WS-9659 A AND B, NOVEL TESTOSTERONE 5α-REDUCTASE INHIBITORS ISOLATED FROM A *STREPTOMYCES*

I. TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL CHARACTERISTICS

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WS-9659 A and B, produced by *Streptomyces* sp. No. 9659, were extracted from cultured broth, purified by solvent extraction followed by chromatography on silica gel and then isolated as prisms ($C_{22}H_{24}N_2O$, mp 161~162°C, $C_{22}H_{23}N_2OCl$, mp 152~153°C). WS-9659 A and B have testosterone 5α-reductase inhibitory activity. The IC_{50} values of WS-9659 A and B for partially purified rat prostate testosterone 5α-reductase were 5.0×10^{-7} M and 1.0×10^{-5} M, respectively.

Benign prostate hyperplasia is the most common disorder of the urinary tract in men over the age of 40 years. It is generally believed that the growth of the prostate is mediated by 5α-dihydrotestosterone. The current view of androgen action in many tissues, such as the prostate and the seminal vesicles, involves the conversion of testosterone, the major circulating androgen in adult males, to 5α-dihydrotestosterone by the enzyme testosterone 5α-reductase[1].

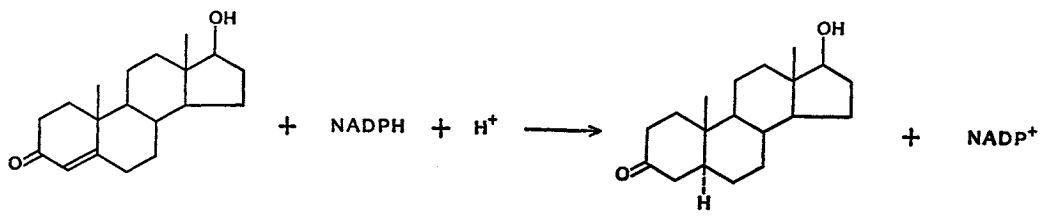
Testosterone 5α-reductase (EC 1.3.1.22) catalyzes the conversion of testosterone to 5α-dihydrotestosterone (Fig. 1). Potent steroidal inhibitors of testosterone 5α-reductase, such as *N,N*-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (4-MA) have recently been developed[2].

Therefore, the aim of our screening is to find nonsteroidal testosterone 5α-reductase inhibitors.

During the course of our research program for discovery of inhibitors which are natural products, we found the new nonsteroidal testosterone 5α-reductase inhibitors, WS-9659 A and B from the fermentation broth of a strain of *Streptomyces*.

In this paper, we describe the taxonomy of the producing strain, the fermentation, the isolation and the physico-chemical properties of WS-9659 A and B. Detailed studies of the biological and pharmacological characteristics of WS-9659 A and B will be discussed in an accompanying paper[3].

Fig. 1. The pathway from testosterone to 5α-dihydrotestosterone.



Testosterone 5α-Dihydrotestosterone

Materials and Methods

Taxonomic Studies

Strain No. 9659 was isolated from a soil sample obtained from Tsukuba, Ibaraki, Japan. The methods described by SHIRLING and GOTTLIEB[4] were employed for the taxonomic study. Morphological observations were made with light and electron microscopes from cultures grown at 30°C for 21 days on yeast extract - malt extract agar, inorganic salts - starch agar, oatmeal agar and glucose - asparagine agar. Production of mature sporulating aerial mycelium was poor on the various media tested.

Cultural characteristics were observed on ten media described by SHIRLING and GOTTLIEB[4], and WAKSMAN[5]. Incubation was at 30°C for 21 days. The color names used in this study were taken from the Methuen Handbook of Colour[6].

Wall analysis was performed by the methods of BECKER *et al.*[7], and YAMAGUCHI[8].

The temperature range for growth was determined on yeast extract - malt extract agar using a temperature gradient incubator (Advantec Toyo Co., Ltd.).

Utilization of carbon sources was examined according to the method of PRIDHAM and GOTTLIEB[9].

