WS-7528, A NEW ISOFLAVANONE WITH ESTROGEN ACTIVITY ISOLATED FROM *STREPTOMYCES* SP. NO. 7528

TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

OSAMU NAKAYAMA, MASASHI YAGI, MIHO TANAKA, SUMIO KIYOTO, ITSUO UCHIDA, MASASHI HASHIMOTO, MASAKUNI OKUHARA and MASANOBU KOHSAKA

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Tsukuba-shi, Ibaraki 300-26, Japan

(Received for publication April 7, 1990)

WS-7528, produced by *Streptomyces* sp. No. 7528, was extracted from cultured broth, purified by solvent extraction followed by chromatography on silica gel and then isolated as pale yellow powder ($C_{16}H_{14}O_5$, mp 95~98°C). WS-7528 inhibited estrogen binding to its receptor protein in rat uterine cytosol. The IC_{50} value of WS-7528 for partially purified rat uterine cytosol receptor was 5.7×10^{-8} M.

This compound was found to induce the growth of the estrogen dependent cell line MCF-7.

WS-7528 was tested orally and subcutaneously in immature rats to confirm its effect on the growth of the uterus.

WS-7528 has also weak anti-inflammatory activity on the carrageenin paw edema of the rat model.

Osteoporosis is a disease which occures frequently in postmenopausal females. Estrogens like diethylstilbestrol have been used in clinical treatment of osteoporosis in Europe and the United States.[1,2] However, these estrogens are so potent that they are toxic to the mammary glands, liver and cardiovascular system.

Therefore, our aim has been to screen for nonsteroidal compounds which display milder estrogenic activities than conventional estrogens. We found a new estrogen-like substance, WS-7528, from the fermentation broth of a strain of *Streptomyces*.

In this paper, we describe the taxonomy of the producing strain, the fermentation, the isolation, the physico-chemical properties and biological activities of WS-7528.

Materials and Methods

Taxonomic Studies

The strain No. 7528 was isolated from a soil sample obtained at Mt. Takami, Nara Prefecture, Japan.

The methods described by SHIRLING and GOTTLIEB[3] were employed for this taxonomic study. Morphological observations were made with light and electron microscopes from cultures grown at 30°C for 14 days on yeast extract-malt extract agar, oatmeal agar and inorganic salts-starch agar.

Cultural properties were observed on ten media described by SHIRING and GOTTLIEB,[3] and WAKSMAN.[4] Incubation was carried out at 30°C for 14 days. Color assignments were made using the Methuen Handbook of Colour.[5]

Cell wall analysis was performed by the methods of BECKER *et al.*[6] and YAMAGUCHI.[7]

Temperature range for growth was determined on yeast extract-malt extract agar using a temperature gradient incubator TN-3 (Advantec Toxo Co., Ltd.).

Utilization of carbon sources was examined according to the methods of PRIDHAM and GOTTLIEB.[8]

Fermentation

A loopful of a mature slant of *Streptomyces* sp. No. 7528 was inoculated to a seed medium (80 ml) containing corn starch 1%, glycerol 1%, glucose 0.5%, Pharmamedia (cotton seed flour, trade mark, purchased from Iwaki Yakuhin) 1%, corn steep liquor 0.5%, Molatin (dried yeast, trade mark, purchased from Kanegafuchi Chemical Ind. Co., Ltd.) 0.5% and calcium carbonate 0.2% (pH 6.5) in a 250-ml Erlenmeyer flask and cultured at 30°C for 72 hours on a rotary shaker with 7.5-cm throw at 200 rpm.

Sixteen hundred ml of the seed culture were inoculated to the production medium containing soluble starch 2%, Pharmamedia 0.5%, gluten meal 0.5%, Molatin 0.25%, and corn steep liquor 0.25% (pH 6.5) in a 200-liter jar fermenter and cultured at 30°C for 72 hours under aeration of 160 liters/minute and agitation of 300 rpm.

Structural Studies

IR spectrum was recorded on a Jasco A-102 spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured on a Bruker AM400WB spectrometer. The chemical shifts are given in ppm (δ) relative to internal TMS. UV spectra were measured on a Hitachi 220A spectrophotometer. EI mass spectra was recorded using a VG ZAB-SE mass spectrometer. MP was measured with a Yanagimoto microscope hot-stage apparatus and is uncorrected. Preparative TLC (PTLC) was performed on pre-coated Silica gel 60 F_{254} plates (E. Merck).

