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Microbial transformation of androst-4-ene-3,17-dione by three fungal species *Absidia griseolla* var. *igachii*, *Circinella muscae* and *Trichoderma virens*

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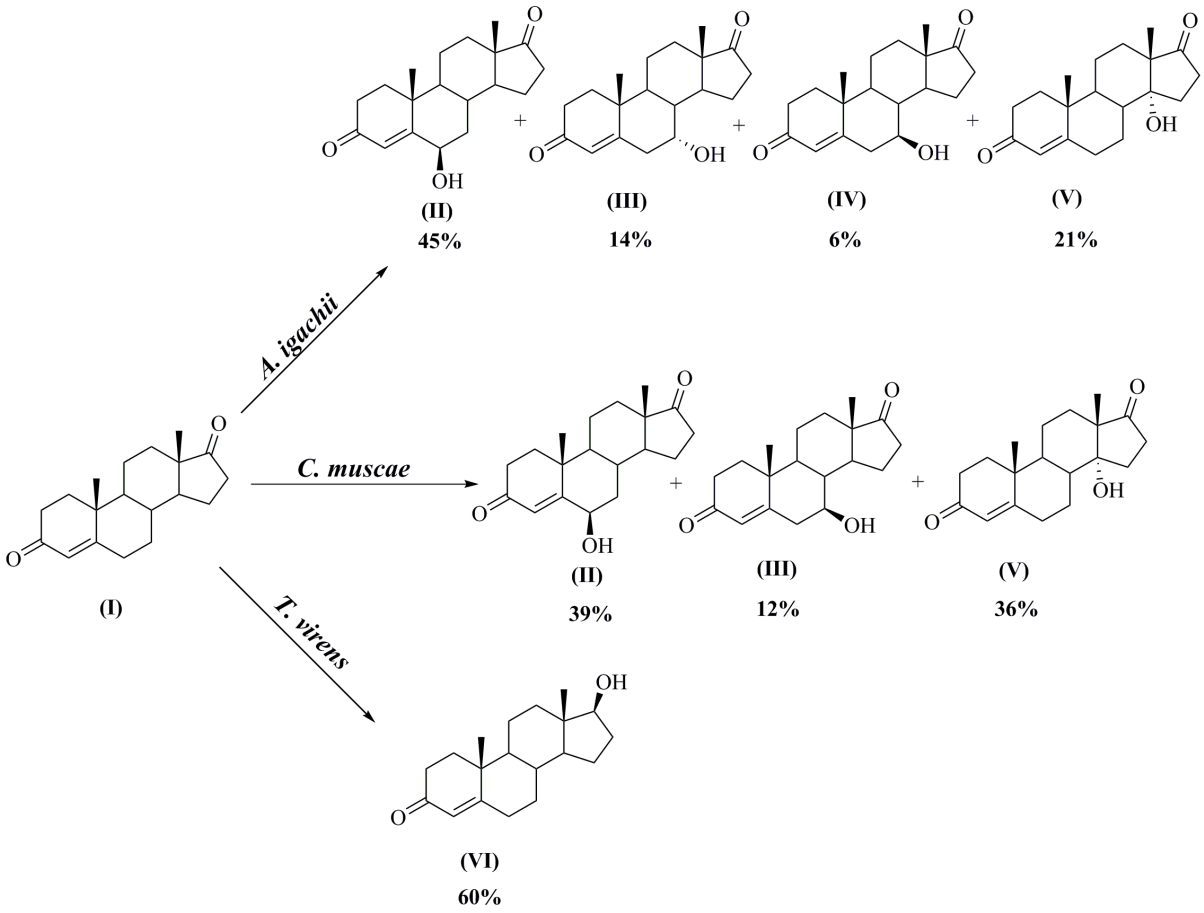
Graphical Abstract



Highlights

- Biotransformation of androst-4-ene-3,17-dione (AD) was investigated.
- Three filamentous fungi were used for the biotransformation.
- *A.griseolla* produced 6β, 7α-, 7β-, and 14α-hydroxy-AD.
- *C. muscae* produced 6β-, 7β-, and 14α-hydroxy-AD.
- Testosterone as only product was obtained in biotransformation of AD by *T. virens*.

