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PREPARATION OF TESTOSTERONE USING MICROBIAL METABOLITE OF CHOLESTEROL

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ABSTRACT

Natural sterols are potential raw materials for the prepartion of pharmacologically active steroid products. This paper details preparation of testosterone, one of the steroidal hormones, through microbial metabolism of cholesterol followed by chemical reduction. The side chain of cholesterol was selectively cleaved by *Mycobacterium smegmatis PTCC 1307*, and then selective reduction of resulting 17-keton group of androst-4-ene-3,17-dione (AD), the main product of microbial transformation, with sodium borohydride at -10°C gave testosterone in high yeild (90%). Assignment of structures were made by IR, NMR, Mass, and UV spectroscopic data.

Keywords: *Mycobacterium smegmatis*, Testosterone, Cholesterol, Sodium borohydride

INTRODUCTION

Cholestrol, adrenocorticoids, and sex hormones have much in common. All are streoids having a similar stereochemical relationship. Sex hormones are mainly biosynthesized from cholesterol. Commercially, testosterone is prepared from various steroids, including sarsapogenin, diosgenin, and androgens (1). Owing to its androgenic and anabolic activities it is desirable to prepare it easily and sufficiently. Quantitatively, natural sources are not sufficient to satisfy all necessities. Furthermore, synthetic methods, which involves several steps are difficult, expensive, and sometimes impossible.

Microbial conversions compared to synthetic methods are more regio- and stereo-selective and have been used for a long time in the production of pharmacologically active compounds (2). The most important problem in the prepartion of steroid hormones from sterols is selective side chain cleavage. Some microorganisms such as *Mycobacterium, Corynebacterium* and *Arthrobacter* have shown the ability to utilize sterols as sole source of carbon and energy. It has been found that sterol ring structure and the side chain are metabolized by different mechanisms (3,4). Marsheck reported the production of substantial amounts of 17-keton compounds without appreciable degradation of the steroid nucleus by *Mycobacterium sp.* NRRL B-3805 and B-3683 (5). The latter microorganism was shown in 1976 by Conner et al. to be able to convert tall oil sterols to C_{19} steroids (6). Wovcha used *Mycobacterium fortuitum* to convert sitosterol to androst-4-ene-3,17-dione and other intermediates (7). Microbial transformation of Polish tall oil was performed by *Mycobacterium sp.* MB 3683 (8). Testosterone production by *Mycobacterium sp.* NRRL B-3683 and isolation of a new mutant for sterol biotransformation were reported by a research group from Cuba (9,10). They also studied the effect of glucose and lactose on steroid biotransformation by the same strain (11). In this paper, we would like to report preparation of testosterone from AD, a microbial metabolite of cholesterol, followed by chemical reduction.

MATERIALS AND METHODS

Materials

Mycobacterium smegmatis PTCC 1307 was obtained from the Persian Type Culture Collection. All chemicals were purchased from Sigma (USA) and culture media were obtained from Oxoid (England).

For analytical thin layer chromatography (TLC), 250 μm thick silicagel precoated plates with 254

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nm fluorescent indicator from Sigma Chemical Co. (USA) and for preparative TLC Kieselgel 60 HF from Merck (German) were used. Mass, FTIR, HNMR and ^{13}CNMR 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 are not corrected. The ultra violet spectra of solutions in $CHCl_3$ were recorded on a Shimadzu Model 160-A UV spectrophotometer

Fermentation

The microorganisms were grown in 100 ml of a preculture medium (nutrient broth 1.3 g, myo-inositol 0.1 g) in shake flasks by incubation at 30 °C and 150 (rpm). After 24 hours, this medium was used as inoculum. To 100 ml of the production medium (containing nutrient broth 1.3 g, myo-inositol 0.1 g at pH 7) was added 10 ml of the inoculum. Cholesterol in concentration about 0.1% in ethanol was added to the production medium as substrate. After 24 hours, 1 mM 2, 2'-dipyridyl was used as enzyme inhibitor. The incubation was carried out at 30 °C and 150 rpm for one week.

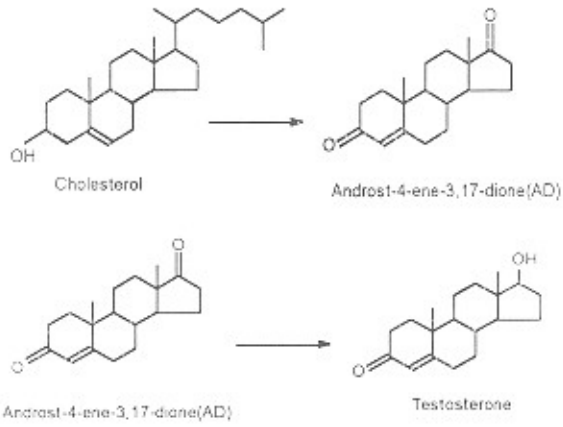

Fig 1. Preparation of testosterone through conversion of cholesterol by *Mycobacterium smegmatis* followed by chemical reduction.

Purification

After the incubation period the broth was extracted three times by chloroform. Following complete removal of the solvent, the residue was subsected to determine the best solvent system for separation of mixture. Separation and purification of the microbial metabolites were performed on the preparative 20x45 cm silicagel plates using ethyl acetate/hexan (30 :70) as mobile phase.