Fermentation

A loopful of *Streptomyces* sp. No. 9659 on mature slant culture was transferred into twenty five 500-ml Erlenmeyer flasks each containing 160 ml of sterile seed medium composed of corn starch 1%, cotton seed flour 1%, dried yeast 0.5%, corn steep liquor 0.5% and $CaCO_3$ 0.2%. The medium was adjusted to pH 6.5 prior to the addition of $CaCO_3$. The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm-throw) for 4 days at 30°C. The content of the flasks was used to inoculate 160 liters of fermentation medium in a 200-liter jar fermenter. The composition of the production medium was as follows: Soluble starch 2%, corn starch 1%, cotton seed flour 1%, corn steep liquor 0.5%, dried yeast 0.5%, NaCl 0.1% and $MgSO_4 \cdot 7H_2O$ 0.05%. pH was adjusted to 6.5 and then $CaCO_3$ 0.2% was added before sterilization.

Preparation of Testosterone 5α-Reductase

Mature Sprague-Dawley male rats (7~8 weeks old) were sacrificed by diethyl ether. The ventral prostates were dissected to be free of their capsules and their combined volume was measured by displacement in several ml of ice-cold medium (0.32 M sucrose, 0.1 mM dithiothreitol and 20 mM sodium phosphate, pH 6.5). Unless specified, all the following procedures were carried out at 0~4°C. The prostates were minced and then homogenized in 3~4 tissue volumes of medium A with Pyrex-glass homogenizer.

The homogenate was fractioned by differential centrifugation at $3,000 \times g$ for 15 minutes. The resulting pellets were resuspended in medium A. The suspension (20~30 mg protein/ml) was stored at −80°C.

Testosterone 5α-Reductase Assay

Reaction solution contains 1 mM dithiothreitol, 40 mM sodium phosphate pH 6.5, 50 μM NADPH, [1,2,6,7-^{3}H]testosterone (85~105 Ci/mmol, New England Nuclear)/testosterone (2.2×10^{-8} M) and the suspension prepared above (0.8 mg of protein) in a total volume of 565 μl. WS-9659 A and B were added each in 10 μl of 10% ethanol whereas control tubes received the same volume of 10% ethanol. The reaction was started with the addition of the suspension. After incubation at 37°C for 30 minutes, the reaction was extracted with 1 ml of ethyl acetate. Fifty μl of ethyl acetate phase was chromatographed on a Merck silica plastic sheet Kieselgel 60 F_{254} using ethyl acetate - cyclohexane (1 : 1) as the developing solvent system. The plastic sheet was air dried and then cut into the testosterone and the 5α-dihydrotestosterone areas. The radioactivity was counted in 5 ml of Aquazol-2 (New England Nuclear) with a Packard scintillation counter (Packard TRI-CARB 4530).

Results

Taxonomic Studies on Strain No. 9659

The aerial mycelium branched monopodially and formed spiral chains of spores with 10 to 50

Fig. 2. Scanning electron micrograph of spore chains of strain No. 9659 on inorganic salts - starch agar.

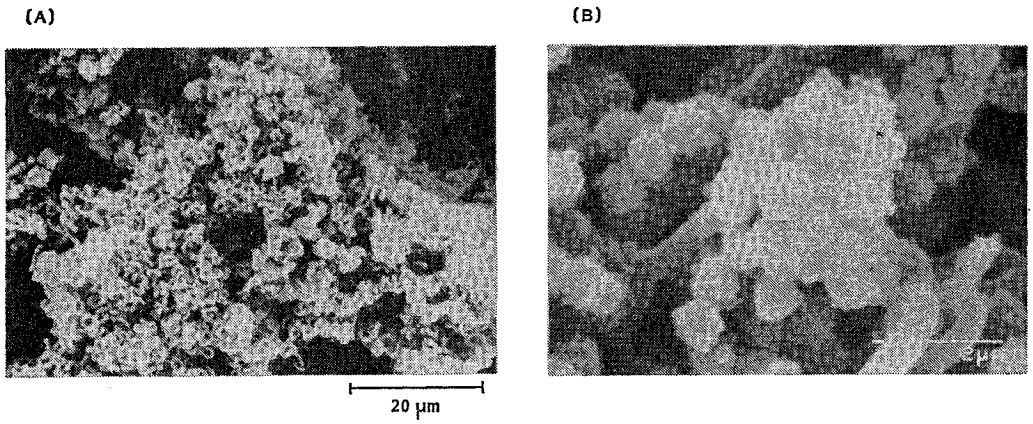


Table 1. Cultural characteristics of strain No. 9659.

