Applied Mechanics and Materials
ISSN: 1662-7482, Vol. 540, pp 347-351
doi:10.4028/www.scientific.net/AMM.540.347
Online: 2014-04-28

Research on Biochemical Materials with Hydroxylation of Androst-4-en-3,17-dione by *Colletotrichum lini* AS3. 4486

Jiajia Ren[1,a], Yanbing Shen[1,b], Rile Ge[1,c] and Min Wang[1,*]

[1] Key Laboratory of Industrial Fermentation Microbiology, Ministry of Education; College of Biotechnology, Tianjin University of Science and Technology, No. 29, 13th Avenue, TEDA, Tianjin, PR China

[a]jiabarhmily@163.com, [b]shenyb@tust.edu.cn, [c]gerileyy@163.com, [*]minw@tust.edu.cn

Keywords: Androst-4-en-3,17-dione; Biotransformation; *Colletotrichum lini*; Hydroxylation

Abstract. Microbial transformation of androst-4-en-3,17-dione (AD; **1**) using *Colletotrichum lini* AS3. 4486 resulted in the production of two metabolites **2** and **3**. The structures of these compounds were elucidated by spectroscopic analysis (LC-MS, FTIR and NMR) as 15α-hydroxyandrost-4-en-3,17-dione (15α-OH-AD; **2**) and 11α,15α-dihydroxyandrost-4-en-3,17-dione (11α,15α-diOH-AD; **3**). AD underwent regioselective hydroxylation at 15α position, subsequently hydroxylated at 11α position and converted to compound **3**. 11α,15α-diOH-AD as an important metabolic product was pharmaceutical intermediate and the yield was up to 97.58% when the concentration of substrate was 4 g L^{-1}.

Introduction

Steroids are biologically and pharmaceutically important compounds and are involved in many physiological activities. Over 300 approved steroid based drugs are known and are widely used as anti-inflammatory, anti-allergic, anti-diabetic and anti-convulsant agents [1]. Androst-4-en-3,17-dione (AD) is an important intermediate in the pharmaceutical industry [2,3]. The production of several high value steroid drugs are mostly derived from key compounds such as AD by chemical synthesis [4,5]. Microbial transformations of steroids provide an important method of obtaining synthetic intermediates and novel steroid derivatives of potential pharmaceutical activity due to its high chemo-, regio- and stereoselectivity, which additionally fulfills green chemistry principles and meets demands of “ white biotechnology ” [6-10]. It has already been shown that a large number of bacterial, fungal and algal species are capable of metabolizing steroids [1,11]. Among them, fungi have been shown to mediate hydroxylation, oxidation, and desaturation by the double bond formation and epoxidation of various steroid substances [11,12]. However, hydroxylation is the most important and common type, widely used in pharmaceutical industry. Hydroxylation at positions 7α and 15α of DHEA offered the production of pharmaceutical intermediates for the synthesis of drospirenone [13]. Our previous research showed that the strain of *Colletotrichum lini* AS3. 4486 contained active hydroxylase, converted progesterone to dihydroxylated derivatives [14]. In this study, the transformation of androst-4-en-3,17-dione (AD; **1**) by *C. lini* AS3. 4486 was investigated.

Experimental

Materials. Substrate AD was obtained from Tianjin Pharmaceutical Company. Kieselgel 60 HF254 was purchased from Merck (Darmstadt, Germany). Silica for column chromatography was performed on silica gel (200-300 mesh, Qingdao Marine Chemical Corporation, Qingdao, China). The microorganism *C. lini* AS3. 4486 used in this study was obtained from Institute of Microbiology, Chinese Academy of Sciences. All chemical solvents and salts used were of analytical grade or higher.

Instruments and methods. Melting points (m.p.) were determined on a Stuart melting point apparatus (SMP30, United Kingdom). Infrared (IR) spectra were recorded using KBr disks on a Bruker Tensor-27 FTIR spectrometer (Rheinstetten, Germany). The LC-MS spectra were obtained with Agilent Technologies 1260 and Agilent Technologies 6120 (USA) instruments by electrospray ionization (ESI). HPLC analysis was performed with a C18 column (4.6mm×250mm). Isocratic column elution was monitored by UV detector, the wavelength was 240 nm and the mobile phase was methanol–water (80:20, v/v) at a flow rate 1ml/min. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained using a Bruker Avance DPX-400 spectrometer (Rheinstetten, Germany) at 400 MHz, with tetramethylsilane (TMS) asinternal standard in $CDCl_3$ or CD_3OD.

