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REVIEW

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Studies on the microbial transformation of androst-1,4-dien-3, 17-dione with *Acremonium strictum*

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Abstract The strain of *Acremonium strictum* PTCC 5282 was applied to investigate the biotransformation of androst-1,4-dien-3,17-dione (**I**; ADD). Microbial products obtained were purified by preparative TLC and the pure metabolites were characterized on the basis of their spectroscopic features (^{13}C NMR, ^{1}H NMR, FTIR, MS) and physical constants (melting points and optical rotations). The 15α-Hydroxyandrost-1,4-dien-3,17-dione (**II**), 17β-hydroxyandrost-1,4-dien-3-one (**III**), androst-4-en-3,17-dione (**IV**; AD), 15α-hydroxyandrost-4-en-3,17-dione (**V**), 15α,17β-dihydroxyandrost-1,4-dien-3-one (**VI**) and testosterone (**VII**) were produced during this fermentation. Formation of the 15α,17β-dihydroxy derivative of ADD is reported for the first time during steroid biotransformation. The bioconversion reactions observed were 1,2-hydrogenation, 15α-hydroxylation and 17-ketone reduction. From the time course profile of this biotransformation, ketone reduction and 1,2-hydrogenation were observed from the first day of fermentation while 15α-hydroxylation occurred from the third day. Optimum concentration of the substrate, which gave the maximum bioconversion efficiency, was 0.5 mg ml^{-1} in one batch. The highest yield of the microbial products recorded in this work was achieved within the pH range 6.5–7.3 and at the temperature of 27 °C.

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Introduction

Acremonium Link et Fries 1821 belongs to a very large group of white or pink moulds with wet heads of conidia produced one-by-one from the tips of straight hyphae or lateral nipples. In some species, the conidia buds secondary spores and the colony become yeast-like [1, 2]. They are extremely common in man's environment, are found in soil, decaying vegetation and foodstuffs. It is not commonly associated with human diseases, but it has been identified as a pathogen in cases of mycetoma, keratomycosis, postoperative endophthalmitis, onychomycosis and meningitis [3].

The genus is well known for producing numerous important enzymes including alkaline protease [4], isopenicillin *N* synthetase [5], glucooligosaccharide oxidase [6], protease [7], ascorbate oxidase [8], phenol oxidase [9] and β-glucanases [10]. Xenovulene A, a novel GABA-benzodiazepine receptor binding compound [11]; acremonol and acremodiol, two new fungal bislactones [12]; acremostatins A, B and C, three new lipoaminopeptides [13]; UCS 1025 A and B, new antitumor antibiotics [14] and the polyketide-derived antibiotics [15] are some new secondary metabolites isolated from the genus belonging to *Acremonium*.

Concerning the steroid biotransformation by these fungi, some trends have been identified so far. Hydroxylation in various positions of esterane and pregnane-based steroids using *A. kiliense* [16] and *A. potronii* [17], respectively, has been reported in some studies. *A. alabamensis* and *A. roseum* were found to carry out the side chain cleavage of 3β-hydroxy-24-ethylcholest-5-ene, cholest-5-en-3β-ol, 3β-hydroxy-24-ethylcholest-5,22-diene [18] and pregn-4-en-3,20-dione [19], respectively. The potential of the strains belonging to *Acremonium strictum* for the biotransformation of some steroid substances such as

19-norethisterone [16], androstendione [20], hydrocortisone [21] and progesterone [22, 23] was also studied in some limited publications. However, to date no research has been carried out on the microbial transformation of androst-1,4-dien-3,17-dione (ADD) by this fungus.

This study was performed because of the importance of ADD as a lead compound for preparing some pharmaceutically-interesting steroids. In the present work, the ability of *A. strictum* was evaluated for the biotransformation of ADD as an exogenous substrate.

Materials and methods

Instruments

Melting points (mp) were determined on a Reichert–Jung hot stage melting point apparatus and are uncorrected. Optical rotations were measured in 1-dm cells on a Perkin-Elmer 142 automatic spectropolarimeter. ^{1}H and ^{13}C nuclear magnetic resonances (NMR) spectra were recorded using the FTNMR Varian Unity plus spectrometer at 400 and 100 MHz, respectively, in $CDCl_3$ with tetramethylsilane (TMS) as the internal standard. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS. The coupling constant (J) was given in hertz (Hz). Infrared (IR) spectra were recorded on a Magna-IR 550 Nicolet FTIR spectrometer. Mass spectra (MS) were obtained with a Finnigan MAT TSQ-70 instrument by electron impact (EI) at 70 eV. Thin layer chromatography (TLC) and preparative TLC were performed, respectively, on 0.25 and 0.5 mm thick layers of silica gel G (Kieselgel 60 $HF_{254+366}$, Merck). Layers were prepared on glass plates and activated at 105°C, 1 h before use. Chromatography was performed with acetone/hexane (45:55, v/v) and visualized by spraying the plates with a mixture of methanol/sulfuric acid (6:1, v/v) and heating them in an oven at 100°C for 3 min until the colors developed. The compounds were also visualized under a UV lamp (Strstedt–Gruppe HP-UVIS) at 254 nm.

The high performance liquid chromatography (HPLC) apparatus consisted of a Knauer model K-1001 pump, a K-2600 UV variable-wavelength detector and an online degasser, all from Knauer (Berlin, Germany). Samples were injected to a Knuer D-14163 injector system with a 20 µl sample loop. The data were acquired and processed by means of the Eurochrom chromatography software (Knauer, Berlin, Germany). Chromatographic separation was achieved on a Finepak SIL C18-10 reverse phase column (C18, 25 × 0.46 cm i.d., 10 µm particle size) from Jasco Corporation (Japan).

