WB2838 [3-CHLORO-4-(2-AMINO-3-CHLOROPHENYL)-PYRROLE]: NON-STEROIDAL ANDROGEN-RECEPTOR ANTAGONIST PRODUCED BY A *Pseudomonas*

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(Received for publication January 11, 1993)

In the course of our search for non-steroidal androgen-receptor antagonists of microbial origin, *Pseudomonas* sp. No. 2838 was found to produce an inhibitor of androgen binding to its receptor. This compound, named WB2838, was isolated and identified as 3-chloro-4-(2-amino-3-chlorophenyl)-pyrrole.

The IC_{50} value of WB2838 for partially purified rat prostate cytosol receptor was 8.0×10^{-7} M. However, the IC_{50} value of WB2838 against estrogen-receptor binding was about 90-fold greater than that against androgen-receptor binding. WB2838 inhibited the growth of androgen-responsive mouse mammary carcinoma SC-3 cells in the presence of 10^{-8} M testosterone at IC_{50} value of 4.1×10^{-7} M. This inhibition was reversed by adding 10^{-5} M testosterone to the culture medium. WB2838 also showed the inhibitory activity against the growth of the ventral prostate induced by testosterone propionate in castrated immature rats.

Therefore, it was concluded that WB2838 was a non-steroidal androgen-receptor antagonist.

Androgen plays an important role in the prostatic growth including benign prostatic hyperplasia and prostate cancer. Androgen actions are thought to be mediated through binding to its own receptor. Therefore, androgen-receptor antagonists can be used in the treatment for androgen-responsive diseases.

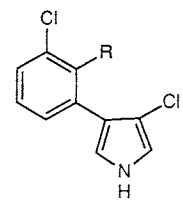
Until now, several androgen-receptor antagonists have been developed and clinically used[1]. They can be divided into two classes based on their chemical structures: steroid (exemplified by cyproterone acetate (CPA) and chlormadinone acetate (CMA)) or non-steroid (exemplified by flutamide).

So, the aim of our screening is to find new non-steroidal androgen-receptor antagonists which do not belong to the groups of the currently known androgen-receptor antagonists.

During the course of our search for androgen-receptor binding inhibitors using receptor binding assay, we isolated WB2838 from the cultured broth of *Pseudomonas* sp. No. 2838. WB2838 was identified as 3-chloro-4-(2-amino-3-chlorophenyl)-pyrrole (Fig. 1)[2]. This compound is known as an antifungal antibiotic[3].

In this paper, we describe new biological and pharmacological activities of WB2838 as a non-steroidal androgen-receptor antagonist.

Fig. 1. Structures of WB2838 and pyrrolnitrin.



WB2838 $R = NH_2$
Pyrrolnitrin $R = NO_2$

Materials and Methods

Isolation of WB2838

Strain No. 2838 was isolated from a soil sample obtained at Shizuoka Prefecture, Japan.

Characterization of the strain was performed

according to the methods mentioned in the Volume 1 of BERGEY's Manual of Systematic Bacteriology[4]. Consequently, strain No. 2838 was classified to the genus *Pseudomonas* and designated *Pseudomonas* sp. No. 2838.

A loopful of slant culture of strain No. 2838 was inoculated into each of three 500-ml flasks containing 160 ml of a sterile seed medium consisting of yeast extract 1%, Nutrient Broth (Kyokuto Seiyaku Kogyo Co., Ltd., Tokyo, Japan) 1% and NaCl 1%. The flasks were shaken on a rotary shaker (220 rpm, 7.5 cm-throw) at 30°C for 24 hours. The resultant seed culture was inoculated into a 30-liter jar fermentor containing 20 liters of a sterile production medium consisting of glucose 2%, glycerin 1%, soybean meal 1%, Nutrient Broth 0.5%, corn steep liquor 1%, $(NH_4)_2SO_4$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.06%, $CaCO_3$ 0.2%, Adekanol (LG109, Asahi Denka Kogyo Co., Ltd., Tokyo, Japan) 0.05% and Silicone (KM-70, Shin-etsu Kagaku Kogyo Co., Ltd., Tokyo, Japan) 0.05% (pH 7.0). The fermentation was carried out at 30°C under aeration of 20 liters/minute and agitation of 200 rpm for 48 hours.