Abstract

Microbial transformation of androst-4-ene-3,17-dione (AD; **I**) by three fungal species including *Absidia griseolla* var. *igachii*, *Circinella muscae* and *Trichoderma virens* was investigated for the first time. While *A. griseolla* and *C. muscae* carried out hydroxylation reactions, the third fungi performed reduction of the 17-carbonyl group in a chemoselective manner. Incubation of AD by *A. griseolla* yielded four metabolites 6β- (**II**), 7α- (**III**), 7*β*- (**VI**) and 14*α*-hydroxy-AD (**V**), among which 6β-hydroxy-AD (**II**) was identified as the major product. Furthermore, the metabolites produced during AD biotransformation by *C. muscae* were 6*β*- (**II**), 7*β*- (**III**) and 14*α*-hydroxy-AD (**V**). On the other hand, *T. virens* remarkably reduced AD into testosterone (**VI**) as the only product with 60% yield. These metabolites were purified by TLC and identified by ^{1}H NMR, ^{13}C NMR and other spectroscopic data.

Keywords: *Absidia griseolla* var. *igachii*; Androst-4-ene-3,17-dione; Biotransformation; *Circinella muscae*; *Trichoderma virens*

1. Introduction

Microbial transformation of steroids, independently or integrated with the other partial syntheses, has been commercially used in the pharmaceutical industry to produce steroid derivatives [1−3]. Among microorganisms which act as the biocatalysts in biotransformation of steroids, fungal species effectively utilize the groups of enzymes for catalyzing different chemical reactions such as hydroxylation and reduction on steroid substances. Fungi under mild condition can easily insert functional groups to chemically inactive carbons with the high regio- and stereospecificity [4,5]. Regarding the advantages of biotransformation, an important stream of researches has been increasingly trying to identify precious steroids derivatives as well as to enhance the yield of the biotransformation process [6−8].

Androst-4-en-3,17-dione (AD) which is a fundamental intermediate for invaluable steroid drugs, belongs to the 17-keto steroid family and has been used in the industrial synthesis of estradiol or estrone [9]. Bioreduction of AD to testosterone has been performed by the enzyme 17β-hydroxysteroid dehydrogenase which is present in most cells of the male organisms. Microbial hydroxylation of AD is broadly used in effective synthesis of novel AD derivatives which is not obtainable through chemical synthesis methods. Most positions in AD molecule have been hydroxylated by different microbial strains. For example, biotransformation of AD by *Beauveria bassiana* was reported, in which the separated products were obtained in the different pH media [10]. Moreover, formation of various hydroxyl steroid derivatives from AD was demonstrated by the filamentous fungus *Mucor racemosus* [11] and *Neurospora crassa* [12]. Additionally, biotransformation of AD by *Curvularia lunata* resulted in four hydroxylation derivatives and one reduction metabolite [13]. Most metabolites obtained from AD bioconversion, either are biologically active compounds or can be applied as the

intermediate to produce invaluable steroid drugs. For instance, one important class of metabolites are 14α-hydroxy-AD derivatives which prevent from aromatase activity and has anti-tumor properties. In addition, 7*α*-hydroxy-AD has applications in the medical industry and particularly in production of diuretics [9,14].

Fungi belonging to the *Trichoderma* and *Circinella* genera are widely available in the nature and have been utilized in the efficient biotransformation of exogenous steroids and demonstrated high catalytic activity in the hydroxylation, lactonization, hydrogenation and ester bond hydrolysis of steroids [15−18]. However, to the best of our knowledge, none of the *Circinella* species have been used in the bioconversion of AD. In particular, neither *C. muscae* nor *T. virens* has been incorporated in the biotransformation of any steroids. Beside this, in our previous studies, the application of *Absidia griseolla* var. *igachii* in the biotransformation of progesterone and testosterone was investigated which resulted in the corresponding 14α-hydroxy derivatives as the main products with the desirable yields [19−21]. Therefore, unrevealed potential of *C. muscae* and *T. virens* in the biotransformation of steroid structures as well as the desirable results which were gained from application of *A. griseolla* in the biotransformation of mentioned steroids, provide a basis to examine the ability of new microbial biocatalysts in the bioconversion of AD. In the present study, the ability of mentioned three fungal species in biotransformation of AD was investigated which resulted in hydroxylation on the position C-6β, C-7α, C-7β and C-14α as well as reduction on the position of C-17β.

2. Materials and methods

2.1. Chemicals and analytical methods

AD was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The components of culture media were purchased from Scharlau (Barcelona, Spain). Inorganic salts and analytical grade solvents were procured from Merck (Darmstadt, Germany). Nuclear magnetic resonance (NMR) spectra were determined on a Bruker Avance-300 with tetramethylsilane (TMS) as internal standard in $CDCl_3$. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS. Coupling constants (J) are given in hertz (Hz). The EI-MS spectra were obtained with a Finnigan MAT TSQ-70 instrument. The IR spectra were recorded on a Shimadzu IR-470 spectrometer. Column chromatography was conducted with silica gel 60 mesh (Merck, Darmstadt, Germany). Preparative thin layer chromatography (TLC) was performed on silica gel 60 mesh GF_{254} plates (20 × 20 cm).