Chemicals

Androst-1,4-dien-3,17-dione was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sabouraud-2%-dextrose broth (SDB) and Sabouraud-4%-dextrose agar (SDA) were purchased from Merck (Darmstadt, Germany). Analytical chemicals and solvents were from Merck, Sigma and Fluka.

Microorganism

The examined fungus was a strain of *A. strictum* PTCC 5282 [21] that maintained on SDA slope for the long time storage and was freshly subcultured before it was used in a transformation experiment.

Incubation conditions

Preculture of A. strictum Spores of *A. strictum*, which had been preserved at low temperature, were inoculated into 50 ml of the sterilized culture medium of SDB in a conical flask. The flask was then incubated for 24 h in an incubator shaker (150 rpm) at 27 °C.

Time course experiment Precultured *A. strictum* was transferred into a 500-ml Erlenmeyer flask containing 100 ml of the SDB medium supplemented with 50 mg of ADD dissolved in 2 ml of absolute ethanol. The incubation continued for 10 days at the same condition. Sampling was carried out every 24 h. Control was similarly processed without the microorganism.

The effect of temperature, pH and the substrate concentration Studies were performed to determine the suitable pH and temperature as well as the amount of substrate that could be transformed to the products. The temperature was checked between 25 ° and 30 °C. The effect of pH on the biotransformation procedure was studied in non-buffered media by adjusting the pH from 5 to 8.5 with NaOH and HCl. The amount of the substrate varied from 0.05 to 0.5 g 100 ml^{-1}. In each case, one parameter was studied and the others were kept constant.

Production of the metabolites for purification

ADD, 1 g, was dissolved in 20, 500-ml conical flasks, each containing 100 ml of the SDB medium using absolute ethanol (2 ml ethanol for each flask). The final concentration of the substrate was 0.05% in each flask. The pH was adjusted at 7.0. The flasks were then incubated in an orbital incubator shaker at 27 °C and 150 rpm for 10 days.

Product isolation and analyses

At the end of incubation, the fermentation broth was extracted with three volumes of chloroform. The extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was then loaded on preparative TLC and fractionated with an acetone/hexane (45:55, v/v) solvent system and the metabolites were separated from silica gel by a mixture of methanol/chloroform (90:10 and 10:90, v/v; three

times). Purified metabolites were identified by the melting points and spectral data (^{13}C NMR, ^{1}H NMR, FTIR and MS) after crystallization.

Quantification procedures

HPLC was applied to the quantitative studies of time course experiments, the influence of temperature, pH and the substrate concentration and also to obtain the biotransformation yield of each metabolite. A total of 10 µl of the concentrated extract was injected into a C18 reverse phase column. Elution was done by an isocratic method using methanol/acetonitril/water (20:25:55, v/v/v) with a flow rate of 1 ml min^{-1}. Detection was done by UV at 254 nm.

Stock and standard solutions Stock solutions were prepared individually by dissolving 10 mg of the substrate (**I**) as well as purified metabolites **II**–**VII** in 10 ml methanol. Subsequently, standard working solutions were prepared individually in methanol for the substrate and all the metabolites as presented in Table 1.

Calibration Triplicate injections were made for each concentration of the standard solutions and then the mean peak area of each compound was plotted against the concentration to obtain the calibration graphs. All concentrations of each compound were subjected to a regression analysis to calculate the calibration equation and correlation coefficients. Throughout the study, the suitability of the chromatographic system was monitored by calculating the capacity factor (k`) and the resolution (R).

Results

Biotransformation of androst-1,4-dien- 3,17-dione

The result of the biotransformation of ADD (**I**) by *A. strictum* PTCC 5282 led to the formation of six major metabolites (**II**–**VII**) (Fig. 1). The HPLC profile of the fermentation extract presented well-resolved peaks (Fig. 2). Retention times for the major steroids were 7.3 (**VI**), 8.2 (**II**), 10.6 (**V**), 12.6 (**VII**), 23.1 (**I**), 25.7 (**III**) and 33.8 (**IV**) min. According to the HPLC analyses, the yield of each product was found to be 6.2% (**II**), 23.3% (**III**), 3% (**IV**), 19% (**V**), 4.5% (**VI**) and 0.92% (**VII**), respectively. At the end of the fermentation, the percentage of the unconverted substrate (**I**) was 28.3%. A HPLC analysis revealed that few other metabolites were also found in the transformed mixture, which was not purified for characterization, due to trace quantity.

The analytical data of compound **I**–**VII** are mentioned in a respective order as follows. ^{13}C NMR assignments of the substrates as well as the bioproducts are listed in Table 5.

Androst-1,4-dien-3,17-dione (I) mp 139–141 °C, $[\alpha]_D$ + 117° ($CHCl_3$), lit [24] mp 141–142 °C, $[\alpha]_D$ + 115° ($CHCl_3$); IR ν_{max} 2,940, 1,736, 1,656, 1,615 cm^{-1}; MS (EI) *m/z* (%) 284 (M^+, $C_{19}H_{24}O_2$), 264 (10), 159 (22), 122 (100), 91 (22), 79 (15), 67 (6); ^{1}H NMR ($CDCl_3$) δ 0.94 (3H, s, H-18), 1.25 (3H, s, H-19), 6.08 (1H, s, H-4), 6.23 (1H, d, J = 10 Hz, H-2), 7.07 (1H, d, J = 10 Hz, H-1); R_f in acetone/hexane (45:55, v/v): 0.58.

