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Biotransformation of steroidal compounds using *Lignincola laevis*

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Biotransformation of testosterone **1** and stanolone **2** has been investigated with fungus of *Lignincola laevis*. Biotransformaton of **1** gives 15α,17β-dihydroxyandrost-4-en-3-one **3**. In the case of **2**, two products, 5α-androstan-3α,17 β-diol **4** and 5α-androstane-3, 17-dione **5** have been obtained.

Keywords: Biotransformation, Fungus, Steroidal compounds, *Lignincola laevis*

From the viewpoint of green chemistry, biotransformation of steroids constitutes an important method in organic chemistry. Since 1950s, the biosynthesis and conversion of steroids by microorganisms have been studied. Various steroids biotransformation, such as hydroxylation, dehydrogenation, redox reaction, Baeyer-Villiger oxidation, or microbial have been reported. Microbial biotransformation of various steroids has been reviewed[1-8].

Biotransformation of testosterone by fungus has been reported. The fungus *Cephalosporium aphidicola* has been shown to hydroxylate testosterone at the C-6 β and C-14 α-position[9]. Similarly, *Botrytis cinerea* carries out hydroxylation at the 7α-position (7α-hydroxytestosterone) of testosterone[10]. *Absidia glauca* hydroxylation products of testosterone were 7α-hydroxyandrostenedione and 6β, 11α-dihydroxyandrostenedione[11]. In a recent study the biotransformation of testosterone by the six fungi was investigated[12]. In this study that seven of the analogues were novel compounds and five others, namely, 7α,17β-dihydroxyandrost-4-en-3-one, 6β,14α-dihydroxyandrost-4-ene-3,17-dione, 15α,17β-dihydroxyandrost-4-en-3-one, 16β,17α-dihydroxy-androst-4-en-3one and 2β,16β,17β-trihydroxyandrost-4-en-3-one, were fully characterized for the first time. Moreover, Testolactone was isolated as 98% yield in the transformation of testosterone[13].

Recently, we have reported that biotransformation of steroids by fungus. Biotransfomation of androst-4-ene-3,17-dione, 3β-hydroxypregnan-5-en-20-one (pregnenolone), 3β-hydroxyandrost-5-en-17-one and estradiol was investigated[14]. Moreover, we have reported that in the reduction of C_3-α-hydroxyl compounds are obtained preferentially, rather than C_3-β-hydroxyl derivatives[15].

Here, we report on the biotransformation of testosterone **1** and stanolone **2** (Fig. 1) by the fungus *Lignincola laevis*.

Results and Discussion

After 16 days of incubation of testosterone **1** with the fungus *L. laevis* led to the compound **3** in good yield. The mixtures of many compounds were obtained, but only the main product was isolated and identified. The structure of biotransformation product was determined by GCMS, ^{1}H and ^{13}C NMR spectra (Table 1 and Table 2). The GCMS spectra of **3** showed molecular ion peak at *m*/*z* 304, which suggest that it added one oxygen atom, other fragment ions appeared at *m*/*z* 286 (loss of H_2O). The ^{1}H NMR spectra compared with the testosterone (**1**) data. The ^{1}H NMR spectra of product **3** showed a shift in the Me-18 (δ 0.82) and Me-19 (1.23). Moreover, the product spectra also contained a new signal at 4.12 (1H, *td*, *J*=9.2, 3.4 Hz, H-15β) indicating hydroxylation. The ^{13}C NMR spectra for the C-15 alcohol appeared at δ 72.5. The ^{1}H and ^{13}C NMR spectra are similar to those previously reported[12]. The compound was determined to be 15α,17β-dihydroxyandrost-4-en-3-one, **3**.