Preparation of Estradiol Receptor in Uterine Cytosol

Mature Sprague-Dawley female rats (7 to 8 weeks old) were ovariectomized and 2 days later, rats were sacrificed.

The uteri were homogenized using buffer consisting of glycerol 10%, monothioglycerol 0.1%, KH_2PO_4 5 mM, pH 7.4 (1 g/4 ml of buffer) with a Polytron in the cold room. The homogenate was centrifuged at 100,000 × g for 30 minutes in a Beckman ultracentrifuge. The protein concentration in the cytosol was about 1 mg/ml. This cytosol solution was used as source of estradiol receptors.

Estrogen Receptor Binding Assay

The reaction solution contained 20 μl of inhibitor, 200 μl of 2,4,6,7-[^{3}H]estradiol (3.15~4.26 TBq/mmol, New England Nuclear, 14.8 KBq/ml) and 200 μl of the partially purified receptor. After incubation at 0°C for 90 minutes, bound estradiol was separated from unbound estradiol using dextran-coated charcoal by addition of 250 μl of solution containing 5 mg/ml Norit A (Nakarai Chemicals, Ltd.), 0.5 mg/ml Dextran T-70 (Pharmacia Fine Chemicals, Ltd.) and 8 mg/ml NaCl to the reaction mixture and incubating for 5 minutes. Then samples were centrifuged at 1,000 × g for 5 minutes to remove the charcoal.

The radioactivity in 50 μl of supernatant was counted in 5 ml of Aquazol-2 (New England Nuclear) with a Packard scintillation counter (Packard TRI-CARB 4530). Specifically bound estradiol was determined as the amount of [^{3}H]estradiol bound in the absence of the non-radioactive estradiol minus the amount of [^{3}H]estradiol bound in the presence of 200-fold excess concentration of non-radioactive estradiol.

Soft Agar Colony Assay

The assay for colony growth in soft agar was performed in 24-well Costar Multiplates. A 0.5-ml base layer of 0.5% agar (Difco; Noble agar) in DULBECCO's modified EAGLE's medium containing 10% fetal calf serum was added per well.

A 0.5-ml overlay of 0.3% agar containing the same medium-fetal serum mixture, 1×10^{3} MCF-7 cells and the sample to be tested was then added. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Counting, after 7 days, was restricted to these colonies having more than 20 cells.

Estrogenic Activity of WS-7528 on Immature Rats

Groups of 5 Sprague-Dawley rats, only 3 weeks old, were used to ensure absence of endogenous estrogenic activity.

WS-7528 was suspended in a 0.5% solution of methyl cellulose and administered orally or subcutaneously for 3 days, while as a representative example of the conventional drug, 17β-estradiol was dissolved in the same solution and administered orally or subcutaneously for 3 days. On the 4th day, each animal was autopsied and its uterine wet weight was recorded.

Inhibition of the Carrageenin Paw Edema

Wister rats, body weight 180~200 g were used. WS-7528 was suspended in a 0.5% solution of methyl cellulose and administered orally 1 hour before carrageenin injection. The edema was induced by injecting subplantarly 0.1 ml of 1% λ-carrageenin solution (made by Sigma) into the right hind paw.

Inflammation was plethysmometrically recorded.

Results

Taxonomic Studies on Strain No. 7528

The vegetative mycelium developed well without fragmentation. The aerial mycelium branched monopodially and formed spiral chains of spores with 20 to 40 spores per chain (Fig. 1). The spores had a warty surface and were oval in shape with a size of 0.6~0.8 × 0.6~0.9 μm. Sclerotic granules, sporangia and zoospores were not observed.

The aerial mycelium was gray to brownish gray. Part of the colony became black and moist, and showed hygroscopic character on most agar media. Reverse side of growth was yellowish white and grayish yellow. Melanoid pigments were not produced. A pale yellow soluble pigment was found in several media. Reverse mycelium pigment and soluble pigment were not pH sensitive. Results are shown in Table 1.

Fig. 1. Scanning electron micrograph of aerial mycelia of strain No. 7528 grown on yeast extract - malt extract agar.