Medium	Cultural characteristics
Yeast extract - malt extract agar	G: Moderate
	A: Poor, yellowish white (4A2) to orange white (5A2)
	R: Dark blue (22F5)
	S: None
Oatmeal agar	G: Moderate
	A: Poor, orange white (5A2)
	R: Brownish gray (7C2)
	S: None
Inorganic salts - starch agar	G: Moderate
	A: Poor, yellowish white (4A2) to orange white (5A2)
	R: Dark brown (9F5)
	S: Pale pink (8A3)
Glycerol - asparagine agar	G: Moderate
	A: None
	R: Reddish brown (8E6)
	S: Pale pink
Peptone - yeast extract - iron agar	G: Moderate
	A: None
	R: Dark brown (8F6)
	S: Dark brown
Tyrosine agar	G: Moderate
	A: None
	R: Brown (7E6)
	S: Dark brown
Glucose - asparagine agar	G: Moderate
	A: None
	R: Orange white (5A2)
	S: Pale pink
Nutrient agar	G: Moderate
	A: None
	R: Light brown (6D4)
	S: None
BENNET's agar	G: Moderate
	A: None
	R: Brown (6E5)
	S: Pale pink
Sucrose - nitrate agar	G: Moderate
	A: None
	R: Gray (6D1)
	S: None

Abbreviations: G, growth; A, aerial mycelium color; R, reverse side color; S, soluble pigment.

spores in each chain (Fig. 2A). Many ball-like bodies with various shape and size were observed on the aerial mycelium. The spores had a warty surface and were spherical in shape with a size of 0.6~0.7×0.6~0.8 μm (Fig. 2B). Neither fragmentation of hyphae nor formation of spores occurred in the substrate mycelium. Sclerotia and zoospores were not observed.

The aerial mass color may be in the yellow color-series (yellowish or orange white) on yeast extract - malt extract agar, oatmeal agar and inorganic salts - starch agar.

Melanoid pigments were formed in peptone - yeast extract - iron agar, tyrosine agar or Tryptone - yeast broth. Soluble pigment of pale pink was found in the medium in inorganic salts - starch agar, glycerol - asparagine agar, glucose - asparagine agar and BENNET's agar. Results are shown in Table 1.

Analysis of whole-cell hydrolysates showed the presence of LL-diaminopimeric acid. Accordingly, the cell wall of this strain is classified as Type I.

Summarized physiological properties of strain No. 9659 are shown in Table 2. The temperature range for growth was from 22 to 35°C with optimum temperature from 30 to 33°C. Starch hydrolysis was positive. Utilization of carbon sources is shown in Table 3. Referring to the BERGEY's Manual[10] or the International Streptomyces Project (ISP)[8~10] report about the results of the taxonomic studies presented here, we conclude that strain No. 9659 belongs to the genus *Streptomyces* Waksman and Henrici 1943, 339.

The strain is to be deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. No. 9659.

Table 2. Physiological properties of strain No. 9659.

Conditions	Characteristics
Temperature range for growth	22~35°C
Optimum temperature	30~33°C
Starch hydrolysis	Positive
Milk coagulation	Negative
Milk peptonization	Negative
Production of melanoid pigment	Positive
Gelatin liquefaction	Negative
Decomposition of cellulose	Negative

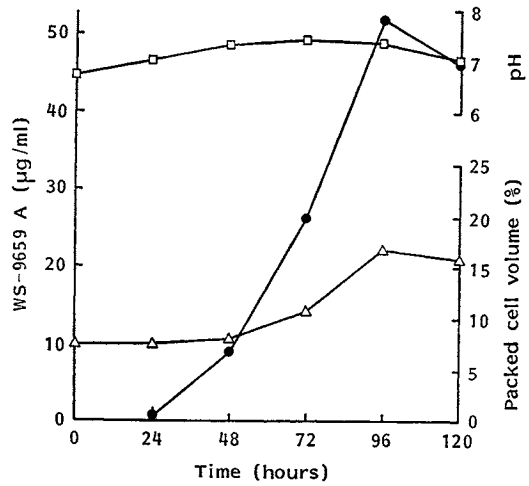
Table 3. Carbon utilization of strain No. 9659.

