Tetrahedron 70 (2014) 41–46

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Biotransformations of steroids to testololactone by a multifunctional strain *Penicillium simplicissimum* WY134-2

Beibei Yang [a,b], Yu Wang [a], Xi Chen [a], Jinhui Feng [a], Qiaqing Wu [a,*], Dunming Zhu [a,*], Yanhe Ma [a]

[a] *National Engineering Laboratory for Industrial Enzymes and Tianjin Engineering Center for Biocatalytic Technology, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China*
[b] *University of Chinese Academy of Sciences, Beijing 100049, China*

ARTICLE INFO

Article history:
Received 10 September 2013
Received in revised form 7 November 2013
Accepted 14 November 2013
Available online 20 November 2013

Keywords:
Steroids
Biotransformation
Testolactone
Testolic acid
Penicillium simplicissimum
Metabolic pathways

ABSTRACT

The biotransformations of a range of steroidal compounds, including 17α-hydroxy progesterone, progesterone, testosterone, androst-4-ene-3,17-dione (AD), pregnenolone, and dehydroepiandrosterone (DHEA), by *Penicillium simplicissimum* WY134-2 have been investigated. In all the cases, testolic acid and testololactone were detected, and the acid was converted to the lactone when pH was adjusted to 1, leading to isolation of testololactone in 25%–96% yields. Especially for progesterone and testosterone, the isolated yields were 93% and 96% with substrate concentration being 3 g/L, suggesting that *P. simplicissimum* WY134-2 may be used for the synthesis of testololactone. The results revealed the multifunctional catalytic activity of *P. simplicissimum* WY134-2 toward steroids for the first time. The possible reaction pathways of steroids promoted by this strain were discussed.

1. Introduction

The derivatives of steroids formed through chemical synthesis and microbial transformation are often potential active pharmaceutical ingredients (APIs) or key intermediates.[1–3] Compared to chemical modification, microbial transformation has high chemo-, regio-, and stereoselectivity, and is more ecologically friendly. As such, microbial transformation offers an effective and economical way to synthesize steroidal derivatives. For example, some strains, such as *Curvularia lunata* have been employed for the production of cortisol from 11-deoxycortisol in industrial processes.[4] However, there is a considerable shortage of effective strains for production of steroidal compounds of pharmaceutical importance. Therefore, we initiated our effort to search for new microbial strains with extraordinary ability of converting steroids. In this course, an isolated strain *Penicillium simplicissimum* WY134-2, obtained from the soil samples collected from Sichuan province of China, was found to convert 17α-hydroxy progesterone to testololactone with high yield.

Up to now, there are numerous literature concerning the biotechnological applications of *P. simplicissimum*, such as the biosorption of lead and copper ions,[5] degradation of polyethylene,[6] one-step conversion of wheat straw to sugars,[7] and so on. Some enzymes, e.g., lipase, xylanase, and vanillyl-alcohol oxidase, from *P. simplicissimum* have been isolated, purified, and characterized.[8–10] However, to the best of our knowledge, *P. simplicissimum* has not been applied in the biotransformation of steroids. In addition, steroidal lactones have conspicuous medicinal properties, such as anticancer, antibacterial, anticarcinogen, and antiandrogen.[11–14] Testololactone can suppress the synthesis of estrogen by inhibiting the aromatase enzyme.[15] Such function indicates that testololactone may be used to treat breast cancer,[16] prostatic hyperplasia, and prostate cancer.[17] It can also be used as therapeutic agents for disorders caused by the imbalance between estrogen and androgen action, such as gynecomastia[18] and precocious puberty.[19] As such, *P. simplicissimum* WY134-2 was examined toward the bioconversion of 17α-hydroxy progesterone (**1**), progesterone (**2**), testosterone (**3**), androstenedione (**4**), pregnenolone (**5**), and DHEA (**6**) in detail and the possible transformation pathways were conjected.

2. Results and discussion

In the more detailed study of transformation of 17α-hydroxy progesterone (**1**) by *P. simplicissimum* WY134-2, testolic acid was also formed. The acid was completely converted to

* Corresponding authors. Tel.: +86 22 84861963; fax: +86 22 84861996; e-mail addresses: wu_qq@tib.cas.cn (Q. Wu), zhu_dm@tib.cas.cn (D. Zhu).

0040-4020/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tet.2013.11.039

testololactone—when the pH of fermentation culture was adjusted to 1.0. In order to improve the efficacy of such conversion, the effects of initial pH and temperature on this bioconversion were investigated by using the cells growing at 30 °C as catalyst and 17α-hydroxy progesterone (**1**) as substrate. The results are presented in Figs. 1 and 2. It is obvious that the optimal initial pH was 8.0 and the yield of testololactone was highest when the biotransformation temperature was 30 °C.

