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Microbial hydroxylation of 16α,17α-dimethyl-17β-(l-oxopropyl) androsta-l,4-dien-3-one to rimexolone by *Curvularia lunata* AS 3.4381

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Abstract

A novel synthetic route to rimexolone, a corticosteroid for treatment of ocular inflammation without significant elevation of intraocular pressure, was described. An investigation has been undertaken of the microbial transformation of 16α,17α-dimethyl-17β-(l-oxopropyl)androsta-l,4-dien-3-one by microorganisms known to hydroxylate conventional steroids, using *Curvularia lunata* AS 3.4381 gave rimexolone, the product of 11β-hydroxylation, respectively. The target compound was characterized with reference substance rimexolone by TLC, HPLC, elemental analysis, MS, IR, and NMR.

Keywords: Steroid 11β-hydroxylation; Biotransformation; *Curvularia lunata* AS 3.4381; Rimexolone

1. Introduction

Rimexolone (**1**, Scheme 1), a corticosteroid first prepared by Organon [1], was introduced in 1995 by Alcon as Vexol® which is a sterile, multi-dose topical ophthalmic suspension for treatment of anterior uveitis and post-operative ocular inflammation. Rimexolone represents an important therapeutic advance because its long-term administration does not significantly elevate intraocular pressure, a side effect of traditional corticosteroids possibly causing the risk of glaucoma [2–5], such as dexamethasone and prednisolone.

It should be the most important steps to form 16α-, 17α and 21-trimethyl in ring D of steroid in the synthetic route of **1**. In previously published literatures which utilized different starting materials and synthetic routes, satisfactory results, especially the balance between synthesis efficiency and economy were hardly achieved. A recently report of Raymond's synthesis from prednisolone [6], acquired about 20% yield with seven steps, but the application of some special regents such as $(CH_3)_2Cu(CN)Li_2$, LHMDS and benzyl-trimethylammonium fluoride limited its production scale.

The hydroxylation of steroids by fungal biocatalysts has been known for many years [7,8], This procedure remains one of the most useful preparative methods for the introduction of hydroxyl groups at sites of the steroid nucleus remote from other functionality, and the value of microbial steroid hydroxylation in the preparation of pharmacologically active steroids is well established.

In our recently study, as described in Scheme 1, androsta-1,4-dien-3,20-dione (ADD, **2**) was propenylated by potassium methyl acetylide, and then the product **3** was dehydrated and hydrolyzed to afford the 16-en-20-one steroid 17-(1-oxopropyl)androsta-1,4,16-trien-3-one (**5**). Compound **5** was treated by methyl Grignard reagent with catalytic amount dry copper(I) chloride followed by reaction of the resulting 17(20)-enolate with methyl iodide to 16α,17α-dimethylated compound 16α,17α-dimethyl-17β-(1-oxopropyl)androsta-1,4-dien-3-one (**6**), which was just the substrate of the biotransformation. A 20% yield was gained in all above-mentioned chemical procedures.

The biotransformation of 16α,17α-dimethyl-17-(1-oxopropyl)androsta-1,4-dien-3-one into 11β-hydroxylated product, rimexolone, has been done by *Curvularia lunata* AS 3.4381, which is a strain of mycelial fungus and performs the 11β-hydroxylation with cytochrome P-450 enzymes systems. The growth of the mycelium and the incubation conditions such as