Compounds	Growth
D-Glucose	+
Sucrose	±
D-Xylose	+
D-Fructose	±
L-Rhamnose	+
Raffinose	+
L-Arabinose	+
Inositol	+
Mannitol	+

+, Utilization; ±, doubtful utilization.

Fig. 3. Time course of fermentation.

□ pH, ● potency, △ packed cell volume.



Fermentation was carried out in a 200-liter jar fermenter under conditions described in Materials and Methods.

WS-9659 A potency was measured as testosterone 5α-reductase inhibition activity. Growth was expressed as the packed cell volume which was determined by centrifugation at 2,000 rpm for 20 minutes.

Production of WS-9659 A

Fig. 3 presents the data from a typical 200-liter fermentation and gives information regarding WS-9659 A production, pH and packed cell volume. The pH of the fermentation broth initially increases from 6.8 to 7.4 and after 72 hours begins to decrease. WS-9659 A production begins after about 30 hours and reaches a maximum of 40~50 μg/ml by 96 hours and then declines slightly.

Isolation and Purification

The isolation scheme is shown in Fig. 4.

The cultured broth was filtrated with the aid of diatomaseous earth (4 kg). The cultured filtrate (100 liters) was adjusted to pH 7.0 and extracted with ethyl acetate (50 liters). The mycelial cake was extracted with acetone (100 liters). The acetone extract was concentrated *in vacuo* to give an aqueous solution (4 liters). After adjusting to pH 7.0, the active component was extracted with ethyl acetate (20 liters). The extracts of the cultured filtrate and mycelia were combined and concentrated *in vacuo* to give an oily residue, which was mixed with 500 g of silica gel (Kieselgel 60, 70~230 mesh, E. Merck) and slurried in methanol. After evaporating the solvent, the resultant dry powder was subjected to column chromatography using the same type of silica gel (1.5 liters, column size; 11 × 16 cm) packed with *n*-hexane. The column was washed with ethyl acetate and eluted with a mixture of ethyl acetate and acetone (4 : 1). The fractions containing active compounds were evaporated and dissolved in a mixture of chloroform and methanol (100 : 1) and applied to silica gel (Kieselgel 60, 230~400 mesh, 200 ml column size: 5 × 40 cm) which was packed with the same solvent.

The column was developed with the same solvent system. The active fractions were evaporated *in vacuo* to give a bluish powder. This powder was dissolved in methanol and was applied to NS gel (Nippon Seimitsu Co., Ltd.).

The active fractions were collected and concentrated under reduced pressure to give WS-9659 A

Fig. 4. Isolation procedure of WS-9659 A and B.

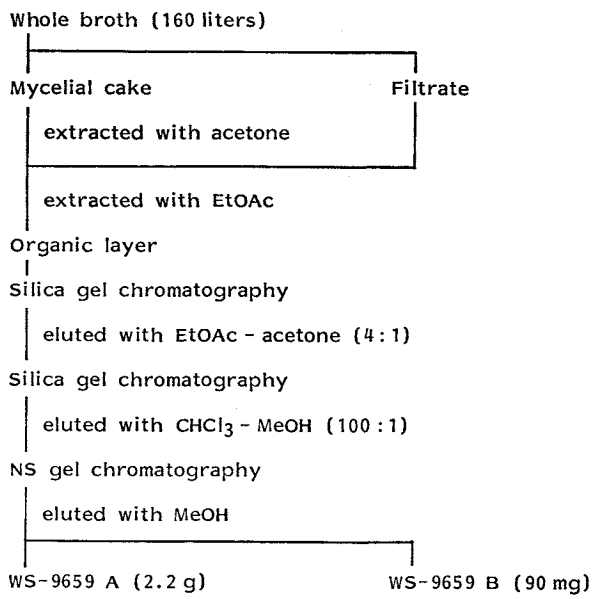


Fig. 5. IR spectrum of WS-9659 A in $CHCl_3$.

