A New Aromatase Inhibitor, FR901537

I. Taxonomy, Fermentation, Isolation, Physicochemical Characteristics and Biological Activities

NOBUTAKA OOHATA, YASUHIRO HORI, YUKIKO YAMAGISHI, TAKASHI FUJITA, SHIGEHIRO TAKASE, MICHIO YAMASHITA, HIROSHI TERANO* and MASAKUNI OKUHARA

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

(Received for publication September 14, 1994)

FR901537 is a new aromatase inhibitor produced by a bacterium *Bacillus* sp. No. 3072. Structural studies of FR901537 suggested that it was a novel naphthol derivative having pantetheine in its structure.

FR901537 showed a potent inhibitory activity against aromatase from human placenta or rat ovary, but did not inhibit the activity of 11β-hydroxylase from bovine adrenal cortex. Lineweaver-Burk plot analysis revealed that FR901537 is a competitive inhibitor.

Aromatase is a cytochrome P450 enzyme which catalyzes the conversion of androgens into estrogens at the last step of estrogen biosynthesis[1]. The ovary is a major site of estrogen biosynthesis in premenopausal women. In postmenopausal women, the sources of estrogen are peripheral tissues such as adipose tissue, skin and muscle. Aromatase is also found in liver, brain, placenta and breast tumor itself[2~5]. Inhibitors with a high specificity for aromatase can be an effective means of treating estrogen-dependent tumors such as breast cancer, endometrial cancer and prostate cancer[6~8].

In the case of the treatment of hormone-dependent breast cancers, it is generally accepted that estrogen receptor antagonist, tamoxifen, is the firstline drug. However, almost all patients eventually become resistant to tamoxifen and often relapse with metastatic tumors that are still hormone sensitive. The mode of action of aromatase inhibitor is different from that of estrogen antagonists[9,10].

Taking this finding into consideration, we screened for new nonsteroidal aromatase inhibitors from microbial products. As a result of the screening, we isolated a new compound FR901537 from the cultured broth of *Bacillus* sp. No. 3072[11].

This paper describes the taxonomy of the producing strain, and the fermentation, isolation, characterization and biological activities of FR901537.

Materials and Methods

Microorganism and Taxonomy

The FR901537 producing organism was isolated from a soil sample obtained from Chiba prefecture.

The morphological, cultural and physiological characterization was carried out by the method described by SNEATH *et al.*[12], and COWAN and STEEL[13].

Culture and Medium Conditions

A loopful of the bacterial strain No. 3072 grown on an agar slant was inoculated into thirty nine 500-ml Erlenmeyer flasks containing 160 ml of a seed medium consisting of glucose 1%, glycerin 1%, peptone 0.5%, corn steep liquor 1%, meat extract 0.25%, $(NH_4)_2SO_4$ 0.1%, L-arginine 0.1%, $MgSO_4 \cdot 7H_2O$ 0.006% (adjusted to pH 7) and $CaCO_3$ 0.2%, and incubated in a rotary shaker (250 rpm) at 30°C for 2 days. The entire seed culture was transferred to five 30-liter jar fermentors containing 20 liters each and one 200-liter jar fermentor containing 160 liters of production medium consisting of glucose 2%, glycerin 7%, corn steep liquor 3%, $(NH_4)_2SO_4$ 0.1%, soybean meal 2% (adjusted to pH 7.5 with 6 N NaOH), and $CaCO_3$ 0.5%, Adecanol LG-109 0.05% (Asahi Denka), and Silicone KM70 0.05% (Shin-Etsu Kagaku). Cultivation was carried out at 30°C for 4 days at 200 rpm rotation, one atm inner pressure, and 20 liters/minute aeration in a 30-liter jar fermentor or 160 liters/minute aeration in a 200-liter jar fermentor.

Assay for FR901537 Production

The amount of FR901537 in the fermentation broth was determined by HPLC (Hitachi model L-6000) using a YMC AM-302, S-5, A-120 column (4.5 mm inner diameter × 150 mm length, YMC Co., Ltd.) at 270 nm with a mobile phase of 55% aqueous methanol and a flow rate of 1 ml/minute. The retention time of FR901537 was 15.7 minutes.