Testosterone preparation

To a suspension of 150 mg of AD in C_2H_5OH at 10 °C, a solution of 70 mg sodium borohydride in ethanol (2 ml) was added over 30 min while the temperature was maintained at -5 to -10 °C. The mixture was stirred for 1 hour and after warming to +5 °C it was treated with small amounts of HCl 3N until to reach to pH of 5-7. The ethanol was evaporated under vacuum and the residue was dissolved in ethylacetate (about 1-2 ml). The solution was washed with water, and organic phase was dried over anhydrous Na_2SO_4 and then evaporated under vacuum. Testosterone was crystallized from the mixture of water and acetone as creamy-white crystal (12).

RESULTS

Mycobacterium smegmatis degraded cholesterol side chain. The structure of AD (the main metabolite isolated in the presence of 2,2'-dipyridyl) and testosteron were established by their melting point and spectral data.

Androst-4-ene-3,17-dione (AD)

MS m/z (%) 286 (M^+, $C_{19}H_{26}O_2$) (100), 244 (73.3), 200 (28.57), 148 (33.3), 124 (22.85).

^{1}H-NMR (400 MHz , $CDCl_3$) δ 5.73 (s, 1H, H_4).

^{13}C-NMR (100MHz,$CDCl_3$): δ 220.27 (C_{17}), 199.05 (C_3), 170.16 (C_5), 123.92 (C_4), 53.60, 50.61, 34.92 (C_8, C_9, C_{14}), 47.30, 38.44 (C_{10}, C_{13}), 35.56, 35.49, 33.73, 32.37, 31.08, 30.54, 21.56, 20.11 (C_1, C_2, C_6, C_7, C_{11}, C_{12}, C_{15}, C_{16}), 17.19, 13.53 (C_{18}, C_{19}).

FT-IR (KBr) 1670 (C=O) 1730 (C=O) cm^{-1}.

UV ($CHCl_3$): λmax = 251 nm, Σ max = 26176

M.P: 173-175 °C (13).

Testosterone

MS m/z (%) 288 (M^+, $C_{19}H_{28}O_2$) (4.76), 246 (17), 147 (33.3), 124 (100), 91.2 (28.5).

^{1}H-NMR (400 MHz , $CDCl_3$): δ 5.7(s, 1H, H_4), 3.6 (t,1H, H_{17}).

IR (KBr) 3400 (OH), 1660 (C=O) cm^{-1}.

UV ($CHCl_3$): λmax = 246 nm, Σ max = 16176

M.P: 148-151 °C (13).

DISCUSSION

Testosterone was isolated first by Laquer in 1935 from animal testes. Since natural sources are not sufficient, researchers have focused on microbial conversions and synthetic methods for the preparation of testosterone. Due to the low specificity, considerable undesired by-products, and low convertion ratio, chemical reactions are not suitable for the preparation of testosterone (14). The formation of seven low molecular weight degradation products of progesterone by *M. smegmatis* was reported in 1965 (15). In one study the cholesterol was esterified to succinate of 26-hydroxy-4-cholestene-3-one and 26-hydroxy-1,4-cholestadiene-3-one using *M.smegmatis* (16). The resulting 19-nortestosterone was hydrogenated to enantiomers by the same microrganism (17). *M. smegmatis* is reported in a German patent for the preparation of 6-hydroxy-3-oxo-$\Delta_{1,2}$-steroids (18). In 1981 Atrat et al. used immobilized preparations of *M. smegmatis* to produce 4-androstene-3,17-dione from sterols (19). *M. smegmatis* PTCC 1307 has degraded cholesterol to androst-4-ene-3,17-dione (AD).

Selective removal of side chain, requires blocking 9α-hydroxylase, a monoxygenase containing ferrous ion, which cleaves the ring. Specific inhibition of this enzyme is possible by removing Fe^{++} from the medium by chelation with 2,2`-dipyridyl (2,20,21).

AD, the main microbial metabolite, is a suitable precursor in the synthesis of testosterone. Conversion of AD to testosterone has previously been reported (22). In this study, we used *Mycobacterium smegmatis* which was obtained from the Persian Type Culture Collection to cleave the side chain of cholesterol. The main microbial metabolite, AD, was purified and converted to testosterone by a different method through reduction with sodium borohydride at -10 °C. Different carbonyl compounds are reduced by hydride ion with different rates. In particular, reduction by $NaBH_4$ in alcohols is a fast and irreversible reaction which has been employed as a criteria for comparison of the reactivity of different carbonyl groups (23). 3-keton group of AD is conjugated with carbon-carbon double bond between positions 4 and 5. The resonance stabilization provided by the 4,5-double bond reduces the electrophilicity of the carbonyl group. Therefore, nucleophilic attack by the strongly basic hydride ion (H^-) occurs on carbonyl carbon of 17-keton group. AD was selectively reduced by $NaBH_4$ at 17-keton group. The ^{1}H-NMR and IR spectra were in agreement with the reduction of 17-keton group of AD by $NaBH_4$. In the NMR spectrum of the product H-4 of double bond appeared as a singlet at 5.7 ppm, and H-17 appeared as a triplet at 3.6 ppm. In addition, as expected, IR spectrum of reduced AD showed only 3-keton group at 1660 cm^{-1}. Because of conjugation of 3-keton group with double bond, in the UV spectra of AD and testosterone, a distinct maximum absorption (λ_{max}) at 251 nm and 246 nm were observed, respectively. All spectral data are in agreement with reduction of AD with $NaBH_4$ at 17-keton group. In conclusion, combination of microbial conversion and chemical reduction provided an easy and simple method for the preparation of testosterone.

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