The cultured broth of 10 jar fermentors (200 liters) was mixed with 300 liters of acetone and filtered with the aid of diatomaceous earth (10 kg). The acetone extract was concentrated to give 180 liters of an aqueous solution, adjusted to pH 7.0 with 6 N HCl and extracted with EtOAc (70 liters × 3). The EtOAc extracts were combined, concentrated to give an oily residue, mixed with 300 ml of Silica gel 60 (70~230 mesh, E. Merck, Darmstadt, F.R.G.) and subjected to silica gel (400 ml) column pre-packed with *n*-hexane. The column was washed with *n*-hexane (2 liters) and *n*-hexane - EtOAc (5 : 1, 1.5 liters). Then, the active fractions were eluted with *n*-hexane - EtOAc (5 : 1, 2.4 liters) and concentrated to give an oily residue. This oily residue was dissolved in 20 ml of CH_2Cl_2 and applied to silica gel (400 ml) column pre-packed with CH_2Cl_2. The column was washed with 600 ml of CH_2Cl_2 and then eluted with 250 ml of CH_2Cl_2. The fractions containing the objective compound were combined and concentrated to give an oily residue. The oily residue was dissolved in 10 ml of MeOH and subjected to YMC gel ODS-A60 (60/200 mesh, YMC Co., Ltd., Kyoto, Japan, 180 ml) column pre-packed with 70% aqueous MeOH. The column was washed with 250 ml of 70% aqueous MeOH and then eluted with the same solvent. The active fractions were combined and concentrated to give a pale yellow powder. This powder was recrystallized from MeOH - H_2O to give WB2838 substance as pale yellow needles (535 mg).

WB2838: FAB-MS *m/z* 227 $(M+H)^+$; elemental analysis (%) C 53.09, H 3.64, Cl 31.09, N 12.18 (C 52.89, H. 3.55, Cl 31.32, N 12.34 calcd for $C_{10}H_8Cl_2N_2$); UV λ_{max}^{MeOH} nm (log ε) 216 (4.33), 298 (3.59); IR ν_{max}^{KBr} cm^{-1} 3480, 3400, 1610, 1590, 1450, 1300, 1100, 1070, 970, 890; ^1H NMR ($CDCl_3$) δ_H 4.13 (2H, br s), 6.71 (1H, t, J=7.5 Hz), 6.78 (1H, t, J=2.5 Hz), 6.82 (1H, t, J=2.5 Hz), 7.08 (1H, dd, J=1.5, 7.5 Hz), 7.23 (1H, dd, J=1.5, 8 Hz), 8.38 (1H, br s); ^{13}C NMR ($CDCl_3$) δ_C 111.9 (s), 116.2 (d), 117.1 (d), 118.0 (d), 119.0 (s), 119.4 (s), 120.2 (s), 128.4 (d), 130.0 (d), 141.6 (s).

The structure of WB2838 was elucidated to be 3-chloro-4-(2-amino-3-chlorophenyl)-pyrrole[2,3] on the basis of above-mentioned physico-chemical properties and spectroscopic data.

Drugs

Flutamide and pyrrolnitrin were synthesized in our laboratories. Testosterone, testosterone propionate and CMA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). For *in vitro* experiments, testosterone, WB2838, pyrrolnitrin, flutamide and CMA were dissolved in ethanol and further diluted in buffer or medium. For *in vivo* experiment, WB2838 and testosterone propionate were dissolved in sesame oil.

Animals

Specific pathogen free (SPF) Sprague-Dawley rats (male and female, 7~8 weeks old) were purchased from Japan S.L.C. (Shizuoka, Japan). SPF Wistar rats (male, 4 weeks old) were purchased from Japan Clea Inc. (Tokyo, Japan).

