Testosterone 5α-Reductase Inhibitors, Menaquinone 7 Produced by a Bacillus and Phenazine Methosulfate

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Menaquinone 7 (MW: 649, C₃₀H₆₀O₇), a natural electron acceptor for steroid ring A dehydrogenations, produced by Bacillus sp. SNU-299, was isolated as a rat prostate testosterone 5α-reductase inhibitor with an IC₅₀ value of 4.0×10⁻⁸ M from the cultured broth. Phylloquinone was as active as the purified microbial metabolite with an IC₅₀ value of 6.6×10⁻⁸ M. On the basis of this evidence, the inhibitory activities of electron carriers, menadione, phenazine methosulfate, and 2,6-dichlorophenolindophenol, for rat prostate testosterone 5α-reductase were tested, and the IC₅₀ values were 3.1×10⁻⁸ M, 4.9×10⁻⁸ M, 8.9×10⁻⁸ M, respectively. A product of the 5α-reductase enzyme reaction and an electron and proton carrier, NADP⁺, inhibited the 5α-reductase by rat prostate testosterone 5α-reductase with an IC₅₀ value of 9.2×10⁻⁷ M. However, the inhibition effect of a proton carrier, carbonylcyaniade-m-chlorophenylhydrazone, for rat prostate testosterone 5α-reductase was substantially inactive.

Key words: electron carrier; testosterone 5α-reductase inhibitor; menaquinone 7; Bacillus; phenazine methosulfate; steroid ring A dehydrogenation

Testosterone, the major circulating androgen in adult males, converts to the more potent androgen, 5α-dihydoxytestosterone, by the catalysis of testosterone 5α-reductase (3-oxo-5α-steroid:NADP⁺ Δ⁵-oxidoreductase (EC 1.3.1.22)) in target tissues such as the prostate. There are at least two isozymes of testosterone 5α-reductase, type 1 isozyme and type 2 isozyme, in human and rat. It is generally believed that benign prostate hyperplasia is associated with an overproduction of 5α-dihydroxytestosterone.¹ Therefore, the inhibition of testosterone 5α-reductase represents an important pharmacological approach toward the treatment of benign prostate hyperplasia.² NADP⁺, a product of the 5α-reductase enzyme reaction, inhibits the conversion by up to 60% when presented with NADPH.³ Several nonsteroidal compounds of microbial origin and their derivatives and analogs, including the phenazine derivatives,⁴-⁶ riboflavin and its analogs,⁷ and the ascomycin derivatives,⁸ were reported as inhibitors of testosterone 5α-reductase by a number of investigators. Among these inhibitors, a simple phenazine antibiotic, WS-9659 A, was shown to have specific inhibitory activities on rat, dog and human prostate testosterone 5α-reductase more than rabbit lens aldose reductase and pig heart lactate dehydrogenase.⁹ Moreover, this compound was also found to be pharmacologically active in vivo in rats.⁹

It is notable that most of these nonsteroidal inhibitors involve an electron carrier in their oxidation-reduction chemistry. In the course of our search for inhibitors of testosterone 5α-reductase, menaquinone 7 was isolated as a nonsteroidal inhibitor from the cultural broth of Bacillus sp. SNU-299, and several electron carriers, including phenazine methosulfate, were found to have potent inhibitory activity. We report here the isolation and identification of menaquinone 7, which has a rat prostate testosterone 5α-reductase inhibition effect. We also describe the inhibitory activities of 5α-reductase on rat prostates in terms of the electron carriers related to this compound, such as phenazine methosulfate.

MATERIALS AND METHODS

General ¹H-NMR spectra were recorded on a JEOL JNM-LA300 spectrometer. The chemical shifts are given in ppm (d) relative to internal TMS. IR spectra were recorded on a Jasco 300E spectrophotometer. UV spectra were measured on a Beckman DU650 spectrophotometer. Electron impact (EI)-MS were recorded using a JEOL JMS AX505WA mass spectrometer. Pre-coated Silica gel 60 F₂₅₄ plates (E. Merck, Darmstadt, Germany) were used for preparative TLC and TLC analysis.