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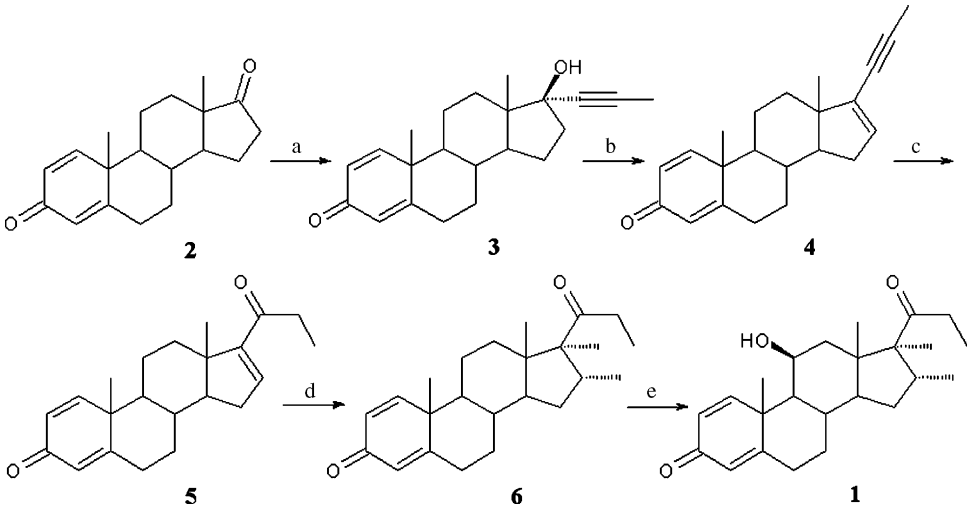

Scheme 1. Reagents and conditions: (a) $CH_3C{\equiv}CK$, tolene, *t*-BuOH, (b) $POC1_3$, (c) H_2SO_4, Hg^{2+}, (d) CH_3MgCl, Cu(I), THF, −20 °C, then CH_3I, r.t. and (e) biotransformation with *Curvularia lunata* AS 3.4381.

pH, temperature, the steroid addition mode and procedure time were studied and experimental results indicated that the optimized two-stage fermentation procedure for 11β-hydroxylation was available.

2. Experimental

2.1. Apparatus, materials, and methods

The reagents were applied as received by market. Temperatures were external unless otherwise indicated. Melting points were uncorrected. NMR spectra for solution in $CDCl_3$ were determined at 400 MHz with a Bruker AC-E200 spectrometer (tetramethysilane as internal standard), and coupling constants (*J*) are reported in Hz. Mass spectra were determined with a Bruker Bio TOF IIIQ high resolution spectrometer. HPLC analysis was performed on Shimadzu 10AVP Series chromatograph equipped with UV–vis detector (in 244 nm), a ODS column (250 mm × 0.46 mm) and methanol–water (70:30) as mobile phase (flow rate = 1.00 ml/min). TLC was carried out by using silica gel GF_{254}, and column chromatography was carried out with silica gel H.

2.2. Maintenance and growth of microorganisms

C. lunata AS 3.4381 was obtained by China General Microbiological Culture Collection Center (CGMCC), and isolated and screened for its high ability of 11β-hydroxylation. All microorganisms were maintained on agar slopes and freshly subculture in liquid medium (g/l: soybean powder 5, yeast extract powder 5, glucose 20, sodium chloride 5, potassium phosphate dibasic 5, adjust pH 6.0–6.5) before using in biotransformation experiments.

2.3. Biotransformation procedures

Microorganisms on sabouraud agar slants were cultured in liquid medium in a 100 ml Erlenmayer flask for 72 h at 28 °C, and 10% of cultures were transferred to fresh liquid medium and incubated for the next 24 h. The obtained homogenous precultures of the mycelium was isolated by filter and introduced again to the phosphate buffer (0.05 mol/l, pH 6.5), and then the steroid substrate 16α,17α-dimethyl-17β-(1-oxopropyl) androsta-1,4-dien-3-one (**6**, 0.5 g) dissolved in 10% calcium chloride methanol solution (0.5 g/1) were added. The flasks were treated by ultrasonic for several minutes and incubated on shake cultivation at a 220 rpm speed in the same conditions. Samples were taken at intervals, filtered, and the filtrate analyzed by HPLC and TLC to follow the course of the biotransformation. The biotransformation was terminated after 48 h.