Analytical Measurement

Optical rotation was measured with a Jasco DIP-140 polarimeter using a 10-cm microcell.

UV and IR spectra were obtained with a Hitachi 220A spectrometer and Jasco A-102 IR spectrometer, respectively.

The mass spectrum was recorded with VG ZAB-SE mass spectrometer. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) were measured with a Bruker AM400wb spectrometer controlled with an ASPECT 3000 computer.

Preparation of Human Placental and Rat Ovarian Aromatase

Human placenta was washed with ice-cold 0.15 M KCl. After the adhering membranes, large blood vessels, and blood were removed, it was cut into fine pieces with scissors.

Wistar rats were subcutaneously administered pregnant mare's serum gonadotropin (PMSG), and the ovaries were removed 90 hours later. The obtained ovaries were washed with ice-cold 0.15 M KCl, and then the connective tissues and adipose tissues were removed.

Microsome fraction as an aromatase source was prepared from a human placenta or rat ovaries according to the method described by STEELE *et al.*[14] and KREKELS *et al.*[15]

Assay of Aromatase Activity

Aromatase activity was determined by measuring the amount of $^{3}H_2O$ formed during aromatization of [^{3}H]androstendione. Briefly, [1β-^{3}H(N)]-4-androstene-3,17-dione (20 nM), NADPH (500 μM), the above-mentioned microsome fraction (100 μl), and an inhibitor solution (10 μl) were reacted in 150 μl of 0.02 M phosphate buffer containing 0.1% bovine serum albumin at 37°C for 1 hour. After the reaction, 25 μl of 10% trichloroacetic acid was added thereto, and the mixture was incubated at ice-cold temperature for 10 minutes to terminate the reaction. Dextran-coated 5% activated charcoal (125 μl) was added to separate $^{3}H_2O$ generated by aromatic ring formation of androstendione from substrate, and the mixture was incubated at ice-cold temperature for 10 minutes to remove the free androstendione. After the incubation, the mixture was centrifuged at 3,000 rpm for 10 minutes, 3 ml of a scintillator was added to 150 μl of the supernatant, and radioactivity was measured by a liquid scintillation counter. Percent inhibition was estimated by the comparison of the results obtained with and without of inhibitor.

Measurement of 11β-Hydroxylase Activity

The activity of 11β-hydroxylase was determined according to the method of BOSSCHE *et al.*[16] by measuring the corticosterone and cortisol synthesis from [^{3}H]-11β-deoxycorticosterone and [^{3}H]-11β-deoxycortisol, respectively.

Mitochondrial fraction as an 11β-hydroxylase source was prepared from bovine adrenal cortices according to the method described by BOSSCHE *et al.*[16]

Determination of Protein Content

Protein content was determined by using the Bio-Rad protein assay kit.

Chemicals

Reagents used in this work were obtained from the following sources: PMSG from UCB-Bioproducts, S. A. (Belgium); NADPH, androstendione, corticosterone, cortisol and aminoglutethimide (AG) from Sigma Chemical Company (St. Louis, MO); [^{3}H]-androstendione, [1β-^{3}H(N)]-4-androstene-3,7-dione, [^{3}H]-11-deoxy-corticosterone, and [^{3}H]-11-deoxycortisol from New England Nuclear (Boston, MA).

Animals

Wistar rats (female, weighing 120~150 g) were obtained from Charles River Japan Inc., and *ddY* mice (female, 4 weeks old) were from Japan SLC Inc.

Results

Taxonomy of the Producing Organism

Morphological observations of strain No. 3072 were made using optical and electron microscopes with cells grown on nutrient agar at 30°C for 24 hours (Fig. 1). Strain No. 3072 was a Gram-positive, endospore-forming bacterium. The rod-shape cells were about 1.0~1.2 × 2.0~2.3 μm in size. Results are summarized in Table 1.

