Arch. Pharm. Res. Vol.20, No.6, pp. 525-528, 1997

Microbial 9α-Hydroxylase: Epoxidation of 9(11)-dehydro-17α-methyl-testosterone

Hee Kyoung Kang and Sang Sup Lee

College of Pharmacy, Seoul National University, Seoul, 151-742, Korea

(Received June 23, 1997)

Steroid 9α-hydroxylase is a key enzyme system in steroid nucleus degradation in company with Δ^1-dehydrogenase. To examine 9α-hydroxylase activity during microbial transformation of steroids, 9(11)-dehydro-17α-methyl-testosterone was adopted as a stable substrate for preventing the rupture of steroid nucleus. Using *Nocardia restrictus* ATCC 14887 capable of introducing a 9α-hydroxyl group into steroids, 9α,11α-oxido-17β-hydroxy-17α-methyl-4-androstene-3-one and 9α,11α-oxido-17β-hydroxy-17α-methyl-1,4-androstadiene-3-one were obtained. These microbiologically transformed products could be used as reference compounds in the enzyme assay.

Key words : Steroid 9α-hydroxylase, 9(11)-Dehydro-17α-methyl-testosterone, 9α,11α-Oxido-17β-hydroxy-17α-methyl-4-androstene-3-one, 9α,11α-Oxido-17β-hydroxy-17α-methyl-1,4-androstadiene-3-one

INTRODUCTION

Steroid 9α-hydroxylase is an enzyme found in microorganisms which can utilize steroids as a sole carbon source (Dodson and Muir, 1961b; Marsheck, 1972). The enzyme catalyzes the production of 9α-hydroxy-4-androstene-3,17-dione, an important intermediate for the semi-synthesis of potent anti-inflammatory drugs such as 9α-fluorocorticoids from 4-androstene-3,17-dione (AD) (Kieslich, 1984; Marsheck *et al.*, 1983). Steroid 9α-hydroxylase in company with Δ^1-dehydrogenase is used in industrial processes. Though studies on the Δ^1-dehydrogenase was progressed, knowledge about 9α-hydroxylase is limited (Chang and Sih, 1964; Strijewski, 1982; Itagaki *et al.*, 1990a; Itagaki *et al.*, 1990b; Drobnic *et al.*, 1993).

One of the mechanisms of steroid degradation by microorganisms involves a 9α-hydroxylation reaction, followed by a Δ^1-dehydrogenation (or vice versa) with the formation of a 9,10-seco-steroid (Dodson and Muir, 1961a; Gibson *et al.*, 1966; Sih and Lee, 1966). Thus, saturated steroids as progesterone, testosterone, and AD cannot be used as a substrate to study 9α-hydroxylase, because 9α-hydroxy steroid, formed as intermediate, would be further metabolized by the degrading enzymes. This disadvantage can be overcome by the application of 9(11)-unsaturated steroid substrates based on the rule of Bloom and Shull (Bloom and Shull, 1955).

To use 9(11)-dehydro-17α-methyl-testosterone (DHMT) as a substrate for determination of 9α-hydroxylase activity, 9α,11α-oxido forms of DHMT (Fig. 1: III and IV) were needed. In the present work described in this article, 9α,11α-oxido-17β-hydroxy-17α-methyl-4-

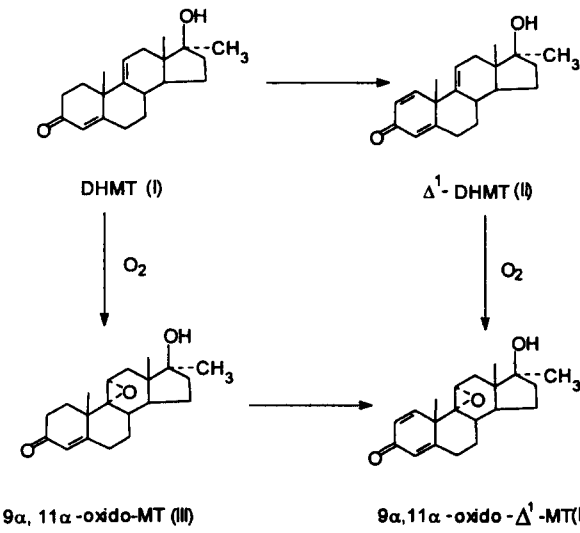

Fig. 1. Products found by bioconversion of 9(11)-dehydro-17α-methyl-testosterone. DHMT: 9(11)-dehydro-17α-methyl-testosterone. Δ^1-DHMT: 9(11)-dehydro-17β-hydroxy-17α-methyl-1,4-androstadiene-3-one. 9α,11α-oxido-MT: 9α,11α-oxido-17β-hydroxy-17α-methyl-4-androstene-3-one. 9α,11α-oxido-Δ^1-MT: 9α,11α-oxido-17β-hydroxy-17α-methyl-1,4-androstadiene-3-one.