2.2. Microorganisms and fermentation conditions

The fungal strains *A. griseolla* var. *igachii*, *C. muscae* and *T. virens* were obtained from the Persian type culture collection (Iranian Research Organization for Science and Technology). The strains were maintained on potato-dextrose agar plates (20 g glucose, 300 g diced potatoes, 15.0 g agar/L) at 4 °C and freshly sub-cultured before using in the transformation experiment. For each fungus**,** the experiment was conducted in ten 250 mL Erlenmeyer flasks, each containing 100 mL of the sterilized autoclaved cultivation medium which were inoculated with freshly obtained spores from agar slope cultures. After cultivation at 24 °C for 24 h in a rotary shaker (120 rpm), 100 mg of AD dissolved in 1 mL of absolute ethanol was added to each flask to achieve a final concentration of 1 mg mL^{-1}. Every day, a sample was collected from the cultures and the progress of the reaction was monitored by TLC. Due to study the catalytic efficiency of

microorganisms, control reactions consisting of cultivation medium without microorganism were similarly incubated.

2.3. Product isolation and purification

At the end of incubation, the fermentation media were extracted three times with chloroform. The organic extract was evaporated under reduced pressure on a rotary evaporator which was then loaded on preparative TLC with n-hexane/ethyl acetate. Incubation of AD (1g) for 4 days with the fungus *A. griseolla* gave products namely 6β-hydroxy-AD (**II**; 475 mg, 45%) as major metabolite and also 14α-hydroxy-AD (**V**; 221 mg, 21%), 7α-hydroxy-AD (**III**; 147 mg, 14%) and 7β-hydroxy-AD (**IV**; 63 mg, 6%). The hydroxylated metabolites of AD obtained after 4 days incubation of *C. muscae* were purified and identified through their spectroscopic data. Chromatographic purification of the products resulted in the isolation of three metabolites namely 6β-hydroxy-AD (**II**; 412 mg, 39%), 14α-hydroxy-AD (**V**; 380 mg, 36%) and 7β-hydroxy-AD (**IV**; 127 mg, 12%). Incubation of AD with *T. virens* produced testosterone (**VI;** 637 mg) as the only metabolite in the desired yields 60%. The yields of each product were calculated based on the percentage of dry weight of each compound. The sites of hydroxylation of the metabolites were determined according to the interpretation of ^{1}H NMR, ^{13}C NMR spectral data. Supplementary evidence for identification of the compounds was obtained by comparing optical rotations and melting points of metabolites with literature. The molecular structures of metabolites are shown in Fig. 1.

2.4. Time course study of the metabolites

In order to investigate time course study of the metabolites, sampling was carried out every 12 h. For this purpose, in each sampling, 20 mL of the broth was taken and

extracted with chloroform (3 × 20 mL), then the combined organic phases were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. Afterwards, the crude product was redissolved in 1 mL HPLC grade acetonitrile and injected to HPLC (20 μL) at room temperature. The mobile phase consisted of A (water–acetonitrile, 45:55) and B, 100% acetonitrile. The HPLC system was operated at a flow rate of 0.5 mL/min with a linear gradient of 100% A to 100% B over 20 min, and then held at 100% B for 15 min.

3. Results and discussion

*3.1. 6β-Hydroxy-AD (**II**)*

Colorless crystals; mp 190–191 °C; (lit [22] 190–192 °C), $[\alpha]_D^{20}$ + 117 ° ($CHCl_3$, *c* = 1.0), (lit [23] $[\alpha]_D^{20}$ + 122 ° ($CHCl_3$, *c* = 1.0)); IR (KBr); $_{\nu max}$: 3470, 1721, 1668, 1615 cm^{-1}; MS *m*/*z* (%) 302 (25) (M^+, $C_{19}H_{26}O_3$), 284 (21), 140 (52), 112 (62), 57 (100); ^{1}H NMR (300.13 MHz, $CDCl_3$) δ (ppm): 0.96 (3H, s, H-18), 1.42 (3H, s, H-19), 4.41 (1H, brs, H-6α), 5.84 (1H, s, H-4); ^{13}C NMR (75.47 MHz, $CDCl_3$, see table 1); R_f in n-hexane/ethyl acetate (1:3 (v/v)): 0.57.

The mass spectrum of compound **II** showed the molecular ion peak at *m*/*z* 302, corresponding to the formula $C_{19}H_{26}O_3$. IR spectrum showed a strong hydroxyl band at 3470 cm^{-1}. Additionally, the appearance of a new downfield signal at δ 4.34 ppm (1H, brs) in the ^{1}H NMR spectrum and a carbon resonance at δ 72.8 ppm in the ^{13}C NMR spectrum of **II** indicated insertion of a hydroxyl group added to a secondary carbon. Comparison of the ^{1}H NMR, ^{13}C NMR, optical rotation and melting point of metabolite **II** with literature was consistent with hydroxylation at the 6β-position [22,23].

