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Synthesis of steroidal lactone by *penicillium citreo-viride*

Hong-Min Liu[a,*], *Heping Li*[a], *Lihong Shan*[a], *Jian Wu*[b]

[a] *New Drug Research & Development Center, Zhengzhou University, Zhengzhou 450052, China*
[b] *Bioengineering Department Zhengzhou University, Zhengzhou 450052, China*

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ABSTRACT

The biotransformations of a series of steroids by the fungus *penicillium citreo-viride* A.C.C.C. 0402 have been investigated, and the conversion to the same product testolactone (1) was observed from progesterone (2), dehydroepiandrosterone (3), 4-androstene-3, 17-dione (4), 5-androstene-3, 17-diol (5) with the exception of pregnenolone (6) and 3β-hydroxy-5, 16-pregnadien-20-one (7). The possible metabolic pathways of the biotransformations were also discussed in the paper and the fungus *penicillium citreo-viride* A.C.C.C. 0402 was isolated during screening stains from samples collected from Zhengzhou, Henan province of China.

1. Introduction

Steroidal lactones possess useful biological activities such as anticancer, antibacterial, anticareinogen and antiandrogenic activites [1–4], and 16-oxa analogues of estrane series express antihypercholestrolemic activities [5]. Plants of genus *Withania* are known to exhibit a variety of pharmacological activites mainly due to the presence of withanolides, which are steroidal lactones. Testolactones are moderately specific first generation inhibitors of human aromatase activity and thereby may contribute to the prevention of hormone dependent tumors such as breast cancer [6], prostatic hyperplasia and prostate cancer [7]. Testolactones are used as therapeutic agents in disorders caused by imbalance between estrogen and androgen action, e.g. gynecomastia [8] or precocious puberty [9]. Aromatase inhibitors are also essential tools for studying the role of estrogens in adults, or during development [10].

Because of the important bioactivities of the testolactones, scientists were prompted to synthesize these steroidal lactones. Although chemical ways of synthesis of steroidal lactones such as Baeyer-Villiger oxidation are possible, the biotransformation is thought to be the more environmental method, especially nowadays, green chemistry is advocated so frequently. In fact a variety of fungi [11–17] have been identified which can stereospecifically degrade the 17β-acetyl side chain of progesterone **2** in high yield to give the ring D testololactone **1** and the metabolic pathway was also discussed [18]. Recently testosterone (**8**) was transformated into **1** by *Penicillium notatum* has been reported [19]. With the aim of synthesizing steroidal lactones and doing some further work, we have endeavored to screen microorganisms capable of producing steroidal lactones, and a common strain of *penicillium citreo-virid* A.C.C.C. 0402 which could transform **2–5** into **1** was isolated during screening stains from samples collected from Zhengzhou, Henan province, while **6** and **7** have not any

* *Corresponding author*. Tel.: +86 371 67767200; fax: +86 371 67767200.
E-mail addresses: liuhm@public2.zz.ha.cn, liuhm@zzu.edu.cn (H.-M. Liu).
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changes. *Penicillium* genus have been reported to be used in the biotransformations using steroids as the substrates, but the attention was focused mainly on 15α-hydroxylation catalyzed by *Penicillium raistrickii* [20,21] and 5α-reductase of *Penicillium decumbens* [22,23] and *Penicillium crustosum* [24]. Here we first reported on the microbial transformation using the strain of *penicillium citreo-viride*.

2. Experimental

2.1. Instrumental methods

Sterilization was carried out in an HVE-50 Hirayama autoclave. Aseptic operation was carried out in ClassIIA/B3 Biological safety cabinet from Forma Scientific. Incubation was carried out on an HZQ-Q orbital shaker. Melting points (mp) were determined on a XT5 melting point apparatus, which was uncorrected. Infrared (IR) spectra was recorded using KBr discs on a Bruker Vectror-22 spectrometer. Mass spectra (MS) was obtained on an Esquire3000 mass spectrometer by electrospray ionization (ESI). The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained using a Bruker Avance DPX-400 spectrometer at 400 and 100 MHz, respectively, with tetramethylsilane (TMS) as internal standard in DMSO-d_6. Chemical shifts (δ) were given in parts per million (ppm) relative to TMS. Coupling constants (J) were given in hertz (Hz). Chromatography was performed with ether acetate/chloroform (1:1) and visualized by spraying the plates with 50% sulfuric acid solution and heating in an oven at 100 °C until the colors developed. Thin layer chromatography (TLC) was performed on 0.25 mm thick layer of silica gel G (Qingdao Marine Chemical Factory, China).

