *Beauveria bassiana* can catalyze reactions including hydroxylation, reduction and oxidation [6–15]. However, little work has been reported on the regulation and control of the biocatalytic abilities of *B. bassiana*. In the present study, two media were used, and after 24–96 h of transformation of **I** by *B. bassiana* CCTCC AF206001, metabolites were isolated. We report here on the details of these investigations.

2. Experimental

2.1. Microorganism

B. bassiana CCTCC AF206001 was isolated from its insect host *Diprion jingyuanensis* by the State Key Laboratory of Agricultural Microbiology in Huazhong Agricultural University, and preserved at China Center for Type Culture Collection.

2.2. Conditions of cultivation and transformation

The strain of *B. bassiana* was maintained on agar slants at 4 °C. Nine days culture on PDA slants at 28 °C were used to preculture with 40 ml liquid medium. The medium consisted of glucose (30 g l^{-1}), corn steep liquor (20 g l^{-1}), K_2HPO_4 (1 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g l^{-1}) and distilled water, either pH 6.0 or 7.0.

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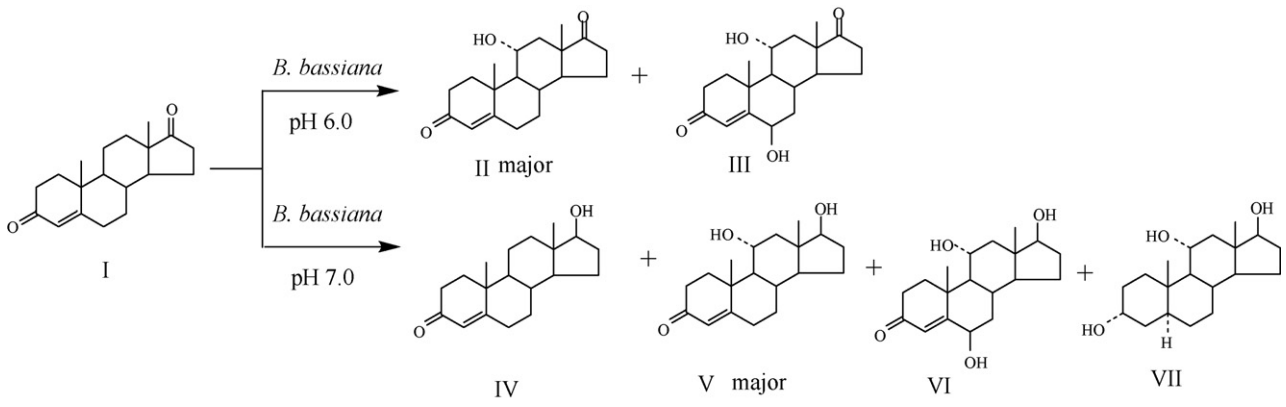

Fig. 1 – The structures of substrate and metabolites.

Cultures were all shaken at 28–30 °C. After 48 h, the preculture (4 ml) was transferred to 40 ml fresh medium (10%, v/v) in 250 ml flasks for an incubation (24 h, 180 rpm). Thereafter 200 mg of the substrate dissolved in 1 ml of ethanol was added to the culture (24–96 h, 160 rpm). The microbial transformations of substrate were monitored by TLC and HPLC.

2.3. *Isolation and identification of transformation products*

The fermentation media were extracted three times with 20 ml ethyl acetate after transformation. After removal of the solvent, the residues were analyzed with TLC, HPLC and separated by silica gel column chromatography. TLC analysis was carried out using 0.25 mm thick layers of silica gel G (silica gel GF254, Qingdao Haiyang Chemical Co., Ltd.). Layers were prepared on glass plates and activated at 105 °C 1 h before use. Chromatography was performed with chloroform/methanol (12:1, v/v) and compounds were visualized by spraying the plates with a solution of sulfuric acid–ethanol (1:10, v/v) and heating in an oven at 105 °C for 3 min until color developed. HPLC analysis was performed using an Agilent 1100 HPLC instrument equipped with a C18 column (4.6 mm × 250 mm). Isocratic column elution was monitored by an Agilent 1100 UV/Visible detector. The wavelength was set at 240 nm and the mobile phase used was methanol–water (60:40, v/v) at a flow rate 1 ml/min.