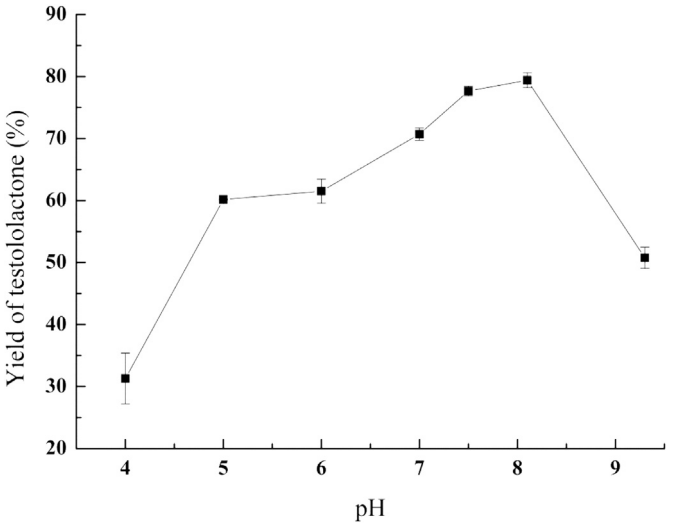


Fig. 1. pH dependence of the biotransformation of 17α-hydroxy progesterone (**1**) by *P. simplicissimum* WY134-2 (1% Tween 80 as emulsifier, 30 °C for 48 h).

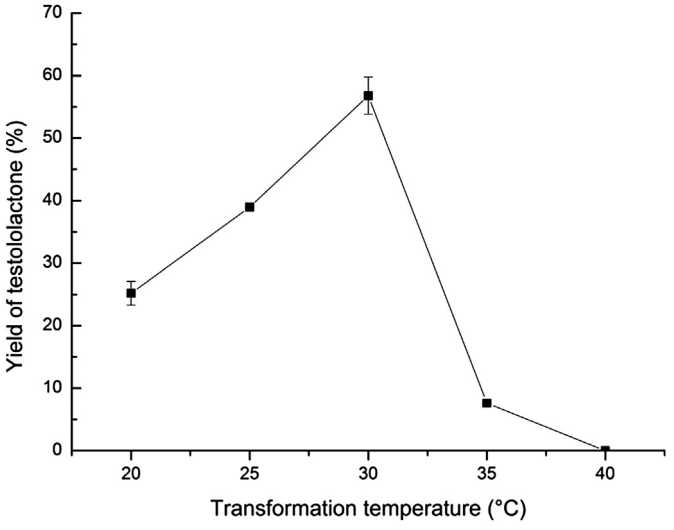


Fig. 2. Temperature dependence of the biotransformation of 17α-hydroxy progesterone (**1**) by *P. simplicissimum* WY134-2 (1% Tween 80 as emulsifier, initial pH 8.0, reaction time 48 h).

Owing to the low solubility of steroidal compounds in aqueous medium, some co-solvents including DMF, DMSO, acetone, and methylene dichloride were used to test the organic solvent tolerance of *P. simplicissimum* WY134-2. The effect of co-solvents on the biotransformation of 17α-hydroxy progesterone (**1**) by *P. simplicissimum* WY134-2 is shown in Fig. 3. It was clear that all the tested solvents inhibited the activity of *P. simplicissimum* WY134-2. Methylene dichloride could completely inhibit the conversion of 17α-hydroxy progesterone (**1**). Owing to the wide use of Tween 80

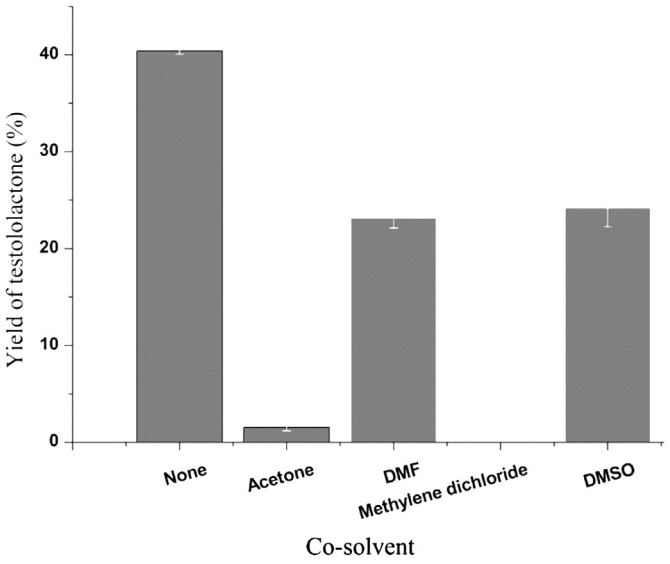


Fig. 3. Effect of co-solvents on the biotransformation of 17α-hydroxy progesterone (**1**) by *P. simplicissimum* WY134-2 (initial pH 8.0, 30 °C for 48 h, final concentration of solvents was 2% (v/v)).

in the biotransformations of steroids,[20–22] the effect of different concentrations of Tween 80 on the bioconversions of 17α-hydroxy progesterone (**1**) by *P. simplicissimum* WY134-2 was studied (Fig. 4). Obviously, the addition of Tween 80 promoted this conversion, and the yield of testololactone was highest when substrate was dissolved in 1% Tween 80.