Fig. 2 shows the time course study of biotransformation for testosterone (**1**) by *L. laevis*. The

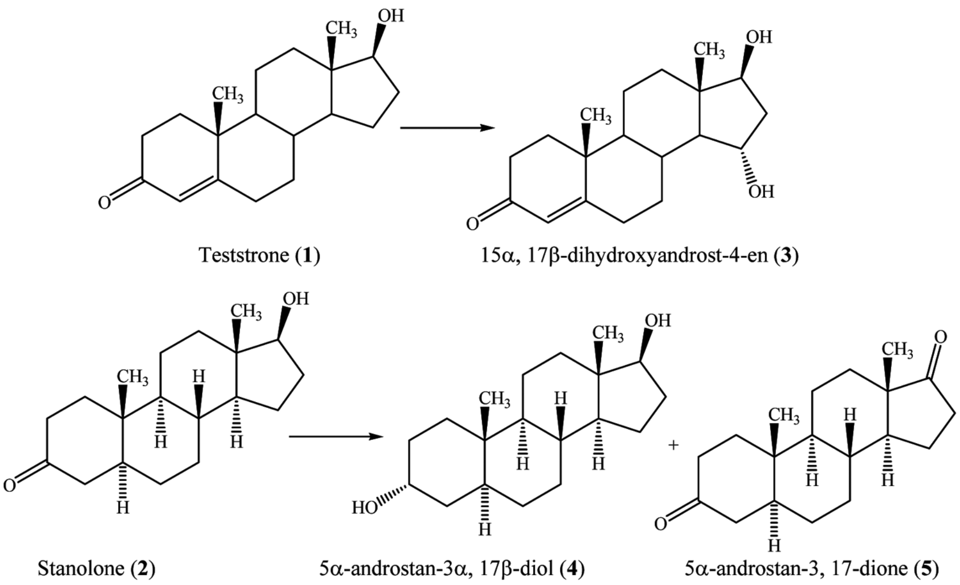

Fig. 1 — The structure of substrates (**1**-**2**) and its metabolites

Table 1 — Product ratio following GC chromatography

Starting material	Metabolites	GC ratio* (%)
Testosterone **1**		40
	15α,17β-Dihydroxyandrost-4-en-3-one 3	50
Stanolone **2**		72
	5α-Androstan-3α,17β-diol 4	18
	5α-Androstan-3,17-dione 5	10

* The products were extracted with ethyl acetate after 18 days.

Table 2 — ^{13}C NMR data for biotransformation products **3**-**5** determined in $CDCl_3$

Carbon atom	Compd		
	3	4	5
1	35.8	32.2	38.5
2	33.9	29.0	38.1
3	199.5	66.6	211.7
4	123.9	36.7	44.6
5	170.9	39.2	46.6
6	32.7	28.4	28.6
7	32.3	31.6	30.5
8	35.3	35.5	34.9
9	53.8	54.4	53.8
10	38.6	36.2	35.8
11	20.5	20.3	20.7
12	36.6	35.8	31.5
13	44.3	43.0	47.7
14	58.5	51.1	51.2
15	72.5	23.4	21.7
16	42.8	30.5	35.8
17	78.8	82.0	221.0
18	12.6	11.1	13.8
19	17.5	11.2	11.4

biotransformation product ratio increased gradually and reaches the maximum amount after 16 days of incubation. After 16 days, the amount of **3** was constant with further incubation time.

The biotransformation of stanolone **2** afforded only two products. The GCMS spectra of products **4** and **5** showed molecular ion peak at *m*/*z* 292 and 288 respectively. The ^{1}H NMR and ^{13}C NMR spectra compared with the stanolone **2** data. This was confirmed by analysis of the product **4** ^{1}H NMR spectra that contained a signal at δ 4.04 and is fully consistent with 3β-H. The product 4 was disappeared of the signal (δ 212.0) indicated by 3-C (C=O) in the starting material **2**. The ^{1}H NMR and ^{13}C NMR spectra are similar to previously report[16]. The compound was determined to be 5α-Androstan-3α,17β-diol **4**. On the other hand, product **5** was devoid of the triplet signal (3.64 ppm) by the 17α-H in the ^{1}H NMR spectra and the appearance of a new signal at 221.0 ppm in the ^{13}C NMR spectra. The ^{1}H and ^{13}C NMR spectra are similar to previously report[17]. The compound was determined to be 5α-androstane-3, 17-dione, **5**.