The FAB-MS spectra were obtained with a ZAB-3F-HF instrument. The ^{1}H NMR Spectra and ^{13}C NMR spectra were recorded at room temperature on a Varian Unity-Inova 600 spectrometer at 600 and 150 MHz, respectively using $CDCl_3$ or CD_3OD as a solvent and TMS as an internal standard. For compound **VI**, standard pulse sequences were used for DEPT, COSY, HSQC and HMBC experiments. IR spectra were recorded in KBr on a Thermo Nicolet Nexus 470 FTIR spectrometer. UV spectra were recorded in methanol on a PerkinElmer Lambda 35 UV/VIS Spectrometer. Melting points (mp) were determined on an XT-4 micromelting point apparatus and are uncorrected. Optical rotations were measured on solutions of methanol in 1-dm cells on a Perkin-Elmer Models 341 spectropolarimeter.

2.4. *Transformation of androst-4-ene-3,17-dione by B. bassiana*

The substrate was incubated with *B. bassiana* in two different media for 24–96 h. In the pH 6.0 medium, after 24 h of incubation, the only isolated product was 11α-hydroxyandrost-4-ene-3,17-dione (**II**), and after 48 h of incubation 6β,11α-dihydroxyandrost-4-ene-3,17-dione (**III**) was also obtained. In the pH 7.0 medium, after 24 h of incubation, 11α,17β-dihydroxyandrost-ene-3-one (**IV**) was obtained whereas after 48 h of incubation the single product observed was 11α,17β-dihydroxyandrost-ene-3-one (**V**), and after 72 h of incubation 6β,11α,17β-trihydroxyandrost-ene-3-one (**VI**) was also obtained. After 96 h of incubation 5α-androstane-3α,11α,17β-triol (**VII**) had also been formed (see Fig. 1).

The results of HPLC analysis and the isolated yields (mol%) of substrate and metabolites are given in Tables 1 and 2.

2.4.1. *11α-Hydroxyandrost-4-ene-3,17-dione (II)*

Colorless crystals (crystallized from methanol); mp 196–197 °C, $[\alpha]_D^{25} + 145^\circ$ ($CHCl_3$, $c = 0.8$), literature [16,17]; mp 196–197 °C;

Table 1 – Transformation of I with the pH 6.0 medium

Compound	24 h		48 h	
	HPLC content (%)	Isolated yield (%)	HPLC content (%)	Isolated yield (%)
I	0	0	0	0
II	99.4	90.9	86.3	80.1
III	0	0	12.8	10.3

A 24 and 48 h denote the reaction time.

Table 2 – Transformation of I with the pH 7.0 medium

Compound	24 h		48 h		72 h		96 h	
	HPLC content (%)	Isolated yield (%)	HPLC content (%)	Isolated yield (%)	HPLC content (%)	Isolated yield (%)	HPLC content (%)	Isolated yield (%)
I	80.6	78.2	0	0	0	0	0	0
IV	18.7	15.4	0	0	0	0	0	0
V	0	0	99.2	91.3	84.6	82.3	82.5	74.6
VI	0	0	0	0	14.2	8.0	15.9	8.7
VII[a]	–	0	–	0	–	0	–	6.0

A 24, 48, 72 and 96 h denote the reaction time.

[a] Compound **VII** could not be detected with the UV detector of the HPLC.

$[\alpha]_D^{25} + 143^\circ$ (MeOH, c = 1.0); UV $\lambda_{max}^{MeOH} = 241$ nm; IR ν_{max} (cm^{-1}): 3430, 1734, 1664, 1609; FAB-MS (3-nitrophenyl methanol, probe), *m/z*: 303 $[M+H]^+$; ^1H NMR ($CDCl_3$): δ (ppm): 0.95 (3H, s, H-18), 1.35 (3H, s, H-19), 4.08 (1H, ddd, *J* = 12, 11, 5 Hz, H-11β), 5.76 (1H, s, H-4); ^{13}C NMR ($CDCl_3$): δ (ppm): 218.2 (C-17), 199.8 (C-3), 169.9 (C-5), 124.9 (C-4), 68.8 (C-11), 59.3 (C-9), 50.2 (C-14), 48.0 (C-13), 43.1 (C-12), 40.1 (C-10), 37.5 (C-16), 35.7 (C-1), 34.7 (C-2), 34.2 (C-8), 33.4 (C-6), 30.4 (C-7), 21.8 (C-15), 18.4 (C-19), 14.7 (C-18); R_f in chloroform/methanol (12:1): 0.72; R_t: 9.9 min.