The mycelium was filtered and the filtrate was extracted for three times with ethyl acetate. After dried at 80 °C and comminuted, the mycelium was suspended in ethyl acetate and desintegrated with sonicator. The HPLC analysis result of the extracts indicated that the biotransformation efficiency was about 43%. All organic extracts were dried over anhydrous sodium sulfate and the solvents were evaporated under reduced pressure at 40 °C. The residue was purified by chromatography (20% ethyl acetate-cyclohexane) giving 57 mg of 16α,17α-dimethyl-17β-(1-oxopropyl)androsta-1,4-dien-3-one (**7**) as recovered and 182 mg of lip-hydroxy-16α,17α-dimethyl-17β-(1-oxopropyl) androsta-1,4-dien-3-one (**1**) (34.8%).

3. Results and discussion

3.1. Structural identification

The substrate of biotransformation was a white solid with mp 163–165 °C (Ref. [9]: 164–165 °C); ^{1}H NMR ($CDC1_3$) *δ*: 0.73 (s, 3H, 19-H), 0.86 (d, *J* = 7.2 Hz, 3H, 16α-Me), 1.00 (s, 3H, 17α-Me), 1.02 (*t*, *J* = 7.2 Hz, 3H, 21-CH_3), 1.22 (s, 3H, 18-H), 1.0–2.9 (m, 15H), 3.04 (m, 1H, 16β-H), 6.06 (s, 1H, 4-H), 6.22 (dd, *J* = 2.0 and 10.4 Hz, 1H, 2-H), 7.04 (d, *J* = 10.4 Hz, 1H, 1-H); ^{13}C NMR ($CDC1_3$) *δ*: 5.058, 14.098, 15.954, 16.990, 18.555, 22.426, 32.519, 32.690, 32.866, 32.934, 33.008, 33.441, 35.524, 43.374, 46.147, 48.930,

Table 1
Rate of flow comparison of the substrate (Sub.), reference substance (RS) and product (Pro.) by three different types of solvent system on TLC

Developing solvent	Sub.	RS	Pro.
Dichlorom ethane–acetone (4:1)	0.855	0.709	0.709
Petroleum ether–acetone (7:3)	0.582	0.473	0.473
Cyclohexane–ethyl acetate (7:3)	0.446	0.268	0.268

51.993, 63.150, 123.730, 127.413, 155.403, 168.752, 186.023, 214.076.

The product of biotransformation was a white solid with mp 264–268 °C (Ref. [10]: 258–268 °C); UV λ_{max} 244 nm (in methanol) (Ref. [10]: 244 nm); $[\alpha]_D$ +98.6° (c = 0.50 in py) (Ref. [10]: +100°, c = 0.92 in py); FT-IR ν_{max} 3395.01 (11-OH), 1697.94 (20-ketone), 1657.36 (3-ketone) and 1612.11 cm^{-1} (4-C=C), 1596.12 (1-C=C); MS 371.6 [M + H]), 393.6 ([M + Na]) (base peak), 409.6 ([M + K]); ^{1}H NMR ($CDC1_3$) δ: 0.91 (d, J = 7.2 Hz, 3H, 16α-Me), 0.95 (s, 3H, 19-H), 1.04 (*t*, J = 7.2 Hz, 3H, 21-CH_3), 1.45 (s, 3H, 18-H), 2.36 (q, 3H, 21-H), 1.1–2.7 (m, 14H), 3.05 (m, 1H, 16β-H), 4.38 (m, 1H, 11α-H), 6.14 (s, 1H, 4-H), 6.38 (dd, J = 1.6 and 10.2 Hz, 1H, 2-H), 7.37 (d, J = 10.2 Hz, 1H, 1-H); Anal. Calcd. for $C_{24}H_{34}O_3$: C, 77.80; H, 9.25. Found: C, 77.81; H, 9.23.

An authentic reference substance of rimexolone was synthesized as Raymond reported [6]. The product had the same retention values with the reference substance of TLC run by three different types of developing systems in Table 1. Infrared spectrograms showed that there was no difference between the product and reference substance not only in characteristic absorption bands such as 11β-hydroxyl, 20-carbonyl and 1,4-dien-3-one structure, but in fingerprint region.