15α-Hydroxyandrost-1,4-dien-3,17-dione (II) mp 245–247 °C, $[\alpha]_D$ + 125° ($CHCl_3$), lit [25] mp 248°C; IR ν_{max} 3,411, 2,922, 1,736, 1,657, 1,605 cm^{-1}; MS (EI) *m/z* (%) 300 (20) (M^+, $C_{19}H_{24}O_3$), 272 (8), 167 (15), 154 (100), 86 (22), 70 (60); ^{1}H NMR ($CDCl_3$) δ 0.91 (3H, s, H-18), 1.22 (3H, s, H-19), 4.38 (1H, m, H-15), 6.09 (1H, s, H-4), 6.20 (1H, d, J = 10 Hz, H-2), 7.02 (1H, d, J = 10 Hz, H-1); R_f in acetone/hexane (45:55, v/v): 0.35.

17β-Hydroxyandrost-1,4-dien-3-one (III) mp 170–173 °C, $[\alpha]_D$ + 30° ($CHCl_3$), lit [25] mp 171 °C, $[\alpha]_D$ + 34° ($CHCl_3$); IR ν_{max} 3,416, 1,647, 1,449 cm^{-1}; MS (EI) *m/z* (%) 286 (40) (M^+, $C_{19}H_{26}O_2$), 253 (8), 227 (8), 185 (8), 159 (15), 147 (44), 122 (100), 91 (64), 79 (30), 67 (22), 55(15); ^{1}H NMR ($CDCl_3$) δ 0.84 (3H, s, H-18), 1.29 (3H, s, H-19), 3.66 (1H, t, J = 7 Hz, H-17), 6.09 (1H, s, H-4), 6.25 (1H, d, J = 8, H-2), 7.08 (1H, d, J = 8 Hz, H-1); R_f in acetone/hexane (45:55, v/v): 0.53.

Androst-4-en-3,17-dione (IV) mp 176–178 °C, $[\alpha]_D$ + 182.5° ($CHCl_3$), lit [24]; mp 171.5–172.5 °C, $[\alpha]_D$ + 194.8° ($CHCl_3$); IR ν_{max} 3,016, 1,741, 1,657, 1,215 cm^{-1}; MS (EI) *m/z* (%) 286 (100) (M^+, $C_{19}H_{26}O_2$), 244 (54), 201 (30), 148

Table 1 Linearity results, limit of detection (LOD) and limit of quantification (LOQ)

Compound	Equation	r^2	Slope (RSD%)	Intercept (RSD%)	Concentration of standard solutions	LOD (µg ml^{-1})	LOQ (µg ml^{-1})
I	$Y = 0.763817X - 0.611905$	0.9938	0.093	15.246	1,10,20,40,60, 80	1	10
II	$Y = 0.673086X - 0.001922$	0.9981	0.269	11.107	0.1,1,10,20,40,80	0.1	1
III	$Y = 0.236021X + 0.106759$	0.9939	1.844	18.438	1,5,10,20,50,80	1	5
IV	$Y = 0.576878X + 1.087968$	0.9915	0.815	8.908	0.1,1,5,10,30,50	0.1	1
V	$Y = 0.242794X + 0.197478$	0.9997	1.942	15.919	0.1,1,5,10,25,50	0.1	1
VI	$Y = 0.091254X + 0.051563$	0.9983	0.883	15.727	0.1,1,10,20,50,80	0.1	1
VII	$Y = 1.302799X + 1.341786$	0.9936	0.091	0.679	0.05, 0.5,1,5,10,20	0.05	0.5

