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REVIEW ARTICLE

Microbial hydroxylation of 17β-estradiol by *Penicillium brevicompactum*

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Abstract

Microbial hydroxylation of 17β-estradiol (**1**) with *Penicillium brevicompactum*, a fungal species not used in biotransformation so far, yielded four metabolites: 1, 3, 5-estratriene-3, 15α-diol-17-one (**2**); 1, 3, 5-estratriene-3, 6α, 17β-triol (**3**); 1, 3, 5-estratriene-3, 15α, 17β-triol (**4**); and 1, 3, 5-estratriene-3, 6α, 15α-triol-17-one (**5**). All the products were determined by ^{1}H NMR, ^{13}C NMR, two-dimensional NMR, and HRMS techniques. Compounds **3**, **4**, and **5** are reported for the first time via microbial transformation, and **5** is a new compound as far as we know. Possible metabolic pathway of 17β-estradiol via *Penicillium brevicompactum* was also proposed.

Keywords: *Biotransformation*, *17β-estradiol*, *hydroxylation*, Penicillium brevicompactum

Introduction

Microorganisms have been widely applied for biotransformation of steroids in order to obtain more functionalized compounds due to high regio- and stereoselectivity of these reactions (Wang et al. 2013). In particular, microbial hydroxylation of steroids provides a useful mild synthetic method for obtaining access to rare steroids (Bartmańska et al. 2005). Meanwhile, hydroxylation products of steroids are important due to their physiological role in mammalian organisms (Li and Bigelow 2010; Bhatti and Khera 2012). Every site in the steroid molecule is accessible for microbial hydroxylation and various microbial strains may introduce hydroxyl group at the same position in a steroid molecule.

Estrogens are gonadal steroidal hormones that have important roles in reproduction (Salci and Biryol 2002). 17β-Estradiol is the most potent and naturally occurring estrogen in human beings followed by estrone and estriol, and is the main isomer that activates the estrogen receptor (Guo et al. 2010). The hydroxylation of β-estradiol has been studied for 2-hydroxyestradiol and 4-hydroxyestradiol possessing putative carcinogenic metabolites (Li et al. 2014). The CYP-450 isoforms are largely responsible for the hydroxylation of estradiol in human body (Federico et al. 1994; Anthony et al. 2003). So far, microbial hydroxylation of 17β-estradiol mainly appears in 2, 4, 6β-, 7α-, 7β-, 15α-, and 16α-positions and some metabolites have certain biological activities (Laskin et al. 1964; Koshimura et al. 2010; Kurisu et al. 2010; Hussain et al. 2011). In continuation of the work with microbial transformation of steroids (Ge et al. 2008; Shan et al. 2009), we screened local soil samples and identified a fungal strain as *Penicillium brevicompactum* through 16S rRNA sequencing with the help of Microbial ID (Newark, DE). As far as we knew, the *Penicillium sp.* used in the biotransformation of steroid mainly resulted in the metabolism of D ring δ-lactones (Bartmańska et al. 2005; Liu et al. 2006; Huang et al. 2010; Swizdor 2013; Yang et al. 2014), occasionally resulted in hydrogenation (Miller and Hessler 1970; Yildirim et al. 2010), side chain degradation

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(Bartmańska et al. 2005). The hydroxylation reaction was also reported, which frequently occurs at 1α (Tweit et al. 1962; Liu et al. 2011), 15α (Irrgang et al. 1992; Bartmańska et al. 2005), 15β, 11α (Capek and Hanc 1962), and 12β-positions (El-Tayeb et al. 1964). But according to our view, the microbial hydroxylation of 17β-estradiol by *Penicillium brevicompactum* has not been reported so far. In this paper, the biotransformation mode of 17β-estradiol by *P. brevicompactum* was studied, and four metabolites were identified, which were mono- or dihydroxyl products in C-6α, C-15α, and sometimes accompanied with oxidization at C-17 position. Metabolite of 1, 3, 5-estratriene-3, 6α, 15α-triol-17-one (**5**) is reported for the first time. The biotransformation pathway of 17β-estradiol by *P. brevicompactum* to main products is also discussed.