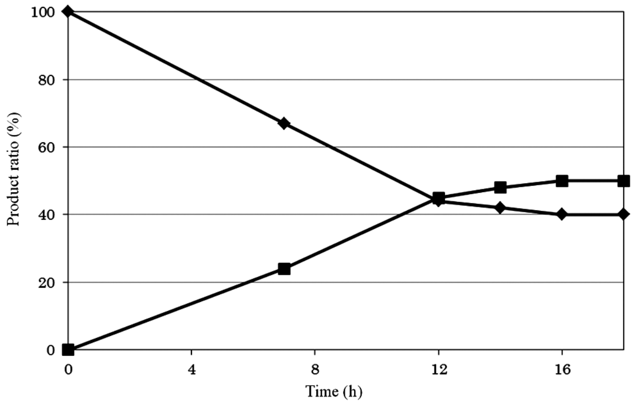

Fig. 2 — Biotransformation of testosterone (**1**) by *Lignincola laevis.* ◆ Testosterone (**1**), ■ 15α,17β-dihydroxyandrost-4-en-3-one (**3**)

Experimental Details

Gas chromatography/mass spectrometry analysis was performed using Shimadzu GCMS-QP5050 [(EI-MS 70 eV) DB1 (0.25 mm×30 m×0.25 mm) capillary column GC; GC: GC-17A]. The NMR spectra were measured on a JEOL GSX 400 spectrometer. $CDCl_3$ with TMS as the internal standard was used. The IR spectra were measured on a JASCO FT/IR 4600 spectrometer. Optical rotations were performed at room temperature using AS ONE shaker SRR-2. Purifications were done by column chromatography using silica gel (Merck silica gel 60). Testosterone **1** and stanolone **2** were purchased from Tokyo Chemical Industry Co., Ltd.

Microorganisms and medium

L. laevis was provided by National Institute of Technology and Evaluation Biological Resource (*L. laevis* : NBRC32130). Yeast and mold medium (YM medium) [0.04% Yeast medium and 0.04% mold medium in artificial sea water] was used for biotransformation.

Biotransformation conditions

An Erlenmeyer flask (200 mL) containing 100 mL of YM medium was inoculated with suspension of *L. laevis*, and incubated at 25°C for 4 days on a rotary shaker (100 rpm) under the dark. After growth of *L. laevis*, 20 mg of substrate with XAD-7 was added to the YM medium. The incubation was them continued for 7-18 days at 25°C. XAD-7 was filtered from the medium. The filtered was extracted with ethyl acetate (2×50 mL), washed with water (2×50 mL) and dried. XAD-7 was washed with ethyl acetate (50 mL) and the extract was concentrated. All the products were determined by ^{13}C NMR and gas chromatographic analysis.

Biotransformation of steroidal compounds

Erlenmeyer flasks (200 mL) containing 100 mL of YM medium were inoculated with suspension of *L. laevis* and them incubated for 4 days at 25°C on a rotary shaker (100 mL) under the dark. Steroidal compounds were added as substrate (20 mg), and the mixture was incubated for 7, 12, 14 16 and 18 days. After incubation, the culture and XAD-7 were separated by filtration, and the filtrated culture medium was extracted with ethyl acetate (2×50 mL), washed with water (2×50 mL). On the other hand, XAD-7 was washed with ethyl acetate (50 mL), and the broth extract was concentrated. The ethyl acetate extracts were analyzed on GCMS analysis. Silica gel chromatography was performed with *n*-hexane/ethyl acetate (1:1) as solvent.