Correspondence to: Sang Sup Lee, College of Pharmacy, Seoul National University, Seoul, 151-742, Korea

androstene-3-one (9α,11α-oxido-MT) and 9α,11α-oxido-17β-hydroxy-17α-methyl-1,4-androstadiene-3-one (9α,11α-oxido-Δ^1-MT) were prepared by a microbiological method.

MATERIALS AND METHODS

Cultures

Nocardia restrictus ATCC 14887 and *Mycobacterium fortuitum* KCTC 1122 (*Mycobacterium fortuitum* ATCC 6842) were obtained from American Type Culture Collection and Korean Collection for Type Cultures, respectively. These microorganisms are able to utilize steroids as carbon and energy source. *N. restrictus* was cultivated in a medium containing per l 8 g nutrient broth (Difco), 10 g yeast extract (Difco), and 10 g glucose. And *M. fortuitum* was grown in a medium containing per l 8 g nutrient broth, 5 g glycerol, 1 g yeast extract, and 1 g tween 80.

Analysis of fermentation

Portions (100 ml) of these medium were inoculated with 10 ml aliquots of 72 h seed cultures of these microorganisms and then incubated at 28°C on a rotary shaker for 40 h. The flasks were supplemented with a N,N'-dimethylformamide solution of DHMT (Sigma) to give a final concentration of 250 μg/ml and further incubated. The progress of each fermentation was checked by extraction 3 ml of the broths with same volume of ethylacetate and chromatographing the extracts on precoated thin-layer silica gel plates with 254 nm fluorescent indicator (Sigma). The solvent system generally used for develping the plates was $CHCl_3$/CH_3OH (96/4, v/v). The developed plates were photographed under short wave ultraviolet illumination.

Isolation and identification of products formed during the bioconversion of DHMT by *N. restrictus*

N. restrictus was grown in 2 l Erlenmeyer flask with 500 ml of the medium. After 40 h of incubation at 28°C on a rotary shaker, DHMT was added to give a final concentration of 500 μg/ml. The fermentation was continued for 96 h, and the whole culture broth was extracted three times with the same volume of ethylacetate. The ethylacetate extract was dried with sodium sulfate and concentrated to dryness. The residue was chromatographed on a silica gel column (2.5 × 20 cm) packed in silica gel (0.06~0.2 mm), eluted with $CHCl_3$/CH_3OH (96/4, v/v), at a flow rate of 90 ml per hour, and fracton volumes of 10 ml were collected. On the basis of TLC results, the fractions that seemed to be 9α,11α-oxido compounds of DHMT by activity of 9α-hydroxylase were pooled into two fractions. Infrared (IR) spectra were obtained on a Perkin Elmer 1710 spectrometer. Ultraviolet (UV) spectra were taken on a Shimadzu UV-Visible recording spectrophotometer model 2100. Mass spectra were obtained by direct sample introduction into VG Trio-2 spectrometer. Proton magnetic resonance spectra were obtained with JEOL JNH-GSX 400 spectrometer and chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane as an internal standard. Using the spectroscopic parameters of UV, IR, ^{1}H-NMR, and EI-MS, 9α,11α-oxido compounds of DHMT, 9α,11α-oxido-MT and 9α,11α-oxido-Δ^1-MT were identified. These 9α,11α-oxido epoxides were monitored by using HPLC (pump: LC-9A, UV detector: SPD-6AV, system controller: SCL-6B, integrator: C-R6A, Shimadzu).

RESULTS

To use DHMT as an ideal substrate for determination of steroid 9α-hydroxylase, 9α,11α-oxido compounds of DHMT (Fig. 1: III and IV) were needed. Firstly, time-dependent bioconversion patterns of DHMT by *N. restrictus* and *M. fortuitum* that utilize steroids as carbon and energy source were examined. With the results of TLC profiles, it was expected that 9α,11α-oxido epoxides of DHMT were effectively accumulated by *Nocardia* than by *Mycobacterium* (Fig. 2). Subsequently, silica gel column chromatography for ethylacetate extract of 96 h fermentation broth after the addition of 250 mg of DHMT to *N. restrictus* culture