Experiments

Instruments and materials

Sterilization was carried out in HVE-50 Hirayama autoclave. Aseptic operation was carried out in Class II A/B_3 biological safety cabinet from Forma Scientific (Marietta, OH). Incubation was carried out on HZQ-Q orbital shaker. Thin layer chromatographic (TLC) plates was visualized under ultraviolet light and by spraying with ethanol–phosphomolybdic acid (20%) reagent, followed by heating for color development. Purifications were carried out by column chromatography using silica gel (200–300 mesh) as the stationary phase. Melting points (Mps) were measured on a WC-1 melting-point apparatus and were uncorrected. Infrared (IR) spectra were recorded on a Thermo Nicolet 200 spectrometer using KBr disks in the 400–4000 cm^{-1} region. High Resolution-Electron Spray Mass Spectra (HR-EMS) were recorded on a Waters Q-Tof MS System (Milford, MA) in positive ion mode. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained using a Bruker Avance DPX-400 spectrometer (Billerica, MA) at 400 and 100 MHz, respectively. Compounds were dissolved in DMSO-d_6 with tetramethylsilane (TMS) as internal standard. High performance liquid chromatography (HPLC) analysis was performed on a Waters HPLC system and the column was Phenomenex C_{18} column (250×4.60 mm, 5 μm). 17β-Estradiol was purchased from Zhejiang Xianju Pharmaceutical Co., Ltd. (Taizhou, China). Solvents were analytical grade.

Microorganism and culture mediums

The fungal strain *Penicillium brevicompactum* was isolated from several collected soil samples of the urban area of Zhengzhou city and identified by morphologic observation and 16s rRNA sequencing. The fungus was maintained on agar slopes composed of peptone (1.2%), dextrose (3.0%), KH_2PO_4 (0.09%), yeast extract (0.1%), agar (3.0%), and distilled water (pH 4.5) at 4 °C and freshly subcultured before using in the transformation experiments. Liquid culture medium contained peptone (1.2%), dextrose (3.0%), KH_2PO_4 (0.09%), yeast extract (0.1%), and distilled water at pH 4.5.

Culture conditions. For screening scale biotransformation of 17β-estradiol, spores freshly obtained from peptone dextrose agar slopes were transferred aseptically into 250 mL Erlenmeyer flasks containing 50 mL of liquid sterile culture medium (2×10^8 spores/mL) and were incubated on rotary shakers for 2 days, operating at 210 r/min and 30 °C. Then, the substrate was diluted with acetone with a concentration of 100 g/L, followed by 0.5 mL of the solution added into every flask. The cultures were continued and 3 mL of broth of every flask harvested respectively every 24 h within 7 days was extracted with the same volume of ethyl acetate, respectively. Then, the solvent was evaporated under reduced pressure to give reaction mixtures for TLC analysis.

For preparative scale of biotransformation of 17β-estradiol by *P. brevicompactum*, spores freshly obtained from peptone dextrose agar slopes were transferred aseptically into every 500 mL Erlenmeyer flasks with 100 mL of liquid sterile culture medium. After 2 days incubation at 210 r/min and 30 °C, 1500 mg of 17β-estradiol (**1**) dissolved in 15 mL of acetone was evenly added to these 15 flasks. Further culture lasted for 5 days, and the mycelium was separated from the broth by filtration under vacuum, and rinsed in ultrasonic with ethyl acetate (3×200 mL) to ensure that all of the available products were isolated from the mycelium. The mycelial broth was extracted with the same volume of ethyl acetate for three times. The organic extract was combined, concentrated into about 200 mL, and then washed with saturated aqueous $NaHCO_3$, brine and distilled water respectively for three times. Subsequently, the organic extract dried over anhydrous sodium sulfate and was concentrated in vacuum to give crude extract.