2.2. Microorganism

The strain of *penicillium citreo-viride* A.C.C.C. 0402 was isolated from the samples collected from the suburb of Zhengzhou, Henan province, PRC, and identified by Bioengineering Department Zhengzhou University. Stock cultures were maintained at 4 °C on agar slopes composed of peptone (1.2%), dextrose (3.0%), yeast extract (0.1%), KH_2PO_4 (0.13%), agar (2.0%) (pH 4.5).

2.3. Conditions of cultivation and transformation

Spores freshly obtained from peptone dextrose agar slopes were transferred aseptically into 10, 500 ml Erlenmeyer flasks containing 100 ml of sterile peptone dextrose broth in a biological safety cabined and were incubated for 48 h at 27.5 °C. The cultures were shaken at 210 rpm on an orbital shaker. Then 1.0 g substrate dissolved in 20 ml acetone was evenly distributed among the flasks under sterile conditions and incubated for a further 5 days at the same conditions after which the metabolites were extracted from the broth.

2.4. Extraction and separation of metabolites

The mycelium was separated from the broth by filtration under vacuum, and rinsed in ultrasonic with ethyl acetate (100 ml × 5) to ensure that all of the available products were isolated from the mycelium. The mycelial broth was then extracted for five times with ethyl acetate. The organic extract altogether was concentrated into about 300 ml, which was then washed with saturated aqueous $NaHCO_3$, brine and water, respectively, for three times, then dried with sodium sulfate and the solvent was evaporated under reduced pressure to give the mixed products. The mixture was separated on silica gel column chromatography by using ether acetate/chloroform (1:1) as eluant, and the solvent was collected in aliquots (10 ml) and analysed by thin layer chromatography to identify the separated metabolite fractions. The solvent systems used for running the TLC plates was also ether acetate/chloroform (1:1), and the TLC was visualized by spraying the plates with 50% sulfuric acid solution and heating in an oven at 100 for 3 min until the colors developed.

Table 1 – The biotransformations of the substrates of 2–7

Substrates	Yield of **1** (%)	Yield of **4** (%)
2	60.9	8.4
3	65.4	<0.5
4	73.6	–
5	32.5	45.2
6	–	–
7	–	–

(–) Means that no product was detected on TLC.

2.5. Incubation of 2–7

The biotransformations of the substrates of **2–7** were, respectively, shown in Table 1 according to Sections 2.3 and 2.4, and the results were shown in Fig. 1. All of the substrates of **2**, **3**, **4** and **5** could be converted into testolactone **1**, mp 201.2–202.1 °C; IR(KBr): 3420.1, 2954.6, 1719.8, 1667.2 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 5.76 (s, H4), 1.18 (s, H18), 1.36 (s, H19) ppm; ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 38.9 (C1), 32.3(C2), 199.1(C3), 171.1(C4), 124.1(C5), 35.5 (C6), 33.8(C7), 37.9 (C8), 52.5 (C9), 38.4 (C10), 19.8 (C11), 30.4 (C12), 82.7 (C13), 45.7 (C14), 21.8 (C15), 28.5 (C16), 169.3 (C17); 17.4 (C18), 20.0 (C19); HRMS(ESI) m/z: $C_{19}H_{26}O_3$[M + H^+], calcd. 303.1960, found 303.1957, [M + Na^+], calcd. 325.1780, found 325.1771, [M + K^+] calcd. 341.1579, found 343.1827.

Compound 4, mp 172.6–173.5 °C; IR(KBr): 2952.1, 2919.2, 1735.3, 1664.3, 1616.3, 1378.2 cm-1; 1H NMR (CDCl3, 400 MHz) δ: 5.75 (s, H4), 0.92 (s, H18), 1.22 (s, H19) ppm; 13C NMR (CDCl3, 400 MHz) δ: 35.5 (C1), 33.8 (C2), 199.1 (C3), 124.0 (C4), 170.2 (C5), 32.4 (C6), 31.2 (C7), 35.0 (C8), 53.7 (C9), 38.5 (C10), 20.2 (C11), 35.6 (C12), 47.4 (C13), 50.7 (C14), 21.6 (C15), 31.7 (C16), 220.2 (C17); 13.6 (C18), 17.6 (C19); HRMS(ESI) m/z: C19H26O2[M + H +], calcd. 287.2006, found 287.2018.

3. Results and discussion

A series of different structural steroids were found to yield the same product **1** in the biotransformation by *penicillium citreo-virid* which has not been reported, so we have investigated the scope of the use of *penicillium citreo-virid* for steroid transformation and discussed the metabolic pathways.