2.4.2. *6β,11α-Dihydroxyandrost-4-ene-3,17-dione (III)*

Colorless crystals (crystallized from ethyl acetate); mp 260 °C, $[\alpha]_D^{25} + 79^\circ$, literature [18]; mp 259–260 °C; $[\alpha]_D^{25} + 72^\circ$ (MeOH, c = 1.0); UV $\lambda_{max}^{MeOH} = 240$ nm; IR ν_{max} (cm^{-1}): 3423, 1735, 1668, 1609; FAB-MS (3-nitrophenyl methanol, probe), *m/z*: 319 $[M+H]^+$; ^1H NMR (CD_3OD): δ (ppm): 0.99 (3H, s, H-18), 1.56 (3H, s, H-19), 4.12 (1H, ddd, *J* = 12, 11, 5 Hz, H-11β), 4.41 (1H, brs, H-6α), 5.84 (1H, s, H-4); ^{13}C NMR (CD_3OD): δ (ppm): 220.7 (C-17), 202.4 (C-3), 170.3 (C-5), 126.2 (C-4), 72.4 (C-6), 68.0 (C-11), 59.0 (C-9), 50.0 (C-14), 47.6 (C-13), 42.2 (C-12), 39.6 (C-10), 39.0 (C-7), 36.5 (C-16), 35.6 (C-1), 34.1 (C-2), 28.2 (C-8), 21.5 (C-15), 19.4 (C-19), 14.0 (C-18); R_f in chloroform/methanol (12:1): 0.48; R_t: 5.7 min.

2.4.3. *17β-Hydroxyandrost-4-ene-3-one (IV)*

Colorless crystals (crystallized from acetone); mp 154–156 °C; $[\alpha]_D^{25} + 106^\circ$ (MeOH, c = 1.0); UV $\lambda_{max}^{MeOH} = 238$ nm; IR ν_{max} (cm^{-1}): 3430, 1664, 1609; ^1H NMR ($CDCl_3$): δ (ppm): 0.80 (3H, s, H-18), 1.20 (3H, s, H-19), 3.66 (1H, t, *J* = 8 Hz, H-17α), 5.73 (1H, s, H-4); ^{13}C NMR ($CDCl_3$): δ (ppm): 199.9 (C-3), 171.5 (C-5), 124.1 (C-4), 81.8 (C-17), 54.1 (C-9), 50.7 (C-14), 43.0 (C-13), 38.9 (C-10), 36.6 (C-12), 35.9 (C-8), 35.8 (C-1), 34.2 (C-2), 33.0 (C-6), 31.7 (C-7), 30.6 (C-16), 23.6 (C-15), 20.8 (C-11), 17.6 (C-19), 11.3 (C-18); R_f in chloroform/methanol (12:1): 0.79; R_t: 11.3 min.

2.4.4. *11α,17β-Dihydroxyandrost-ene-3-one (V)*

Colorless crystals (crystallized from methanol); mp 218–219 °C, $[\alpha]_D^{25} + 97^\circ$ ($CHCl_3$, c = 0.8), literature [16,17]; mp 218–219 °C; $[\alpha]_D^{25} + 91^\circ$ (MeOH, c = 1.0); UV $\lambda_{max}^{MeOH} = 240$ nm; IR ν_{max} (cm^{-1}): 3430, 1668, 1610; FAB-MS (3-nitrophenyl methanol, probe), *m/z*: 305 $[M+H]^+$; ^1H NMR ($CDCl_3$): δ (ppm): 0.81 (3H, s, H-18), 1.31 (3H, s, H-19), 3.68 (1H, t, *J* = 8 Hz, H-17α), 4.02 (1H, ddd, *J* = 12, 11, 5 Hz, H-11β), 5.71 (1H, s, H-4); ^{13}C NMR ($CDCl_3$): δ (ppm): 200.7 (C-3), 171.9 (C-5), 124.6 (C-4), 81.2 (C-17), 69.0 (C-11), 59.4 (C-9), 50.1 (C-14), 48.7 (C-12), 43.9 (C-13), 40.4 (C-10), 37.7 (C-1), 35.6 (C-2), 34.5 (C-8), 34.0 (C-6), 31.5 (C-7), 30.8 (C-16), 23.6 (C-15), 18.7 (C-19), 12.7 (C-18); R_f in chloroform/methanol (12:1): 0.57; R_t: 6.2 min.