Time course of substrate and metabolic products

A time course experiment was conducted in order to determine the metabolic pathway. Conditions were identical to the screening scale biotransformation experiment. The flask was harvested every 24 h within 6 days. Reaction mixtures extracted were dissolved in CH_3OH (3 mL), 20 μL of which was then analyzed by

HPLC. Each analytical determination was performed in triplicate. The mobile phases were CH_3CN/water gradient (26:74, 55:45, v/v) over 30 min, where the mobile phases were held for 16 and 14 min, respectively. The flow rate was 1.0 mL/min and the detection wavelength was 205 nm.

Product isolation and analysis

The crude extract (1570 mg) was subjected to an open silica gel column and eluted with chloroform/methanol gradient (chloroform/methanol, 30:1, 20:1, 10:1, v/v). Purification of the crude extract using chloroform/methanol (30:1, v/v) afforded compound **2** (321 mg). Elution with chloroform/methanol (20:1, v/v) gave two compounds, **3** (267 mg) and **4** (360 mg) sequentially. From chloroform/methanol (10:1, v/v) elution, compound **5** (310 mg) was obtained. All the metabolites were identified by Mps and a combination of IR, MS, and two-dimensional NMR. The solvents used for products crystallization was ethyl acetate/petroleum ether (5:1, v/v).

1, 3, 5-Estratriene-3, 15α-diol-17-one **(2)**, white powder; Yield: 19.4%; mp: 240–242 °C (226–230 °C) (Laskin and Fried 1964); IR (KBr) ν_{max}: 3369, 2924, 1726, 1619, 1508, 1448, 1286, 1241, 1054, 1017, 980, 852, 819, 747, 623 cm^{-1}; ^1H NMR (DMSO-d_6) δ (ppm): 7.05 (1H, d, J= 8.5 Hz, H-1), 6.52 (1H, dd, J= 8.4, 2.6 Hz, H-2), 6.45 (1H, d, J= 2.5 Hz, H-4), 4.29–4.22 (td, J= 14.3, 6.6 Hz, H-15β), 0.85 (3H, s, H-18); ^{13}C NMR data (shown in Table 1); MS: *m/z*: 269.1538 $[M - H_2O + H]^+$ (calcd. 269.1542).

1, 3, 5-Estratriene-3, 6α, 17β-triol **(3)**, white powder; Yield: 16.1%; mp: 240–242 °C (230–235 °C) (Burrows et al. 1973); IR (KBr) ν_{max}: 3358 (br), 2930, 1659, 1612, 1584, 1501, 1453, 1376, 1346, 1252, 1167, 1134, 1050,1013, 969, 866, 820, 796, 774, 623 cm^{-1}; ^1H NMR (DMSO-d_6) δ (ppm): 7.06 (1H, d, J= 8.5 Hz, H-1), 6.73 (1H, d, J= 2.6 Hz, H-4), 6.61 (1H, dd, J= 8.4, 2.6 Hz, H-2), 4.47 (1H, t, J= 4.9 Hz, H-6β), 3.56–3.50 (tt, J= 9.2, 4.6 Hz, H-17α), 0.69 (3H, s, H-18); ^{13}C NMR data (shown in Table 1); MS: *m/z*: 271.1697 $[M - H_2O + H]^+$ (calcd. 271.1698).

1, 3, 5-Estratriene-3, 15α, 17β-triol **(4)**, white powder, Yield: 21.6%; mp: 225.4–230.9 °C (248–250 °C) (Laskin and Fried 1964); IR (KBr) ν_{max}: 3346 (br), 2926, 2868, 1609, 1588, 1502, 1445, 1240, 1135, 1054, 966, 929, 871, 786, 619, 567 cm^{-1}; ^1H NMR (DMSO-d_6) δ (ppm): 7.04 (1H, d, J= 8.5 Hz, H-1), 6.50 (1H, dd, J= 8.4, 2.6 Hz, H-2), 6.43 (1H, d, J= 2.5 Hz, H-4), 3.96–3.81 (1H, m, H-15β), 3.70–3.66 (1H, dd, J= 8.6, 6.1 Hz, H-17α), 0.66 (3H, s, H-18); ^{13}C NMR data (shown in Table 1); MS: *m/z*: 271.1700 $[M - H_2O + H]^+$ (calcd.271.1698).

