

## Microbial Hydroxylation of 16 $\alpha$ , 17 $\alpha$ -Epoxyprogesterone by *Penicillium Decumbens*

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### Abstract

Microbial transformation has been successfully applied in the production of steroid intermediates with therapeutic use and commercial value in pharmaceutical industry due to its high regio- and stereo-selectivity. As such, it is still important to screen microbial strains with novel activity or more efficient abilities in the development of the commercial steroid industry. Biotransformation of steroid: 16 $\alpha$ , 17 $\alpha$ -epoxyprogesterone (1) using *Penicillium decumbens* as biocatalyst was investigated and selective hydroxylation of 1 was observed. The products were separated by silica gel column chromatography, and the structure determination was performed by MS, NMR, and X-ray crystallography. Biotransformation of 1 afforded 7 $\beta$ -hydroxy-16 $\alpha$ , 17 $\alpha$ -epoxyprogesterone (2) and 7 $\beta$ ,11 $\alpha$ -dihydroxy-16 $\alpha$ ,17 $\alpha$ -epoxyprogesterone (3). The two novel metabolic products 2 and 3 were reported for the first time. Moreover, the identified C7 $\beta$ - and C11 $\alpha$ -hydroxylation is a novel reaction of microbial transformation of steroids by *P. decumbens*.

**Keywords:** Steroid; Biotransformation; *Penicillium decumbens*; 16 $\alpha$ ; 17 $\alpha$ -Epoxyprogesterone; Hydroxylation.

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### Introduction

Steroid, an important class of bioactive compounds, has been widely used as anti-inflammatory, anti-microbial, anti-diabetic, anti-allergenic, and anti-cancer drugs in clinical therapy (1-3). It is well known that the structural modification of steroid, such as the introduction of hydroxyl unit to different positions would change the activity and stability of steroid in

different ways (4-6). Hence, there has been increasing interest in the synthesis and structural modification of steroid (4-6). Chemical synthesis has shown many disadvantages as a traditional method for the preparation of drug molecules such as the toxicity of chemical catalysts, complicated reaction steps, long production time, and low yield (7). However, biotransformation can overcome most of the shortcomings of chemical synthesis mentioned above, especially for the production of compounds with complex structures like steroids (8-10). In addition, the biotransformation is more efficient than

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chemical synthesis in reactions that require high stereoselectivity or regioselectivity (11).

16 $\alpha$ , 17 $\alpha$ -Epoxyprogesterone (1) often serves as an important intermediate for many hormone based drugs such as hydrocortisone, cortisone, and megestrol (12).

Although the biotransformation of (1) has already been successfully applied in industry for the production of 11 $\alpha$ -hydroxylation 16 $\alpha$ , 17 $\alpha$ -Epoxyprogesterone (3), the screening of microbial strains with novel catalytic activities still plays an important role in developing more efficient production processes as well as producing novel steroid compounds. For example, 7 $\alpha$ -, 9 $\alpha$ -, 11 $\alpha$ -, 14 $\alpha$ -, 11 $\beta$ - and 20 $\beta$  hydroxylation of (1) has been reported to be conducted by various filamentous fungi (3, 13).

In our study, *Penicillium decumbens* was observed to efficiently transform 16 $\alpha$ , 17-epoxyprogesterone during the screening of fungal strains and two new hydroxylated steroid derivatives were obtained. Since *P. decumbens* already known to have activity in the reduction of double bonds and hydroxylation of steroids (14, 15). The result of our study also indicates that *P. decumbens* can be applied to the production of novel hydroxylated steroids.

## Experimental

All chemical reagents were of analytical grade from commercial suppliers. TLC was conducted on a silica gel plate (Merck GFZZ34, 0.25 mm). With acetate ester/petroleum ester (1: 1, V/V).

As solvent. Spots were detected through UV-light (254 nm). <sup>1</sup>H-NMR spectra were recorded on a JEOL JNM GSX 400M spectrometer in DMSO. <sup>13</sup>C-NMR spectra were measured at 100 MHz in DMSO. Mass spectra were performed on Waters 3100/2767 with electron impact ionization (EI) at 70 eV. X-ray crystallography were carried out with Mo K $\alpha$  radiation ( $k = 0.07$  nm) using a Bruker APEX CCD diffractometer at 293 K.