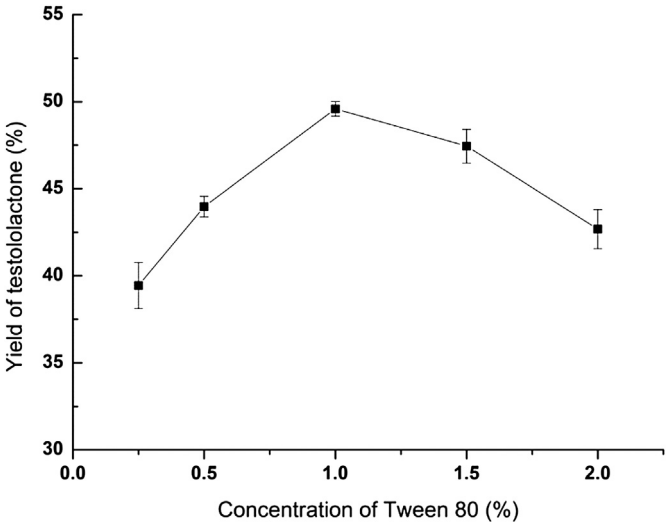


Fig. 4. Effect of different concentrations of Tween 80 on the biotransformation of 17α-hydroxy progesterone (**1**) by *P. simplicissimum* WY134-2 (initial pH 8.0, 30 °C for 48 h).

The bioconversions of six steroids (**1**–**6**) were studied at pH 8.0 and 30 °C by using cells growing at 30 °C. Substrates diffused in 1% Tween 80 were added after cells growing in fermentation medium for 16–20 h (dry cell weight: 9.75 g/L). Different substrate concentrations ranging from 1 g/L to 3 g/L were initially screened by analyzing the reaction mixtures with TLC. The biotransformations were performed at the optimized substrate concentration and the products were isolated. The results are summarized in Table 1. It can be seen that testololactone was isolated in 93% and 96% yields for substrates progesterone (**2**) and testosterone (**3**), respectively.

Table 1
Biotransformation of steroids **1**–**6** to testololactone (**7**)

Substrates	Concentration (g/L)	Reaction time (h)	Isolated yield (%)
17α-Hydroxyprogesterone (**1**)	1	48	56
Progesterone (**2**)	3	24	96
Testosterone (**3**)	3	24	93
Androst-4-ene-3,17-dione (**4**)	1	24	78
Pregnenolone (**5**)	1	48	25
Dehydroepiandrosterone (**6**)	2	24	53

Such effective bioconversion of steroids indicated that *P. simplicissimum* WY134-2 might be a potential strain for the industrial production of testololactone.

During the conversion of progesterone (**2**) to testololactone (**7**), two intermediates were detected at 2 h, which were identified as testosterone (**3**) and androstenedione (**4**) by comparing HPLC chromatographs with authentic samples (Fig. 5). The time course of Scheme 1.[23] Firstly, the side chain was oxidized by Baeyer–Villiger monooxygenase to yield testosterone acetate, which was hydrolyzed to testosterone (**3**). Then AD (**4**) was obtained by the dehydrogenation of testosterone (**3**). Finally, AD (**4**) was oxidized by Baeyer–Villiger monooxygenase to give testololactone (**7**), which could be hydrolyzed to testolic acid (**8**). The hydrolysis of testololactone to testolic acid, which was confirmed by our experiment, was also reported by Holmlund.[24,25]

According to the proposed reaction pathway, AD (**4**) was an intermediate of the conversion from progesterone (**2**) to testololactone (**7**). However, the transformation efficiency of AD (**4**) to testololactone (**7**) was far behind that of progesterone (**2**). There are two possible reasons for such unusual phenomenon. First, the D-ring lactonization may be inhibited by higher concentration of AD (**4**), as evidenced by the lower conversion at higher AD (**4**) concentration. Second, progesterone (**2**) rather than AD (**4**) might be a better inducer for the Baeyer–Villiger monooxygenase produced by *P. simplicissimum* WY134-2, leading to higher enzyme activity of the strain. A similar observation was reported for the bioconversion

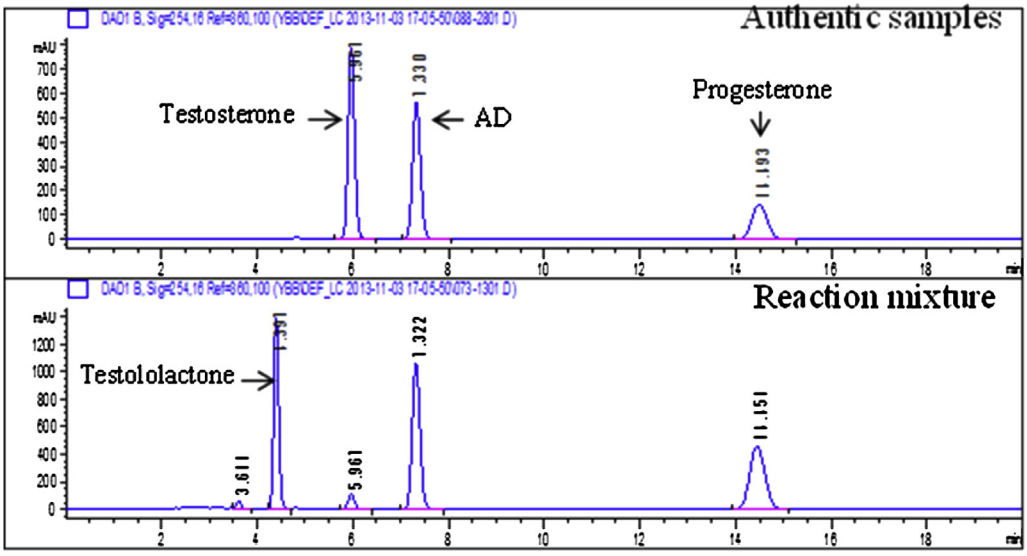


Fig. 5. HPLC analysis of the progesterone (**2**) biotransformation by *P. simplicissimum* WY134-2 (above, authentic samples; below, reaction mixture taken after incubation at 30 °C for 10 h since the introduction of substrate; initial pH 8.0, 30 °C, 1% Tween 80).