1, 3, 5-Estratriene-3, 6α, 15α-triol-17-one **(5)**, colorless needles; Yield: 17.8%; mp: 217–219 °C; IR (KBr) ν_{max}: 3373 (br), 2929, 1727, 1612, 1502, 1453, 1379, 1348, 1288, 1249, 1053, 1019, 971, 864, 824, 750, 626 cm^{-1}; ^1H NMR (DMSO-d_6) δ (ppm): 7.07 (1H, d, J= 8.5 Hz, H-1), 6.72 (1H, d, J= 2.5 Hz, H-4), 6.62 (1H, dd, J= 8.4, 2.7 Hz, H-2), 4.48(1H, s, H-6β), 4.26–4.19 (1H, m, H-15β), 0.87 (3H, s, H-18); ^{13}C NMR data (shown in Table 1); MS: *m/z*: 285.1490 $[M - H_2O + H]^+$ (calcd. 285.1491).

Table 1. ^{13}C NMR data for compounds **1–5** determined in DMSO-d_6 (δ ppm).

	Compounds				
Carbon atom	1	2	3	4	5
1	126.5	126.7	125.9	126.7	125.7
2	113.2	113.3	114.8	113.2	114.4
3	155.3	155.4	155.5	155.3	155.0
4	115.4	115.4	116.7	115.3	116.3
5	137.6	137.7	140.6	137.8	140.0
6	29.6	29.8	66.0	29.9	65.4
7	27.4	27.6	33.2	27.9	31.3
8	39.2	38.8	37.1	39.4	32.0
9	44.3	44.1	44.0	44.5	43.4
10	130.9	130.3	130.9	130.8	129.7
11	26.6	26.2	26.2	26.7	25.2
12	37.1	32.0	36.9	37.5	36.3
13	43.3	50.5	43.5	44.3	50.0
14	50.0	56.7	49.8	57.5	55.9
15	23.3	68.8	23.2	71.1	68.3
16	30.4	46.5	30.4	43.1	46.0
17	80.5	217.0	80.6	78.0	216.4
18	11.7	15.5	11.8	13.3	15.0

Results and discussions

The fungus of *Penicillium brevicompactum* was screened from the north-east of the Zhengzhou urban area for its potential biotransformation ability of steroid substrates. The PCR product was sequenced and BLAST analysis of 16S rRNA gene was performed. Sequences having maximum similarities were retrieved, aligned with that of isolate C28030-1-4-1. The alignment was subjected to neighbor-joining analysis indicated the fungus as *Penicillium brevicompactum* (shown in Figure 1).

When 17β-estradiol was fed to the mature mycelium of *P. brevicompactum*, four new plots were observed on the TLC plate, and thus was selected for scale up study. The isolate mixture after 4 days incubation with 17β-estradiol yielded four metabolites **2**–**5**, and the structure of the transformation products is shown in Figure 2.

Product **2**, R_f=0.43 (chloroform/methanol =30:1, v/v), was obtained as white powder with 19.4% yield

