

MICROBIAL TRANSFORMATION OF CHOLESTEROL BY *MYCOBACTERIUM SMEGMATIS*

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Abstract

Mycobacterium smegmatis PTCC 1307 (CIP 73.26) was used as a microbial agent to produce androsta-1,4-diene-3,17-dione (ADD) and androst-4-ene-3,17-dione, two useful precursors in the synthesis of steroid drugs. The side chain of cholesterol, as the substrate, was selectively cleaved in the presence of five enzyme inhibitors. An intermediate structure with intact side chain, cholest-4-ene-3-one, was also detected and purified.

Keywords: *Mycobacterium smegmatis*; Biotransformation; Cholesterol; Androsta-1,4-diene-3,17-dione; Androst-4-ene-3,17-dione; Cholest-4-ene-3-one

Introduction

Microbial conversions compared to chemical reactions are more regio- and stereoselective and have been used in the production of pharmaceutical products for a long time [1]. The most essential problem in preparing steroid hormones from sterols is the selective cleavage of the side chain. Chemical approach is not effective because of its low conversion rate and has proved to be far short of economical profit. This is due to low specificity of the reaction, causing a considerable contamination of undesired by-products [2].

The ability of some microorganisms, represented by *Mycobacterium*, *Corynebacterium* and *Arthrobacter* to

utilize sterols as sole sources of carbon and energy, was first discovered by Soehngen [3]. It was found that sterol ring structure and the side chain were metabolized by different mechanisms [4].

In 1969 cholesterol decomposing ability of 1589 microbial strains was examined by Arima and his coworkers [3]. Marsheck reported the production of substantial amounts of 17-ketonic compounds without appreciable degradation of the steroid nucleus by *Mycobacterium* sp. NRRL B-3805 and B-3683 [5]. In 1976 the latter was shown by Conner to be able to convert tall oil sterols to C₁₉ steroids [6]. Wovcha used *Mycobacterium fortuitum* to convert sitosterol to androst-4-ene-3,17-dione and other intermediates [7].

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Microbial transformation of Polish tall oil was performed using *Mycobacterium sp.* MB 3683 [8]. Testosterone production using *Mycobacterium sp.* NRRL B-3683 and isolation of a new mutant for sterol biotransformation were reported by research groups from Cuba [9,10]. They also studied the effect of glucose and lactose on the steroid biotransformation by the same strain [11].

In a preceding paper [12], the effect of six different agents acting as enzyme inhibitors on selective side chain degradation of cholesterol by *Arthrobacter simplex* PTCC 1036 was reported. In the present paper, the results of selective side chain degradation of cholesterol using *Mycobacterium smegmatis* PTCC 1307 in the presence of five separate enzyme inhibitors are described.

Experimental

Mycobacterium smegmatis PTCC 1307 was obtained from the Persian Type Culture Collection. All chemicals and culture media were obtained from Sigma Chemicals and Oxoid, respectively.

For analytical thin layer chromatography, 250 μm thick silicagel precoated plates with 254 nm fluorescent indicator (Sigma Chemical Co), and for preparative thin layer chromatography, Kieselgel 60 HF (Art. 7741, Merck) were used.

The microorganism was grown in 100 ml of a preculture medium (nutrient broth 1.3 g, myo-inositol 0.1 g per 100 ml) in shake flasks by incubating at 30°C and 150 round per minute for 24 h. To 100 ml of the production medium (containing nutrient broth 1.3 g, myo-inositol 0.1 g, cholesterol 0.2 g per 100 ml at pH 7), 10 ml of the preculture medium was inoculated and incubated at 30°C and 150 rounds per minute. The enzyme inhibitor was added after 24 h. Five chemicals tested for their enzyme inhibition activity (added in separate shake flasks) were: 1 mM 2,2'-dipyridyl; 1 mM 8-quinolinol; 0.1 mM 1,10-dihydroxy phenanthroline; 1 mM NiSO₄ and 10 mM CoSO₄.

After the incubation period (eleven days), the broth was extracted three times by chloroform. Complete removal of the solvent was followed by silicagel thin layer chromatography to detect the products. Separation and purification of the products were performed by preparative silicagel plates (20 \times 40 cm) using ethyl acetate:hexan 3:7 as solvent system.