Culture Media and Transformation. Stock cultures were maintained at 4 °C on agar slopes composed of potato infusion (20%), glucose (2%) and agar (2%). The strain was cultivated in shake flasks in two stages. Firstly, mycelium was grown on 50 mL seed medium (glucose 3%, corn steep liquor 1%, pH 7.0) for 48 h on a rotary shaker (210 r/min) at 25 °C. At the second stage, 10% (v/v) of the first mycelium obtained were added to 50ml fresh medium containing (%): glucose 3, corn steep liquor 1, soy meal 1, $NaNO_3$ 0.2, KH_2PO_4 0.1, K_2HPO_4 0.2, $MgSO_4·7H_2O$ 0.05, KCl 0.05, $FeSO_4·7H_2O$ 0.002 (pH 7.0) in 250ml flasks for an incubation (24 h, 210 r/min). Thereafter 200mg of the substrate dissolved in 1ml of methanol was added to every Erlenmeyer flasks (24-48 h, 210 r/min). The microbial transformations of substrate were monitored by TLC and HPLC.

Extraction, Separation and Purification of Metabolites. After the biotransformation, mycelium was separated from the fermentation broth by filtration and extracted three times with twice the volume of ethyl acetate, while the broth was extracted with the same solvent. The biomass rinsed in ultrasonic with ethyl acetate to ensure that all of the available products were isolated from the mycelium. All extracts were combined and washed with saturated aqueous NaCl, then dried with anhydrous Na_2SO_4. At last the solvent was evaporated under reduced pressure to give crude extract. For biotransformation system of *C. lini* AS3. 4486, the crude extract was subjected to open silica gel column (40 mm × 457 mm) and eluted with dichloromethane/ether/methanol gradiently (50:2:1, 25:2:1, 8:2:1, v/v/v). The white powder was diffused with petroleum ether/acetone at room temperature.

Result and discussion

Analysis of Biotransformation Product. As shown in Fig.1, *C. lini* AS3. 4486 could transform AD into two kinds of products. The compound **2** (R_f = 0.71) and compound **3** (R_f = 0.54) were showed identical with the transformation of 12 h (lane 1). Then the compound **2** decreased and generated a large proportion of compound **3** with the transformation of 48 h (lane 3). It was also demonstrated that the compound **2** was transformed into compound **3** by *C. lini* AS3. 4486 when use the product **2** as substrate (lane 5). The bioconversion medium without substrate was showed in lane 6. The yields (%) of metabolites were analysed by HPLC and the yield of compound **3** was up to 97.58% when AD concentration was 4 g/L (Tables 1).

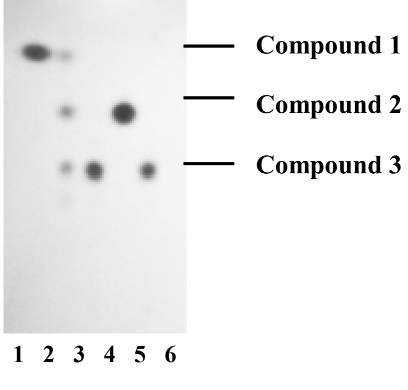

Fig.1 TLC of androst-4-en-3,17-dione (AD) transformation. lane1: Standard AD; lane 2: the bioconversion medium of 12h with the addition of 0.2% AD; lane 3: the bioconversion medium of 48 h with the addition of 0.2% AD; Lane 4: Standard 15α-hydroxyandrost-4-en-3,17-dione (15α-OH-AD); lane 5: the bioconversion medium of 48h with the addition of 0.2% 15α-OH-AD; lane 6: the bioconversion medium without substrate.

Table 1. Transformation of AD by *C. lini* AS3. 4486

Reaction time	HPLC content (%)	
	Compound **2**	Compound **3**
12	38.09	35.54
48	0	97.58

Identification of products. Bioconversion products were purified by column chromatography and recrystallization, Two metabolites **2** and **3** were isolated and purified from fermentation broth containing *C. lini* AS3. 4486 (Fig. 2). No transformation occurred in the control media. Structure elucidation was accomplished by FTIR, ESI-MS and NMR techniques.