Fig. 1. Scanning electron micrograph of *Bacillus* sp. No. 3072.

Bar represents 5 μm.

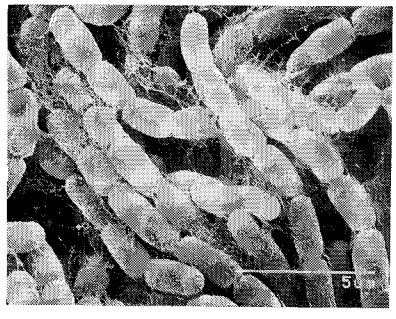


Table 1. Morphological characteristics of the strain No. 3072.

color of colony	yellowish white
cell shape	rod
cell size	1.0-1.2 x 2.0-2.3 μm
spore formation	positive
spore position	central
spore shape	ellipsoidal

Physiological characteristics are as follows; The growth temperature was from 17 to 38°C. Strain No. 3072 was catalase-positive, oxidase-negative and nitrate reduction negative. This strain also was esculin hydrolysis positive, gelatin liquefaction positive and casein hydrolysis positive. This strain did not hydrolyze starch. Acid formation was observed from D-glucose, D-fructose, D-galactose and salicine.

According to Bergey's Manual of Systematic Bacteriology, this strain was classified as a *Bacillus* sp. Strain No. 3072 was deposited in the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Tsukuba, Japan with accesion No. FERM BP-4124.

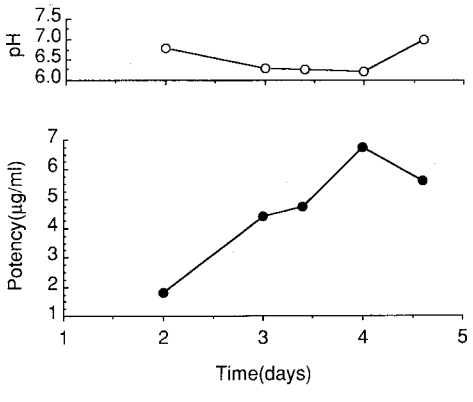
Fermentation of FR901537

Fig. 2 shows a typical example of the time course FR901537 production by *Bacillus* sp. No. 3072 in a 200-liter jar fermentor, along with change in the pH of the medium. Further, by microscopic observation during the time course of fermentation, the morphological changes of *Bacillus* sp. No. 3072 were examined. After the 33 hours incubation vegetative cells of *Bacillus* sp. No. 3072 formed endospores and free spores were released at 40 hours. Refractile appearance of spores was confirmed by a phase-contrast microscope. During this sporulation, the production of FR901537 was very low or undetectable (less than 0.2 μg/ml). At 48 hours the spores lost the refractility, darkened and swelled, the germination was initiated and vegetative growth commenced. The amount of FR901537 in the fermentation broth reached about 6.5 μg/ml at 96 hours during the germination and outgrowth.

Isolation and Production

An equivalent amount of methanol (240 liters) was added to the cultured broth and filtered with the aid of diatomaceous earth (5 kg). The filtrate was concentrated *in vacuo* and adjusted to pH 5 with 6 N HCl. The concentrate was applied on a Diaion HP-20 column and eluted with 80% aqueous methanol. The eluate was concentrated *in vacuo* to remove methanol and the concentrate obtained was subjected to chromatography on an Alumina AC12. The column was eluted with 80% aqueous methanol and the eluted fraction was added distilled water to give a final concentration of 45% aqueous methanol. The active materials was charged on a Diaion HP-20SS column and eluted with 60% aqueous methanol. The eluate was concentrated *in vacuo* to remove methanol and the concentrate was charged on a Diaion HP-20 column and eluted with methanol. The active fractions were collected and concentrated *in vacuo* to give colorless needles of FR901537 (100 mg).

Fig. 2. Time course of FR901537 production and pH change.



