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Microbial transformation of 17α-ethynyl- and 17α-ethylsteroids, and tyrosinase inhibitory activity of transformed products

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Abstract

The microbial transformation of the 17α-ethynyl-17β-hydroxyandrost-4-en-3-one (**1**) (ethisterone) and 17α-ethyl-17β-hydroxyandrost-4-en-3-one (**2**) by the fungi *Cephalosporium aphidicola* and *Cunninghamella elegans* were investigated. Incubation of compound **1** with *C. aphidicola* afforded oxidized derivative, 17α-ethynyl-17β-hydroxyandrosta-1,4-dien-3-one (**3**), while with *C. elegans* afforded a new hydroxy derivative, 17α-ethynyl-11α,17β-dihydroxyandrost-4-en-3-one (**4**). On the other hand, the incubation of compound **2** with the fungus *C. aphidicola* afforded 17α-ethyl-17β-hydroxyandrosta-1,4-dien-3-one (**5**). Two new hydroxylated derivatives, 17α-ethyl-11α,17β-dihydroxyandrost-4-en-3-one (**6**) and 17α-ethyl-6α,17β-dihydroxy-5α-androstan-3-one (**7**) were obtained from the incubation of compound **2** with *C. elegans*. Compounds **1–6** exhibited tyrosinase inhibitory activity, with compound **6** being the most potent member (IC_{50} = 1.72 μM).
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Keywords: 17α-Ethynyl-17β-hydroxyandrost-4-en-3-one; 17α-Ethyl-17β-hydroxyandrost-4-en-3-one; Microbial transformation; *Cephalosporium aphidicola*; *Cunninghamella elegans*; Tyrosinase inhibition

1. Introduction

Tyrosinase inhibitors have become increasingly important for cosmetics and medicines primarily in relation to hyperpigmentation and associated conditions. Since the enzyme plays a key role in melanin biosynthesis, its inhibitors may help in the prevention of excessive dermal melanin production [1]. In addition, tyrosinase is known to be involved in the molting process of insects and adhesion of marine organisms [2].

In continuation of our work on microbial transformation of bioactive compounds [3–7], we describe here the biotranformation of 17α-ethynyl-17β-hydroxyandrost-4-en-3-one (**1**), 17α-ethyl-17β-hydroxyandrost-4-en-3-one (**2**) and tyrosinase inhibitory activity of transformed products.

First fungal transformation of compound **1** was reported by Zakelj-Mavric et al., resulting in the formation of 7β-hydroxyl derivative [8]. It was reported that 17α-ethynyl group prevents steroid–enzyme binding in the normal mode and thus inhibits the formation of 11α-hydroxylated product [9]. However, in this paper we report the formation of 11α-hydroxylated products of 17α-ethynyl- and 17α-ethyl steroids, along with other oxidized products.

2. Experimental

2.1. General experimental procedures

Column chromatography on silica gel, 70–230 mesh. Precoated silica gel plates (Si gel 60 F_{254}, *E. Merck*) were used for TLC, UV_{254} active: detection at 254 nm, and by ceric sulphate spray reagent, optical rotations JASCO DIP 360 digital polarimeter. UV and IR spectra: Hitachi-UV-3200 and Jasco-320-A spectrophotometer, respectively. ^{1}H and ^{13}C NMR (δ ppm, J in Hz) COSY, HMQC, HMBC and NOESY spectra, Bruker spectrometers operating at 500 and 400 MHz. EI, FAB, and HREIMS were recorded on a JEOL JMS-HX110

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and JMS-DA-500 mass spectrometers, *m/z* (rel. int.). PAAR Hydrogenation apparatus 3916.EA was used for hydrogenation.

2.2. Microorganisms and chemicals

Cunninghamella elegans (TSY-0865) and *Cephalosporium aphidicola* (IMI 68689) were maintained on Sabouraud-Glucose-Agar slants and stored in a refrigerator at 4 °C. 17α-Ethynyl-17β-hydroxyandrost-4-en-3-one (**1**) was purchased from E. Merck, Germany.

2.3. Preparation of medium

Medium (3 L) for *C. elegans* (TSY-0865) was prepared by mixing glucose (60 g), peptone (15 g), KH_2PO_4 (15 g) and yeast extract (9 g). The medium for *C. aphidicola*: (IMI 68689) was prepared by mixing these ingredients in distilled H_2O (2 L): glucose 100 g, KH_2PO_4 (2 g), KCl (2 g), $MgSO_4{\cdot}7H_2O$ (4 g), glycine (4 g) and *Gibberella* trace element solution (4 mL).