Androgen-receptor Binding Assay

The androgen-receptor binding assay was performed according to the method of NAKAYAMA *et al.*[5], which was modified as described below. The details are as follows. Prostate cytosol fraction was used as source of androgen-receptors. Mature Sprague-Dawley male rats (7~8 weeks old) were castrated and 24 hours later, the rats were sacrificed. Unless specified, all the following procedures were carried out at

0~4°C. Ventral prostates excised from the rats were dissected to be free of their capsules, minced and homogenized in ice-cold buffer consisting of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM Na_2MoO_4, 5 mM dithiothreitol, 10% (v/v) glycerol, 25 μg/ml α_1-antitrypsin and 25 μg/ml leupeptin with a Polytron homogenizer[6]. Cytosol was obtained by centrifuging the homogenate at 108,000 × *g* for 60 minutes and stocked at −80°C. The protein concentration of cytosol was determined using BioRad Protein Assay Kit (Bio-Rad, Richmond, CA, U.S.A.).

The reaction mixture consisting of 10 μl of a test sample, 100 μl of [^{3}H]mibolerone (3.22 TBq/mmol, New England Nuclear, Wilmington, DE, U.S.A., 64.3 KBq/ml)[7] and 100 μl of rat prostate cytosol (15~20 mg protein/ml) was incubated in a test tube at 0°C for 4 hours. Five μM triamcinolone acetonide was also included to inhibit the binding of radioactive ligand to both progesterone- and glucocorticoid-receptor[7]. After incubation, 200 μl of dextran-coated charcoal solution consisting of 0.5% Norit A (Nakarai Chemicals, Ltd., Kyoto, Japan) and 0.05% Dextran T-70 (Pharmacia Fine Chemicals, Ltd., Uppsala, Sweden) was added to the reaction mixture and further incubated at 0°C for 10 minutes. Then, the mixture was centrifuged at 3,000 rpm for 5 minutes. The radioactivity in 100 μl of the supernatant was counted in 10 ml of Aquazol-2 (New England Nuclear) with a liquid scintillation counter. The specific binding was calculated by subtracting the non-specific binding of [^{3}H]mibolerone in the presence of 1,000-fold molar excess of unlabeled testosterone from the total binding. All *in vitro* experiments were performed in triplicate.

Estrogen-receptor Binding Assay

The estrogen-receptor binding assay was performed according to the method described previously[8].

Effect on the Growth of SC-3 Cells

Androgen-responsive mouse mammary carcinoma SC-3 cells (one of the cloned cell lines obtained from Shionogi carcinoma 115 cells was kindly gifted from Dr. K. MATSUMOTO, Osaka University Medical School, Osaka, Japan) were maintained *in vitro* by serial passage in EAGLE's minimum essential medium (Flow Laboratories, Rockville, MD, U.S.A.) supplemented with 50 U/ml benzylpenicillin, 50 μg/ml streptomycin, 2% dextran-coated charcoal treated-fetal bovine serum (Gibco, Grand Island, NY, U.S.A.) and 10^{-8} M testosterone[9]. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 - 95% air.

Effects on the growth of SC-3 cells were tested in 96-well microtiter plates, with each well containing 4×10^3 SC-3 cells in 100 μl of the serum-free medium [HAM's F-12 (Flow Laboratories): EAGLE's minimum essential medium (1 : 1, v/v) containing 0.1% bovine serum albumin] in the presence of 10^{-8} M, 10^{-5} M or no testosterone[10]. The cells were cultured at 37°C for 4 days and then the growth of the cells was measured by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay described previously[8].

Effect on the Growth of the Ventral Prostate in Immature Rats

Wistar male rats (4 weeks old, weighing approximately 90 g) were castrated. Three days later, 320 μg/kg of testosterone propionate suspended in sesame oil were injected subcutaneously to the rats. At the same time, WB2838 dissolved in sesame oil were administered orally to the rats. The administration of testosterone propionate and WB2838 were repeated once a day for 5 consecutive days. Five rats were used in each group. Six hours after the last administration, the rats were sacrificed by decapitation. Then the ventral prostates and seminal vesicles were excised and weighed.