Chemicals Pyocyanine was isolated from the broth cultures of Pseudomonas aeruginosa (KCTC 1636) as described by the literature.¹ ¹-Hydroxyphenazine was prepared from pyocyanine by treatment with 1.3% NaOH and purified by preparative TLC.⁵,¹⁰ Riboflavin was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Phylloquinone was purchased from E. Merck. Menadione was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phenazine methosulfate, 2,6-dichlorophenolindophenol and carbonylcyaniade-m-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NADP⁺ was purchased from Aldrich Chem. Co. (Milwaukee, WI, U.S.A.).

Testosterone 5α-Reductase Assay The enzyme suspension of testosterone 5α-reductase from the prostate of rats was prepared and the enzyme inhibition assay was carried out in principle according to the methods described by Nakayama et al.,¹² Takamatsu et al.¹³ and Moore and Wilson.¹⁴ Type 2 isozyme of testosterone 5α-reductase was targeted in this study due to differences in tissue distribution and biochemical properties of testosterone 5α-reductase isozymes. A brief description follows: Sprague-Dawley male rats (Laboratory Animal Center, Seoul National University) 7–8 weeks old were sacrificed by diethyl ether, and the ventral prostates were dissected and weighed. The prostates were homogenized in medium A (0.32 M sucrose, 0.1 mM dithio-

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threitol and 20 mM sodium phosphate, pH 6.5) to about 20% (w/v) with a Pyrex glass homogenizer. The homogenate was filtered over 8 layers of surgical gauze and centrifuged at 3000 × g for 15 min. The resulting pellets were resuspended in medium A at a protein concentration of 20 mg/ml by triturating the suspension sequentially through an 18-gauge and then 20-gauge needle. The reaction mixture, consisting of the test sample in 10 μl of 10% ethanol (control tubes received the same volume of 10% ethanol), 525 μl of reaction solution (1 mM dithiothreitol, 40 mM sodium phosphate buffer (pH 6.5), 50 μM NADPH and 2.2 mM [1,2,6,7-3H]testosterone (3.15—3.89 TBq/mmol, New England Nuclear) and 40 μl of the enzyme suspension prepared above, was incubated at 37°C for 30 min. After extraction with 1 ml of ethyl acetate, 50 μl of ethyl acetate phase was chromatographed on a silica plastic sheet (Kieselgel 60 F254, Merck) using ethyl acetate–cyclohexane (1:1) as the developing solvent system. After the testosterone and 5α-dihydrotestosterone areas were cut from the sheet, the corresponding radioactivity of each was counted in 5 ml of Aquasol-2 (New England Nuclear) with a liquid scintillation counter (Wallac 1410).

RESULTS AND DISCUSSION

Strain SNU-299 was isolated from a soil sample collected in Seoul, Korea, and was identified as a *Bacillus* sp. from morphological observations with light and electron microscopes.[12]