Compound **2** a white power; m.p. 203.4-204.5 ºC; R_f(dichloromethane/ether/methanol, 385:60:40 v/v/v): 0.71; ESI-MS m/z $[M+H]^+$ 303.4; IR νmax 3375.03, 1746.29, 1631.99, 1605.77 cm^{-1}; ^1H NMR ($CDCl_3$, 400 Hz): δ 0.96 (3H, s, H-18), 1.23 (3H, s, H-19), 4.42 (1H, q, J = 8 Hz, H-15β), 5.75 (1H, s, H-4). For ^{13}C-NMR data, see table 2.

Compound **3** a white power; m.p. 204.9-206.0 ºC; R_f(dichloromethane/ ether/methanol, 385:60:40 v/v/v) : 0.54; ESI-MS m/z $[M+H]^+$ 319.4; IR νmax: 3448.99, 3432.99, 1745.07, 1649.27, 1610.48 cm^{-1}; ^1H NMR (CD_3OD, 400 Hz): δ 1.01 (3H, s, H-18), 1.41 (3H, s, H-19), 4.01 (1H, td, J = 12, 4 Hz, H-11β), 4.35 (1H, q, J = 8 Hz, H-15β), 5.75 (1H, s, H-4). For ^{13}C-NMR data, see table 2.

The ESI-MS spectrum of compound **2** showed the $[M+H]^+$ at m/z 303.4, indicative of the addition of 16 mass units to **1** in agreement with the formula $C_{19}H_{26}O_3$. The FTIR spectrum of compound **2** showed characteristic absorptions at 3375.03, 1746.29, 1631.99, 1605.77 cm^{-1} for hydroxyl and saturated ketone and conjugated ketone groups with double bonds, respectively. The ^1H-NMR spectrum of compound **2** showed a complex profile and the only signals easily assigned were those of H-4 (δ 5.75) and H-15 (δ 4.42). The singlets at δ0.96 and δ1.23 were readily assigned to the methyl groups at C-18 and C-19. Definitive confirmation of the structure of compound **2** was given by comparison with NMR data previously reported for 15α-hydroxyandrost-4-en-3,17-dione [15].
The ESI-MS spectrum of compound **3** had the $[M+H]^+$ ion peak at m/z 319.4, which indicated the

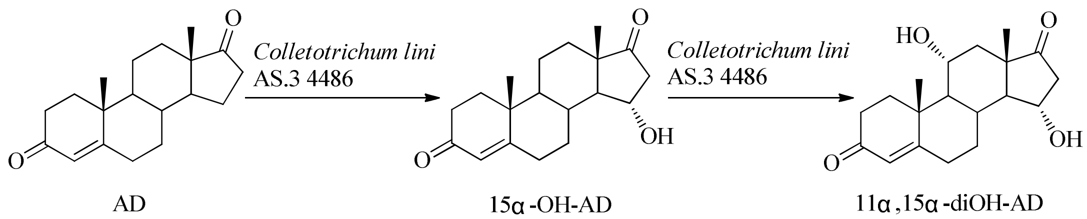

Fig. 2 Proposed biotransformation pathway of ADD by *C. lini* AS3. 4486.

Table 2 ^{13}C-NMR assignments of biotransformation products (**2** and **3**) by *C. lini* AS3. 4486

Carbon	Chemical shifts [δ (ppm)]		Carbon	Chemical shifts [δ (ppm)]	
	2	**3**		**2**	**3**
1	35.68	38.54	11	20.22	68.94
2	33.88	34.93	12	31.90	41.51
3	199.67	202.94	13	50.47	48.96
4	123.83	124.55	14	57.29	60.36
5	170.85	175.32	15	70.18	70.11
6	32.68	34,72	16	46.29	48.62
7	31.28	34.94	17	216.31	217.54
8	35.42	36.25	18	15.32	16.49
9	53.73	57.65	19	17.49	18.91
10	38.69	47.19			

addition of 32 mass units to **1** in agreement with the formula $C_{19}H_{26}O_4$. The IR spectrum of compound **3** confirmed that the characteristic absorption for the hydroxyl groups had appeared. According to ^{1}H NMR spectrum, the natures of hydroxyl groups were secondary confirmed by the presence of two signals at δ 4.01 ppm (1H, td, J = 12, 4 Hz, H-11) and 4.35 ppm (1H, q, J = 8 Hz, H-15). The major difference of ^{13}C-NMR spectra between compound **3** and compound **2** was the presence of the carbon signal change, C-11 shifted from δ20.22 downfield to δ 68.94. These data are consistent with compound **3** being a 11α-hydroxylation product of compound **2**.