Results

Effect of WB2838 on Androgen-receptor Binding

WB2838 inhibited androgen-receptor binding in a dose dependent manner (Fig. 2). The IC_{50} value of WB2838 was 8.0×10^{-7} M. The activity of WB2838 was more potent than that of flutamide, but less potent than that of CMA (Table 1). The activity of pyrrolnitrin[11], which is known to be potent antifungal agent and replaces amino group of WB2838 with nitro group, was less potent than that of WB2838.

Fig. 2. Inhibitory effects of WB2838, pyrrolnitrin, flutamide and chlormadinone acetate (CMA) on androgen-receptor binding.

○ WB2838, ● pyrrolnitrin, □ flutamide, ■ CMA.

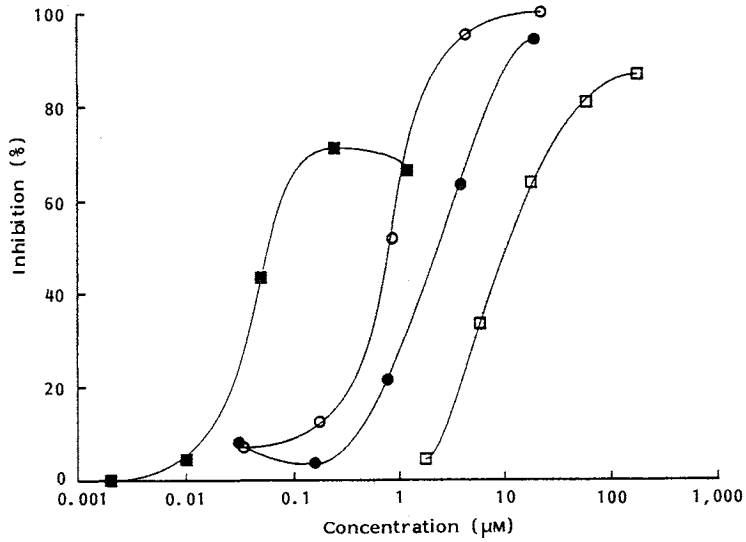
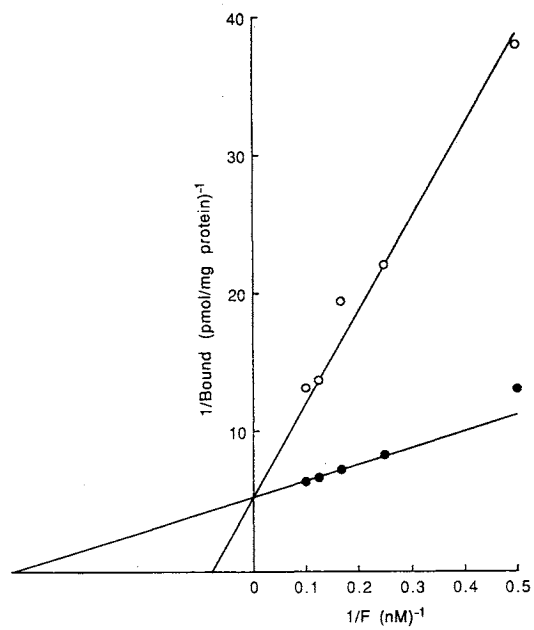


Table 1. Inhibition of androgen- and estrogen-receptor binding by WB2838, pyrrolnitrin, flutamide and chlormadinone acetate (CMA).

Drug	IC_{50} (M)	
	Androgen-receptor	Estrogen-receptor
WB2838	8.0×10^{-7}	7.5×10^{-5}
Pyrrolnitrin	2.4×10^{-6}	1.3×10^{-5}
Flutamide	1.2×10^{-5}	$>1.0\times10^{-4}$
CMA	7.4×10^{-8}	$>1.0\times10^{-4}$

Fig. 3. Lineweaver-Burk plot for inhibition of androgen-receptor binding by WB2838.

● No WB2838, ○ WB2838 1.1×10^{-6} M.



WB2838 showed very weak inhibitory activity against estrogen-receptor binding as shown in Table 1. The IC_{50} value of WB2838 against androgen-receptor binding was about 90-fold lower than that against estrogen-receptor binding. Thus, androgen-receptor binding of WB2838 has a high degree of selectivity.