Bar represents 5 μm.

Analysis of whole cell hydrolysates of strain No. 7528 showed the presence of LL-diaminopimelic acid. Accordingly, the cell wall of this strain is classified as type I.

Summarized physiological properties of strain No. 7528 are shown in Table 2.

This strain could utilize many carbon sources tested for growth (Table 3).

Based on the taxonomic properties described above, strain No. 7528 is considered to belong to the genus *Streptomyces* and to be a strain of the gray series of the PRIDHAM and TRESNER grouping.[9]

Isolation and Purification

The isolation scheme is shown in Fig. 2.

The cultured broth (150 liters) was filtered with the aid of diatomaceous earth (4 kg). To the mycelia containing diatomaceous earth, were added 100 liters of acetone and the mixture was stirred for 30 minutes. The suspension of mycelia was filtered and the filtrate was concentrated *in vacuo* to a volume of 20 liters. This concentrate was extracted with ethyl acetate at pH 7.0 (20 liters × 2). The ethyl acetate layer was washed with water, dried with anhydrous Na_2SO_4 and evaporated to give an oily residue.

Table 1. Cultural properties of strain No. 7528.

Medium	Cultural properties	Medium	Cultural properties
Yeast extract-malt extract agar	G: Good A: Abundant, brownish gray (6E2) R: Brownish yellow (5C7) S: Grayish yellow (3B5)	Tyrosine agar	G: Moderate A: Scant, yellowish white (1A2) R: Brownish orange (5C6) S: Grayish yellow (4B6)
Oatmeal agar	G: Good A: Abundant, grayish brown (7E3) R: Yellowish white (3A2) S: None	Glucose-asparagine agar	G: Good A: Abundant, brownish gray (7C2) R: Yellowish white (4A2) S: Pale yellow (3A3)
Inorganic salts-starch agar	G: Good A: Thick, brownish gray (10E2) R: Grayish yellow (3A3) S: Pale yellow (3A3)	Nutrient agar	G: Poor A: Scant, white R: Yellowish white (3A2) S: None
Glycerol-asparagine agar	G: Good A: Thin, brownish gray (6C2) R: Yellowish white (3A2) S: None	Potato-glucose agar	G: Good A: Thick, gray (C1) R: Yellowish brown (5E8) S: Light yellow (3A5)
Peptone-yeast extract-iron agar	G: Poor A: Scant, white R: Light yellow (5C6) S: None	Sucrose-nitrate agar	G: Good A: Scant, white R: Yellowish white (3A2) S: None

G: Growth, A: aerial mass color, R: reverse side color, S: soluble pigment.

Table 2. Physiological properties of strain No. 7528.

Conditions	Characteristics
Temperature range for growth	16~38°C
Optimum range	31~36°C
Gelatin liquefaction	Positive
Milk coagulation	Negative
Milk peptonization	Positive
Starch hydrolysis	Negative
Production of melanoid pigments	Negative
Decomposition of cellulose	Negative
Nitrate reduction	Negative

Table 3. Carbon utilization of strain No. 7528.

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

+: Utilization, ±: doubtful utilization.

The oily residue was mixed with 300 g of silica gel (Kieselgel 60, 70~230 mesh, made by Merck Co., Ltd.), and this mixture was slurried in methanol. After evaporating the solvent, the resultant dry powder was subjected to column chromatography using the same silica gel (1.5 liters, column size: 11 × 16 cm) which was packed with chloroform. The column was washed with a mixture of chloroform and methanol (50 : 1) and eluted with a mixture of chloroform and methanol (20 : 1).

The fractions containing the active compound were evaporated, dissolved in a mixture of *n*-hexane and ethyl acetate (4 : 1) and applied to silica gel (Kieselgel 60, 230~400 mesh, 200 ml). The column was washed with the same solvent, then developed with a mixture of *n*-hexane and ethyl acetate (3 : 2). The active fractions were evaporated *in vacuo* to give a yellow powder. This powder was dissolved in methanol and was treated to HPLC. The column (Cosmosil C_{18}, 10 × 250 mm, purchased from Nakarai Chemicals, Ltd.) was developed with 60% aq methanol containing 0.1% acetic acid. The active fractions were collected and concentrated under reduced pressure to a volume of 50 ml. This solution was neutralized with 1 N NaCl and extracted with ethyl acetate. The ethyl acetate extract was concentrated to dryness and subsequently obtained as a pale yellow powder (400 mg) after recrystallization from a mixture of *n*-hexane

and diethyl ether.