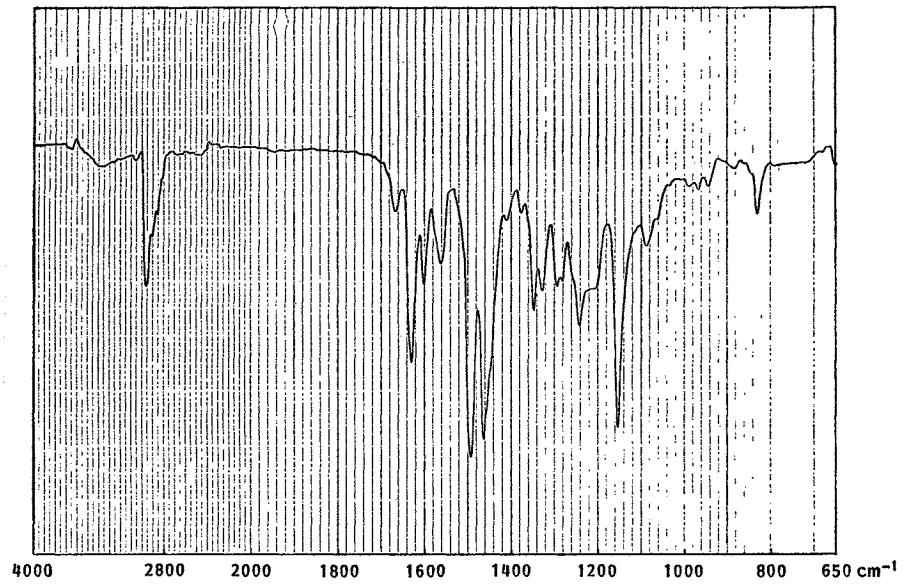
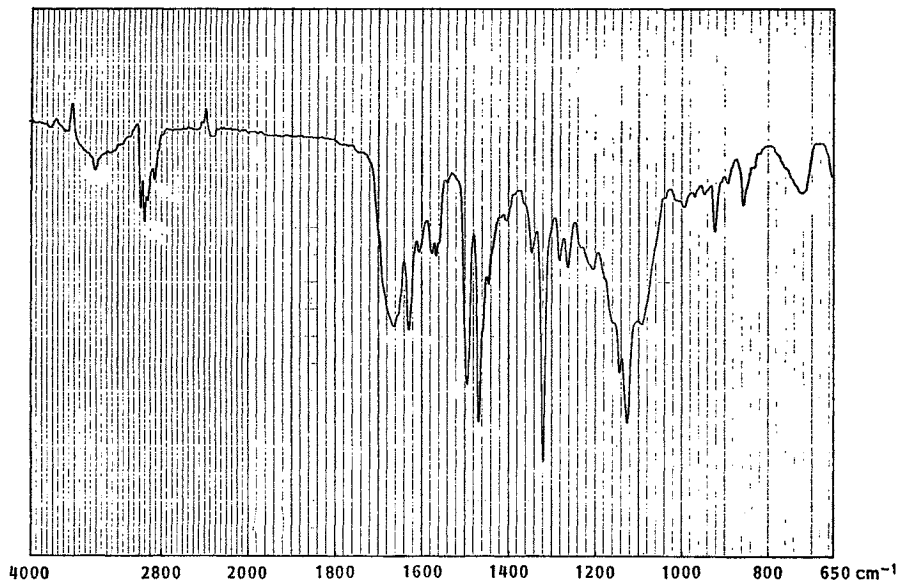


Fig. 6. IR spectrum of WS-9659 B in $CHCl_3$.



and B in the form of bluish powders. Each of the powders was dissolved in diethyl ether and methanol and concentrated under reduced pressure. These concentrates were kept at 4°C. Purified WS-9659 A (2.2 g) was obtained as deep blue prisms and the purified WS-9659 B (90 mg) was obtained as bluish prisms.