2.4.5. *6β,11α,17β-Trihydroxyandrost-ene-3-one (VI)*

Colorless crystals (crystallized from chloroform); mp 235–236 °C; $[\alpha]_D^{25} + 34^\circ$ (MeOH, c = 1.0); UV $\lambda_{max}^{MeOH} = 240$ nm; IR ν_{max} (cm^{-1}): 3430, 1668, 1609; FAB-MS (3-nitrophenyl methanol, probe), *m/z*: 321 $[M+H]^+$; ^1H NMR (CD_3OD): δ (ppm): 0.83 (3H, s, H-18), 1.45 (3H, s, H-19), 3.60 (1H, t, *J* = 8 Hz, H-17α), 4.01 (1H, ddd, *J* = 12, 11, 5 Hz, H-11β), 4.25 (1H, brs, H-6α), 5.77 (1H, s, H-4); ^{13}C NMR (CD_3OD): δ (ppm): 201.5 (C-3), 169.8 (C-5), 125.2 (C-4), 79.9 (C-17), 71.9 (C-6), 67.5 (C-11), 58.4(C-9), 49.1 (C-14), 48.0 (C-12), 42.8 (C-13), 38.9 (C-10), 38.4 (C-7), 36.7 (C-1), 33.4 (C-2), 28.9 (C-16), 28.2 (C-8), 22.3 (C-15), 18.6 (C-19), 10.8 (C-18); R_f in chloroform/methanol (12:1): 0.32; R_t: 4.4 min.

2.4.6. *3α,11α,17β-Trihydroxy-5α-androstane (VII)*

Colorless crystals (crystallized from chloroform); mp 261–263 °C; $[\alpha]_D^{25} - 21^\circ$ (MeOH, c = 1.0); IR ν_{max} (cm^{-1}): 3490, 3400; FAB-MS (3-nitrophenyl methanol, probe), *m/z*: 307 $[M-H]^-$; ^1H NMR (CD_3OD): δ (ppm): 0.71 (3H, s, H-18), 1.08 (3H, s, H-19), 3.58 (1H, t, *J* = 8 Hz, H-17α), 3.80 (1H, m, H-3β), 4.00 (1H, m, H-11β); ^{13}C NMR (CD_3OD): δ (ppm): 80.9 (C-17), 68.4 (C-11), 66.9 (C-3), 59.8 (C-9), 50.2 (C-14), 48.2 (C-12), 47.4 (C-5), 43.3 (C-13), 38.4 (C-4), 36.4 (C-10), 35.2 (C-8), 33.9 (C-7), 32.6 (C-1), 29.5 (C-2), 28.4 (C-6), 27.0 (C-16), 23.6 (C-15), 23.1 (C-19), 11.3 (C-18); R_f in chloroform/methanol (12:1): 0.43.

3. Results

The structures of the transformation products were determined from FAB-MS, IR, ^1H NMR and ^{13}C NMR spectra. The positions and configurations of the introduced hydroxyl groups were determined mainly from changes in ^1H NMR spectra compared with the starting material and data in literature [19].

The FAB-mass spectrum of compound **II** showed the $[M+1]^+$ at *m/z* 303, indicative of the addition of 16 mass units to **I** in agreement with the formula $C_{19}H_{26}O_3$. The IR spectrum showed characteristic absorptions at 3430, 1734, 1664, 1609 cm^{-1} for hydroxyl and saturated ketone and conjugated ketone groups, respectively. The ^1H NMR spectrum showed a new downfield signal for the oxygen-bearing methine proton at δ 4.08 ppm (ddd, *J* = 12, 11, 5 Hz), which indicated introduction of a C-11α hydroxyl group in compounds **II**. The ^{13}C

NMR chemical shifts were assigned by comparison with literature data for **I** [20]. For hydroxylation at C-11α, a new signal indicative of an oxygen-bearing methine carbon appeared at δ 68.8 ppm, and downfield shifts were observed for C-9 (δ 59.3 ppm) and C-12 (δ 43.1 ppm) and a γ-gauche upfield shift for C-8 (δ 34.2 ppm). The NMR data were in agreement with those reported in literature [16,17].