Physico-chemical Properties

The IR spectrum of WS-7528 is shown in Fig. 3. ^{13}C NMR spectrum of WS-7528 is shown in Fig. 4. The Rf value of WS-7528 on silica gel TLC developed with *n*-hexane-ethyl acetate (3:2) was 0.25.

The other physico-chemical properties are summarized in Table 4. The structure is shown in Fig. 5.

WS-7528 (**1**) is a weakly acidic, optically active substance. The molecular formula ($C_{16}H_{14}O_5$) of **1** was established by elemental analysis and EI-MS (Table 4). The ^{13}C NMR spectrum of **1** in CD_3OD showed 14 carbon signals including one carbonyl (δ 202.3 (s)), 12 aromatic carbons (δ 168.2 (s), 165.9 (s), 164.4 (s), 158.3 (s), 129.3 (s), 129.3 (d) (2×C), 116.2 (d) (2×C), 101.9 (s), 97.3 (s), 96.1 (d)), two methylens (δ 82.2 (d), 47.1 (d)), and one methyl (δ 10.5(q)). The ^1H NMR spectrum (CD_3OD) showed 11 protons (Table 5), thus indicating that three of the 14 protons in **1** are exchangeable. These three protons were found to be attributable to those of three hydroxy groups as follows. Acetylation of **1** with AC_2O in pyridine gave triacetate, in which the newly introduced acetyl groups were observed at δ 2.39 (3H, s), 2.31 (3H, s), and 2.305 (3H, s) in the ^1H NMR spectrum (CD_3OD). The 11 protons observed in the ^1H NMR spectrum of **1** were assignable to those of 4-substituted phenol, 5,6-disubstituted resorcinol, tetrasubstituted ethylene, and methyl groups as shown in Table 5. The UV spectrum

Fig. 2. Isolation procedure of WS-7528.

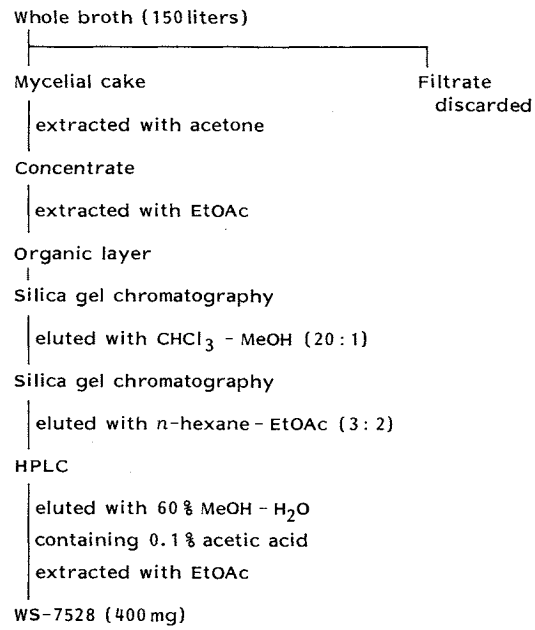


Fig. 3. IR spectrum of WS-7528 in Nujol.

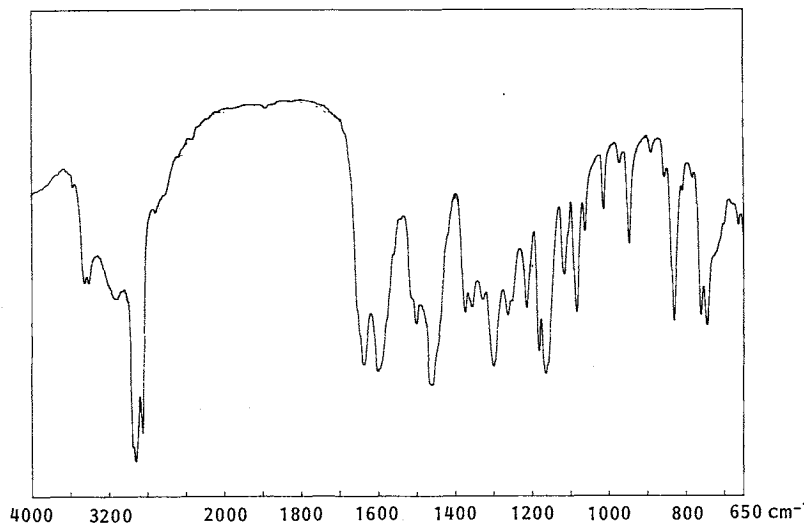


Fig. 4. ^{13}C NMR spectrum of WS-7528 in CD_3OD.