Mass, FTIR, ¹H NMR and ¹³C NMR spectra of compounds were recorded with Finnigan mat TSQ-70, Magna IR Spectrometer 550 (Nicolet) and V-NMR 400 (Varian), respectively. Melting points were taken on a Kofler hot stage apparatus and were not corrected.

Results

Mycobacterium smegmatis degraded cholesterol side chain in the presence of all tested enzyme inhibitors. Three steroid metabolites were isolated using preparative thin layer chromatography.

The first metabolite had the following properties:

- Mass spectrum, m/e (EI): m/z 384 (M⁺, C₂₇H₄₄O), 342, 299, 260, 229, 124

- ¹H NMR (400 MHz, CDCl₃) δ 5.708 (s, 1H, H₄)

- ¹³C NMR (100 MHz, CDCl₃) δ 199.50 (C₃), 171.54 (C₅), 123.73 (C₄),

- 56.05, 55.84, 53.77, 42.34, 39.59, 39.47, 38.55, 36.08, 35.73, 35.66, 33.95, 32.92, 32.01, 28.17, 27.99, 24.16, 23.79, 22.84, 22.57, 20.99, 18.63, 17.35, 11.94

- Infrared spectroscopy FTIR (KBr) λ_{max} 1680 cm⁻¹ (s, C=O)

- Melting point: 82-84°C

The second metabolite showed the following properties:

- Mass spectrum, m/e (EI): m/z 286 (M⁺, C₁₉H₂₆O₂), 244, 200, 148, 124

- HNMR (400 MHz, CDCl₃) δ 5.73 (s, 1H, H₄)

- ¹³C NMR (100 MHz, CDCl₃) δ 220.27 (C₁₇), 199.05 (C₃), 170.16 (C₅), 123.92 (C₄), 53.60, 50.61, 34.92 (C₈, C₉, C₁₄), 47.30, δ 38.44 (C₁₀, C₁₃), 35.56, 35.49, 33.73, 32.37, 31.08, 30.54, 21.56, 20.11 (C₁, C₂, C₆, C₇, C₁₁, C₁₂, C₁₅, C₁₆), 17.19, 13.53 (C₁₈, C₁₉)

- Infrared spectroscopy: FTIR (KBr) λ_{max} 1670 cm⁻¹ (s, C=O) 1730 cm⁻¹ (s, C=O)

- Melting point: 173-175°C

The third metabolite had the following properties:

- Mass spectrum, m/e (EI): m/z 284 (M⁺, C₁₉H₂₄O₂), 266, 172, 159, 122

- ¹HNMR (400 MHz, CDCl₃) δ 6.970(d, 1H, H₁), 6.128 (d, 1H, H₂), 5.978 (s, 1H, H₄)

- ¹³C NMR (100 MHz, CDCl₃) δ 219.79 (C₁₇), 186.01 (C₃), 168.26 (C₅), 155.25, 127.52, 123.93, 52.11, 50.23, 34.92 (C₁, C₂, C₄, C₈, C₉, C₁₄), 47.54, 43.29 (C₁₀, C₁₃), 35.51, 32.42, 32.17, 31.05, 21.95, 21.78 (C₆, C₇, C₁₁, C₁₂, C₁₅, C₁₆), 18.62, 13.70 (C₁₈, C₁₉)

- Infrared spectroscopy: FTIR (KBr) λ_{max} 1720 cm⁻¹ (s, C=O)

- Melting point: 141-143°C

Spectral and chromatographic data of the above compounds were identical with those of cholest-4-ene-3-one (first metabolite) and androst-4-ene-3,17-dione (second metabolite) and androsta-1,4-diene-3,17-dione (third metabolite), respectively (Fig. 1).