these compounds present in reaction mixture was investigated and the results are shown in Fig. 6. Thus, the bioconversion course of progesterone to testolic acid and testololactone was proposed as in

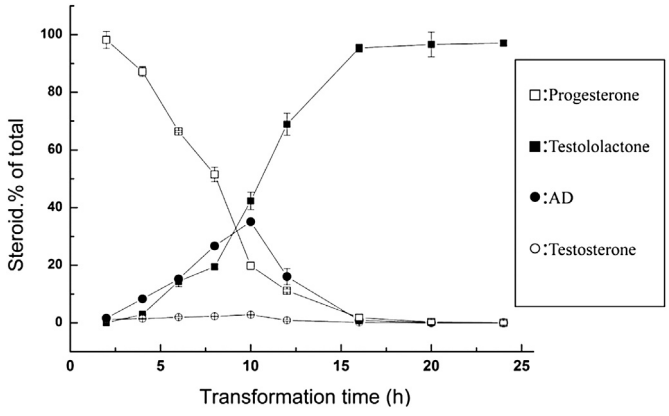
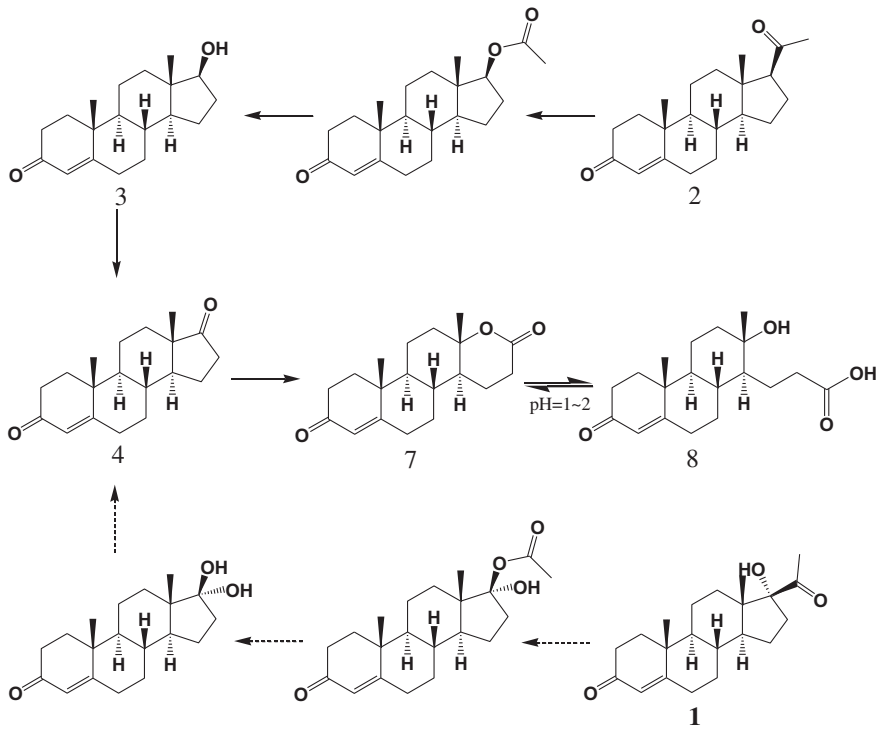


Fig. 6. Time course profile for the biotransformation of progesterone (**2**) by *P. simplicissimum* WY134-2 (initial pH 8.0, 30 °C, 1% Tween 80).

of progesterone (**2**) to Δ^1-testololactone catalyzed by *Septomyxa affinis*, in which androst-1,4-diene-3,17-dione (ADD) was proposed as an intermediate. Whereas ADD couldn't be transformed to Δ^1-testololactone when it was the starting substrate for *S. affinis* under identical conditions except when progesterone (**2**) was either present at the initiation of bioconversion or the cells had been pre-induced with progesterone (**2**).[26] It was suggested that progesterone (**2**) rather than ADD might be a better inducer of the enzyme responsible for the formation of Δ^1-testololactone.