*3.2. 7α-Hydroxy-AD (**III**)*

Colorless crystals; mp 240–242 °C; (lit [12] 245–248 °C), $[\alpha]_D^{20}$ + 155 ° ($CHCl_3$, *c* = 1.0), (lit [24] $[\alpha]_D^{20}$ + 143 ° ($CHCl_3$, *c* = 1.0)); IR (KBr); $_{vmax}$: 3421, 1733, 1661 and 1611 cm^{-1}; MS *m*/*z* (%) 302 (28) (M^+, $C_{19}H_{26}O_3$), 284 (14), 136 (58), 109 (62), 57 (100); ^{1}H NMR (300.13 MHz, $CDCl_3$) δ (ppm): 0.90 (3H, s, H-18), 1.25 (3H, s, H-19), 4.13 (1H, brs, H-7β), 5.85 (1H, s, H-4); ^{13}C NMR (75.47 MHz, $CDCl_3$, see table 1); R_f in n-hexane/ethyl acetate (1:3 (v/v)): 0.21.

The mass spectrum showed the molecular ion peak at *m*/*z* 302. Analysis of the NMR spectra indicated the appearance of a broad singlet resonance at δ 4.13 ppm (1H, brs) and a carbon resonance at δ 67.1.

*3.3. 7β-Hydroxy-AD (**IV**)*

Colorless crystals; mp 214–216 °C; (lit [22] 215–217 °C), $[\alpha]_D^{20}$ + 182 ° ($CHCl_3$, *c* = 1.0), (lit [22] $[\alpha]_D^{20}$ + 178 ° ($CHCl_3$, *c* = 1.0)); IR (KBr); $_{vmax}$: 3464, 1689 and 1620 cm^{-1}; MS *m*/*z* (%) 302 (71) (M^+, $C_{19}H_{26}O_3$), 282 (10), 134 (50), 109 (100); ^{1}H NMR (300.13 MHz, $CDCl_3$) δ (ppm): 0.94 (3H, s, H-18), 1.26 (3H, s, H-19), 3.51 (1H, brs, H-7β), 5.80 (1H, s, H-4); ^{13}C NMR (75.47 MHz, $CDCl_3$, see table 1); R_f in n-hexane/ethyl acetate (1:3 (v/v)): 0.34.

A new downfield methine proton appeared at δ 3.51 ppm (1H, brs) in the ^{1}H NMR spectrum. The ^{13}C NMR spectrum also supported the presence of a hydroxyl group (δ 75.1 ppm). Definitive confirmation of the structure of the compound was given by comparison with NMR data previously reported for 7β-hydroxy-AD [22].

*3.4. 14α-Hydroxy-AD (**V**)*

Colorless crystals; mp 258–260 °C; (lit [24] 259–262 °C), $[\alpha]_D^{20}$ + 160 ° ($CHCl_3$, *c* = 1.0), (lit [24] $[\alpha]_D^{20}$ + 155 ° ($CHCl_3$, *c* = 1.0)); IR (KBr); $_{vmax}$: 3420, 1721, 1670, 1607,

1151 cm^{-1}; MS *m/z* (%) 302 (25) (M^+, $C_{19}H_{26}O_3$), 220 (15), 116 (21), 78 (33), 69 (40), 57 (100); ^{1}H NMR (300.13 MHz, $CDCl_3$) δ (ppm): 1.06 (3H, s, H-18), 1.23 (3H, s, H-19), 5.76 (1H, s, H-4); ^{13}C NMR (75.47 MHz, $CDCl_3$, see table 1); R_f in n-hexane/ethyl acetate (1:3 (v/v)) 0.67.

The ^{13}C NMR displayed the presence of a new oxygen-bearing methine carbon signal at δ 80.7, but no downfield proton resonance was observed in ^{1}H NMR spectrum in the range of δ 3.5–4.5 ppm. The data obtained confirmed that the hydroxyl group should be attached to a tertiary carbon. Finally, assignment of ^{1}H and ^{13}C NMR signals by comparison of the shift data obtained with that reported was consistent with hydroxylation at the 14α-position [24].