2.4. Culture and fermentation procedure

The following procedures were used for each fungus: 2-day-old spores were aseptically transferred into broth medium flask (250 mL) containing 100 mL of freshly prepared and autoclaved media. The seed flasks thus obtained were incubated on a shake table at 30 °C for 2 days. Two-day-old broth cultures from 100 mL seed flask were inoculated aseptically into 30 media flasks (250 mL) for *C. elegans* and 20 media flasks (250 mL) for *C. aphidicola* containing 100 mL of medium and fermentation was continued for further 2 days.

Compounds **1** and **2** were dissolved in EtOH and solutions were evenly distributed among 30 flasks (10 mg/0.25 mL in each flask) for *C. elegans*, and 20 flasks (10 mg/0.25 mL in each flasks) for *C. aphidicola* containing 48-h-old stage II cultures. Fermentation was carried out for further 12 days on a rotatory shaker (200 rpm) at 29 °C. In all experiments, one control flask without biomass (for checking substrate stability) and one flask without exogenous substrate (for checking endogenous metabolite) were used. The culture media and mycelium were separated by filtration. The mycelium was washed with EtOAc (1 L) and the filtrate was extracted with EtOAc (5 L). The combined organic extract was dried over anhydrous Na_2SO_4, evaporated under reduced pressure, and analyzed by thin layer chromatography. Control flasks were also harvested and compared with test by TLC to confirm the bio-transformed products.

2.5. Hydrogenation of 17α-Ethynyl-17β-hydroxyandrost-4-en-3-one (*1*)

Sample (1.0 g) dissolved in 50 mL dry MeOH reduced in a PAAR hydrogenator with a catalyst (5% palladium on carbon) yielded a compound **2**. Recovered yield was 95%.

2.6. Incubation of 17α-Ethynyl-17β-hydroxyandrost-4-en-3-one (*1*)

The substrate **1** (200 mg), distributed in 20 conical flasks, was incubated as above for *C. aphidicola*. Chromatography of the extract on Si gel yielded starting material (90 mg) and compound **3** (11 mg).

2.6.1. 17α-Ethynyl-17β-hydroxyandrosta-1,4-dien-3-one (*3*)

White solid; mp 161–162 °C, $[\alpha]_D^{25}$ +14.2 (*c* 0.02, $CHCl_3$); UV (MeOH) λ_{max} (log ∈) 244 nm (3.4); lit value mp 163–164 °C, $[\alpha]_D^{26}$ 0 (*c* 1.150, $CHCl_3$); λ 245 nm (15,600) [10].

Again the substrate **1**, (300 mg) in 30 conical flasks was incubated in the culture media of *C. elegans* as above. Chromatography of the extract on Si gel gave; starting material (160 mg) and 17α-ethynyl-11α,17β-dihydroxyandrost-4-en-3-one (**4**) (5.5 mg).

2.6.2. 17α-Ethynyl-11α,17β-dihydroxyandros-4-en-3-one (*4*)

Gummy $[\alpha]_D^{25}$ +4 (*c* 0.05, $CHCl_3$); UV (MeOH) λ_{max} (log ∈) 240 nm (3.4); IR ($CHCl_3$) ν_{max} 3407, 1710, 1659 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz), see Table 1; ^{13}C NMR (CD_3OD, 100 MHz), see Table 1; EIMS *m/z* (rel. int.) 328 [M^+] (10), 310 (7) [M^+-18], 261 (17), 187 (15),145 (17), 121 (35), 119 (25), 109 (28), 91 (45), 79 (40), 55 (100); HREIMS *m/z* 328.2045 (calcd for $C_{21}H_{28}O_3$, 328.2038).

2.7. Incubation of 17α-Ethyl-17β-hydroxyandrost-4-en-3-one (*2*)

The substrate **2** (200 mg) in 20 conical flasks was incubated as described above for *C. aphidicola*. The extract was chromatographed on Si gel affording; starting material **2** (120 mg) and compound **5** (4.4 mg).