The CCDC numbers of the crystal of 2 and 3 were obtained after their crystallographic data was deposited with the Cambridge Crystallographic Data Centre. Further details of the crystallographic parameters can be

obtained for free on application to CCDC as supplementary publication, 12 Union Road, Cambridge CB2 1EZ, UK

### *Microorganism cultivation and substrate biotransformation*

*P. decumbens* TCCC 41604, stored in our laboratory, was cultured on potato-dextrose-agar medium in a test tube at 28 °C for 4-5 d. 8 mL of sterilized water was added subsequently and 1 mL of the suspension was inoculated into a 50 mL medium containing 20 g L<sup>-1</sup> glucose, 20 g L<sup>-1</sup> peptone and 10 g L<sup>-1</sup> yeast extract.

After 24 h. cultivation on a rotary shaker (180 min<sup>-1</sup>) at 28 °C, 50 mg of 16 $\alpha$ , 17 $\alpha$ -epoxyprogesterone (1) was added to the flask and the biotransformation was conducted for 24 h. under the same condition. The culture medium was analyzed by TLC.

### *Separation and purification of the products*

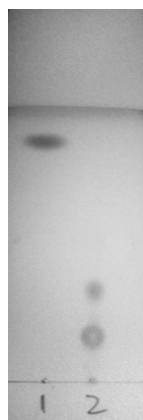
The products were extracted from the medium by equal volume of ethyl acetate for three times. After the evaporation of ethyl acetate in vacuum, white powder was obtained. After purification through silica gel column chromatography, two white products were obtained. Re-crystallization of products was performed and crystals were obtained, respectively

### *7 $\beta$ -hydroxy-16 $\alpha$ , 17 $\alpha$ -epoxyprogesterone (2)*

White crystal. MS: Calcd. for C<sub>21</sub>H<sub>28</sub>O<sub>4</sub>: m/z 345.35 [M]<sup>+</sup>. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  (ppm): 5.64 (s, 1H); 4.70 (d, 1H, *J* = 6.4 Hz); 3.90 (s, 1H); 3.15-3.13 (m, 1H); 2.45-2.38 (m, 3H); 2.35-2.12 (m, 2H); 2.01-1.89 (m, 5H); 1.63-1.54 (m, 4H); 1.53-1.42 (m, 1H); 1.41-1.25 (m, 1H); 1.22-1.00 (m, 7H); 0.89-0.88 (m, 1H). <sup>13</sup>C NMR (DMSO): 205.5 (C(20)); 198.5 (C(3)); 168.7 (C(5)); 124.2 (C(4)); 73.5 (C(17)); 70.1 (C(7)); 61.1 (C(16)); 50.4 (C(6)); 44.4 (C(10)); 39.8 (C(2)); 39.6 (C(13)); 39.4 (C(8)); 38.1 (C(9)); 35.3 (C(1)); 34.1 (C(12)); 31.4 (C(14)); 30.3 (C(15)); 26.2 (C(11)); 20.4 (C(19)); 17.2 (C(21)); 15.3 (C(18)).

### *7 $\beta$ , 11 $\alpha$ -dihydroxy-16 $\alpha$ , 17 $\alpha$ -epoxyprogesterone (3)*

White crystal. MS: Calcd. for C<sub>21</sub>H<sub>28</sub>O<sub>5</sub>: m/z 361.39 [M]<sup>+</sup>. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)



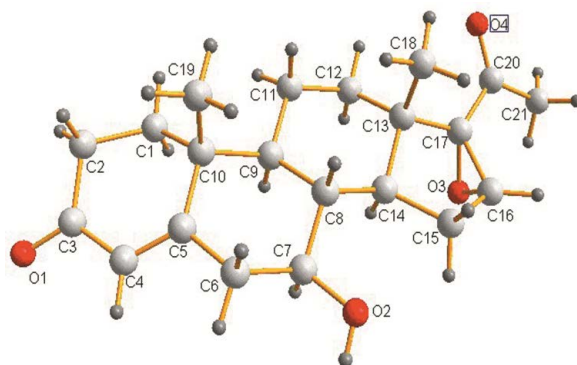
**Figure 1.** TLC analysis of transformation products by *P. decumbens*.

$\delta$  (ppm): 5.62 (s, 1H); 4.71 (d, 1H,  $J = 6.4$  Hz); 4.40 (d, 1H,  $J = 6.8$  Hz); 3.91 (s, 1H); 3.87-3.84 (m, 1H); 3.17-3.14 (m, 1H); 2.50-2.33 (m, 4H); 2.19-2.15 (m, 3H); 2.01-1.96 (m, 4H); 1.64-1.55 (m, 2H); 1.26-1.17 (m, 5H); 1.04-0.97 (m, 4H).  
 $^{13}\text{C}$  NMR (DMSO): 204.3 (C=O(20)); 198.0 (C(3)); 167.8 (C(5)); 123.5 (C(4)); 72.1 (C(17)); 68.8 (C(7)); 66.1 (C(11)); 60.3 (C(16)); 54.9 (C(9)); 42.5 (C(6)); 41.0 (C(2)); 39.1 (C(12)); 38.9 (C(10)); 38.7 (C(13)); 38.5 (C(8)); 35.8 (C(1)); 33.3 (C(14)); 29.2 (C(15)); 25.1 (C(19)); 17.5 (C(21)); 15.2 (C(18)).