Biotransformation of androstenedione to other steroid medicines or intermediates has been widely studied[16,17]. The present work showed that fermentation of AD with *Curvularia lunata* for 10 days obtained five oxidation and reduction products [12]. *Curvularia lunata* VKM F-644 is able to convert AD into C-6β, C-7α and C-14α hydroxylated derivatives [18]. It was also demonstrated that *Mucor* 881 can introduce hydroxy to AD at C-6β and C-11α position [1]. All the products transformed from AD can be used as steroid medicines or their intermediates [19]. In this study，the fungus *C. lini* AS3. 4486 was used to investigate the microbial transformations of AD. The strain can stereoselectively hydroxylate AD at C-11α and C-15α. AD underwent regioselective hydroxylation at 15α position, subsequently hydroxylated at 11α position and the product of 11α,15α-diOH-AD (**3**) can be obtained in high yield.

Acknowledgements

This work was supported by the National High Technology Research and Development of China (2011AA02A211), the National Natural Science Foundation of China (21276196) and Tianjin High School Science & Technology Fund Planning Project (20120629).

References

[1] S.P. Kolet, S. Haldar and H.V. Thulasiram, in: Steroids, Vol. 78 (2013), p. 1152-1158.

[2] V.V. Kollerov, A.A. Shutov, V.V. Fokina, G.V. Sukhodolskaya and M.V. Donova, in: J. Ind. Microbiol. Biotechnol, Vol. 148 (2006), p. 719-723.

[3] C. Perez, A. Falero, and B.R. Hung, in: J. Mol. Catal. B Enzym, Vol. 55 (2008), p. 61-68.

[4] S. Ahmad, S.K. Garg and B.N. Johri, in: Biotechnol. Adv, Vol. 10 (1992), p. 1-67.

[5] A. Malaviya and J. Gomes, in: Bioresource Technol, Vol. 99 (2008), p. 6725-6737.

[6] H.L. Holland, in: Steroids Vol. 64 (1999), p. 178-186.

[7] S. Hu, G. Genain and R. Azerad, in: Steroids, Vol. 60 (1995), p. 337-352.

[8] S.B. Mahato and S. Garai, in: Steroids, Vol. 62 (1997), p. 332-345.

[9] B. Mahato and I. Mazumder, in: Phytochemistry, Vol. 34 (1989), p. 883-898.

[10] T. Janeczko, A. Świzdor, J. Dmochowska-Gładysz, A. Białońska, Z. Ciunik and E. Kostrzewa-Susłow, in: J Mol Catal B: Enzym, Vol. 82 (2012), p. 24-31.

[11] M.A. Faramarzi, M. Tabatabaei Yazdi, H. Jahandar, M. Amini and H.R. Monsef-Esfahani, in: J Chem Technol Biotechnol, Vol. 84 (2009), p. 1021-1025.

[12] M.I. Choudhary, S. Sultan, A. Yasin and S. Farzana, in: Nat Prod Res, Vol. 18 (2004), p. 529-535.

[13] A. Romano, D. Romano, E. Ragg, F. Costantino, R. Lenna, R. Gandolfi and F.S. Molinari, in: Steroids, Vol. 71 (2006), p. 429-434.

[14] Y.J. Shen, H. Sun, Y.W. Fu and M. Wang, in: Adv. Mater. Res, Vol. 343 (2012), p. 70-73.

[15] L.H. Huang, J. Li, G. Xu and H.M. Liu, in: Steroids, Vol. 75 (2010), p. 1039-1046.

[16] Z.G. Xiong, Q. Wei, H.M. Chen, W. J. Xu and X.M. Hu, in: Steroids, Vol. 71 (2006), p. 979-983.

[17] M. Koshimura, A. Hara and M. Kuniyoshi, in: J Mol Catal B: Enzym, Vol. 67 (2010), p. 72-77.

[18] V.V. Kollerov, A.A. Shutov, V.V. Fokina, S.A. Gulevskaya and M.V. Donova, in: Appl. Biochem. Micro, Vol. 46 (2010), p. 198-205.

[19] Y.L. Lin, X. Song J. Fu, J.Q. Lin and Y.B. Qu, in: J. Chem. Technol. Biotechnol., Vol. 84 (2009), p. 789-793.