*3.5. Testosterone (**VI**)*

Colorless crystals; mp 151–152 °C; (lit [24] 154–156 °C), $[\alpha]_D^{20}$ +105 ° (MeOH, *c* = 1.0) (lit [22] $[\alpha]_D^{20}$ +106 ° (MeOH, *c* = 1.0)); IR (KBr); $_{vmax}$: 3441, 1668, 1612; MS m/z (%) 288 (47) (M^+, $C_{19}H_{28}O_2$), 246 (35), 167 (38), 55 (100);^{1}H NMR (300.13 MHz, $CDCl_3$) δ (ppm): 0.81 (3H, s, H-18), 1.20 (3H, s, H-19), 3.66 (1H, t, *J* = 8.5 Hz, H-17α), 5.74 (1H, s, H-4); ^{13}C NMR (75.47 MHz, $CDCl_3$, see table 1); R_f in n-hexane/ethyl acetate (1:2): 0.40.

The mass spectrum of metabolite had a molecular ion peak at m/z 288, two mass units higher than the molecular ion of substrate (286). The ^{13}C NMR spectrum for this metabolite showed 19 carbon signals, that signal at δ 220 ppm corresponding to 17-carbonyl had been disappeared and an oxygenated carbon at δ 81.6 ppm was observed. The ^{1}H NMR spectrum showed downfield methylene protons resonating at δ 3.66 ppm (t, *J* = 8.5 Hz). The NMR data were in agreement with those reported in literature [22].

3.6. Time course study

A time course study was separately performed for each microorganism and the results indicated that there were four products including **II−V** from bioconversion of AD by *A. griseolla* which 6β-, 7β- and 14α-hydroxylation appeared in the broth from the first day while 7α-hydroxy-AD was produced from the second day. The biotransformation yield increased gradually and reaches the maximum amount after 4 days of incubation. After 4 days, the amount of metabolites appeared to be constant with further incubation time (Fig. 2a). In incubation of AD by *C. muscae* showed that all the products were accumulated from the 12 hour of fermentation (Fig. 2b). The last time course study was constructed to investigate the bioconversion of AD by *T. virens.* The obtained results indicated that AD was not hydroxylated and only testosterone was obtained (Fig. 2c). The metabolite was quickly established in the very first 12 hours and was increased until the day 3 in which the metabolite reached its maximum amount. Afterward, there was not any meaningful change in the amount of the metabolite.

Although due to the useful biological activity of AD derivatives, different microorganisms have been applied in the bioconversion process of AD (see table 2), the endeavor for identifying more economical microorganisms are still in progress with the aim of producing metabolites with the higher yields as well as more stereo- and regiospecificity [2,5].

17β-reduction of AD is one of the key reactions for synthesizing testosterone, however in the most microbial transformation of AD, the oxidated products have also been observed beside the reduction of carbonyl group (see table2 - entries 2, 6 and 14). Faramarzi et al. through applying *Nostoc muscorum* have reported the selective reduction of AD to testosterone in which in order to increasing the yields of reaction, microalgal cell immobilization and organic solvent have been used (see table 2, entry

12). In the current study, *T. virens* acted as the regio-selective biocatalyst in the biotransformation of AD and merely produced testosterone with the desirable yield of 60% which nominated *T. virens* as the industrial candidate of reducing steroid compounds.

Furthermore, the biotransformation of AD by *A. griseolla* and *C. muscae* has been resulted in hydroxylation at some specific sites in the molecule of AD, which the major products of both strains were 6β- and 14α-hydroxy-AD. It is necessary to mention that the obtained result for *A. griseolla* is consistent with our previous studies in which *A. griseolla* was effectively used in the biotransformation of other steroids [19−21].

4. Conclusion

Examining different microorganisms in biotransformation of steroid derivatives has become an interesting field of research. In this study, the potential of three fungi species, *A. griseolla*, *C. muscae* and *T. virens* in biotransformation of AD was examined. Microbial hydroxylation of AD by *A. griseolla* effectively resulted in the formation of products **II**, **III**, **IV** and **V**. Furthermore, biotransformation of AD by *C. muscae* produced metabolites **II**, **IV** and **V**. The above fungi showed the high ability in stereoselective hydroxylation. In contrast with the two mentioned fungi, no hydroxylation took place in the biotransformation of AD by *T. virens*, but this strain was able to reduce a ketone in C-17 to the corresponding hydroxyl group. To the best of our knowledge this is the first report on biotransformation of AD by these fungal species.