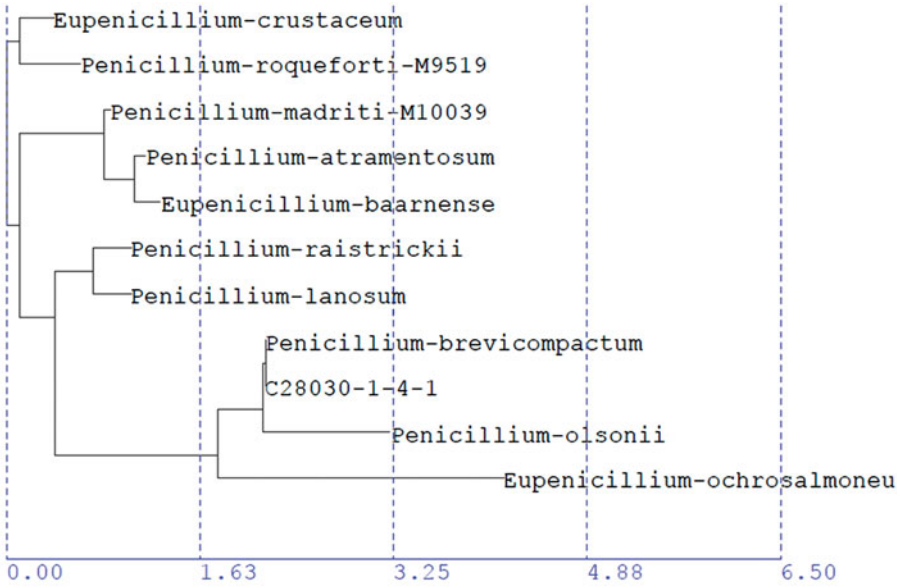

Figure 1. Phylogenetic tree constructed from the 16S rRNA gene of strain *P. brevicompactum*.

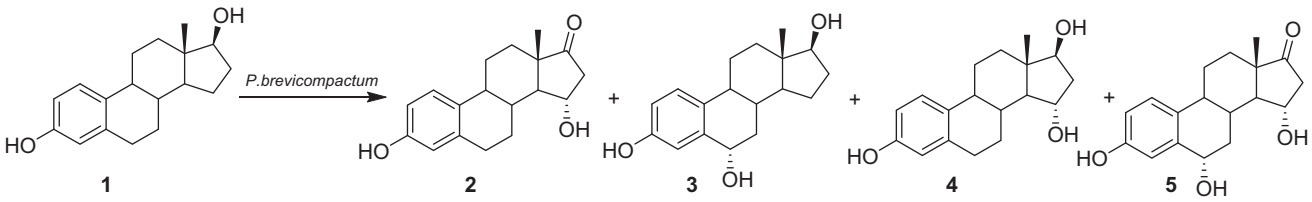

Figure 2. Biotransformation of 17β-estradiol (**1**) by *P. brevicompactum*.

having 98.8% HPLC purity. It showed IR absorption bands at v_{max} 3369 cm^{-1} for hydroxyl and the characteristic absorption for the carbonyl signal at v_{max} 1726 cm^{-1}. The ^{13}C NMR spectrum showed absorption at δ 217.0 for C-17 instead of absorption at δ 80.5 for the substrate, due to the presence of carbonyl group on the five member rings that caused angle strain. The ^{1}H NMR spectrum further confirmed this by the disappearance of absorption at δ 3.52 in compound **2** which appeared in compound **1**. The ^{13}C NMR spectrum exhibited a new oxygen-bearing methine at δ 68.8 along with the loss of a methylene at δ 23.2 (C-15), coupled with downfield shift for the carbon signal at C-14 (δ 56.7) and C-16 (δ 46.5) when compared to ^{13}C NMR spectrum of compound **1**. The HMBC data showed that H-14 (δ 1.42) and H-16 (δ 2.93, 1.91, respectively) were coupled to this carbon, indicating of oxygen insertion at C-15 (Figure 3). The signal of 1.42 was single, which showed that H-15 was on the opposite direction to H-14. In other words, an α-oriented hydroxyl group was introduced to C-15. The NOE effect observed between H-15 and H-18 confirmed the α-orientation of 15-hydroxyl furtherly. Based on the above analysis, compound **2** was identified as 1, 3, 5-estratriene-3, 15α-diol-17-one.