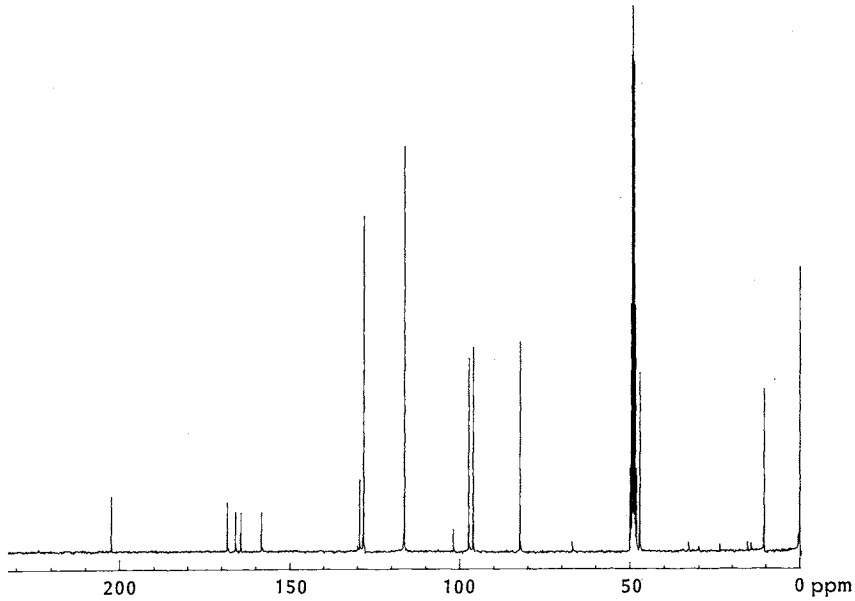


Table 4. Physico-chemical properties of WS-7528.

Appearance	Pale yellow powder
MP	95~98°C
Molecular formula	$C_{16}H_{14}O_5$
EI-MS $(M+H)^+$	287
$[\alpha]_D^{22}$ (10 mg/ml, MeOH)	−126.8°
Elementary analysis	Calcd for $C_{16}H_{14}O_5$
Calcd:	C 66.90, H 4.90
Found:	C 66.72, H 4.63
UV λ_{max}^{MeOH} nm (ε)	212 (23,140), 225 (23,430), 286 (16,570), 325 (sh)
Color reaction	
Positive:	Cerium sulfate, ferric chloride
Negative:	Ninhydrin, Molisch
Solubility	
Soluble:	MeOH, EtOH, Me_2CO, EtOAc
Insoluble:	Water, *n*-hexane

Fig. 5. Structure of WS-7528 (**1**).

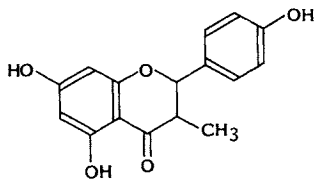
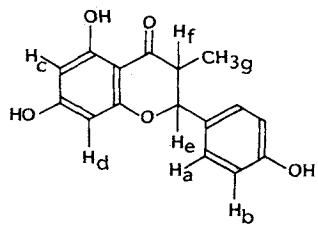
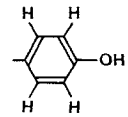
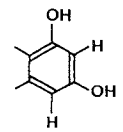


Table 5. ^1H NMR data of WS-7528 in CD_3OD.



Proton	Chemical shift[a]	m	Coupling constants[b]	Assignment
H_a	7.24 (2H)	d	8.6	
H_b	6.82 (2H)	d	8.6	
H_c	5.98	d	2.3	
H_d	5.92	d	2.3	
H_e	5.45	d	3.0	(O)>CH–CH<(CH$_3$)
H_f	2.67	dq	3.0, 7.3	
H_g	0.95 (3H)	d	7.3	(>CH)–CH$_3$

m: Multiplicities.
[a] δ: ppm.
[b] J=Hz.