The yield of testololactone from 17α-hydroxy progesterone (**1**) was lower than that from progesterone (**2**), which might indicate that the existence of 17α-OH may increase the steric hindrance, which hindered the Baeyer–Villiger monooxygenation of side chain. During the conversion of 17α-hydroxy progesterone to testololactone catalyzed by *P. simplicissimum* WY134-2, no intermediate was detected. In accordance with the biotransformation of 17α-hydroxy progesterone (**1**) to testololactone (**7**) catalyzed by *Cylindrocarpon radicicola* ATCC 11011,[27] a possible transformation mechanism of 17α-hydroxy progesterone (**1**) to testolic acid (**8**) by *P. simplicissimum* WY134-2 was proposed (Scheme 1). With the oxidation by Baeyer–Villiger monooxygenase and hydrolysis by esterase, an unstable intermediate was obtained, which was



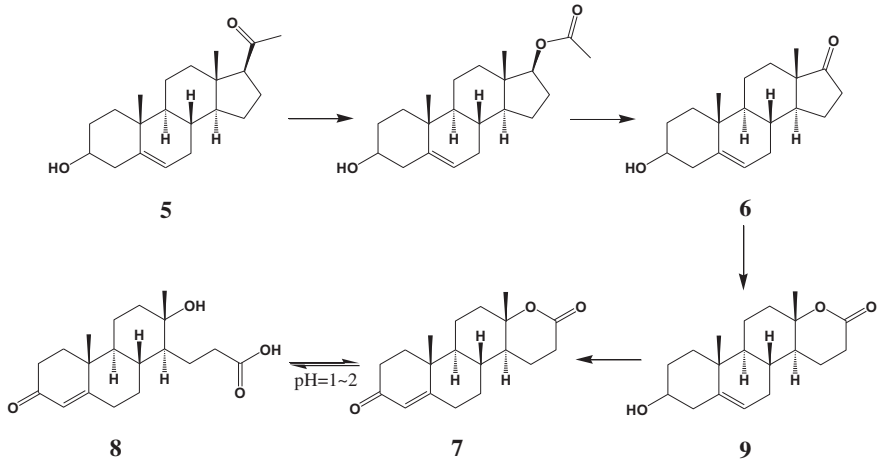
Scheme 1. D-Lactonization pathways of steroidal 4-en-3-ketones.

quickly transformed to AD (**4**). Then the transformation of AD (**4**) to testolic acid (**8**) proceeded as described above.

Among these steroidal substrates, the yield of testololactone from pregnenolone (**5**) was lowest. Comparing the structure of pregnenolone (**5**) and progesterone (**2**), it is possible that the oxidation of 3β-OH and/or $\Delta^{5\rightarrow4}$ isomerization in those two compounds might be the rate-determining steps.

There were different reported transformation pathways for the conversion of pregnenolone (**5**) and DHEA (**6**) to testololactone (**7**).[28] In the bioconversion process of pregnenolone (**5**) catalyzed by *P. simplicissimum* WY134-2, 3β-Hydroxy-17a-oxa-D-homo-androst-5-en-17-one (**9**) was detected as an intermediate, which also could be converted to testololactone (**7**). Therefore, the bioconversion course of pregnenolone (**5**) was proposed in Scheme 2. Firstly, the side chain was cleaved through Baeyer–Villiger monooxygenation, followed by the hydrolysis of ester and dehydrogenation of C-17 hydroxy group yielded DHEA (**6**). Baeyer–Villiger monooxygenation of DHEA (**6**) gave 3β-hydroxy-17a-oxa-D-homo-androst-5-en-17-one (**9**), which was oxidized by 3-β-hydroxysteroid dehydrogenase (3β-HSD) to give testololactone (**7**). Finally, testololactone was hydrolyzed to testolic acid (**8**).

Some other microbial strains have been reported to transform steroidal compounds to testololactone (**7**). For example, *Aspergillus tamari* KITA transformed 17α-hydroxy progesterone (**1**) and deoxycorticosterone to testololactone at substrate concentration of 1 g/L in 5 days. Some byproducts, including 17,20(*R*)-dihydroxypregna-4-en-3-one and Δ^1-testololactone, were observed in the bioconversion of 17α-hydroxy progesterone to testololactone by this fungi.[29] *Rhizopus stolonifer* (ATCC 10404) converted testosterone to give testololactone with substrate final concentration being 0.7 g/L



Scheme 2. D-Lactonization pathways of steroidal 5-en-3-alcohols.

in 5 days. In this conversion, four byproducts were detected.[30] There were some penicillium genus, which could give rise to testololactone. For instance, *Penicillium camembertii* AM83 could convert steroids **2**–**6** to give testololactone (**7**). Among these steroidal substrates, the highest yield of testololactone was 94% as analyzed by GC. However, the final concentration of substrates was only 0.2 g/L.[28] Such low substrate concentration limited the application of *P. camembertii* AM83 for the industrial production of testololactone. *Penicillium notatum* could transform steroids **2**, **5**, and **6** to yield testololactone (**7**) with substrate final concentration being 0.33 g/L. Yet the presence of a substituent at 17α-position is a barrier to lactonization by *P. notatum*.[31] In our study, *P. simplicissimum* WY134-2 could transform six steroidal compounds to testololactone. Especially, the isolated yield reached 96% and 93% for substrates progesterone and testosterone at 3 g/L in 24 h, respectively. Testololactone was the sole isolated product for substrates **1**–**4**. Only trace of byproduct 3β-Hydroxy-17a-oxa-D-homo-androst-5-en-17-one was detected when the substrates were **5** and **6**.