Discussion

Mycobacterium smegmatis is found in this research

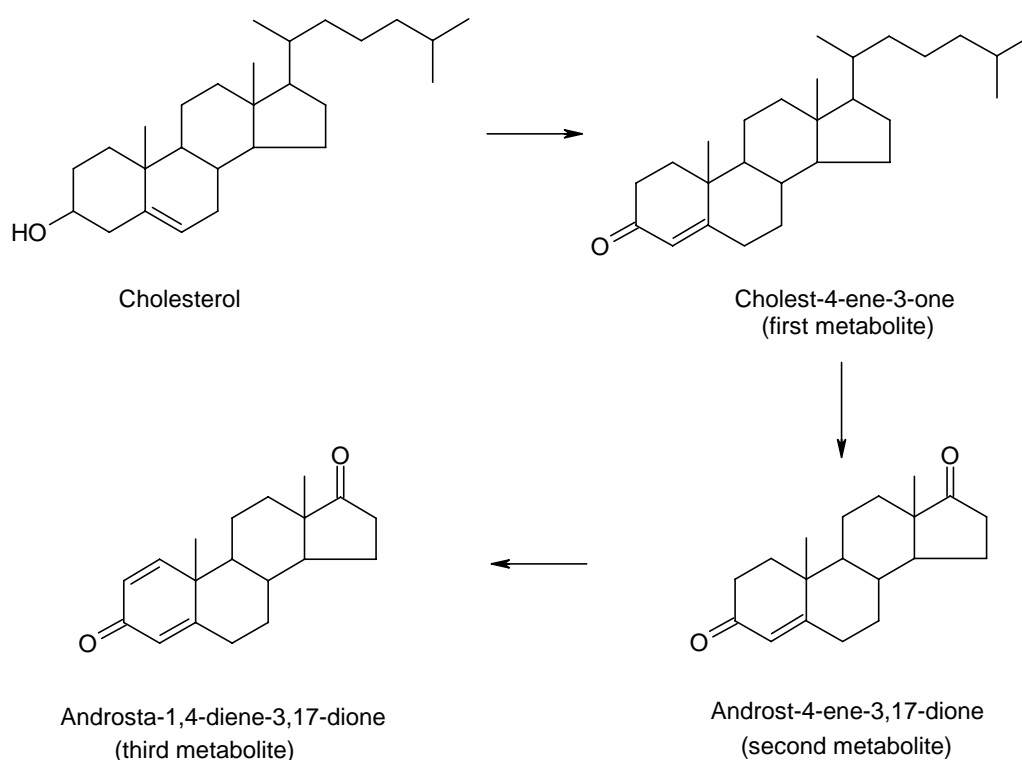


Figure 1. Metabolic pathway of cholesterol in *Mycobacterium smegmatis*.

to be efficient in cleaving the side chain of sterols selectively in the presence of enzyme inhibitors.

The formation of seven low molecular weight degradation products of progesterone by *M. smegmatis* was reported in 1965 [13]. Schubert and coworkers esterified cholesterol to succinate of 26-OH-4-cholestene-3-one and 26-OH-1,4-cholestadiene-3-one using *M. smegmatis* [14]. They also hydrogenated 19-nortestosterone enantiomers by the same microorganism [15]. As reported by a German patent, *M. smegmatis* was used to prepare 6-OH-3-oxo- Δ 1,4-steroids [16]. In 1981 Atrat used immobilized preparations of *M. smegmatis* to produce 4-androstene-3,17-dione from sterols [17].

M. smegmatis PTCC 1307 degrades cholesterol and the biotransformation products were identified as androsta-1,4-diene-3,17-dione (ADD), androst-4-ene-3,17-dione (AD), and an intermediate steroid with intact side chain, cholest-4-ene-3-one. AD and ADD are suitable precursors in the synthesis of steroid hormones such as testosterone and estrogens.

Five different inhibitors were tested separately for their effect on biotransformation. The results of biodegradation as tested qualitatively by TLC were the same. Selective removal of side chain requires blocking

of the enzymes essential for ring cleavage. 9- α -hydroxylase, a monooxygenase containing ferrous ion, is the most important enzyme in ring fission. Specific inhibition of this enzyme is possible by removing this ion from the medium [1,18]. The mode of action of Ni^{++} and Co^{++} might be replacement of ferrous ion which is required for the function of 9- α -hydroxylase. While 2,2'-dipyridyl, 1,10-phenanthroline and 8-quinolinol inhibit the same enzyme by chelating Fe^{++} [19].

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