Referred to the literatures [6,10,11] and according to the consequences of the structural identifications, the product had a structure of 11β-hydroxy-16α,17α-dimethyl-17β-(1-oxopropyl) androsta-1,4-dien-3-one, thus it could be inferred the product was rimexolone.

3.2. Biotransformation conditions

Because of the variable metabolic activity of *C. lunata* during its growth, its growth characteristic was firstly studied for the highest efficiency of 11β-hydroxylation (Fig. 1). With a starting condition of pH 6.5, the mycelium was filtered each 6 h, dried at

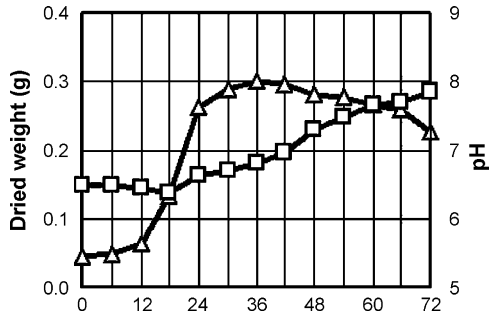

Fig. 1. Time course of pH value of the incubation medium (□) and net mass (g) of the mycelium (△).

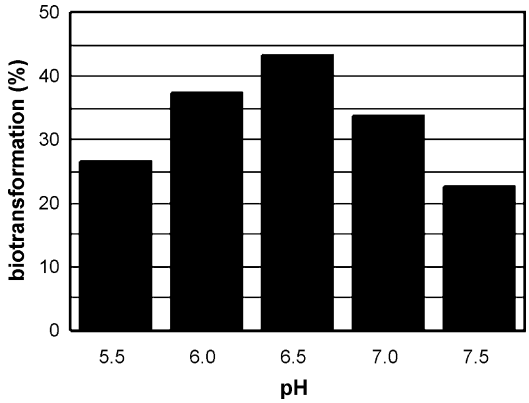

Fig. 2. pH value course of biotransformation efficiency (%).

80 °C under reduced pressure overnight and its weight (g) was measured. After an exponential growth phase, the growth of *C. lunata* AS 3.4381 reached the upper limit and got in a plateau from 30 to 44 h, and then the mycelium began to wither.

Other incubation conditions being fixed, phosphate buffers (0.05 M) with pH 5.5, 6.0, 6.5, 7.0, 7.5 were experimented (Fig. 2). HPLC analysis was performed after 48 h of biotransformation and the consequences showed that the highest conversion was obtained when the phosphate buffer with pH 6.5 was employed. As Fig. 2 indicated, the pH value of the medium had a slow ascensus with the microorganisms' growth, a pH 6.5 phosphate buffer (0.05 M) could keep the cultivation environment stable.

The cultivation media were kept in different temperatures (26, 28, 30 and 32 °C), and the corresponding incubation production was analyzed respectively by HPLC. The analysis consequences shown as in Fig. 3 that 30 °C was the best temperature and 11β-hydroxylase would be inactivated at either higher or lower temperature.

Steroid substrate dissolved in different dissolvent (buffer with β-cyclodextrin, methanol, ethanol and acetone) was added to the medium with a concentration of 0.5 g/1 and the flasks were treated or not treated by ultrasonic parallelly and the incubation production was analyzed by HPLC after 48 h. Contrasting with other substrate addition modes, the process of methanol dissolvent and application ultrasonic was better possibly because it would disperse steroid better in the cultivation medium and have less toxicity to the microorganism and enzyme (Fig. 4).

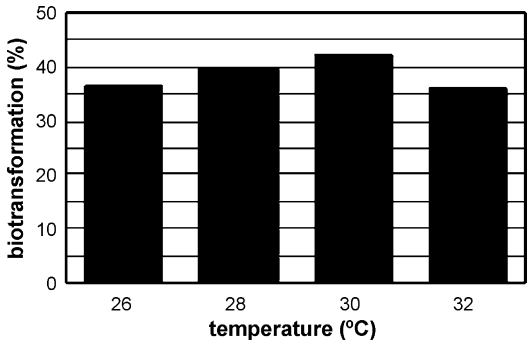

Fig. 3. Temperature (°C) course of biotransformation efficiency (%).