X Concentration (µg ml^{-1}), Y area

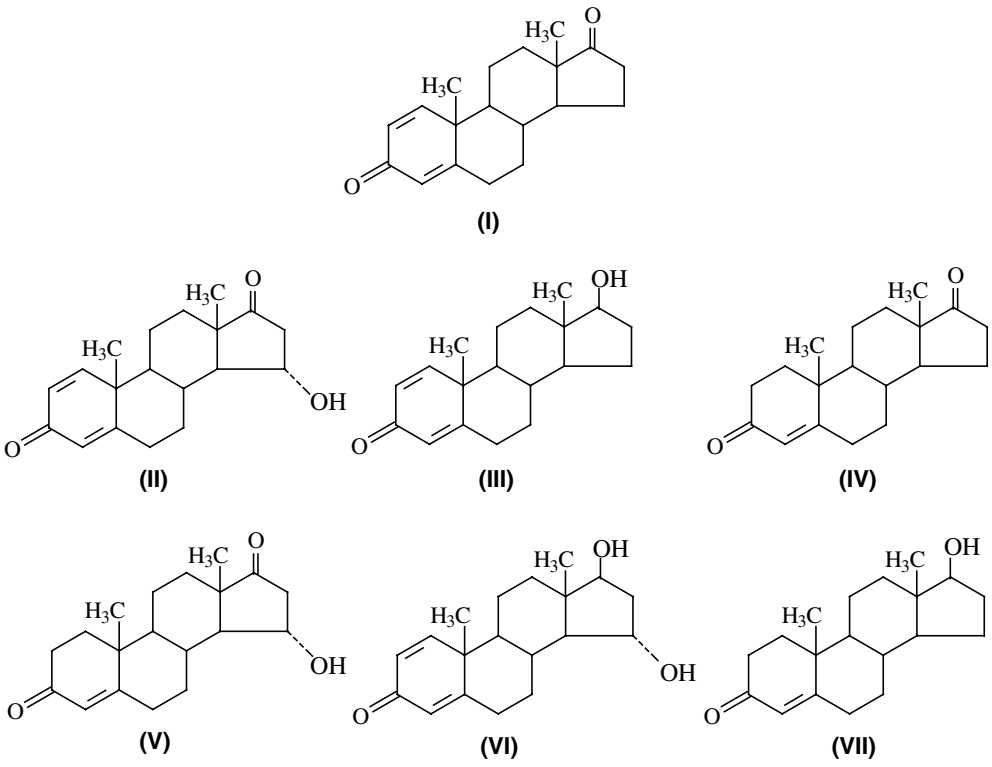

Fig. 1 The structures of ADD (**I**) and its biometabolites: 15α-hydroxyandrost-1,4-dien-3,17-dione (**II**), 17β-hydroxyandrost-1,4-dien-3-one (**III**), androst-4-en-3, 17-dione (AD) (**IV**), 15α-hydroxyandrost-4-en-3, 17-dione (**V**), 15α,17β-dihydroxyandrost-1, 4-dien-3-one (**VI**), testosterone (**VII**)

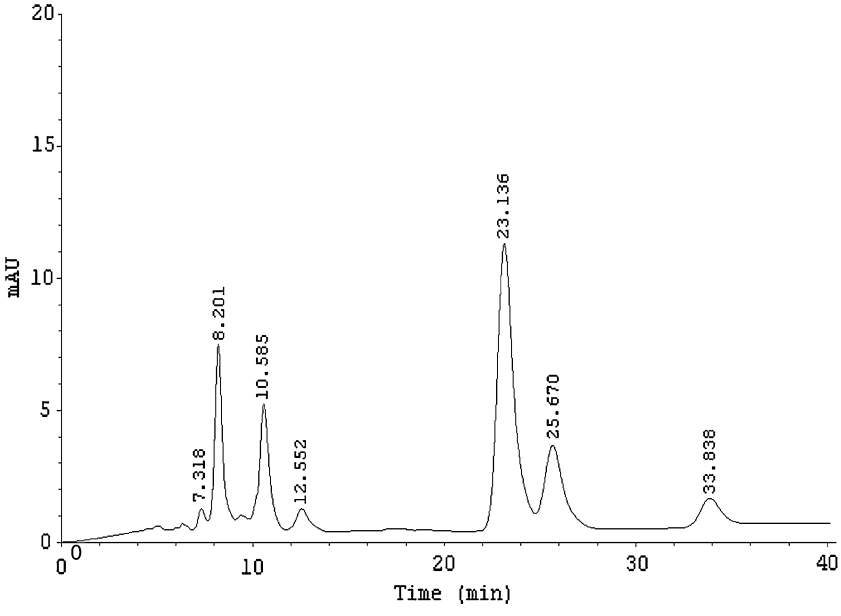

Fig. 2 HPLC profile of ADD (**I**) transformation by *A. strictum*: 15α,17β-dihydroxyandrost-1,4-dien-3-one (**VI**), 7.3 min; 15α-hydroxyandrost-1,4-dien-3,17-dione (**II**), 8.2 min; 15α-hydroxyandrost-4-en-3, 17-dione (**V**), 10.6 min; testosterone (**VII**), 12.6 min; androst-1,4-dien-3,17-dione (ADD) (**I**), 23.1 min; 17β-hydroxyandrost-1, 4-dien-3-one (**III**), 25.7 min; androst-4-en-3,17-dione (AD) (**IV**), 33.8 min

(50), 124 (100), 97 (74), 69 (64); ^{1}H NMR ($CDCl_3$) δ 0.96 (3H, s, H-18), 1.24 (3H, s, H-19), 5.80 (1H, s, H-4); R_f in acetone/hexane (45:55, v/v): 0.65.

15α-Hydroxyandrost-4-en-3,17-dione (V) mp 200–201 °C, $[\alpha]_D$ + 220° ($CHCl_3$), lit [25] mp 200–202 °C, $[\alpha]_D$ + 218° ($CHCl_3$); IR ν_{max} 3,431, 2,924, 1,735, 1,662, 1,615 cm^{-1}; MS (EI) *m/z* (%) 302 (14) (M^+, $C_{19}H_{26}O_3$), 284 (10), 257 (15), 232 (9), 56 (21), 47 (16), 43 (24), 35 (25), 31 (100), 30 (94); ^{1}H NMR ($CDCl_3$) δ 0.97 (3H, s, H-18), 1.25 (3H, s, H-19), 4.45 (1H, m, H-15), 5.78 (1H, s, H-4); R_f in acetone/hexane (45:55, v/v): 0.44.

15α,17β-Dihydroxyandrost-1,4-dien-3-one (VI) mp 126 –129 °C, $[\alpha]_D$ + 57° ($CHCl_3$); IR ν_{max} 3,432, 2,936, 1,659, 1,615 cm^{-1}; MS (EI) *m/z* (%) 302 (11) (M^+, $C_{19}H_{26}O_3$), 287 (9), 276 (12), 213 (7), 212 (11), 188 (32), 154 (16), 138 (9), 83 (20), 49 (100); ^{1}H NMR ($CDCl_3$) δ 0.84 (3H, s, H-18), 1.27 (3H, s, H-19), 3.91 (1H, t, J = 9.6 Hz, H-17), 4.13 (1H, sx, J = 8.8 Hz, and J = 3.6 Hz, Hβ-15), 6.09

(1H, s, H-4) 6.25 (1H, d, $J = 9.4$, H-2), 7.07 (1H, d, $J = 9.4$ Hz, H-1); R_f in acetone/hexane (45:55, v/v): 0.16.