Physico-chemical Properties

The IR spectra of WS-9659 A and B are shown in Figs. 5 and 6. ^1H NMR spectra of WS-9659

Fig. 7. ^{1}H NMR spectrum of WS-9659 A in $CDCl_3$.

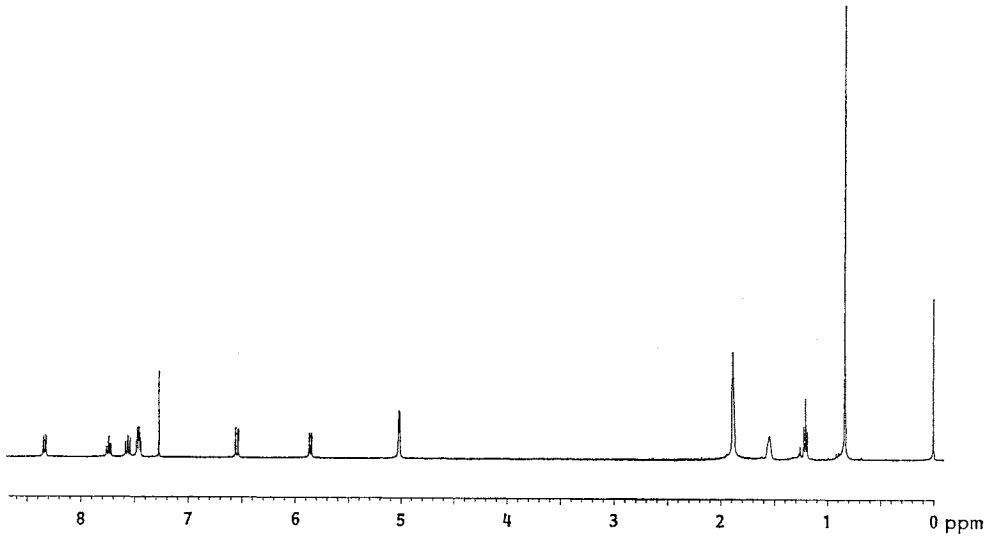
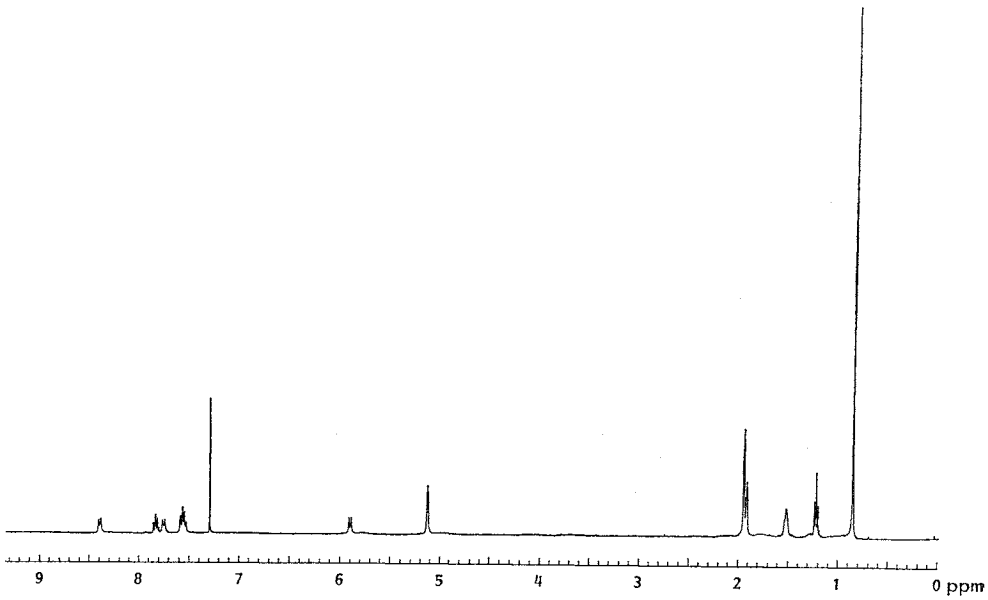


Fig. 8. ^{1}H NMR spectrum of WS-9659 B in $CDCl_3$.