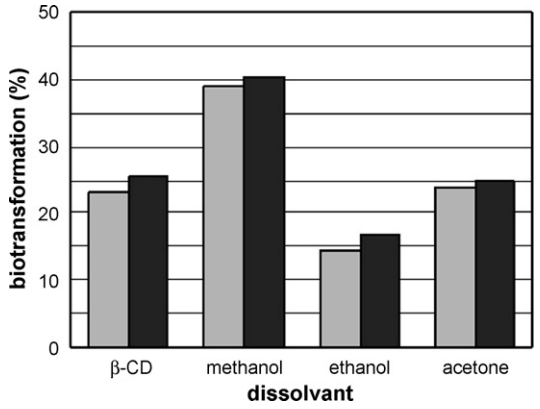

Fig. 4. Steroid addition mode course of biotransformation efficiency (%) and the flasks were not treated ( ) or treated (■) by ultrasonic parallelly.

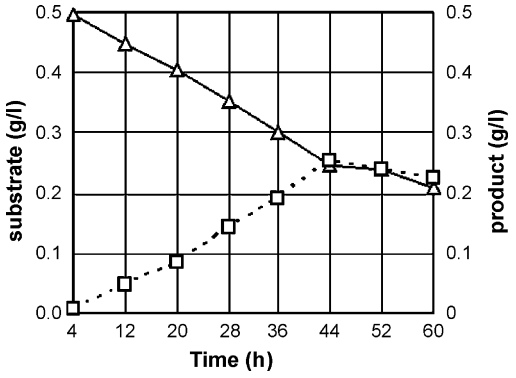

Fig. 5. Time course of concentration (g/l) of the substrate (△) and product (□).

With the above-determined starting conditions, the incubation was analyzed each 8 h by HPLC. As is indicated in Fig. 1, the concentration of the product increased stably at the exponential growth phase of the mycelium and reached the peak at about the 44th h (Fig. 5).

However, if the procedure went on, the concentration of the substrate and product had little change and the by-products increased dramatically (Fig. 5), and therefore the biotransformation should be terminated at the 48th h.

Mycelial fungus *C. lunata* was generally applied in steroids 11β-hydroxylation, and a 55–60% yield was obtained with *C. lunata* in hydrocortisone industrial production. For the steric hindrance of steroids C_{10}β- and C_{13}β-methyl groups,steroids 11β-hydroxylation was more difficult than C_{11}α-hydroxylation, and in addition, there was always side reactions concomitantly which principally included C_7α-, C_{11}α- and C_{14}α-hydroxylation, therefore, the yield of 11β-hydroxylation was lower than the C_{11}α-hydroxylation's, and more by-products would be produced including corresponding none-C_{14}α-hydroxyl steroids. In our study, compared to the microbial 11β-hydroxylation to hydrocortisone with *C. lunata*, there were few by-products in the biotransformation of 11-dehydroxy rimexolone (**7**).This possibly because the steric hindrance of the 16α,17α-dimethyl groups interfered the C_{14}α-hydroxylation. As the consequence in Fig. 5, the biotransformation efficiency was close to 50%, but limited by the reaction scale, the obtained pure rimexolone was only about one-third of the substrate, furthermore, about 20% substrate was unreacted.

4. Conclusion

The selective microbial 11β-hydroxylation of 16α,17α-dimethyl-17β-(1-oxopropyl)androsta-1,4-dien-3-one by *C. lunata* AS 3.4381 was performed and the product of biotransformation was identified as rimexolone. After optimized the conditions of incubation, a 50% biotransformation efficiency was obtained.

The starting material androsta-1,4-dien-3,20-dione (ADD, **2**) was a usual and economical steroids substance, and using microbial hydroxylation reduced the risk of environmental pollution. Compared to the reported methods, the procedure in this article afforded a novel oecumenical and efficient synthetic route to rimexolone.

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