Physico-chemical Properties

The physico-chemical properties of FR901537 are summarized in Table 2. FR901537 is soluble in methanol, ethanol and acetone, slightly soluble in ethyl acetate, dichloromethane and *n*-hexane, and insoluble in water. Color reactions are as follows: FR901537 gave positive reactions to iodine vapor, ceric sulfate and Ehrlich tests, but was negative in Ninhydrin, Molisch and ferric chloride tests. The molecular formula was determined to be $C_{23}H_{29}N_3O_6S_2$ by elemental analysis, X-ray microanalyzer and FAB-MS. The Rf values of FR901537 on silica gel TLC (Silica gel $60F_{254}$, E. Merck) developed with dichloromethane-methanol (6:1) and on RP-18

Table 2. Physico-chemical properties of FR901537.

Appearance	colorless needles
MP	144 - 146 ℃
$[\alpha]_D^{26}$	+13.7° (*c* 1.0, DMSO)
Molecular Formula	$C_{23}H_{29}N_3O_6S_2$
FAB-MS(m/z)	508$(M+H)^+$
Elemental analysis	
calcd for $C_{23}H_{29}N_3O_6S_2 \cdot 1/2H_2O$:	C 52.56, H 5.94, N 7.99, S 12.20
found:	C 52.15, H 6.03, N 7.81, S 11.47
UV	
λ_{max}^{MeOH} nm(ε)	215(28,600), 255(sh, 26,000), 265(28,600), 335(7,000)
$\lambda_{max}^{NaOH\text{-}MeOH}$ nm(ε)	215(31,700), 230(sh, 20,200), 278(26,000), 370(10,600)
Color test	
Positive:	$Ce(SO_4)_2$-H_2SO_4, I_2, Ehrlich
Negative:	Ninhydrin, Molisch, $FeCl_3$
Solubility	
Soluble:	MeOH, Acetone, DMSO
Slightly soluble:	EtOAc, CH_2Cl_2, *n*-Hexane
Insoluble:	H_2O
TLC Rf value	
System I[a]	0.5
System II[b]	0.46

[a] Plate; Silica gel 60 F_{254} (E.Merck, Art.5715), CH_2Cl_2:MeOH=6:1
[b] Plate; RP-18 WF_{254S} (E.Merck, Art.13124), 70% aq. MeOH

Fig. 3. IR spectrum of FR901537 (KBr).

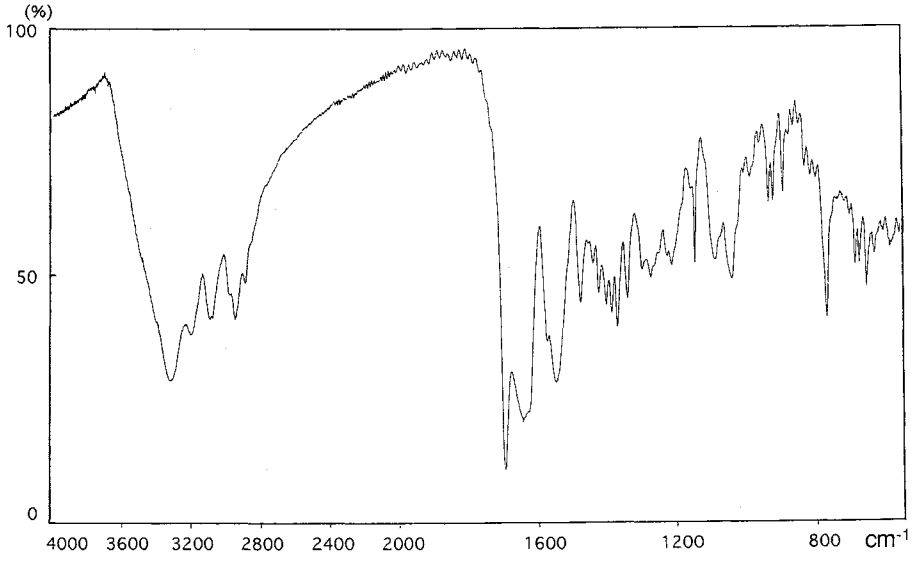


Fig. 4. ^{1}H NMR spectrum of FR901537 in DMSO-d_6 (400 MHz).