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Figure Captions

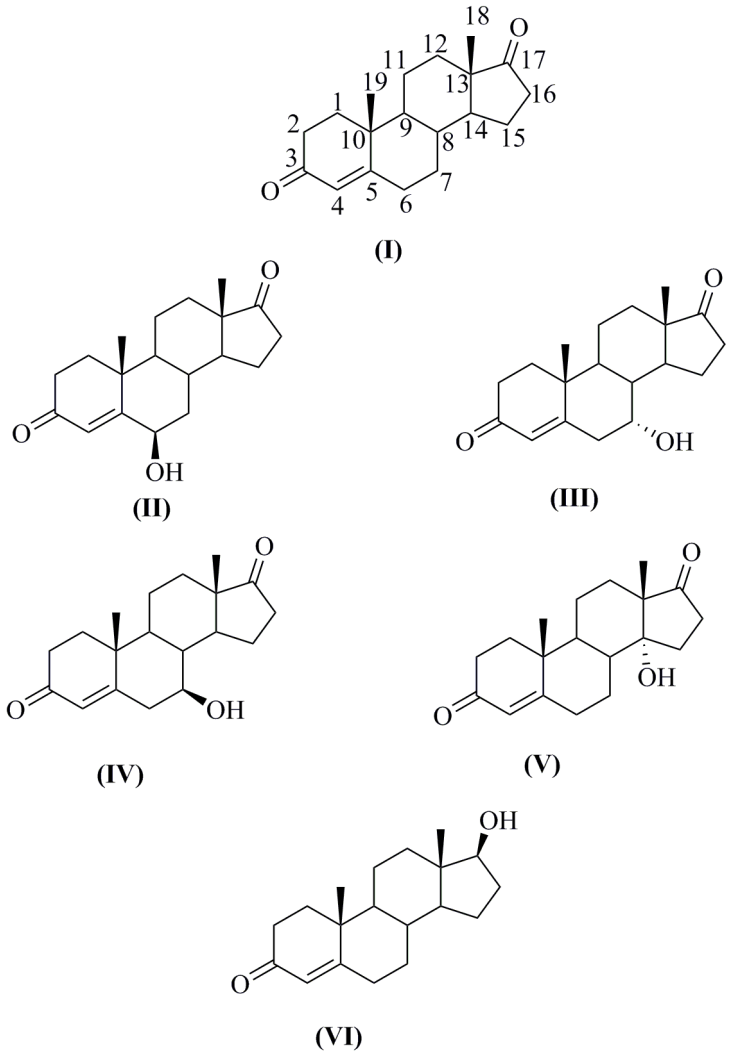
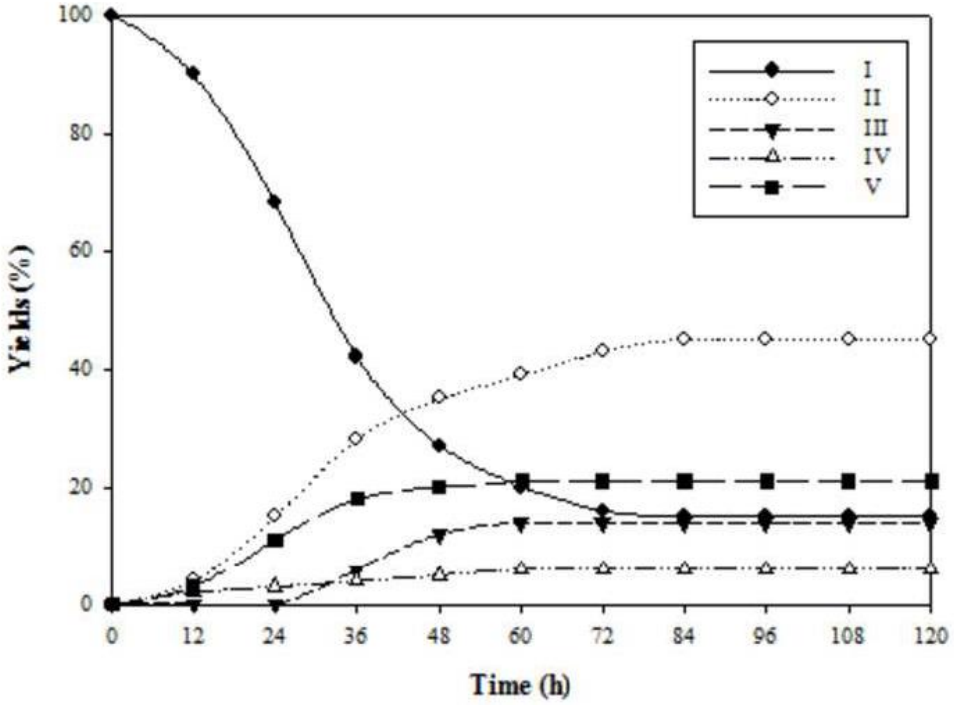


Fig. 1. The structure of AD and its metabolites.