Lineweaver-Burk plot for inhibition of androgen-receptor binding by WB2838 is shown in Fig. 3. This kinetic analysis suggested that WB2838 was a competitive inhibitor of androgen-receptor binding. An apparent *Ki* value of WB2838 was calculated to be 6.8×10^{-7} M by assessing Dixon plot for inhibition of androgen-receptor binding (data not shown).

Effects of WB2838 on the Growth of SC-3 Cells *In Vitro*

In an attempt to determine whether WB2838 was androgen-receptor antagonist or agonist, we

investigated the effects of WB2838 on the growth of androgen-responsive mouse mammary carcinoma SC-3 cells *in vitro*[9,10]. As shown in Fig. 4, WB2838 stimulated slightly the growth of SC-3 cells in the absence of testosterone, but inhibited the growth of them in the presence of 10^{-8} M (physiological concentration) testosterone in a dose dependent manner. Its IC_{50} value was 4.1×10^{-7} M. The inhibitory activity of WB2838 was more potent than those of pyrrolnitrin and flutamide, but slightly less potent than that of CMA (Table 2). The inhibitory activity of WB2838 against the growth of SC-3 cells was dramatically reversed in the presence of 10^{-5} M (1,000-fold excess of physiological concentration) testosterone (Fig. 4). The same reverse of the inhibitory activities were observed with both flutamide and CMA (Table 2). The activity of pyrrolnitrin was slightly reversed. These results

Table 2. Effects of WB2838, pyrrolnitrin, flutamide and CMA on the growth of SC-3 cells.

Drug	T: free	IC_{50} (M)	
		T: 10^{-8} M	T: 10^{-5} M
WB2838	Weak agonist ($>1.4 \times 10^{-7}$ M)	4.1×10^{-7}	1.8×10^{-5}
Pyrrolnitrin	No effect	2.1×10^{-6}	1.1×10^{-5}
Flutamide	No effect	3.0×10^{-6}	3.3×10^{-5}
CMA	Weak agonist ($>2.5 \times 10^{-6}$ M)	7.2×10^{-8}	4.8×10^{-6}

T: Testosterone.

Fig. 4. Effects of WB2838 on the growth of mouse mammary carcinoma SC-3 cells.

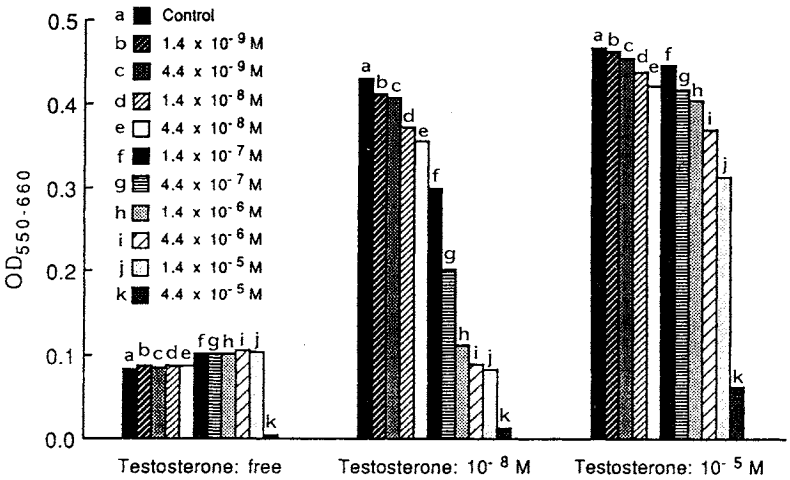


Table 3. Effects of WB2838 on the weight of rat ventral prostates and seminal vesicles.