In our previous report, we found that *Fusarium oxysporum* SC1301 could convert steroids **2**–**6** and ADD to give testolactone, which is also called as Δ^1-testololactone, with final concentration of substrates being 1 g/L.[32] Compared to *F. oxysporum* SC1301, *P. simplicissimum* WY134-2 lacks the Δ^1-dehydrogenase. *F. oxysporum* SC1301 could not transform 17α-hydroxy progesterone (**1**) to testolactone, it might be due to the steric hindrance resulting from the hydroxyl group at 17α-position.

3. Conclusions

P. simplicissimum WY134-2, isolated from soil samples, could convert steroidal compounds 17α-hydroxy progesterone (**1**), progesterone (**2**), androst-4-ene-3,17-dione (**3**), testosterone (**4**), pregnenolone (**5**), and dehydroepiandrosterone (**6**) to furnish the same product, testololactone. Such biotransformations include oxidation of C-3 OH group and isomerization of $\Delta^{5\rightarrow4}$, a selective Baeyer–Villiger oxidation of steroidal side chain and ring-D, dehydrogenation and hydrolysis of ester. It has been demonstrated for the first time that *P. simplicissimum* has great multi-functional catalytic properties toward the transformation of steroidal compounds. Compared with other microorganisms reported previously, such as *P. camembertii* AM83, *P. notatum* and *A. tamari*, *P. simplicissimum* WY134-2 exhibited higher activity and specificity to give product testololactone (**7**). The results revealed that *P. simplicissimum* WY134-2 may be a useful strain for production of testololactone (**7**).

4. Experimental

4.1. General procedures

Steroids **3**, **4**, and **5** were kindly donated by Tianjin Jinyao Group Co., LTD. Other steroidal compounds and solvents were obtained from commercial sources. Silica gel (Qingdao Haiyang ChemicalCo., 200–300 mesh) was used for the column chromatography. The thin-layer chromatography (TLC) was performed by developing in solvent mixture of ethyl acetate and petroleum ether (1/3, v/v), and visualizing by UV light (254 nm). The high performance liquid chromatography (HPLC) analysis was performed on an Agilent 1200 system with an Eclipse XDB-C18 column (4.6×150 mm, eluent: acetonitrile/water 6:4, flow rate: 0.5 ml/min, detector: UV254 nm).

4.2. Microorganism, maintenance, and cultivation

P. simplicissimum WY134-2 was isolated from soil samples collected from Sichuan province of China. The fungus was maintained at 4 °C on Potato Dextrose Agar slants. Spores was washed with 3 ml of sterilized water and added to flat-bottomed flask (250 ml) with 50 ml of the seed culture consisting of glucose (3%), corn steep liquor (1.5%), $NaNO_3$ (0.3%), $MgSO_4{\cdot}7H_2O$ (0.05%), KCl (0.05%), $FeSO_4{\cdot}4H_2O$ (0.002%), $K_2HPO_4{\cdot}3H_2O$ (0.2%), and KH_2PO_4 (0.1%) (pH 6.5). The seed culture was shaken at 200 rpm and 30 °C for 14–16 h. Then 1 ml of fungus suspension was transferred to the flat-bottomed flask (100 ml) with 20 ml of transformation culture medium, whose components were the same as seed culture. The resulting mixture was cultured for 24 h and the substrate was added for biotransformation.

4.3. Biotransformation

4.3.1. Optimization of biotransformation conditions. The initial pH of transformation culture was adjusted to different pH ranging from 4 to 9, and the cell suspension was transferred to transformation culture medium, which was cultured for 24 h as described in Section 4.2. 17α-Hydroxy progesterone (**1**, final concentration of 1 g/L) dissolved in 1% Tween-80 (v/v 4%) was then added to the transformation culture and the bioconversion was implemented at 30 °C, 200 rpm for 48 h. The pH of the reaction mixture was adjusted to 1.0 with concentrated hydrochloric acid and the reaction mixture was vibrated at 40 °C for 24 h. The mixture was extracted with ethyl acetate (20 ml×3). The yield of testololactone was measured by HPLC analysis.

Similar to the optimization of initial pH, after 17α-hydroxy progesterone (**1**) was added to the transformation culture, the bioconversion was carried out at different temperature from 20 to 40 °C and the optimal pH obtained above. After incubation for 48 h, the products were extracted and analyzed as described above to measure the yield of testololactone.

Similar to the previous optimization of initial pH, 17α-hydroxy progesterone (**1**) dissolved in different co-solvents (2% v/v) was added to the transformation culture with substrate final concentration being 1 g/L. The transformation was implemented at optimal initial pH and optimal temperature for 48 h. The products were extracted and analyzed as described above to measure the yield of testololactone.

Similar to the optimization of initial pH, 17α-hydroxy progesterone (**1**) dissolved in different concentrations of Tween 80 (4% v/v) was added to the transformation culture with substrate final concentration being 1 g/L. The transformation was implemented at optimal initial pH and optimal temperature for 48 h. The products were extracted and analyzed as described above to measure the yield of testololactone.

All of the following bioconversions of steroidal compounds (**1**–**6**) were carried out under the optimal conditions.