(a)



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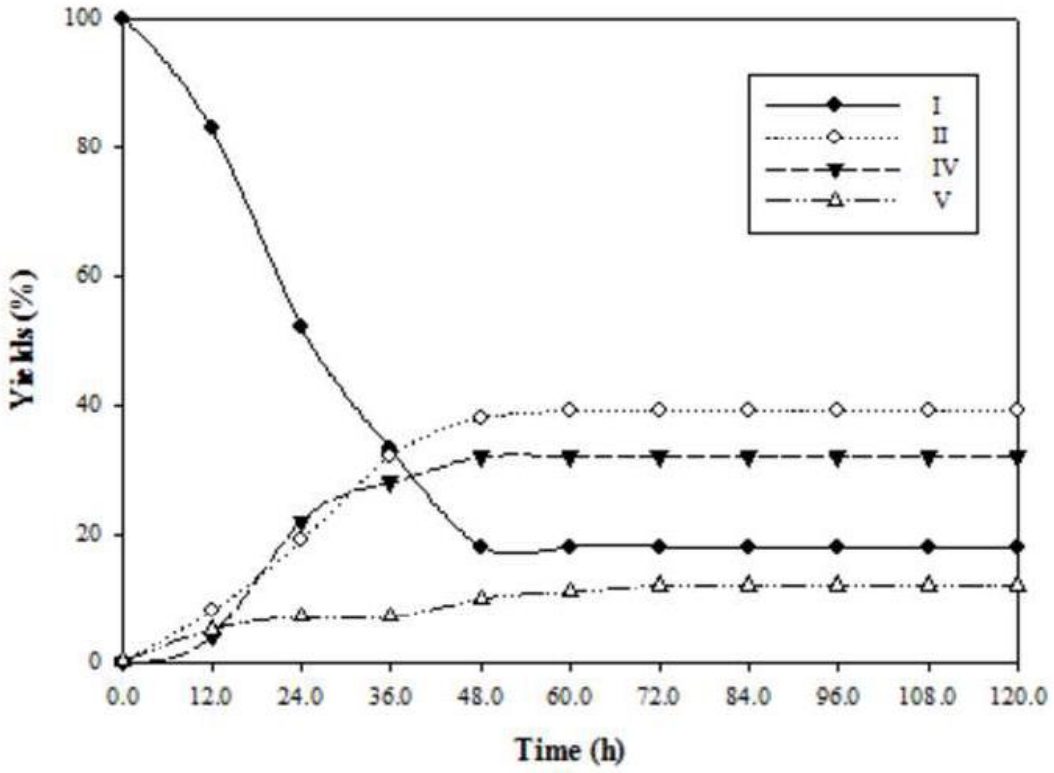

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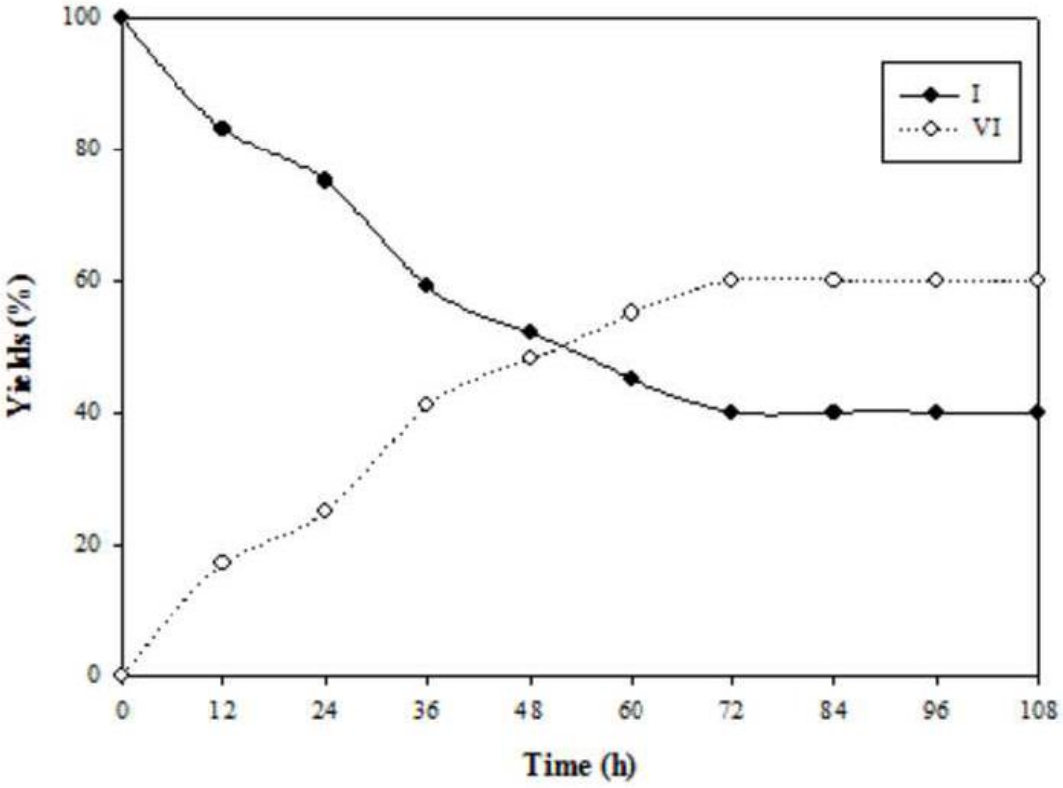