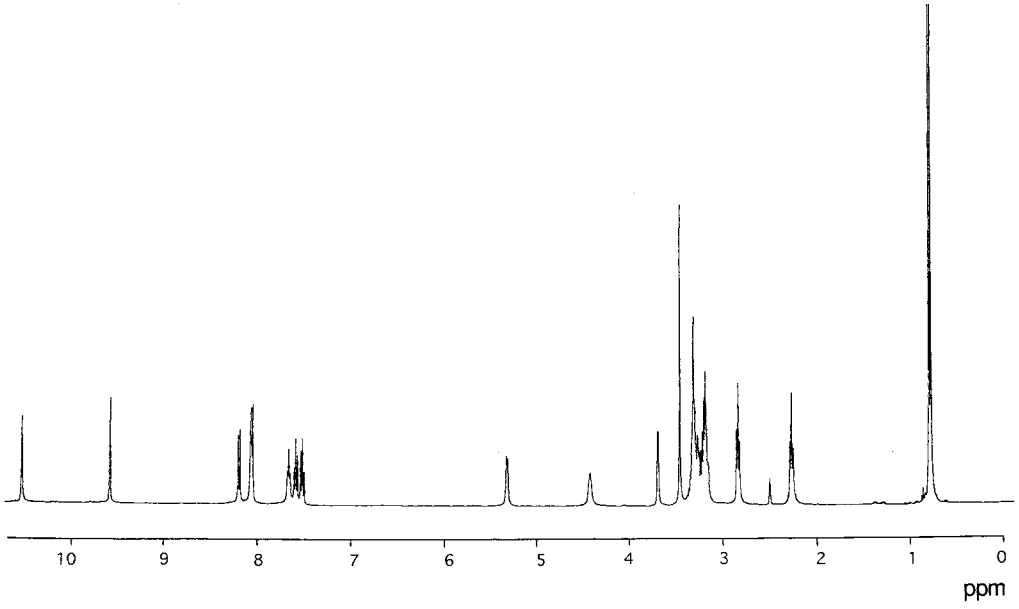
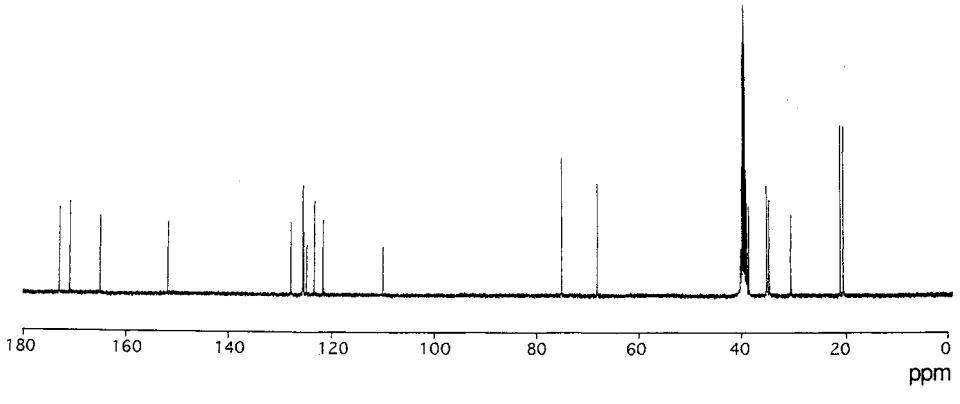


Fig. 5. ^{13}C NMR spectrum of FR901537 in DMSO-d_6 (100 MHz).



$WF_{254}S$ TLC (E. Merck) developed with 70% aqueous methanol were 0.50 and 0.46, respectively.