2.7.1. 17α-Ethyl-17β-hydroxyandrosta-1,4-dien-3-one (*5*)

White solid; mp 168–170 °C (uncorrected); $[\alpha]_D^{25}$ +11.1 (*c* 0.07, $CHCl_3$); UV (MeOH) λ_{max} (log ∈) 242 nm (2.8).

The substrate **2** (300 mg) in 30 conical flasks was also incubated as described above for *C. elegans*. The extract was chromatographed on Si-gel affording starting material (90 mg); compounds **6** (8.5 mg) and **7** (4.8 mg).

2.7.2. 17α-Ethyl-11α,17β-dihydroxyandrost-4-en-3-one (*6*)

Gummy; $[\alpha]_D^{25}$ +45 (*c* 0.06, $CHCl_3$); UV (MeOH) λ_{max} (log ∈) 241 nm (3.5); IR ($CHCl_3$) ν_{max} 3401, 1708, 1658 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz), see Table 1; ^{13}C NMR (CD_3OD, 125 MHz), see Table 1; EIMS *m/z* (rel. int.)

Table 1
^{1}H NMR (500 MHz; CD_3OD) and ^{13}C NMR (100 MHz; CD_3OD) data for compounds **4**, **6** and **7**

Carbon no.	Compound **4**		Compound **6**		Compound **7**	
	^{1}H NMR (δ)	^{13}C NMR (δ)	^{1}H NMR (δ)	^{13}C NMR (δ)	^{1}H NMR (δ)	^{13}C NMR (δ)
1	2.2, m, 2.07, dt (4.3, 14.0)	38.5	2.24, m, 2.04, dt (4.1, 13.8)	34.8	2.02, m, 1.98, m	38.4
2	2.70, dt (4.5, 14.0) 2.27, m	34.9	2.68, dt (4.5, 9.0) 2.41, m	38.6	2.04, dt (13.8, 4.1) 2.41, m	36.4
3	–	202.3	–	202.3	–	215.0
4	5.73, s	124.4	5.70, s	124.0	2.32, m, 2.14, m	42.8
5	–	175.4	–	175.5	1.92, m	51.5
6	2.23, m, 2.0, m	34.7	1.60, m, 1.01, m	33.5	3.60, br s, (W 1/2 = 12.5)	72.6
7	1.92, m, 1.88, m	32.6	1.90, m, 1.78, m	32.8	1.71, m, 1.28, m	34.2
8	1.65, m	37.0	1.86, m	37.4	1.50, m	41.0
9	1.12, m	60.0	1.08, m	60.0	1.08, m	51.2
10	–	41.3	–	41.5	–	38.3
11	3.98, dt (4.9, 10.5)	69.8	4.0, dt (4.6, 10.4)	70.0	1.50, m, 1.34 m	20.8
12	1.94, m	45.2	1.82, m, 1.77, m	44.1	1.36, m, 1.22, m	33.5
13	–	48.3	–	33.6	–	42.2
14	1.67, m	50.6	1.28, m	50.8	1.26, m	50.5
15	1.68, m, 1.35, m	23.8	1.78, m, 1.50, m	24.8	1.74, m, 1.47, m	23.5
16	2.25, m, 1.95, m	40.0	1.65, m, 0.97, m	34.9	1.64, m, 0.97, m	35.2
17	–	79.7	–	83.8	–	84.8
18	0.90, s	14.3	0.93, s	29.7	0.90, s	15.2
19	1.35, s	18.7	1.35, s	19.8	1.21, s	25.1
20[a]	–	88.4	1.85, m, 1.84, m	29.7	1.55, m, 1.41, m	29.8
21[a]	2.90, s	74.9	0.97, t (7.2)	9.8	0.96, t (7.1)	8.2

[a]The numbering for C-20 and C-21 denotes ethynyl and ethyl moieties.

332 [M^+] (22), 314 [M^+-18], (11), 191 (3), 149 (17), 124 (27), 123 (23), 111 (86), 109 (82), 83 (23), 79 (23), 57 (95), 55 (100); HREIMS m/z 332.2302 (calcd for $C_{21}H_{32}O_3$, 332.2351).