*4.3.2. Biotransformation of 17α-hydroxy progesterone (**1**).* 17α-Hydroxy progesterone (180 mg) dissolved in 1% Tween 80 (8 ml) was added to the culture (180 ml) prepared as described above at 30 °C. The reaction was analyzed by TLC and HPLC. After incubation for 48 h, the substrate was consumed and a single product was detected. Taking into consideration the unstability of this product (testolic acid), the pH of reaction mixture was adjusted to 1.0, and the mixture was shaken at 200 rpm and 40 °C for 24 h to ensure the conversion of testolic acid to testololactone. The reaction mixture was extracted with ethyl acetate (180 ml×3), and the organic extract was dried over sodium sulfate. The solvent was evaporated under reduced pressure to give yellow crude product, which was purified by silica gel chromatography with ethyl acetate/petroleum ether (1:3) as eluent to give testololactone (**7**) as a white solid (91.6 mg, 56% yield), which was identified as testololactone (**2**). ^{1}H NMR: (600 MHz, $CDCl_3$) δ: 1.09–1.16 (1H, m), 1.17 (3H, s, 18-H3), 1.25–1.36 (2H, m), 1.38 (3H, s, 19-H3), 1.40–1.41 (1H, m), 1.53–1.59

(1H, m), 1.65–1.83 (4H, m), 2.00–2.08 (4H, m), 2.33–2.46 (4H, m), 2.56–2.62 (1H, m), 2.68–2.73 (1H, m), 5.76 (1H, s, 4-H); ^{13}C NMR: $CDCl_3$, 150 MHz, δ (ppm): 199.3, 171.3, 169.4, 124.3, 82.4, 52.7, 45.9, 39.2, 38.7, 38.2, 35.7, 34.0, 32.5, 30.6, 28.7, 22.0, 20.3, 20.1, 17.6. HRMS (ESI$^+$): $C_{19}H_{26}O_3Na$ [M+Na]$^+$: calcd 325.1780, found 325.1766.

*4.3.3. Biotransformation of progesterone (**2**).* The bioconversion of progesterone followed the same procedure as described in Section 4.3.2. Progesterone (420 mg) was added to the transformation culture (140 ml). After incubation for 24 h, testololactone (**7**) was obtained (387.7 mg, 96% yield).

During the transformation, two intermediates were detected at 2 h by TLC and HPLC, and identified as testosterone (**3**) and androstenedione (**4**) by comparing HPLC chromatographs with authentic samples.

*4.3.4. Biotransformation of testosterone (**3**).* The bioconversion of testosterone followed the same procedure as described in Section 4.3.2. Testosterone (360 mg) was added to the fermentation culture (120 ml). After incubation for 24 h, testololactone (**7**) was obtained (351.3 mg, 93% yield).

*4.3.5. Biotransformation of AD (**4**).* The bioconversion of AD followed the same procedure as described in Section 4.3.2. AD (120 mg) was added to the culture (120 ml). After incubation for 24 h, testololactone (**7**) was obtained (98.9 mg, 78% yield).

*4.3.6. Biotransformation of pregnenolone (**5**).* The bioconversion of pregnenolone followed the same procedure as described in Section 4.3.2. Pregnenolone (260 mg) was added to the culture (260 ml). After incubation for 48 h, testololactone (**7**) was obtained (62.8 mg, 25% yield).

During the conversion, an intermediate, which was isolated and identified as 3β-Hydroxy-17a-oxa-D-homo-androst-5-en-17-one (**9**) in a separate experiment, was detected at 3 h by HPLC. ^{1}H NMR: $CDCl_3$, 600 MHz, δ (ppm): 0.96 (3H, s, 19-H3), 1.30 (3H, s, 18-H3), 3.51 (1H, m, 3-H), 5.33 (1H, m, 6-H); ^{13}C NMR: $CDCl_3$, 150 MHz, δ (ppm): 171.5, 140.7, 120.6, 83.2, 71.5, 49.0, 46.7, 41.9, 38.9, 36.9, 36.6,34.5, 31.5, 31.1, 28.8, 22.0, 20.1, 19.9, 19.3. HRMS (ESI$^+$): $C_{19}H_{28}O_3Na$ [M+Na]$^+$: calcd 327.1936, found 327.1943.

*4.3.7. Biotransformation of DHEA (**6**).* The bioconversion of DHEA followed the same procedure as described in Section 4.3.2. DHEA (240 mg) was added to the transformation culture (120 ml). After incubation for 24 h, testololactone (**7**) was obtained (133.4 mg, 53% yield). During the transformation, an intermediate was detected at 2 h by HPLC. Its retention time analyzed by HPLC was the same as the intermediate of pregnenolone (**5**). In a separate experiment, this intermediate was isolated and identified as 3β-Hydroxy-17a-oxa-D-homo-androst-5-en-17-one (**9**).

*4.3.8. Time course for the biotransformation of progesterone (**2**).* The time course for the bioconversion of progesterone followed the same procedure as described in Section 4.3.2. Progesterone (final concentration being 3 g/L) was added to the transformation cultures in flasks (20 ml each). Every 2 or 4 h since the introduction of substrate, three flasks samples of reaction mixture were taken, pH was adjusted to 1.0 and the flasks were shaken for 24 h at 200 rpm and 40 °C. The reaction mixtures were extracted with ethyl acetate. The steroid contents in the extract were measured by HPLC analysis.