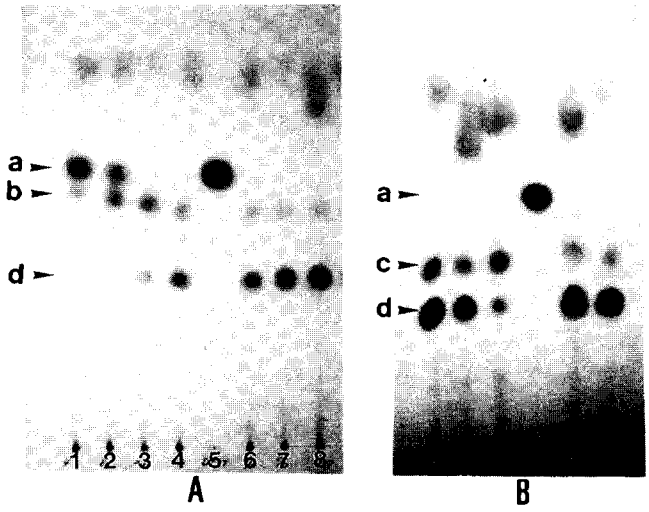

Fig. 2. The TLC profiles for time-dependent conversion patterns of DHMT by *M. fortuitum* (A) and *N. restrictus* (B). In *Mycobacterium* (A), lane 1, 2, 3, 4, 6, 7 and 8 exhibited the TLC profile of 4 h, 8 h, 24 h, 48 h, 72 h, 96 h and 120 h fermentation after the addition of DHMT. In *Nocardia* (B), lane 1, 2, 3, 5 and 6 designated the TLC data of 4 h, 8 h, 24 h, 48 h and 96 h fermentation, respectively. [a]DHMT as standard; [b]expected to be Δ^1-DHMT; [c]expected to be 9α, 11α-oxido-MT; dexpected to be 9α,11α-oxido-Δ^1-MT

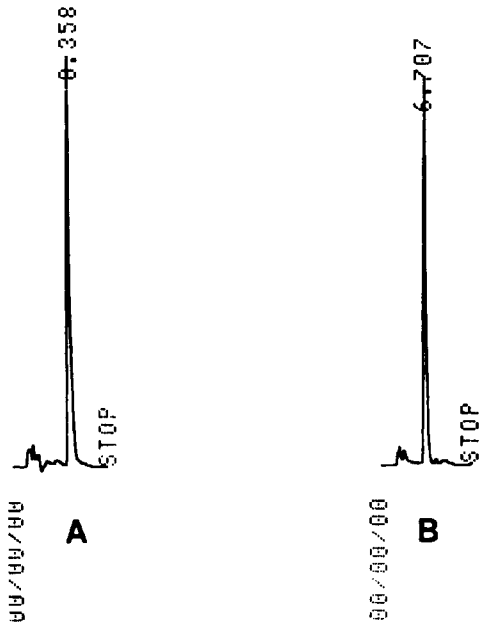

Fig. 3. The HPLC chromatograms of 9α,11α-oxido-MT(A) and 9α,11α-oxido-Δ^1-MT(B).

broth was performed. Compounds expected to be 9α, 11α-oxido-MT and 9α,11α-oxido-Δ^1-MT were isolated and identified by the spectroscopic parameters of UV, IR, ^{1}H-NMR, and EI-MS. The early fractions (R_f=0.44) afforded 55 mg (22%) of 9α,11α-oxido-MT: UV_{MeOH} λ_{max} 237.6 nm (E=14,900 $M^{-1}cm^{-1}$); IR ν_{OH} 3430 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 5.87 (s, C_4-^{1}H); EI-MS m/e 316, 135. The latter fractions (R_f=0.37) gave 103 mg (41%) of 9α,11α-oxido-Δ^1-MT: UV_{MeOH} λ_{max} 238.4 nm (E=13,900 $M^{-1}cm^{-1}$); IR OH 3440 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 6.65 (d, J=10.4, C_1-^{1}H), 6.23 (d, J=10.4, C_2-^{1}H), 6.15 (s, C_4-^{1}H); EI-MS m/e 314, 134. The UV spectra exhibited maximum peaks at 238 nm, characteristic of a steroid 4-ene-3-one. These data were consistent with the reports that 9α,11α-oxido-cortexolone, 4-pregnene-9α,11α-oxido-17β,21-diol-3,20-dione, and 9α,11α-oxido-androstene-3,17-dione had maximum peaks at 236~238 nm (Sih, 1961; Sih, 1962). The mass spectra of them indicated molecular weights of 316 ($C_{20}H_{28}O_3$) and 314 ($C_{20}H_{26}O_3$), and exhibited intense ions at 135, characteristic of a steroid 9α,11α-oxido-4-ene-3-one. Isolated 9α,11α-oxido epoxides of DHMT were monitored at 238 nm using HPLC with a solvent system of 50% actonitrile-water on an ODS column (2.5×150 mm, particle size 4 μm: Cosmosil) at a flow rate of 0.5 ml/min. Under these conditions, the retention times of 9α,11α-oxido-MT and 9α,11α-oxido-Δ^1-MT were 8.3 and 6.7 min, respectively (Fig. 3).