2.7.3. 17α-Ethyl-6α,17β-dihydroxy-5α-androstan-3-one (7)

White solid; mp 198–202 °C (uncorrected), $[\alpha]_D^{25}$ 16 (c 0.03 $CHCl_3$); IR ($CHCl_3$) ν_{max} 3405, 1709, 1657, cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) see Table 1; EIMS m/z (rel. int.) 334 [M^+] (11), 316 [M^+-18] (63), 193 (3), 153 (20), 151 (12), 141 (22), 122 (12), 109 (16), 107 (23), 105 (26), 93 (25), 55 (17), 53 (1); HREIMS m/z 334.2140 (calcd for $C_{21}H_{34}O_3$, 334.2143).

2.8. Tyrosinase inhibition assay

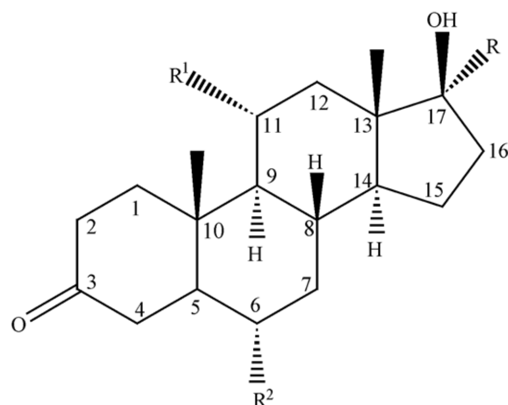
Tyrosinase inhibition assays were performed in 96-well microplate format using SpectraMax® 340 (Molecular Devices, CA, USA) microplate reader according to the method developed by Hearing (1987). Briefly, all the compounds were dissolved in DMSO and finally the solvent mixture (2.5%). 30 Units mushroom tyrosinase (28 nM) was first preincubated with the compounds in 50 nM Na–phosphate buffer (pH 6.8) for 10 min at 25° C. Then the L-DOPA (0.5 mM) was added to the reaction mixture and the enzyme reaction was monitored by measuring the changes in absorbance at 475 nm (at 37 °C) due to the formation of the DOPA chrome for 10 min. The percent inhibition of the enzyme and IC_{50} values of the active compounds were calculated using a program developed with Java and Macro Excel® 2000 (Microsoft Corp., USA) for this purpose. All the studies have been done at least in triplicates and the results here represents the mean ± S.E.M. (standard error of the mean). All the reagents, enzyme, substrate and reference compounds were purchased from Sigma Chem. Co., MO, USA.

3. Results and discussion

Fermentation of 17α-ethynyl-17β-hydroxy-4-androsten-3-one (**1**) with *C. aphidicola* for 12 days yielded a known metabolite, 17α-ethynyl-*17*β-hydroxylandrosta-1,4-dien-3-one (**3**) [10,11], (Scheme 1).

Further fermentation of compound **1** with *C. elegans* for 12 days yielded a new hydroxylated metabolite 17α-ethynyl-11α,17β-dihydroxyandrost-4-en-3-one (**4**) (Scheme 1).

The HREIMS of **4** displayed the molecular ion (M^+) at m/z 328.2045 ($C_{21}H_{28}O_3$, calcd 328.2038) indicating an additional oxygen atom. The ^{1}H NMR spectrum of **4** showed a downfield signal for oxygen-bearing methine proton at δ 3.98 (dt, $J = 10.5$ Hz, $J = 4.9$ Hz) suggesting hydroxylation of a methylene group. A downfield methine carbon signal at δ 69.8 (11-CHOH) further supported this inference. The α-stereochemistry of OH-11 as equatorial was inferred from the multiplicity of the geminal H-11 signal (δ 3.98, dt, $J_{11ax,\,9ax} = J_{11ax,\,12ax} = 10.5$ Hz, $J_{11ax,\,12eq} = 4.9$ Hz) and the NOESY correlations of H-11β (δ 3.98) with CH_3-18β (δ 0.90) and CH_3-19β (δ 1.35). The presence of OH at C-11 was also inferred from HMBC correlations of H-11 (δ 3.98) with C-9 (δ 60.0), and C-11 (δ 69.8) with H-9 (δ 1.12) and H-12 (δ 1.94), as well as the COSY 45°



1. R = -C≡C, Δ^4, $R_1 = R_2$ = H
2. R = -H_2C—CH_3, Δ^4, $R_1 = R_2$ = H
3. R = -C≡C, $\Delta^{1,4}$, $R_1 = R_2$ = H
4. R = -C≡C, Δ^4, R_1= OH, R_2 = H
5. R = -H_2C—CH_3, $\Delta^{1,4}$, $R_1 = R_2$ = H
6. R = -H_2C—CH_3, Δ^4, R_1= OH, R_2 = H
7. R = -H_2C—CH_3, Δ^4, R_1= H, R_2 = OH

Scheme 1. Microbial transformation of 17α-ethynyl-17β-hydroxyandrost-4-en-3-one (**1**) and 17α-ethyl-17β-hydroxyandrost-4-en-3-one (**2**).

correlations of H-11 (δ 3.98) with H-12 (δ 1.94) and H-9 (δ 1.12).