From the ethyl acetate extracts of the cultural broth, compound 1 was isolated as the active component by repeated preparative TLC with the guidance of rat prostate testosterone 5α-reductase inhibitory activity. Compound 1 was insoluble in water, sparingly soluble in methanol and soluble in ethanol, acetone, ethyl acetate, petroleum ether, n-hexane and chloroform. The inhibitory potency of this compound was lost by a solution of alkaline hydroxide, but was unaffected by dilute acids. Compound 1 in petroleum ether showed characteristic UV absorption peaks at 218 nm (ε, 12090), 241 nm (ε, 15490), 267 nm (ε, 15100) and 322 nm (ε, 4900) corresponding to the electronic transition of a 2,3-disubstituted 1,4-naphthoquinone chromophore, indicating the existence of this chromophore within the compound. The EI-MS of the compound showed characteristic fragmentations at m/z 648 (M+, 34%), 225 (100%), 187 (8%), 121 (12%), 69 (41%) and 68 (6%), and fragmentations with very low relative intensities at m/z 633 (M-15), 443 ((M-69)=(68×2)), 376, 375 (M-(69)=(68×3)), 307 (M-(69)=(68×4)) and 239 (M-(69)=(68×5)). The fragmentation at m/z 225 (100%) can be assigned to the peak corresponding to the 2-(1-butenyl)-3-methyl-1,4-naphthoquinone moiety. Furthermore, the fragmentations at less than a multiple of 68 mass units from the molecular ion can be assigned to peaks corresponding to the molecular ion minus isoprene units. These data suggested that compound 1 is a menaquinone with a side chain of 7 repeating isoprene units. Compound 1 was finally identified as menaquinone 7 (Fig. 1) by inspection of the IR spectrum and 1H-NMR spectrum.

Menaquinone 7 (1) was evaluated in vitro for the inhibition of rat prostate testosterone 5α-reductase, and the IC50 value of the compound is shown in Table 1. The results were given in comparison with those of pyocyanine, 1-hydroxyphenazine and riboflavin. Another similar compound, phylloquinone (Fig. 1), was also tested and showed the activity (Table 1). Menaquinone 7 is produced by spore forming soil *Bacillus* as the major homolog of menaquinones.[13] Phylloquinone, a major dietary source of vitamin K, occurs widely in green plants, algae and photosynthetic bacteria as a photosynthetic electron carrier. Menaquinones possessing varying chain lengths have been isolated from gram-positive bacteria, anaerobic and facultative nonphotosynthetic gram-negative bacteria and photosynthetic bacteria, and have the physiological activity of vitamin K.

Menaquinone 7 has been isolated from *Pseudomonas,诺卡菌属 restrictus* and *Arthrobacter simplex* and is shown to act as a natural electron acceptor for steroid ring A dehydrogenases, Δ1-dehydrogenase, Δ4,5α-dehydrogenase, Δ4,5β-dehydrogenase,[14,15] and the widely distributed microorganisms are known to contain these enzymes.[16] Furthermore, electron carriers, menadione, phenazine methosulfate, and

Cultivation of Strain SNU-299 and Isolation of Menaquinone 7 A loopful of stock culture of the strain SNU-299 was inoculated into two 250 ml Erlenmeyer flasks, each containing 50 ml of sterile 3% tryptic soy broth (soybean-casein digest medium, Difco) composed of trypotype (pancreatic digest of casein) 1.7%, soytone (papain digest of soybean meal) 0.3%, dextrose 0.25%, NaCl 0.5%, and K2HPO4 0.25% (pH 7.3 before sterilization). The flasks were incubated at 28°C for 4 d on a rotary shaker (200 rpm). The medium was transferred to ten 2000 ml Erlenmeyer flasks each containing 400 ml of the same type of sterile 3% tryptic soy broth. The flasks were shaken on a rotary shaker (200 rpm) at 28°C for 4 d. The whole broth (4 l, pH 8.5) was centrifuged at 10000 × g for 15 min, and the supernatant was filtered with filter paper. The filtrate was extracted with ethyl acetate (2 l), and the ethyl acetate extracts were concentrated in vacuo to give an oily residue. The residue was purified by preparative TLC performed on precoated Silica gel 60 F254 plates (E. Merck) using a mixture of ethyl acetate–methanol (9:1) as the developing solvent system. The Rf value of the biologically active component was ca. 0.67—0.72 in this solvent system. The eluate was concentrated in vacuo and the residue was further purified by preparative TLC using the same type of plates, and eluted with a mixture of ethyl acetate–cyclohexane (1:1) as the developing solvent system. The Rf value of the active component was ca. 0.78 in this solvent system. The eluate was concentrated in vacuo, dissolved in ethyl acetate, and concentrated under reduced pressure to give active fractions in the form of a yellowish solid. Finally, compound 1 (13.3 mg) was obtained as yellowish plates from the petroleum ether concentrates kept at 4°C.