DISCUSSION

In order to determine steroid 9α-hydroxylase activity, DHMT would be chosen as an ideal substrate. Because it is transformed to 9α,11α-oxido epoxide by 9α-hydroxylase according to the rule of Bloom and Shull, degradation of steroid B nucleus with the concomitant Δ^1-dehydrogenase activity is not expected to occur (Bloom and Shull, 1955). Moreover, the presence of 17α-methyl group prevents 17β-dehydrogenation and lactonization of steroid D nucleus (Kieslich, 1984). From the results of various experiments, Bloom and shull (1955) postulated that "a microorganism capable of introducing an axial hydroxyl function at C_n of a saturated steroid also effect the introduction of an epoxide grouping axial at C_n in the corresponding unsaturated substrate". Therefore, 9α-hydroxylase activity in the cell free systems of various microorganisms would be measured by the transformation of DHMT to the corresponding 9α,11α-oxido epoxides with TLC or HPLC. In this way, isolation and characterization of the enzyme have been performed.

REFERENCES CITED

Bloom, B. M. and Shull, G. M., Epoxidation of unsaturated steroids by microorganisms. *J. Am. Chem. Soc.*, 77, 5767-5768 (1955).

Chang, F. N. and Sih, C. J., Mechanisms of steroid oxidation by microorganisms. VII. Properties of the 9α-hydroxylase. *Biochem.*, 3, 1551-1557 (1964).

Dodson, R. M. and Muir, R. D., Microbiological transformations: VI The microbiological aromatization of steroids. *J. Am. Chem. Soc.*, 83, 4627-4631 (1961a).

Dodson, R. M. and Muir, R. D., Microbiological transformations: VII The hydroxylation of steroids at C-9. *J. Am. Chem. Soc.*, 83, 4631-4635 (1961b).

Drobnic, K., Krizaj, I., Gubensek, F. and Komel, R., Improved purification of steroid 1:2-dehydrogenase from *Nocardia opaca* and partial characterization of its cloned gene sequence. *Biochem. Biophys. Res. Commun.*, 190, 509-515 (1993).

Gibson, D, T., Wang, K.,C., Sih, C. J. and Whitlock, H., Mechanisms of steroid oxidation by microorganisms. *J. Biol. Chem.*, 241, 551-559 (1966).

Itagaki, E., Matushita, H. and Hatta, T., Steroid transhydrogenase activity of 3-ketostercid-Δ^1-dehydrogenase from *Nocardia corallina. J. Biochem.*, 108, 122-127 (1990a).

Itagaki, E., Wakabayashi, T. and Hatta, T., Purification and characterization of 3-ketosteroid-Δ^1-dehydrogenase from *Nocardia corallina. Biochim. et Biophys.* Acta, 1038, 60-67 (1990b).

Kieslich, K.; Smith, L. L. and Martin, C. K. A., Steroids and Sterols, In Rehm, H. J. and Reed, G. (Eds). *Biotechnology,* 6a; Verlag chemie, Weinheim, Florida, pp. 31-95, 1984.

Marsheck, W. J., Microbial degradation of sterols.

Appl. Microbiol., 23, 72-77 (1972).
Marsheck, W. J., Mentor, O., Morton, G. III. and Wang, P. T., Microbial Process for 9α-hydroxylation of steroids. U.S. 4,379,947 (1983).
Sih, C. J., The synthesis of 9α-hydroxy steroids. *J. Org. Chem.*, 26, 4716-4718 (1961).
Sih, C. J., Microbiological epoxidation of steroids. *J. Bacteriol.*, 84, 382 (1962).
Sih, C. J., Lee, S. S., Tsong, Y. Y. and Wang, K. C., Mechanisms of steroid oxidation by microorganisms. *J. Biol. Chem.*, 241, 540-550 (1966).
Strijewski, A., The steroid 9α-hydroxylation system from *Nocardia* species. *Eur. J. Biochem.*, 128, 125-135 (1982).