Drug	Ventral prostate		Seminal vesicle	
	Organ weight (mg/100 g body)	Inhibition (%)	Organ weight (mg/100 g body)	Inhibition (%)
Testosterone propionate	43.6±2.4	0.0	55.4±1.6	0.0
Castration only	11.6±0.9	100.0	14.3±0.8	100.0
Testosterone propionate + WB2838 3.2 mg/kg	41.5±2.5	6.6	44.3±1.3***	27.0
Testosterone propionate + WB2838 10 mg/kg	43.6±2.5	0.0	45.9±2.2**	23.1
Testosterone propionate + WB2838 32 mg/kg	34.4±1.5*	28.8	46.7±2.4*	21.2

For statistical significance, the STUDENT's *t*-test was analyzed against the group given testosterone propionate alone, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

suggested that WB2838 was androgen-receptor antagonist just as flutamide and CMA, but also characteristic of fairly weak partial agonist.

Effect of WB2838 on the Growth of Ventral Prostate in Immature Rats

The androgen-receptor antagonistic activity of WB2838 was evaluated *in vivo*. Injection of testosterone caused the hypertrophy of ventral prostate and seminal vesicle to the castrated immature male rats. When WB2838 was orally administered, the testosterone-induced increase in the weight of ventral prostate and seminal vesicle was reduced slightly but significantly (Table 3). At this time, the decrease in the body weight of the rats was not observed (data not shown).

Discussion

Androgen-receptor antagonists can be used in various androgen-dependent diseases. They may be divided into two groups based on their chemical structures, that is, steroid such as CPA and CMA, and non-steroid such as flutamide[1]. CPA and CMA have been extensively studied. Although they are potent androgen-receptor antagonists, they also show progestational and glucocorticoid properties. Furthermore, they may act as very weak androgen-receptor agonists in some cases. Flutamide is a non-steroidal androgen-receptor antagonist which shows pure-antagonistic properties. However, it also inhibits androgen activity at pituitary and consequently increases in luteinzing hormone and testosterone. It is also said that the half-life of flutamide in blood is short.

Therefore, the aim of our screening is to find new non-steroidal androgen-receptor antagonists which have new chemical structures and show improved properties, that is, no hormonal activities, no agonistic activity, peripheral selectivity and long half-life in blood.

The assay for androgen-receptor binding using partially purified rat prostate cytosol is disturbed by two main causes. One is the instability of androgen-receptors and the other is the presence of androgen binding proteins besides androgen-receptors. The instability is mainly due to the presence of proteolytic enzymes which are capable of rapidly denaturing the receptors, while the non-specific binding is partially due to the presence of progesterone- and glucocorticoid-receptors in the cytosol.

The stability of androgen-receptors was improved by addition of Na_2MoO_4 and dithiothreitol to prevent non-enzymatic denaturations, and by addition of α_1-antitrypsin and leupeptin to prevent degradation by proteolytic enzymes[6]. The binding specificity was optimized by using mibolerone as a radioactive ligand since it binds tightly to androgen-receptor and is stable both chemically and enzymatically[7]. Non-specific binding was reduced by supplementing of triamcinolone acetonide in the assay system to saturate both progesterone- and glucocorticoid-receptors which could otherwise act as binding sites for androgen[7].

As a result of the screening by receptor binding assay mentioned above, we isolated WB2838 as a non-steroidal androgen-receptor antagonist from the cultured broth of *Pseudomonas* sp. No. 2838. WB2838 was identified as 3-chloro-4-(2-amino-3-chlorophenyl)-pyrrole[2,3]. This compound has so far been noted only for its antifungal activity[3]. In the present study, it was revealed for the first time that it also had the property of androgen-receptor antagonist. Furthermore, its structure differed from those of the currently known non-steroidal androgen-receptor antagonists.

WB2838 inhibited not only androgen-receptor binding, but also the growth of androgen-responsive mouse mammary carcinoma SC-3 cells *in vitro*. WB2838 also showed the partial agonistic activity, but it was very weak. Furthermore, although it was a preliminary experiment, WB2838 inhibited the growth of the ventral prostate *in vivo*. Based on the experiments mentioned above, it was concluded that WB2838 was an androgen-receptor antagonist.