Testosterone (VII) mp 165–168 °C, $[\alpha]_D$ + 97° (EtOH), lit [26] mp 165–168 °C, $[\alpha]_D$ + 97° (EtOH); IR ν_{max} 3,413, 2,936, 1,661, 1,607 cm^{-1}; MS (EI) *m/z* (%) 288 (60) (M^+, $C_{19}H_{28}O_2$), 246 (42), 228 (15), 203 (21), 164 (35), 147 (38), 124 (100), 109 (25), 81 (19); ^1H NMR ($CDCl_3$) δ 0.80 (3H, s, H-18), 1.19 (3H, s, H-19), 3.65 (1H, t, $J = 8.4$ Hz, H-17), 5.73 (1H, s, H-4); R_f in acetone/hexane (45:55, v/v): 0.61.

Spectra interpretation

The molecular formula of the compound **II** was deduced as $C_{19}H_{24}O_3$ from MS in combination with ^{13}C NMR spectroscopy. From these data it was found to be 300, which indicated an increase of 16 units as compared to that of the substrate (**I**). The IR spectrum revealed absorption bands for the hydroxyl group (3,411 cm^{-1}), 17-ketone (1,736 cm^{-1}) and 3-ketone (1,657 cm^{-1}) conjugated with double bonds. The ^1H NMR spectrum contained signals for two methyl groups (δ 0.91, H-18 and δ 1.22, H-19; each as s), a singlet at δ 6.09 for H-4 and two doublets at δ 6.20 and δ 7.02 ($J = 10$ Hz) for H-2 and H-1, respectively. The 15α-hydroxy group was confirmed through the absence of a chemical shift for C-15 at δ 21.8 in the ^{13}C NMR spectrum of compound **I**, which was replaced by a singlet at δ 69.9 and also by the appearance of a multiplet at δ 4.38 in ^1H NMR.

In compound **III**, the MS showed the molecular ion at *m/z* 286, which suggested the addition of two units to ADD in agreement with the formula $C_{19}H_{26}O_2$. The IR spectrum indicated only one carbonyl group at 1,647 cm^{-1}, which was conjugated to the C-1 and C-4 double bonds. The lack of an absorption band at 1,736 cm^{-1} (17-ketone) and the existence of a peak at 3,416 cm^{-1} in the IR spectrum of compound **III** conducted to a reduction of the carbonyl to a hydroxyl group at the C-17 position. This alteration was confirmed by chemical shifts of H-17 at δ 3.66 in ^1H NMR and δ 81.2 in ^{13}C NMR.

Metabolite **IV**, $C_{19}H_{26}O_2$, exhibited in its MS the molecular ion peak at *m/z* 286 again. The IR spectrum indicated absorbance at 1,741 cm^{-1} for the 17-carbonyl group and at 1,657 cm^{-1} for 3-ketone conjugated with the double bond on C-4. Two doublet peaks at δ 6.20 and 7.02 in the ^1H NMR spectrum of ADD disappeared. These data were supported with ^{13}C NMR, which indicated the replacement of δ 35.0 and 32.5 with δ 155.2 and 127.6 for C-1 and C-2, respectively. Therefore, in this compound reduction of the C1–C2 olefinic moiety was observed. Optical rotations and melting points of the metabolites were identical with those reported in the literature.

MS of metabolite **V** revealed an ion peak at *m/z* 302 in agreement with the formula $C_{19}H_{26}O_3$. It was 18 units more than ADD (**I**). The IR spectrum showed 3,431 (OH), 1,735 (17-ketone), 1,662 (3-keton-4-ene) and 1,615 (4-ene) cm^{-1}, respectively. The ^1H NMR spectrum exhibited a proton signal (4.45 ppm, as multiplet), two methyl groups (δ 0.97, H-18 and δ 1.25, H-19; each as s) and a singlet at δ 5.78 for H-4. Comparison of the chemical shift of C-15 at δ 70.2 in the ^{13}C NMR spectrum with that of androst-4-en-3,17-dione (AD) (δ 21.7) gave an incremental $\Delta\delta$ value of + 48.5 ppm, which suggests hydroxylation at the 15-position (see Table 5). The stereochemistry of the 15α-hydroxy group was confirmed through the literature of Kirk et al. [27].

In compound **VI** appeared a molecular ion at *m/z* 302 with the formula $C_{19}H_{26}O_3$. The mentioned molecular weight together with the peak at 3,432 cm^{-1} in the IR spectrum suggested addition of the hydroxyl group(s) into the substrate skeleton. According to the ^1H NMR spectrum, one hydroxyl group was appeared as a sextet (δ 4.13) and the other was presented as a triplet (δ 3.91). This was also supported by the ^{13}C NMR spectral data that showed signals at 72.3 and 78.5 ppm (Table 5) attributed to the oxygen atoms. The positions of those two hydroxyl groups were deduced from the comparison with the data for **V** and **VII** relative to the 15α and 17β-positions. The stereochemistry of C-15 in compound **VI** was determined by comparing the coupling constant of H-15 in 15α-hydroxytestosterone [28] which shows a sextet with $J = 9.9$ and 4 Hz signal at δ 4.12 for 15β-H in its ^1H NMR spectrum. An observation of the same coupling constant as a sextet with $J = 9.6$ and 3.6 Hz at δ 4.13 for 15β-H corroborated the presence of the 15α-hydroxyl group in compound **VI**. Thus, product **VI** was characterized as 15α,17β-dihydroxyandrost-1,4-dien-3-one.