Testosterone, 1

White solid. ^1H NMR (400 MHz, $CDCl_3$): δ 0.78 (3H, s, H-18), 3.74 (1H, brs, H-17α); ^{13}C NMR (400 MHz, $CDCl_3$): δ 11.1 (C-18), 17.4 (C-19), 20.6 (C-11) 23.3 (C-15), 30.5 (C-16), 31.5 (C-7), 32.8 (C-6), 34.0 (C-2), 35.7 (C-1), 35.7 (C-8), 36.4 (C-12), 38.7 (C-10), 42.8 (C-13), 50.5 (C-14), 53.9 (C-9), 81.6 (C-17), 123.9 (C-4), 171.3 (C-5), 199.6 (C-3) ; GCMS: [M^+] *m/z* 288 ($C_{19}H_{28}O_2$).

Stanolone, 2

White solid. ^1H NMR (400 MHz, $CDCl_3$): δ 0.76 (3H, s, H-18), 1.02 (3H, s, H-18), 3.64 (1H, *t*, *J*=8.3Hz, 17α-H); ^{13}C NMR (400 MHz, $CDCl_3$): δ 11.1 (C-18), 11.5 (C-19), 21.0 (C-11), 23.4 (C-15), 28.8 (C-6), 31.2 (C-7), 30.5 (C-16), 35.4 (C-8), 35.7 (C-10), 36.6 (C-12), 38.5 (C-1), 38.2 (C-2) 43.0 (C-13), 44.7 (C-4), 46.7 (C-5), 50.8 (C-14), 53.9 (C-9), 81.8 (C-17), 212.0 (C-3); GCMS: [M^+] *m/z* 290 ($C_{19}H_{30}O_2$).

15α,17β-Dihydroxyandrost-4-en-3-one, 3 (Ref. 12)

After 18 days incubation **1**, the main metabolite was 15α,17β-dihydroxyandrost-4-en-3-one **3**. White solid. ^1H NMR (400 MHz, $CDCl_3$): δ 0.82 (3H, s, H-18), 1.23 (3H, s, H-19), 3.90 (1H, *t*, *J*=8.8Hz, H-17α), 4.12 (1H, *td*, *J*=9.3, 3.4 Hz, H-15 β), 5.74 (1H, s, H-4); ^{13}C NMR (400 MHz, $CDCl_3$) data see Table 2 and supplementary information; GCMS: [M^+] *m/z* 304 ($C_{19}H_{28}O_3$).

5α-Androstan-3α, 17β-diol, 4 (Ref. 16)

After 18 days incubation **2**, the minor metabolite was 5α-androstan-3α, 17β-diol **4** (18%). White solid. ^1H NMR (400 MHz, $CDCl_3$); δ 0.73 (3H, s, H-18), 0.79 (3H, s, H-19), 3.63 (1H, t, J=8.5 Hz, H-17α), 4.04 (1H, *t*, *J*=2.4 Hz, H-3β); ^{13}C NMR (400 MHz, $CDCl_3$) data see Table 2 and supplementary information; GCMS: [M^+] *m*/*z* 292 ($C_{19}H_{32}O_2$).

5α-Androstan-3, 17-dione, 5 (Ref. 17)

After 18 days incubation **2**, the minor metabolite was 5α-androstane-3, 17-dione **5**. White solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (3H, s, H-18), 1.03 (3H, s, H-19); ^{13}C NMR (400 MHz, $CDCl_3$) data see Table 2 and supplementary information; GCMS: [M^+] *m*/*z* 288 ($C_{19}H_{28}O_2$).

Conclusion

The biotransformation of testosterone 1 using *L. laevis* gave 15α,17β-dihydroxyandrost-4-en-3-one **3** that was hydroxylated at 15α-position with good yield. However, in the case of stanolone **2**, no hydroxylation occurred and the stereoselective reduction or oxidation reaction proceeded to products 5α-androstan-3α, 17β-diol **4**, 5α-androstane-3,17-dione **5**. This is the first report on steroid compounds conversion using *L. laevis*.

Supplementary Information

Supplementary information is available in the website http://nopr.niscpr.res.in/handle/123456789/58776.

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