## Results and Discussion

TLC was used to analyze the extracts from the culture of fungus. Figure 1 indicated the formation of two biotransformation products. Structures of the target products (2) and (3) were separated and confirmed by X-ray crystallography. The occurrence of hydroxylation at 7 $\beta$  and 7 $\beta$ , 11 $\alpha$  of steroid nucleus was shown in Figure 2 and Figure 3, respectively. Characterization of (2) and (3) was further studied by MS and NMR spectroscopy. Characteristic chemical shifts of (1) (2) and (3) were summarized in Table 1 and Table 2, respectively. Compared with substrate (1) the formation of the significant H signals of (2) at ppm values of 4.70 (d, 1H, 7-OH,  $J = 6.4$  Hz) and 3.15-3.13 (m, 1H, 7-CH) implied the 7 $\beta$ -hydroxylation of (1). In case of compound (3) the occurrence of new H signals at ppm values of 4.71 (d, 1H, 7-OH,  $J = 6.4$  Hz), 3.17-3.14 (m, 1H, 7-CH), 4.40 (d, 1H, 11-OH,  $J = 6.8$  Hz) and 3.87-3.84 (m, 1H, 11-CH) suggested the 7 $\beta$ , 11 $\alpha$ -dihydroxylation of (1). Moreover, other chemical shifts of protons and carbons in methylene groups, methine groups, methyl groups, C=CH units and aryl rings were in reasonable ranges, and the data reflected the crystal structure of (2) and (3). Further MS data was in good accordance with (2) and (3).

The biotransformation process of 16 $\alpha$ , 17 $\alpha$ -epoxyprogesterone (1) using *P. decumbens*



**Figure 2.** Crystal structure of 7 $\beta$ -hydroxy-16 $\alpha$ , 17 $\alpha$ -epoxyprogesterone (2).

Crystal data:  $\text{C}_{21}\text{H}_{28}\text{O}_4$ ,  $M_r = 344.43$ , crystal size 0.18  $\times$  0.17  $\times$  0.15 mm, orthorhombic, space group  $P2(1)2(1)2(1)$ ,  $a = 6.7034(7)$  Å,  $b = 12.6895(12)$  Å,  $c = 20.805(2)$  Å,  $\alpha = 90^\circ$ ,  $\beta = 90^\circ$ ,  $\gamma = 90^\circ$ ,  $V = 1769.8(3)$  Å $^3$ ,  $Z = 4$ ,  $D_{\text{calcd}} = 1.293$  mg/m $^3$ , absorption coefficient = 0.088 mm $^{-1}$ , measured temperature 173(2) K, reflection collected 9106, independent reflections 3092, final R indices  $R1 = 0.0392$ ,  $wR2 = 0.0865$ , R indices (all data)  $R1 = 0.0458$ ,  $wR2 = 0.0902$ ,  $\text{GOF} = 1.053$ . CCDC No: 967634.

**Table 1.** Characteristic chemical shifts in <sup>1</sup>H NMR spectra of steroids (1)(2)(3) No. d (ppm).

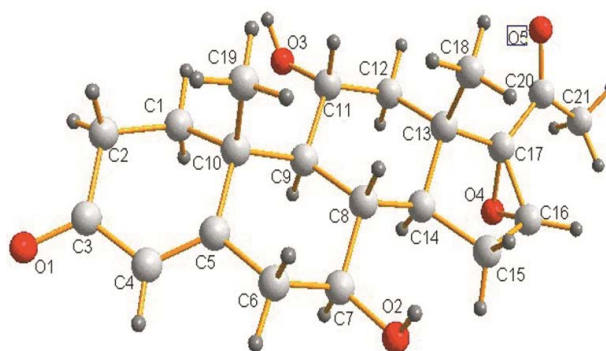
Substrate	$\delta$ (ppm)			
	H-C(7)	H-O(7)	H-C(11)	H-O(11)
1	1.29	/	1.40	/
2	3.15-3.13	4.70	1.40	/
3	3.17-3.14	4.71	3.87-3.84	4.40