Product **3**, R_f=0.30 (chloroform/methanol =20:1, v/v), was obtained as white powder with 16.1% yield having 99.3% HPLC purity. It showed IR absorption bands at v_{max} 3358 cm^{-1} for hydroxyl. The ^{1}H NMR spectrum of compound **3** contained a new signal at δ 4.47 (1H, t, J= 4.9 Hz) confirming the presence of a hydroxyl group as did the new oxygen-bearing methine resonance signal in the ^{13}C NMR spectrum at δ 66.0. Compared with the ^{13}C NMR spectrum of the substrate, the signal at δ 29.6 for C-6

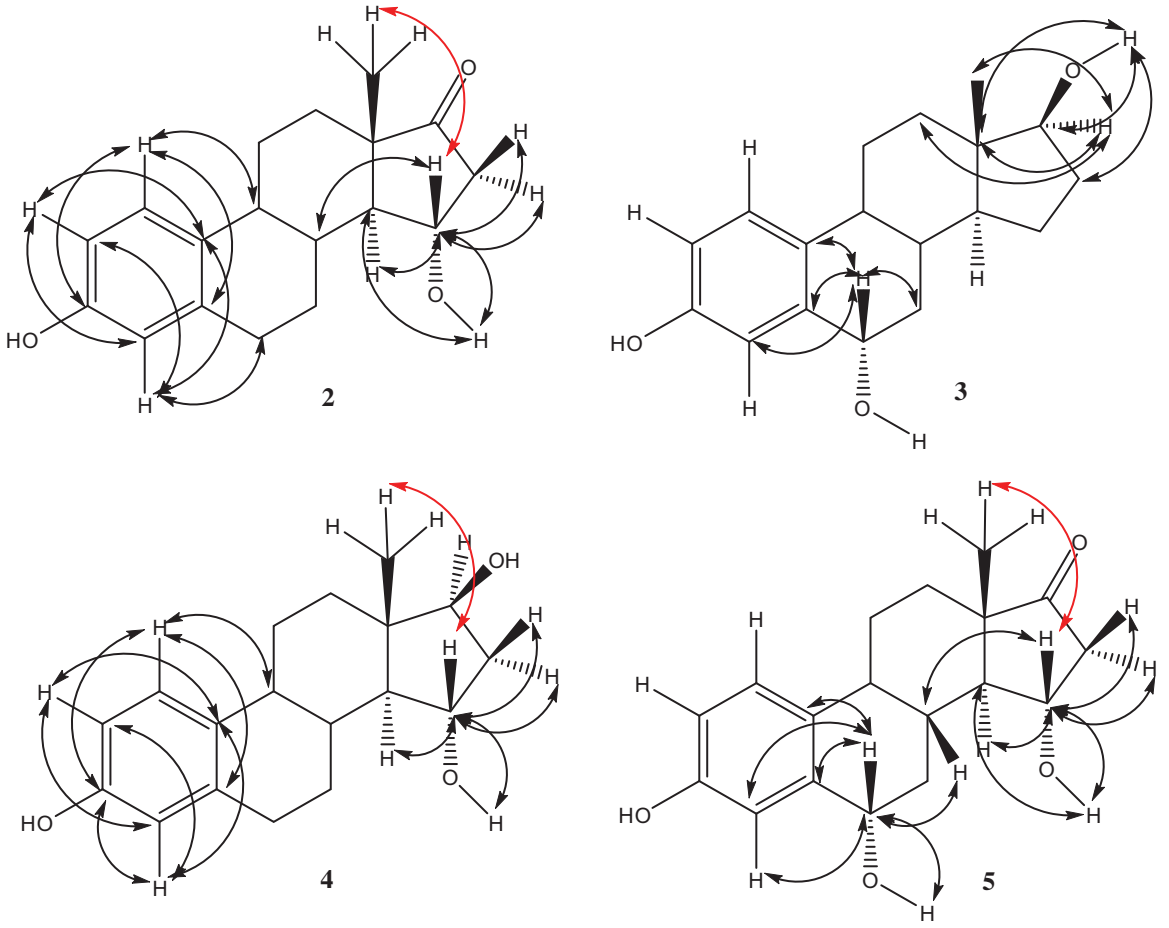

Figure 3. Selected HMBC of products **2–5** and the NOE (red double-headed arrow) between 15β-H and 18-H of products **2**, **4**, and **5**, respectively.