Acknowledgements

This work was financially supported by National High-Tech Research & Development Program of China (863 Program, No. 2011AA02A211), Tianjin Municipal Science & Technology Commission (10ZCZDSY06700).

References and notes

1. Segaloff, A.; Weeth, J. B.; Rongone, E. L.; Murison, P. J.; Bowers, C. Y. *Cancer* **1960**, *13*, 1017–1020.
2. Pribluda, V. S.; Gubish, E. R.; LaVallee, T. M.; Treston, A.; Swartz, G. M.; Green, S. J. *Cancer Metastasis Rev.* **2000**, *19*, 173–179.
3. Akselsen, Ø. W.; Hansen, T. V. *Tetrahedron* **2011**, *67*, 7738–7742.
4. Petzoldt, K.; Annen, K.; Laurent, H.; Wiechert, R. U.S. Patent 4,353,985, 1982.
5. Li, X. M.; Liao, D. X.; Xu, X. Q.; Yang, Q.; Zeng, G. M.; Zheng, W.; Guo, L. *J. Hazard. Mater.* **2008**, *159*, 610–615.
6. Yamada-Onodera, K.; Mukumoto, H.; Katsuyaya, Y.; Saiganji, A.; Tani, Y. *Polym. Degrad. Stab.* **2001**, *72*, 323–327.
7. Yuan, L.; Chen, Z.; Zhu, Y.; Liu, X.; Liao, H.; Chen, D. *Appl. Biochem. Biotechnol.* **2012**, *167*, 39–51.
8. Sztajer, H.; Lünsdorf, H.; Erdmann, H.; Menge, U.; Schmid, R. *Biochim. Biophys. Acta* **1992**, *1124*, 253–261.
9. Schmidt, A.; Schlacher, A.; Steiner, W.; Schwab, H.; Kratky, C. *Protein Sci.* **1998**, *7*, 2081–2088.
10. De Jong, E.; van Berkel, W. J. H.; van der Zwan, R. P.; de Bont, J. A. M. *Eur. J. Biochem.* **1992**, *208*, 651–657.
11. Fried, J.; Thoma, R. W. U.S. Patent 2,744,120, 1956.
12. Cella, J. A.; Kagawa, C. M. *J. Am. Chem. Soc.* **1957**, *79*, 4808–4809.
13. Wolf, M. E.; Zanati, G.; Shanmugasundrum, G.; Gupte, S.; Aadahl, G. *J. Med. Chem.* **1970**, *13*, 531–534.
14. Séralini, G.; Moslemi, S. *Mol. Cell. Endocrinol.* **2001**, *178*, 117–131.
15. Cocconi, G. *Breast Cancer Res. Treat.* **1994**, *30*, 57–80.
16. Budnick, R. M.; Dao, T. L. *Steroids* **1980**, *35*, 533–541.
17. Li, S.; Parish, E. J. *J. Am. Oil Chem. Soc.* **1996**, *73*, 1435–1451.
18. Braunstein, G. D. *Endocr. Relat. Cancer* **1999**, *6*, 315–324.
19. Feuillan, P.; Merke, D.; Leschek, E. W.; Cutler, G. B. *Endocr. Relat. Cancer* **1999**, *6*, 303–306.
20. Zhu, Y.; Huang, W.; Ni, J. *J. Clean. Prod.* **2010**, *18*, 242–247.
21. Bing, H.; Li, W. *Res. J. Biotechnol.* **2013**, *8*, 56–61.
22. Avramova, T.; Spassova, D.; Mutafov, S.; Momchilova, S.; Boyadjieva, L.; Damyanova, B.; Angelova, B. *World J. Microbiol. Biotechnol.* **2010**, *26*, 1009–1014.
23. Mihovilovic, M. D.; Müller, B.; Stanetty, P. *Eur. J. Org. Chem.* **2002**, 3711–3730.
24. Holmlund, C. E.; Blank, R. H.; Sax, K. J.; Evans, R. H. *Arch. Biochem. Biophys.* **1963**, *103*, 105–110.
25. Holmlund, C. E.; Blank, R. H. *Arch. Biochem. Biophys.* **1965**, *109*, 29–35.
26. Miller, T. L. *Biochim. Biophys. Acta* **1972**, *270*, 167–180.
27. Itagaki, E. *J. Biochem.* **1986**, *99*, 825–832.
28. Kołek, T.; Szpineter, A.; Świzdor, A. *Steroids* **2009**, *74*, 859–862.
29. Hunter, A. C.; Carragher, N. E. *J. Steroid Biochem. Mol. Biol.* **2003**, *87*, 301–308.
30. Al-Aboudi, A.; Mohammad, M. Y.; Musharraf, S. G.; Choudhary, M. I.; Atta-ur-Rahman. *Nat. Prod. Res.* **2008**, *22*, 1498–1509.
31. Bartmańska, A.; Dmochowska-Gładysz, J.; Huszcza, E. *Steroids* **2005**, *70*, 193–198.
32. Zhang, H.; Ren, J.; Wang, Y.; Sheng, C.; Wu, Q.; Diao, A.; Zhu, D. *Tetrahedron* **2013**, *69*, 184–189.