The molecular formula of the compound **VII** was deduced as $C_{19}H_{28}O_2$ from MS in combination with ^{13}C NMR spectroscopy. It was found to be 288, which indicated an increase of 4 units compared to that of the substrate. The IR spectrum revealed absorption bands for the hydroxyl group (3,413 cm^{-1}) and 3-ketone (1,661 cm^{-1}) conjugated with a double bond at the C-4 position (1,607 cm^{-1}). The ^1H NMR spectrum contained signals for two methyl groups (δ 0.8, H-18 and δ 1.19, H-19; each as singlet) and a singlet at δ 5.73 for H-4. The 17β-hydroxyl group was confirmed through the absence of a chemical shift for the carbonyl group at δ 219.6 in the ^{13}C NMR spectrum, which was replaced by a signal at δ 81.5 and also by the appearance of a distinctly visible triplet at δ 3.65 ($J = 8.4$ Hz) [27].

HPLC method development

The mobile phase was chosen after several trials with methanol, acetonitrile and water in various proportions. A mobile phase consisting of methanol/acetonitrile/water (20:25:55, v/v/v) was selected to achieve the maximum separation and sensitivity. Flow rates between 0.5 and 1.8 ml/min were studied. A flow rate of 1 ml/min gave an optimal signal to noise with a reasonable separation time. The chromatogram at 254 nm showed a complete resolution for all peaks (Fig. 2).

Linearity Table 1 presents the equation of the regression line, correlation coefficient (r^2) and relative standard deviation (RSD) values of the slope and intercept for each compound. Excellent linearity was obtained for metabolites between peak areas and concentrations of steroid compounds as presented in Table 1. An RSD% less than 20% was accepted.

Limits of detection and quantification Limits of detection (LOD) were established at a signal-to-noise ratio (S/N) of 3. Limits of quantification (LOQ) were established at a S/N of 10. The LOD and LOQ were experimentally verified by six injections of compounds at the LOD and LOQ concentrations. The calculated LOD and LOQ for all compounds are shown in Table 1.

Suitability of the method The chromatographic parameters such as resolution, capacity factor and retention time were satisfactory for these compounds (Table 2).

Precision The precision of the method (intra-day variations of replicate determinations) was checked by injecting nine times the steroidal compounds at the LOQ level (three replicates in three consecutive days). The intra-day precision of the method, expressed as the RSD % at the LOQ level, for each compound is present in Table 3. Inter-day precision of the method was determined by the injection of nine replicates of each compound at the LOQ level in 1 day and calculation of the RSD% for compounds (Table 3). An RSD% less than 20% was accepted.

Accuracy A standard working solution containing compounds **I**–**VII**, to give final concentrations of 40, 40, 50, 50, 50, 80, 20 mg ml^{-1}, respectively, was prepared. The prepared mixture of metabolites was injected nine times as a test sample. From the respective area counts, the concentrations of the compounds were calculated. The accuracy, defined in terms of percent deviation of the calculated concentrations from the actual concentrations, is listed in Table 4. The results are obtained from a range of ± 5%, a method deemed to be accurate (Table 5).

Time course profile of the microbial transformation

For a time course study, the productions of **II**–**VII**, as a function of incubation time, were detected by TLC and determined quantitatively by HPLC (Fig. 3). The starting material, ADD 0.5 mg ml^{-1}, was transformed into various metabolites and *ca* 71.7% of **I** was

Table 2 System performance parameters

Compound	t_r ($n = 9$, mean)	Peak area ($n = 9$, mean)	K'	R
I	26.738	29.127	12.369	1.562
II	8.725	12.196	3.367	1.416
III	24.354	11.109	11.177	7.867
IV	36.936	28.093	15.97	4.277
V	11.419	12.211	4.709	1.323
VI	7.735	4.73	2.867	1.096
VII	13.719	28.041	5.859	2.074

t_r Retention time, k capacity factor, R resolution

Table 3 Precision of the developed method at the LOQ level ($n = 9$)

Compounds	Intra-day		Inter-day	
	Peak area (mean)	RSD (%)	Peak area (mean)	RSD (%)
I	8.598	1.559	8.487	0.834
II	1.417	0.967	1.402	1.372
III	1.17	6.521	1.038	3.086
IV	0.627	6.844	0.621	6.341
V	0.475	1.413	0.484	0.921
VI	0.115	3.174	0.1203	2.827
VII	1.953	2.906	1.898	1.686

Table 4 Accuracy of the developed method ($n = 9$)

Compound	Spiked concentration (μg ml^{-1})	Measured concentration (μg ml^{-1}) (mean ± SD)	Deviation (%)
I	40	37.99589 ± 1.9325	5.01
II	40	39.23563 ± 0.53834	1.191
III	50	47.74928 ± 1.47733	4.501
IV	50	48.63126 ± 3.08375	2.737
V	50	49.94305 ± 0.46036	0.114
VI	80	79.87022 ± 2.25810	0.162
VII	20	20.40527 ± 0.06828	−2.026

Table 5 ^{13}C NMR signals of the substrate and the metabolites (δ in ppm downfield from TMS, in $CDCl_3$)