The inhibitory activities of WB2838 against both androgen-receptor binding and the growth of SC-3 cells were about 15-fold and about 7-fold, respectively, more potent than those of flutamide, which was clinically studied on the treatment for prostatic diseases. Further, the IC_{50} value of WB2838 against androgen-receptor binding was about 90-fold lower than that against estrogen-receptor binding. WB2838 was also a competitive inhibitor. From these results, it seemed to us that WB2838 was a promising, potent,

specific androgen-receptor antagonist, which could be used in the treatment for androgen dependent diseases. Further studies on WB2838 including the precise *in vivo* evaluation, peripheral selectivity and half-life in blood are necessary and currently in progress.

On the other hand, the activities of WB2838 was 6- to 10-fold less potent than those of CMA for inhibitions of both receptor binding and cell growth. Since the activities of WB2838, which replaced nitro group of pyrrolnitrin with amino group, were more potent than those of pyrrolnitrin, chemical modification of WB2838 might lead to more potent compounds than CMA and is now ongoing in our laboratories.

To the best of our knowledge, this is the first report of androgen-receptor antagonist of microbial origin.

References

1) NAMER, M.: Clinical applications of antiandrogens. J. Steroid Biochem. 31: 719~729, 1988
2) HAMIL, R.; R. ELANDER, J. MABE & M. GORMAN: Metabolism of tryptophans by *Pseudomonas aureofaciens*. V. Conversion of tryptophan to pyrrolnitrin. Antimicrob. Agents Chemother. -1967: 388~396, 1968
3) ROITMAN, J. N.; N. E. MAHONY, W. J. JANISIEWICZ & M. BENSON: A new chlorinated phenylpyrrole antibiotic produced by the antifungal bacterium *Pseudomonas cepasia*. J. Agric. Food Chem. 38: 538~541, 1990
4) PALLERONI, N. J.: *Genus* I. *Pseudomonas Migula* 1894. *In* BERGEY's Manual of Systematic Bacteriology. Volume 1. *Eds.*, N. R. KRIEG & J. G. HOLT, pp. 141~199, Williams & Wilkins Co., 1984
5) NAKAYAMA, O.; H. ARAKAWA, M. YAGI, M. TANAKA, S. KIYOTO, M. OKUHARA & M. KOHSAKA: WS-9659 A and B, novel testosterone 5α-reductase inhibitors isolated from a *Streptomyces*. III. Biological characteristics and pharmacological characteristics. J. Antibiotics 42: 1235~1240, 1989
6) PRINS, G. S. & C. LEE: Effect of protease inhibitors on androgen receptor analysis in rat prostate cytosol. Steroids 40: 189~201, 1982
7) SCHILLING, K. & S. LIAO: The use of radioactive 7α,17α-dimethyl-19-nortestosterone (mibolerone) in the assay of androgen receptors. The Prostate 5: 581~588, 1984
8) HORI, Y.; Y. ABE, M. EZAKI, T. GOTO, M. OKUHARA & M. KOHSAKA: R1128 substances, novel non-steroidal estrogen-receptor antagonists produced by a *Streptomyces*. I. Taxonomy, fermentation, isolation and biological properties. J. Antibiotics 46: 1055~1062, 1993
9) NOGUCHI, S.; Y. NISHIZAWA, N. NAKAMURA, N. UCHIDA, K. YAMAGUCHI, B. SATO, Y. KITAMURA & K. MATSUMOTO: Growth-stimulating effect of pharmacological doses of estrogen on androgen-dependent Shionogi carcinoma 115 *in vivo* but not in cell culture. Cancer Res. 47: 263~268, 1987
10) HIRAOKA, D.; N. NAKAMURA, Y. NISHIZAWA, N. UCHIDA, S. NOGUCHI, K. MATSUMOTO & B. SATO: Inhibitory and stimulatory effects of glucocorticoid on androgen-induced growth of murine Shionogi carcinoma 115 *in vivo* and in cell culture. Cancer Res. 47: 6560~6564, 1987
11) ARIMA, K.; H. IMANAKA, M. KOUSAKA, A. FUKUDA & G. TAMURA: Pyrrolnitrin, a new antibiotics substance produced by *Pseudomonas*. Agric. Biol. Chem. 28: 575~576, 1964