The FAB-mass spectrum of compound **III** showed the $[M+1]^+$ at *m/z* 319, consistent with the addition of 32 mass units to **I** in agreement with the formula $C_{19}H_{26}O_4$. The IR spectrum showed characteristic absorptions at 3423, 1735, 1668, 1609 cm^{-1} for hydroxyl and saturated ketone and conjugated ketone groups, respectively. In the ^1H NMR spectrum two new downfield signals were observed for the oxygen-bearing methine protons at δ 4.12 ppm (ddd, *J* = 12, 11, 5 Hz) and 4.41 ppm (brs), which indicated that the hydroxyl groups introduced in compound **III** were at C-11α and C-6β. The ^{13}C NMR chemical shifts were assigned by comparison with the data of **I** and 6β-hydroxylated steroids [20]. For hydroxylation at C-11α, a new assigned to an oxygen-bearing methine carbon signal appeared at δ 68.0 ppm, and there were downfield shifts for C-9 (δ 59.0 ppm) and C-12 (δ 42.2 ppm) and a γ-gauche upfield shift for C-8 (δ 28.2 ppm). Similarly, for hydroxylation at C-6β, a new oxygen-bearing methine carbon signal appeared at δ 72.4 ppm, and downfield shifts were observed for C-7 (δ 39.0 ppm) and a γ-gauche upfield shift for C-8 (δ 28.2 ppm). The NMR data were in agreement with those reported in the literature [18].

The IR spectrum of compound **IV** 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 81.8 ppm in place of the characteristic signal for the 17-carbonyl group, which indicated that the 17-carbonyl group had been reduced. The presence of 17β-hydroxyl group was confirmed by the presence of a characteristic signal for 17α-H at δ 3.66 ppm (t, *J* = 8 Hz). The NMR data were in agreement with those reported in literature [20].

The FAB-mass spectrum of compound **V** showed the $[M+1]^+$ at *m/z* 305, which indicated the addition of 18 mass units to **I** in agreement with the formula $C_{19}H_{28}O_3$. The IR spectrum showed characteristic absorptions at 3430, 1668, 1610 cm^{-1} for hydroxyl and conjugated ketone groups, respectively. The ^1H NMR spectrum showed two new signals for the oxygen-bearing methine protons at δ 3.68 ppm (1H, t, *J* = 8 Hz) and 4.02 ppm (1H, ddd, *J* = 12, 11, 5 Hz), which indicated compound **V** had C-11α and C-17β hydroxyl groups. The ^{13}C NMR showed two new oxygen-bearing methine carbon signals at δ 69.0 and 81.2 ppm and the characteristic signal for the 17-carbonyl group had disappeared, which also indicated that hydroxylation had taken place at C-11α and that the 17-carbonyl group had been reduced. The ^{13}C NMR chemical shifts were assigned by comparison with the data of **IV**. The signal at δ 81.2 ppm was assigned to C-17β. Hydroxylation at C-11α was indicated by a new oxygen-bearing methine carbon signal that appeared at δ 69.0 ppm, and there were downfield shifts for C-9 (δ 59.4 ppm) and C-12 (δ 48.7 ppm) and a γ-gauche upfield shift for C-8 (δ 34.5 ppm). The NMR data were in agreement with those reported in the literature [16,17].