disappeared, coupled with slightly downfield shift for the carbon signal at C-5 and C-7 (δ 140.6, 33.2, respectively). Additionally, the signal of vinyl proton at δ 6.73 (H-4) was correlated with C-6 position in HMBC spectrum. These spectral data suggested the existence of a hydroxyl group at C-6 (Figure 3). So, compound **3** was proposed to be 1, 3, 5-estratriene-3, 6α, 17β-triol.

Product **4**, R_f=0.24 (chloroform/methanol =20:1, v/v), was obtained as white powder with 21.6% yield having 98.6% HPLC purity. It showed IR absorption bands at v_{max} 3346 cm^{-1} for hydroxyl. The disappearance of δ 23.3 (C-15) of the substrate and the presence of a new oxygen-bearing methine carbon signal at δ 71.1 in the ^{13}C NMR spectrum indicated the oxygen insertion at C-15. Furthermore, in HMBC spectra, the carbon signal at δ 71.1 had long-range correlations with the proton signal of H-16 (δ 1.90, 1.70, respectively, Figure 3). In addition, C-14 and C-16 (δ 57.5, 43.1, respectively) signals significantly shifted to downfield. All these shifts confirmed that the addition hydroxyl group was at C-15 position. The NOE effect observed between H-15 and H-18 confirmed the α-orientation of 15-hydroxyl. Therefore, compound **4** was identified as 1, 3, 5-estratriene-3, 15α, 17β-triol.

Product **5**, R_f=0.173 (chloroform/methanol =10:1, v/v), was obtained as white powder with 17.8% yield having 98.5% HPLC purity. It showed IR absorption bands at v_{max} 3373 cm^{-1} for hydroxyl and the characteristic absorption for the carbonyl signal at v_{max} 1727 cm^{-1}. The NMR data of compound **5** was the same to compound **2** but only was different in absorption at H-6 in ^1H NMR spectrum and C-6 in ^{13}C NMR spectrum. The ^{13}C NMR spectrum showed absorption at δ 66.0 for C-6 instead of absorption at δ 29.8 for compound **2**, coupled with a new signal at 4.48 (1H, s) in the ^1H NMR spectrum which suggested the oxygen insertion at C-6. The α-orientation hydroxyl group at C-6 was the same to that of the compound **3**. Based on the above analysis, compound **5** was identified as 1, 3, 5-estratriene-3, 6α, 15α-triol-17-one.

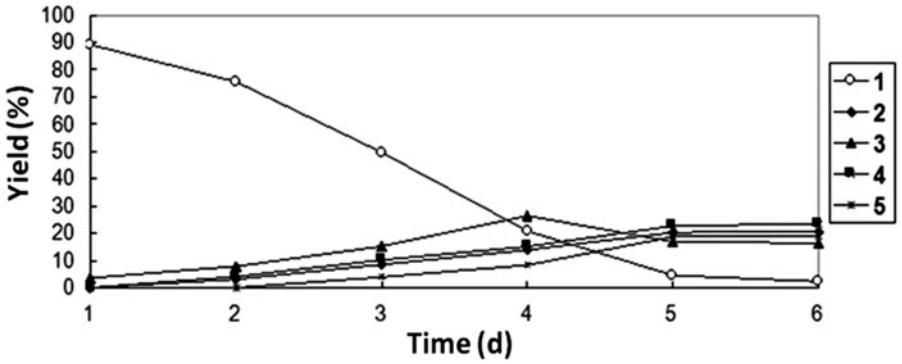

Figure 4. Time course profile for the biotransformation of 17β-estradiol by *P. brevicompactum*.