A and B are shown in Figs. 7 and 8. The Rf values of WS-9659 A and B on silica gel TLC developed with benzene - acetone (1 : 1) were 0.3 and 0.47, respectively.

WS-9659 A and B were, amphoteric, deep bluish prisms, which are soluble in alcohol, acetone, ethyl acetate and chloroform, while only slightly soluble in *n*-hexane. These substances showed positive reactions to iodine and cerium sulfuric acid on silica gel TLC plates. These results as well as other physico-chemical properties are summarized in Table 4. The structures are shown in Fig. 9.

Biological Properties

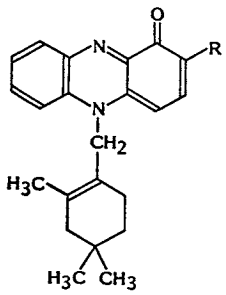
The effect of WS-9659 A and B on rat prostate testosterone 5α-reductase was tested. The IC_{50}

Table 4. Physico-chemical properties of WS-9659 A and B.

	WS-9659 A	WS-9659 B
Appearance	Deep blue prisms	Deep blue prisms
MP (°C)	161~162	152~153
Molecular formula	$C_{22}H_{24}N_2O$	$C_{22}H_{23}N_2OCl$
FAB-MS (M+H)	333	367
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	238 (650), 320 (700)	241 (420), 328 (560)
Elementary analysis	Calcd for $C_{22}H_{24}N_2O$	Calcd for $C_{22}H_{23}N_2OCl$
Calcd:	C 79.48, H 7.28, N 8.43	C 72.02, H 6.32, N 7.64, Cl 9.66
Found:	C 79.13, H 7.13, N 8.40	C 71.82, H 6.37, N 7.86, Cl 9.60
Color reaction		
Positive:	Cerium sulfate, iodine vapor	Cerium sulfate, iodine vapor
Negative:	Ninhydrin, Molisch, Ehrlich	Ninhydrin, Molisch, Ehrlich
Solubility		
Soluble:	MeOH, EtOH, Me_2CO, EtOAc, $CHCl_3$	MeOH, EtOH, Me_2CO, EtOAc, $CHCl_3$
Slightly soluble:	Diethyl ether, benzene	Diethyl ether, benzene
Insoluble:	Water, *n*-hexane	Water, *n*-hexane

FAB: Fast atom bombardment.

Fig. 9. Structures of WS-9659 A and B.



WS-9659 A R=H
WS-9659 B R=Cl

Table 5. Antimicrobial activities of WS-9659 A and B.

Strains	MIC (μg/ml)	
	WS-9659 A	WS-9659 B
Bacillus subtilis ATCC 6633	1	2
Staphylococcus aureus 209P JC-1	4	8
Aureobasidium pullulans IFO 4466	2	4
Escherichia coli NIHJ JC-2	>100	>100
Candida albicans	>100	>100

values of WS-9659 A and B were 5.0×10^{-7} M and 1.0×10^{-5} M, respectively.

Antimicrobial activities of WS-9659 A and B were evaluated by a serial broth dilution method. Table 5 shows the antimicrobial activities. WS-9659 A and B revealed antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Aureobasidium pullulans*, but was not effective against *Escherichia coli* and *Candida albicans* at 100 μg/ml. The LD_{50} of WS-9659 A was above 100 mg/kg (ip) when tested in *dd*Y mice.

Discussion

Enzyme inhibitors such as protease inhibitors, glucosidase inhibitors have been identified as pharmacological agents from microbial origin. We have searched for new compounds which have testosterone 5α-reductase inhibition activity from microbial origin using a rat prostate preparation. WS-9659 A and B were isolated as a highly potent testosterone 5α-reductase inhibitors through our screening program.

It is very interesting that 4-MA steroids, which are known to be highly potent inhibitors of testosterone 5α-reductase, have steroidal structures, while WS-9659 A has a nonsteroidal structure and is highly potent in the testosterone 5α-reductase inhibition assay.

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