(λ_{max}^{MeOH} nm 286 (ε 16,570)) showed bathochromic shifts in basic medium ($\lambda_{max}^{MeOH-NaOH}$ nm 321 (ε 28,000)), suggesting the presence of a flavonone nucleus such as that of naringenin[10] (λ_{max}^{EtOH} nm 290, $\lambda_{max}^{EtOH-NaOH}$ nm 328). The structure of WS-7528 was thus presumed to be **1**.

Acetylation of WS-7528

To a solution of WS-7528 (100 mg) in pyridine (2 ml) was added AC_2O (1 ml) and the mixture was allowed to stand overnight at room temperature. The reaction mixture was evaporated to dryness under reduced pressure. The residue was taken in CH_2Cl_2, washed with H_2O, dried over $MgSO_4$, and evaporated to give a residue, which was purified by preparative TLC ($CHCl_3$ - MeOH, 50 : 1) to give triacetate (60 mg): oily.

EI-MS $(M+H)^+$ 413; ^1H NMR (CD_3OD) 7.41 (2H, d, J=8.5 Hz), 7.15 (2H, d, J=8.5 Hz), 6.85 (1H, d, J=2.3 Hz), 6.56 (1H, d, J=2.0 Hz), 5.59 (1H, d, J=2.6 Hz), 2.72 (1H, dq, J=2.97 and 7.2 Hz), 2.39 (3H, s), 2.31 (3H, s), 2.305 (3H, s), 0.99 (3H, d, J=7.5 Hz).

Biological Activities

WS-7528 was evaluated for its effect on [^{3}H]estradiol binding by the estrogen receptor protein of uterine cytosol.

The receptor binding of [^{3}H]estradiol (3×10^{-9} M) was inhibited by 70% by a 10-fold excess (3×10^{-8} M) of nonradioactive estradiol, and it was not affected by the same concentration of progesterone or spironolactone. WS-7528 was titrated for its ability to inhibit [^{3}H]estradiol binding to the uterine cytosol receptor. The concentration of WS-7528 required to give 50% inhibition (IC_{50}) was estimated from the titration curve to be 5.7×10^{-8} M.

Using some flavones up to 10^{-6} M, did not significantly inhibit the receptor binding of [^{3}H]estradiol.

The IC_{50} values of compounds we tested are summarized in Table 6.

When included in the growth medium for the cell line MCF-7, this compound was capable of inducing cell growth (Table 7). This effect was dose dependent, at 10 μg/ml the growth observed was twice that of control.

Table 6. Comparison of IC_{50} values of estrogen receptor binding substances.

Compound	IC_{50} (M)
17β-Estradiol	8.1×10^{-9}
Diethylstilbestrol	3.0×10^{-9}
Estrone	3.2×10^{-8}
Estriol	1.1×10^{-7}
WS-7528	5.7×10^{-8}
Tamoxifen	1.5×10^{-6}
Fisetin	$>1.0 \times 10^{-5}$
Naringenin	$>1.0 \times 10^{-5}$

Table 7. The effect of WS-7528 on MCF-7 cell colony formation.

Compound	Concentration (μg/ml)	Number of colonies
WS-7528	0.01	39.7± 5.8
	0.1	55.3±15.2
	1.0	76.3±13.6**
	10.0	81.0± 8.9***
17β-Estradiol	0.01	62.3± 3.8*
Control	0	42.7±11.0

* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Table 8. Estrogenic activity of WS-7528 on immature rats when dosed orally.

Compound	Daily dose (mg/kg)	Number of animals	Body weight (g+SD)	Uterine wet weight (mg+SD)
WS-7528	10	5	53.8+1.2	102.8+ 6.5***
	3.3	5	55.3+0.6	85.2+ 9.1*
17β-Estradiol	2.0	5	54.0+1.1	281.4+47.3***
	0.67	5	55.3+1.5	239.9+30.0***
	0.22	5	53.2+1.4	225.9+19.2***
Control	0	5	56.8+0.7	64.5+ 4.3

*-*** See Table 7.

Table 9. Estrogenic activity of WS-7528 on immature rats when dosed subcutaneously.