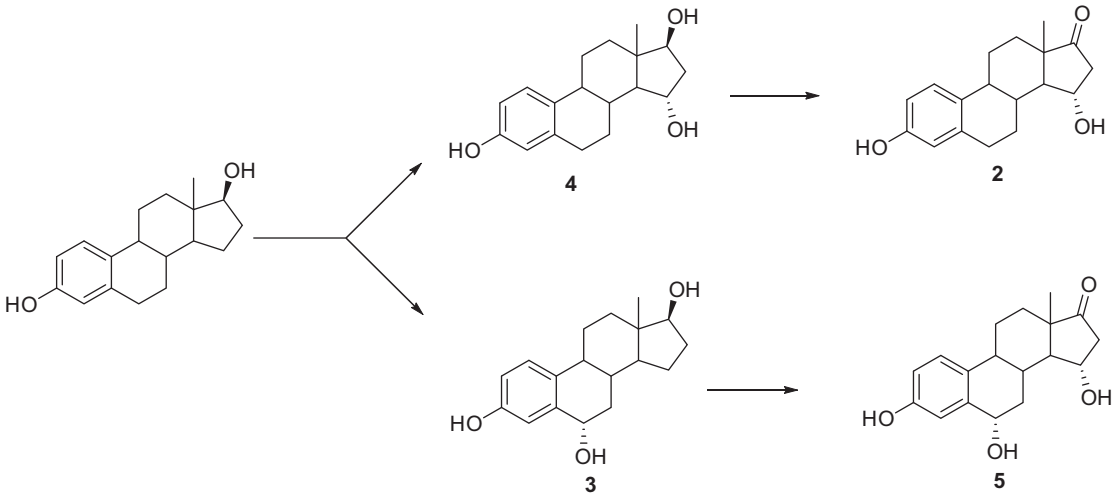

Figure 5. A proposed biotransformation pathway of 17β-estradiol by *P. brevicompactum*.

Time course for biotransformation of 17β-estradiol with Penicillium brevicompactum

The time course for biotransformation of **1** to the metabolites **2**, **3**, **4**, and **5** is summarized in Figure 4. The results showed that: (i) after 5 days incubation, the substrate was almost completely transformed and the amount of every metabolic product remained unchanged; (ii) product **3** peaked to about 26% at the 4th day, then declined to about stable 16% after 5 days of incubation, which suggested that **3** was further transformed into other metabolic product; (iii) after 5 days of incubation, products **4** and **2** steadily increased to 23% and 20%, respectively; (iv) product **5** could be detected after 2 days and experienced a steady rise to about 19% after 5 days.

Obviously, there were 17β-OH oxidases and 6α, 15α hydroxylases to carry out transformation of 17β-estradiol in *Penicillium brevicompactum*. Initially, the hydroxylase of *Penicillium brevicompactum* introduces hydroxyl group at 6α, 15α, position of 17β-estradiol (**1**) respectively, coupled with oxidation of 17β-OH by the oxidase. But the oxidation reaction was only observed in products **2** and **5**. After 2 days incubation, the 6α-hydroxy metabolite (**3**) was gradually transformed into 6α, 15α-dihydroxy product (**5**). Therefore, a possible biotransformation pathway for the metabolism of β-estradiol (**1**) was proposed as shown in Figure 5.

Conclusion

In conclusion, a strain of *Penicillium brevicompactum* was identified and employed to transformation of 17β-estradiol for the first time. Four metabolites were separated from the biotransformation broth and confirmed by detailed spectroscopy methods. The metabolite **3**, **4**, and **5** are reported for the first time via microbial transformation, and **5** (1, 3, 5-estratriene-3, 6α, 15α-triol-17-one) is a new compound as far as we know.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper. This research work was supported by National Natural Sciences Foundations of China (Nos. 21402178, 21372206) and Scientific Research Fund of Henan province (No. 132300410195).

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