The FAB-mass spectrum of compound **VI** showed the $[M+1]^+$ at *m/z* 321, which indicated the addition of 34 mass units to **I** in agreement with the formula $C_{19}H_{28}O_4$. The IR spectrum showed characteristic absorptions at 3430, 1668, 1609 cm^{-1} for hydroxyl and conjugated ketone groups, respectively. The ^1H NMR spectrum showed three new signals for the oxygen-bearing methine protons at δ 3.60 ppm (1H, t, *J* = 8 Hz), 4.01 ppm (1H, ddd, *J* = 12, 11, 5 Hz) and 4.25 ppm (1H, brs), and the ^{13}C NMR spectrum showed three new signals for the oxygen-bearing methine carbon at δ 67.5, 71.9 and 79.9 ppm. The presence of a 17-hydroxyl group was deduced from HMBC correlation between the carbon at δ 79.9 ppm and H-18 (δ 0.83 ppm), and COSY correlation between H-17 (δ 3.60 ppm) and H-16α (δ 1.54 ppm), H-16β (δ 2.02 ppm). The chemical shift and coupling constants of H-17 further supported the α-orientation of C17-hydroxyl group. Although no correlations with the proton at δ 4.01 ppm were observed, there were several correlations with the carbon at δ 67.5 ppm in the HMBC spectrum, and H-9, H-12α and H-12β also showed HMBC correlations with this carbon. These data coupled with the COSY data showed correlations between δ 4.01 ppm and H-9 (δ 1.05 ppm), H-12α (δ 1.16 ppm) and H-12β (δ 2.08 ppm), respectively, which suggested that this carbon should be assigned to the 11-position. Two large coupling constants ($^3J_{HH}$ = 12, 11, 5 Hz) for H-11 indicated that this proton was in an axial position. Therefore, this hydroxyl group was assigned to C11-α. Similarly no correlations with the proton at δ 4.25 ppm were observed, but there was a correlation between the carbon at δ 71.9 ppm and H-4 (δ 5.77 ppm) in the HMBC spectrum. These data coupled with the COSY data showed correlations between δ 4.25 ppm and H-7α (δ 1.19 ppm) and H-7β (δ 1.92 ppm), which suggested this carbon should be assigned as C-6. The chemical shift and small coupling constants of H-6 further supported the β-orientation of C6-hydroxyl group.

The FAB-MS spectrum of compound **VII** showed the $[M-H]^-$ at *m/z* 307, which established the addition of 22 mass units to **I** in agreement with the formula $C_{19}H_{32}O_3$. The IR spectrum showed characteristic absorptions at 3490 and 3400 cm^{-1} for hydroxyl group whereas the ^{13}C NMR showed that the characteristic signals for 17-carbonyl, 3-carbonyl and alkene had disappeared from their usual positions consistent with reduction. The ^1H NMR spectrum showed three oxygen-bearing methine protons at δ 3.58 ppm (1H, t, *J* = 8 Hz), 3.80 ppm (1H, m), and 4.00 ppm (1H, m), which indicated this compound had three hydroxyl groups at C-3α, C-11α and C-17β, respectively. The ^{13}C NMR chemical shifts were assigned by comparison with the data for monohydroxy androstanes [21] and 3α,17β-dihydroxy-5α-androstane [22]. The signals at δ 66.9 and 80.9 ppm were assigned to C-3α and C-17β. Consistent with hydroxylation at C-11α, a new oxygen-bearing methine carbon signal appeared at δ 68.4 ppm, and there were downfield shifts for C-9 (δ 59.8 ppm) and C-12 (δ 48.2 ppm) and a γ-gauche upfield shift for C-8 (δ 35.2 ppm).

4. Discussion

The fungus *B. bassiana* CCTCC AF206001 has been used to investigate the microbial transformations of androst-4-ene-3,17-dione. The strain can stereoselectively hydroxylate **I** at C-11α and C-6β, and the rate of hydroxylation at C-11α is much greater than that of hydroxylation at C-6β under our

conditions of cultivation and transformation. If the time of fermentation is controlled properly, two products (**II** and **V**) hydroxylated at C-11α can be obtained in high yield (both 91%).

It was also observed that the strain has strong reductive abilities which can be regulated. If the initial pH value of medium is 6.0, reduction is not observed. However, if the initial pH value is increased to 7.0, the reduction of 17-carbonyl is faster than the hydroxylation at C-11α, but much more time is required to reduce the 3-carbonyl group and C4, C5-double bond, which indicates the reduction of 17-carbonyl is regioselective.

To sum up, this strain of *B. bassiana* may have very useful prospects in the pharmaceutical industry, because it can lead to two valuable 11α-hydroxylated intermediates (**II** and **V**) for steroidal drugs in high yield.

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