Compound	Daily dose (mg/kg)	Number of animals	Body weight (g+SD)	Uterine wet weight (mg+SD)
WS-7528	2.5	5	61.2+1.5	133.4+ 6.8**
	0.63	5	63.5+1.2	102.4+ 7.0
	0.16	5	62.3+0.8	102.0+ 2.5
17β-Estradiol	0.5	5	61.4+1.1	261.9+18.2***
Control	0	5	64.0+0.6	80.8+ 6.0

,* See Table 7.

Estrogenic activities of WS-7528 on immature rats are shown in Tables 8 and 9. WS-7528 at the daily dose levels of 3.3 mg/kg, 10 mg/kg (orally), and 2.5 mg/kg (subcutaneously) resulted in uterine weight increasing effect with a dose-response curve of moderate gradient.

Weak anti-inflammatory activity by WS-7528 was observed.

An injection of 100 mg/kg of the compound reduced an induced inflammation by 23%.

Antimicrobial activities of WS-7528 was evaluated by a serial broth dilution method. WS-7528 was not effective against some strains we tested.

Acute toxicity of WS-7528 was examined in *dd*Y mice (female, 5 weeks old) by a single intraperitoneal or subcutaneously injection of graded doses of the compound into five mice. The LD_{50} value of WS-7528 was above 500 mg/kg.

Discussion

We have routinely examined compounds of microbial origin for estrogen receptor binding activity. As a result of our screening program, the highly potent estrogen receptor binding compound WS-7528 has been isolated.[11,12]

In vivo studies using immature female rats and *in vitro* studies using the estrogen dependent cell line MCF-7 show that WS-7528 has agonistic activity. A potential use of estrogen agonist is in the treatment of osteoporosis. Osteoporosis is an almost universal phenomenon in aging people, especially postmenopausal women, and poses a great risk to those suffering from the disorder.

Estrogens are known to be capable of preventing osteoporosis, however, they also have undesirable effects on other body functions, thus limiting their use in treatment.

The development of milder estrogenic substances with no side effects remains a possible strategy to combat osteoporosis. In this regard, this preliminary study suggests that WS-7528 may be a possible candidate for further development. Detailed studies on WS-7528 and other compounds are currently ongoing in our laboratory.

After preparation of this paper, we noticed that Banyu-group intend to report a compound BE-14348-B which is identical with WS-7528.

References

1) AITKIN, J. M.; D. M. HART & R. LINDSAY: Oestrogen replacement therapy for prevention of osteoporosis after oophorectomy. Brit. Med. J. 3: 515~518, 1973
2) NICHOLS, K. C.; L. SCHENKEL & H. BENSON: 17β-Estradiol for postmenopausl estrogen replacement therapy. Obstet. Gynecol. Surv. 39: 230~235, 1984
3) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
4) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Vol. 2. Classification, Identification and Description of Genera and Species. Williams & Wilkins Co., 1961
5) KORNERUP, A. & J. H. WANSCHER (*Ed.*): Methuen Handbook of Colour. Methuen, 1978

6) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
7) YAMAGUCHI, T.: Comparison of the cell-wall composition of morphologically distinct actinomycetes. J. Bacteriol. 89: 444~453, 1965
8) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bacteriol. 56: 107~114, 1948
9) PRIDHAM, T. G. & H. D. TRESNER: *Genus* I. *Streptomyces* Waksman and Henrich 1943. *In* BERGEY's Manual of Determinative Bacteriology, 8th Ed., *Eds.*, R. E. BUCHANAN & N. E. GIBBONS, pp. 748~829, Williams & Wilkins Co., 1974
10) HOROWITZ, R. M. L. & JURD: Spectral studies on flavonoid compounds. II. isoflavones. J. Org. Chem. 26: 2446~2449, 1961
11) NAKAYAMA, O.; M. YAGI, S. KIYOTO & M. OKUHARA (Fujisawa Pharm.): WS-7528 substance and its preparation. Jpn. Pat. Appl. 316311 ('89), Dec. 5, 1989
12) NAKAYAMA, O.; M. YAGI, S. KIYOTO & M. OKUHARA (Fujisawa Pharm.): WS-7528 substance and the preparation thereof. Brit. 8828413.8, Dec. 6, 1988