Carbon atom	Compounds						
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**
1	155.2	155.6	156.1	35.0	35.4	155.6	35.7
2	127.6	127.6	127.4	32.5	32.5	127.5	32.8
3	186.0	186.4	186.5	199.3	199.0	186.3	199.4
4	124.0	123.7	123.7	124.1	124.0	123.7	123.9
5	168.2	168.9	169.5	170.2	170.0	168.9	171.1
6	32.3	33.5	32.7	31.2	31.2	32.7	31.5
7	31.2	32.7	33.1	30.8	29.6	29.6	30.5
8	35.6	35.3	35.5	33.8	33.8	35.2	32.8
9	52.3	52.4	52.6	53.7	53.6	52.5	53.9
10	43.4	43.5	43.6	38.5	38.6	43.5	38.6
11	22.0	22.0	22.5	20.2	20.2	22.3	20.6
12	32.5	31.2	36.3	35.7	35.4	33.6	36.4
13	47.6	46.0	43.1	47.4	46.2	44.5	42.8
14	50.4	56.9	50.2	50.7	57.3	58.0	50.5
15	21.8	69.9	23.5	21.7	70.2	72.3	23.3
16	35.5	50.6	30.2	36.2	50.6	42.4	28.5
17	219.6	216.0	81.2	220.4	215.9	78.5	81.5
18	13.8	15.3	11.1	13.6	15.3	12.6	11.1
19	18.7	18.7	18.6	17.3	17.4	18.7	17.7

I Androst-1,4-dien-3,17-dione (ADD), **II** 15α-hydroxyandrost-1,4-dien-3,17-dione, **III** 17β-hydroxyandrost-1,4-dien-3-one, **IV** androst-4-en-3,17-dione (AD), **V** 15α-hydroxyandrost-4-en-3,17-dione, **VI** 15α,17β-dihydroxyandrost-1,4-dien-3-one, **VII** testosterone

consumed within 10 days. The major metabolite, compound **III**, was detected in a concentration of 33.4 μg ml^{-1} on the sixth day and decreased to 11.7 μg ml^{-1} at the end of the fermentation. Following the decrease of metabolite **III**, the concentration of metabolite **V** increased. The highest concentration of compound **V** was 9.6 μg ml^{-1} on the ninth day. Compound **II** appeared after 2 days and reached 3.1 μg ml^{-1} on the tenth day. Compounds **VI** and **VII** were accumulated in the medium in 3 days and reached the maximum level of 2.3 and 0.75 μg ml^{-1} on the ninth and seventh day, respectively. According to Fig. 3, ketone reduction and 1,2-hydrogenation were observed from the first day of biotransformation while 15α-hydroxylation occurred from the third day.

Influence of fermentation conditions and the substrate concentration on steroid compounds production

The highest bioconversion rates of ADD were obtained within the pH range 6.5–7.5 (Fig. 4). The transformation process was markedly retarded at pH values below 6.5 or above 7.5 for some products (**II**, **III**, **V** and **VI**). The production of compounds **IV** and **VII** was not affected by changing the pH within 5–8.5. The optimal pH for the production of nearly all metabolites was seven. The bioconversion reaction proceeded well at 27 °C for production of all the metabolites except **II** and **VII** that had concentrations higher at 25 and 30 °C, respectively (Fig. 5). Increasing the concentration of ADD from 0.5 to 5 mg ml^{-1} showed that higher concentrations of the substrate (2, 4 and 5 mg ml^{-1}) decreased the microbial conversion (Fig. 6). For the production of all metabolites, the optimum substrate concentration within the range of 0.05–0.5 mg ml^{-1}, which gave the maximum efficiency in the microbial conversion of ADD by *A. strictum*, was 0.5 mg ml^{-1} except for compound **IV** which was 1 mg ml^{-1}.

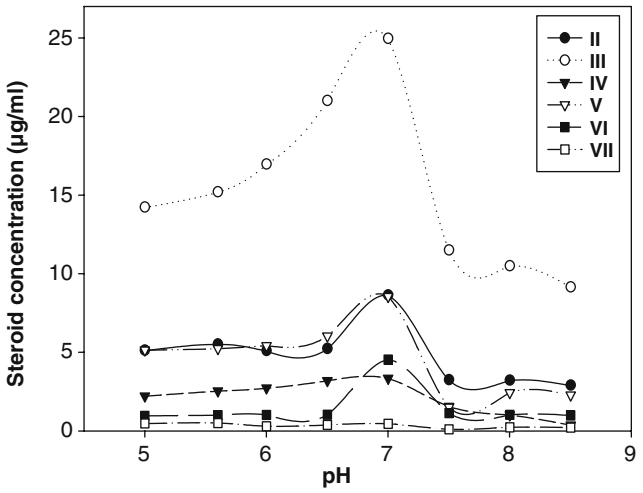

Fig. 4 Influence of the various pH values on the transformation studies

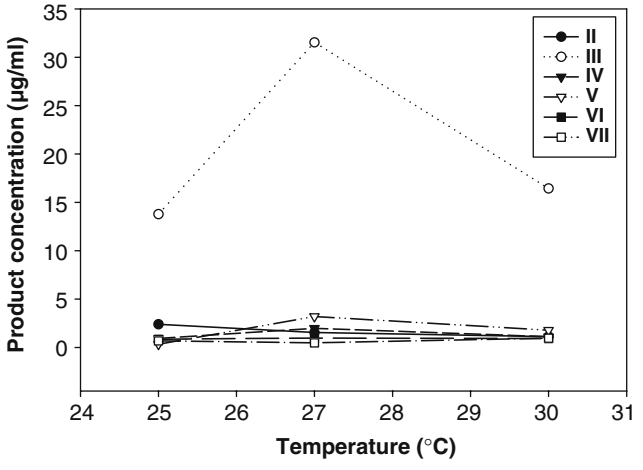

Fig. 5 Influence of three different temperatures (25, 27 and 30 °C) on the bioconversion studies