Fermentation of compound **2** with *C. aphidicola* for 12 days yielded a metabolite which was structurally characterized as 17α-ethyl-17β-hydroxyandrosta-1,4-dien-3-one (**5**) and found to be a known compound [12]. Further two new metabolites of compound **2** were obtained from biotransformation by *C. elegans* which were characterized as 17α-ethyl-11α,17β-hydroxyandrost-4-en-3-one (**6**) and 17α-ethyl-6α,17β-dihydroxy-5α-androstan-3-one (**7**) (Scheme 1).

The HREIMS of **6** displayed the M^+ at *m/z* 332.2302 ($C_{21}H_{32}O_3$ calcd 332.2351) indicating an additional oxygen atom. The ^{1}H NMR spectrum showed methine proton signal at δ 4.0 (dt, J = 10.4 Hz, J = 4.6 Hz) suggesting a hydroxylation at C-11 as in compound **4**. The α-stereochemistry of C-11 OH was inferred from the multiplicity of the H-11 signal (δ 4.0, dt, $J_{11\text{ax}, 9\text{ax}} = J_{11\text{ax}, 12\text{ax}}$ = 10.4 Hz and $J_{11\text{ax}, 12\text{eq}}$ = 4.6 Hz) and the NOESY correlations of H-11 β (δ 4.0) with H-18β (δ 0.93) and H-19β (δ 1.35). The methine signal resonated at δ 69.9 was assigned to hydroxyl-bearing C-11. The HMBC correlation of C-11 (δ 69.9) with H-12α (δ 1.82) and COSY 45° correlations of H-11 (δ 4.0) with H-12 (δ 1.82), H-9 (1.08) and H-19 (1.35) were also observed.

The HREIMS of compound **7** displayed the M^+ at *m/z* 334.2140 ($C_{21}H_{34}O_3$ calcd. 334.2143). Only terminal UV absorption indicated the lack of conjugation in the molecule. The ^{1}H NMR spectrum of **7** was very similar to that of compound **2** with no olefinic proton and an additional hydroxyl-bearing methine proton signal at δ 3.60. Hydroxylation at C-6 was further inferred from a downfield hydroxyl-bearing carbon signal at δ 72.6. The stereochemistry of C-6 proton was deduced to be β (axial) on the basis of coupling constants of H-6 signal ($W_{1/2}$ = 12.5 Hz). The C-6 hydroxyl group must therefore have an α (equatorial) orientation. The α-stereochemistry of C-6 hydroxyl was further supported by NOESY correlation of H-6 (δ 3.62) with CH_3-19 (δ 0.90). The position of the newly introduced hydroxyl group (C-6) was further confirmed by 2D-NMR spectra. The HMBC spectra of compound **7** showed correlations of H-6 (δ 3.60, br s, $W_{1/2}$ = 12.5 Hz) with C-5 (δ 51.1) and C-7 (δ 34.2), and the COSY 45° correlations of H-6 (δ 3.60) with H-5α (δ 1.92) and H-7 (δ 1.73).

Table 2
Tyrosinase inhibitory activity of compounds **1–6**

Compounds	IC_{50} ± S.E.M. (in μM)
1	2.61 ± 0.037328
2	1.53 ± 0.001088
3	7.89 ± 0.00128
4	5.95 ± 0.00078
5	3.46 ± 0.01046
6	1.72 ± 0.00089
Kojic acid	16.92 ± 0.5268
L-Mimosine	3.68 ± 0.02234

Metabolite **7** could not be screened because of its insufficient amount, S.E.M. is the standard error of the mean.

Compounds **1–6** exhibited potent inhibition against tyrosinase with some compounds (**1–2** and **5–6**) more potent than the standards kojic acid and L-mimosine. Results of tyrosinase inhibition studies on compounds **1–6** have been shown in the Table 2.

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