**Table 2.** Characteristic chemical shifts in <sup>13</sup>C NMR spectra of steroids (1)(2)(3) No. d (ppm)

No.	$\delta$ (ppm)										
	C(1)	C(2)	C(3)	C(4)	C(5)	C(6)	C(7)	C(8)	C(9)	C(10)	C(11)
1	31.6	38.7	198.5	123.7	171.1	34.0	32.3	33.0	53.8	45.0	20.4
2	35.3	39.8	198.5	124.1	168.7	50.4	70.1	39.4	38.1	44.4	26.2
3	35.8	41.0	198.0	123.5	167.8	42.5	68.8	38.5	54.9	38.9	66.1

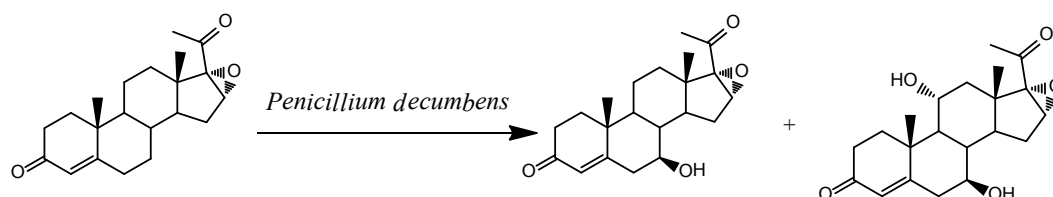
**Table 3.** Characteristic chemical shifts in <sup>13</sup>C NMR spectra of steroids (1)(2)(3) No. d (ppm)

No.	$\delta$ (ppm)									
	C(12)	C(13)	C(14)	C(15)	C(16)	C(17)	C(18)	C(19)	C(20)	C(21)
1	27.2	35.4	31.7	26.2	60.6	70.6	15.4	19.0	205.3	17.2
2	34.1	39.6	31.4	30.3	61.1	73.5	15.3	20.4	205.5	17.2
3	39.1	38.7	33.3	29.2	60.3	72.1	15.2	25.1	204.3	17.5



**Figure 3.** Crystal structure of 7 $\beta$ , 11 $\alpha$ -dihydroxy-16 $\alpha$ , 17 $\alpha$ -epoxyprogesterone (3).

Crystal data: C<sub>21</sub>H<sub>28</sub>O<sub>5</sub>, Mr=360.43, crystal size 0.18×0.17×0.16 mm, orthorhombic, space group P2(1)2(1)2(1), a=7.1849(4)Å, b=13.3360(7)Å, c=19.1506(10)Å,  $\alpha$ = 90°,  $\beta$ = 90°,  $\gamma$ = 90°, V= 1834.97(17)Å<sup>3</sup>, Z=4, D<sub>calcd</sub>=1.305 mg/m<sup>3</sup>, absorption coefficient=0.092 mm<sup>-1</sup>, measured temperature 173(2) K, reflection collected 9414, independent reflections 3207, final R indices R1 = 0.0338, wR2 = 0.0794, R indices (all data) R1 = 0.0371, wR2 = 0.0814, GOF = 1.053. CCDC No: 967633.



Scheme 1. Biotransformation of 16 $\alpha$ , 17 $\alpha$ -epoxyprogesterone by *P. decumbens*

in 24 h of incubation was also investigated. As shown in Table 3, products (2) was first detected in 2 hours. The yield of (2) increased and reached a plateau at around 44.5% after 12 h of incubation and then decreased to 22.8% after 18 h and continued going down. Product (3) was found after 6h incubation and its yield increased and finally reached around 79.7% after 24 h incubation. In control experiment, it was found that fermentation of compound (2) with *P. decumbens* also produced compound (3). This suggested during the biotransformation of (1), the first step is the conversion from (1) to (2) through 7 $\beta$  hydroxylation and the second is the conversion from (2) to (3) through 11 $\alpha$  hydroxylation (Scheme 1). *Penicillium* species have been reported to carry out different types of steroid transformations, mainly including hydroxylation, lactonization and hydrogenation [14-16]. In this study, *P. decumbens* was found to selectively hydroxylation of steroid substrate with a different type of catalysis. Although microbial hydroxylation of steroids has been studied over the decades, *P. decumbens* can be a novel microorganism to perform the selective C7 $\beta$  and C11 $\alpha$  hydroxylation of steroid.

### Conclusions

Two new steroidal compounds were prepared from 16 $\alpha$ , 17 $\alpha$ -epoxyprogesterone by *P. decumbens* for the first time. Their structures were characterized by <sup>1</sup>H NMR, <sup>13</sup>C NMR, X-ray crystallography and mass spectra. The biological activity of two new steroids will be studied in the near future.

### Acknowledgement

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