Fig. 2. Time course profile of AD biotransformation by (a) *A. griseolla*, (b) *C. Muscae* and (c) *T. virens.*

Tables

Table 1. ^{13}C NMR data for AD and its metabolites (δ in ppm downfield from TMS, in $CDCl_3$).

Carbon atom	**I**	**II**	**III**	**IV**	**V**	**VI**
1	35.6	37.1	35.4	36.6	35.7	35.6
2	33.8	34.2	33.9	34.2	33.1	35.5
3	199.1	200.2	198.0	202.1	199.5	199.6
4	124.0	126.6	127.2	125.2	124.2	123.8
5	170.2	167.8	166.7	171.2	170.0	171.3
6	32.4	72.8	41.0	39.5	33.1	33.9
7	31.1	35.8	67.1	75.1	25.5	32.7
8	35.0	29.4	39.3	43.5	37.9	35.7
9	53.7	53.6	45.4	52.1	46.8	53.9
10	38.5	38.1	38.5	39.5	38.6	38.6
11	20.2	20.2	20.1	21.2	19.1	20.6
12	30.6	31.3	30.9	32.8	24.5	36.4
13	47.4	47.7	47.3	49.4	52.5	42.8
14	50.7	50.9	45.6	51.8	80.7	50.4
15	21.6	21.7	21.2	25.6	30.2	23.3
16	35.6	37.2	35.7	36.6	33.9	31.5
17	220.0	220.1	220.3	224.2	218.3	81.6
18	13.6	13.8	13.5	14.6	17.9	11.0
19	17.2	19.6	17.0	17.6	17.3	17.4

Table 2. Microbial transformation of AD by various microorganisms

Entry	Microorganism	Products	References
1	*Aspergillus ochraceus*	11α-Hydroxy-AD	[25]
2	*Beauveria bassiana*	11α-Hydroxy-AD Testosterone 11α,14α-Dihydroxy-AD 6β,11α,17β-Trihydroxy-AD 3α,11α,17β-Trihydroxy-5α-AD	[10]
3	*Bordetella sp.*	Androsta-1,4-diene-3,17-dione (ADD) 9α-Hydroxy-AD 3-OH-SADT	[26]
4	*Botryosphaeria obtusa*	6β,7β-Dihydroxy-AD 6β-Hydroxy-AD 7α-Hydroxy-AD 15α-Hydroxy-AD	[27]
5	*Colletotrichum lini*	15α-Hydroxy-AD 11α,15α-Dihydroxy-AD	[28]
6	*Curvularia lunata*	ADD Testosterone 11α-Hydroxy-AD 11α,17β-Dihydroxy-AD	[13]
7	*Mucor griseocyanus*	7α-Hydroxy-AD 14α-Hydroxy-AD 14α,17β-Dihydroxy-AD	[29]
8	*Mucor (M881)*	6β-Hydroxy-AD 11α-Hydroxy-AD 7β-Hydroxy-AD 6β,11α-Dihydroxy-AD	[22]
9	*Mucor racemosus*	14α-Hydroxy-AD 7α-Hydroxy-AD 14α,17β-Dihydroxy-AD 6β,14α-Dihydroxy-AD 6β,11α,17β-Trihydroxy-AD	[11]
10	*Mucor sp.*	6,14α-dihydroxyandrost-4-ene-3-one	[30]
11	*Neurospora crassa*	6β,14α-Dihydroxy-AD 6β,9α-Dihydroxy-AD 7α-Hydroxy-AD 9α-Hydroxy-AD 14α-Hydroxy-AD Androst-4,6-dien-3,17-dione	[12]
12	*Nostoc muscorum*	Testosterone	[31]
13	*Rhizopous nigricans*	7α-Hydroxy-AD 11α-Hydroxy-AD	[32]
14	*Thamnostylum piriforme*	Testosterone 6β-Hydroxy-AD 14α-Hydroxy-AD 6β-Hydroxy-AD 7α-Hydroxy-AD	[33]