Menaquinone 7 (1): Light yellow plates. 1H-NMR (CDCl3, δ: 1.52—1.98 (48H, 3'-Me, 4', 5', 7'-Me, 8', 9', 11'-Me, 12', 13', 15'-Me, 16', 17', 19'-Me, 20', 21', 23'-Me, 24', 25', 27'-Me, 28'), 2.19 (8, 3H, 3-Me), 3.37 (d, J=7.3 Hz, 2H, 1'), 5.11 (brs, 7H, 2', 6', 10', 14', 18', 22', 26'), 7.53 (m, 2H, 6, 7), 7.70 (m, 7H, 5, 8). IR (KBr) cm⁻1: 1660 (C=O), 1290, 670 (C-H). UV λmax (petroleum ether) nm (ε): 218 (12090), 241 (15490), 267 (15100), 322 (49040). MS m/z (rel. int. %): 648 (M⁺, 34), 646 (4), 633 (3), 551 (1), 550 (1), 476 (1), 474 (1), 443 (1), 376 (1), 375 (1), 368 (1), 307 (2), 239 (4), 225 (100), 187 (8), 149 (32), 121 (12), 81 (27), 69 (41), 68 (6).

[12]
2,6-dichlorophenolindophenol (Fig. 1) were able to replace menaquinone 7 in the reaction.\textsuperscript{1,12} Therefore, the inhibitory effect of these electron carriers on rat prostate testosterone 5α-reductase was tested, and the IC\textsubscript{50} values of the compounds are shown in Table 1. It is notable that all the compounds tested showed inhibitory activities, more or less. Menadione and phenazine methosulfate showed activities considerably more active than that of menaquinone 7 (1) itself, and the inhibitory activity of phenazine methosulfate was found to be more than 10-fold greater than that of pyrocyanine. It is notable that these electron carriers have a proportional relationship between inhibitory activities against testosterone 5α-reductase and stimulatory activities toward steroid ring A dehydrogenases\textsuperscript{13,17} in the order of phenazine methosulfate, menadione, and 2,6-dichlorophenolindophenol.

We also assayed a product of the 5α-reductase enzyme reaction and an electron and proton carrier, NADP\textsuperscript{+}, and the result is consistent with an earlier study (Table 1).\textsuperscript{19}

It is notable, however, that a proton carrier, CCCP, an uncoupler of oxidative phosphorylation,\textsuperscript{10} showed substantially inactive inhibitory activity (Table 1).

The above results indicate that the inhibitory effect of these inhibitory compounds is associated with the electron transfer process rather than proton transfer. One study showed that testosterone 5α-reductase would not catalyze the back reaction, dehydrogenation of reduced steroids,\textsuperscript{19} thus the inhibitory effect of these inhibitory compounds is thought to be due to the inhibition of the process of conversion of testosterone to 5α-dihydrotestosterone rather than stimulation of the back process. Another experiment showed that the electrons are transferred from NADPH to coenzyme Q\textsubscript{10} via NADPH:cytochrome oxidoreductase and then to testosterone by a testosterone 5α-reductase,\textsuperscript{20} thus these electrons being transferred from NADPH to testosterone are thought to be transferred to these inhibitory compounds, which passes them over to an electron transport chain. The fact that the co-factor-binding domain of the rat and human testosterone 5α-reductase isoforms represents a novel structure which does not contain NADPH selectivity residues identified in other reductase enzymes\textsuperscript{21} may explain why these compounds are inhibitors of testosterone 5α-reductase. Therefore, it may be worthwhile investigating the activity of other analogs of these inhibitory compounds in order to study the structure-activity relationship for increased testosterone 5α-reductase inhibition activity. It would be of interest to examine these inhibitory compounds in vivo experiments.

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