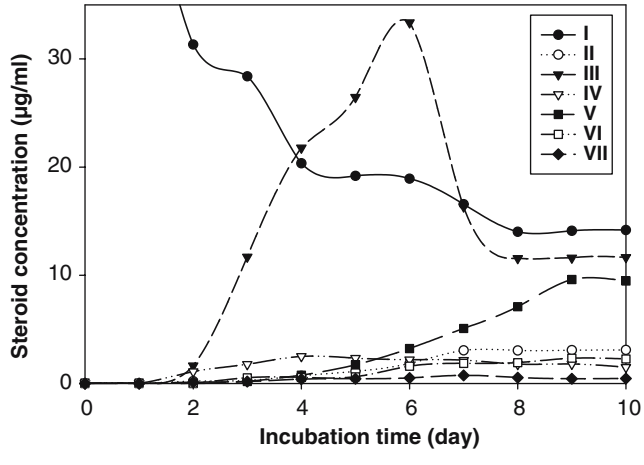

Fig. 3 Time course profile for the biotransformation of ADD (**I**) by *A. strictum*

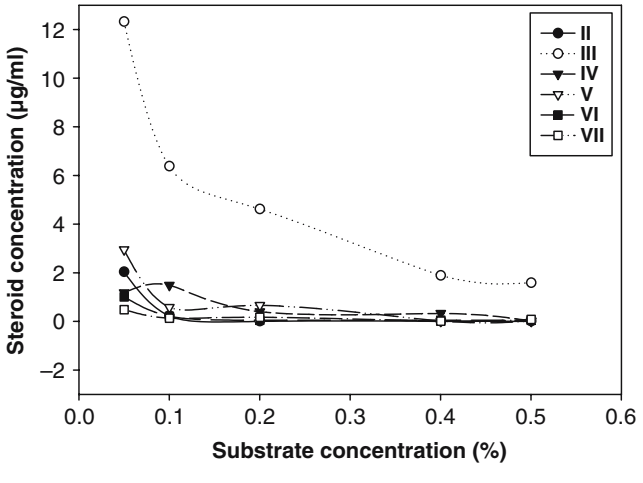

Fig. 6 Effect of ADD (**I**) concentration on production of the metabolites

Discussion

From the results of the experiment, it appears that the *A. strictum* transformation of ADD led to the formation of six bioproducts. The bioconversion characteristics observed were 17-ketone reduction, 1,2-hydrogenation and 15α-hydroxylation. In three cases, ketone reduction occurred (**III**, **VI**, **VII**). The C1–C2 double bond reduction of the substrate led to the accumulation of androst−4-en-3-one (**IV**). We also found three 15-hydroxylated metabolites in the fermentation medium (**II**, **V**, **VI**). Spectroscopic analysis proved that the stereochemistry of the 15-hydroxyl group was as the α-form.

In the previous studies on this strain, 20-ketone reduction, side chain cleavage and 21-acetylation of hydrocortisone [21], 15α- and/or 21-hydroxylation of progesterone [23], side chain cleavage and dimethoxylation of prednisolone (unpublished data) and ester hydrolysis, 17-oxidation and 15α-hydroxylation of nanderolone decanoate (unpublished data) were observed.

ADD is one of the most useful intermediates for producing some valuable pharmaceutical steroid compounds [29] and has been used in many studies as a substrate of the biotransformation experiments. Holland and Chenchaiah [30] isolated 1,2-epoxyandrost-4-en-3,17-dione from the culture of *Rhizopus orrhizus* ATCC 11145 containing ADD. Androst-1,4-dien-3,11,17-trione is a derivative of ADD that was purified in the culture of *Pellicularia filamentosa* f.s. *microsclerotia* [31]. Δ^1-Hydrogenation of ADD was also reported by *Mycobacterium* sp. and *Marchantia polymorpha* [32, 33, respectively]. 17-Ketone reduction was observed during several studies [31–33]. It seems that Δ^1-hydrogenation and 17-keto reduction are common bioreactions on ADD by some microorganisms. There are also some reports on the hydroxylation of the same substrate at C-7 [34], C-11 [31, 33, 34], C-14 [31, 34] and C-15 [34]. This study showed that C-15 is a suitable site for the hydroxylation of ADD by *A. strictum*. Although 15α-hydroxylation together with 17-ketone reduction of AD for production of 15α,17β-androst-1,4-dien-3-one has been reported in the literature [35], this is the first report on the formation of 15α,17β-androst-1,4-dien-3-one during steroid biotransformation.

A. strictum was also examined for the conversion of ADD at pH levels between 5 and 8.5. No change was observed in the biotransformation pattern among the different pH values; the concentrations of all the produced metabolites were high in a neutral pH value (pH 7). The same result was reported by Al-Awadi et al. [36] on the biotransformation of progesterone by *Bacillus stearothermophilus* and by Sallam et al. [37] on the bioconversion of 19-nortestosterone by *Rhodococcus* sp. They also found the optimum pH as 7.

The utilization of high amounts of the steroidal substrates is one of the important factors affecting the economy of the transformation process [38]. In this study, increasing the concentration of ADD showed that the high concentration of the substrate (2, 4 and 5 mg ml^{-1}) retarded the biotransformation process. The optimum substrate concentration, which gave the maximum bioconversion efficiency, was 0.5 mg ml^{-1} (Fig. 6). This may be due to the toxicity of the substrate on the activity of the microorganism. The same phenomena were discussed by Constantinides [39], Goetschel and Bar [40] and Arinbasarova [41].

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