The IR spectrum, ^1H NMR spectrum and ^{13}C NMR spectrum of FR901537 are shown in Figs. 3, 4 and 5, respectively. On the basis of NMR spectral analyses of FR901537 and its derivatives and chemical degradation product, the structure was elucidated as (*R*)-*N*-[2-[2-(2,3-Dihydro-6-hydroxy-2-oxo-1*H*-naphtho[2,1-b]-[1,4]thiazin-5-ylthio)ethylaminocarbonyl]ethyl]-2,4-dihydroxy-3,3-dimethyl-butyramide as depicted in Fig. 6. The full accounting of the structure determination will be reported in due course.

Fig. 6. Structure of FR901537.

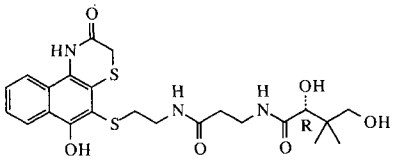


Fig. 7. Effect of FR901537 and aminoglutethimide (AG) on aromatase activity (*in vitro*).

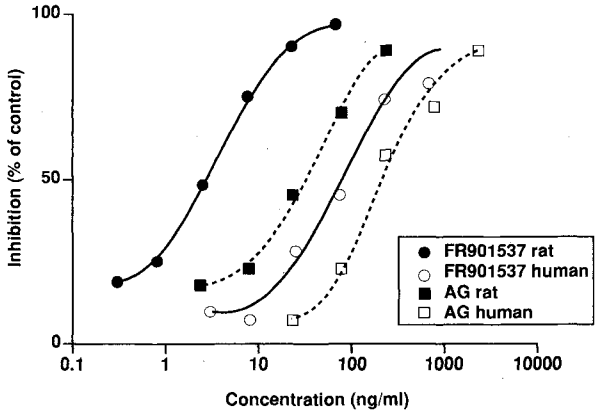
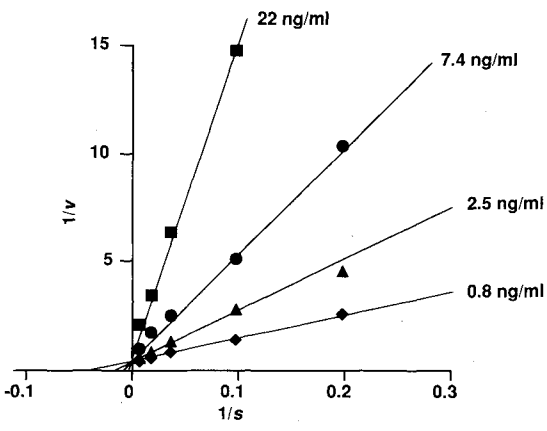


Fig. 8. Lineweaver-Burk plot of effect of FR901537 on aromatase activity (rat ovary).



Biological Activities

Inhibition of placental and ovarian aromatase activity by FR901537 was measured. The concentration of FR901537 required for 50% inhibition of human and rat enzymes (IC_{50}) was 131 ng/ml (2.6×10^{-7} M) and 3.1 ng/ml (6.1×10^{-9} M), respectively. In a comparative study with AG, the aromatase inhibitory activity of FR901537 was shown to be more potent than that of AG (Fig. 7). The kinetics of the inhibition by FR901537 was examined by the use of microsomal fraction of rat ovary. From the Lineweaver-Burk plots obtained with different concentrations of FR901537, a *Ki* value of 1.1 ng/ml was calculated (Fig. 8) and it revealed a competitive type of inhibition.

FR901537 does not inhibit the activity of 11β-hydroxylase.

Acute Toxicity

The acute toxicity of FR901537 was determined in *ddY* mice (5 weeks old, female) by a single intraperitoneal injection of graded doses of FR901537 into a group of 5 mice. The LD_{50} was over 330 mg/kg.

Discussion

FR901537 was isolated from the fermentation broth of *Bacillus* sp. No. 3072. It has a unique chemical structure; FR901537 is a novel naphthol derivative, having pantetheine as its side chain unit. Further, FR901537 was produced during germination and outgrowth of *Bacillus* sp. No. 3072 spores. This is the first report of production of as a unique bioactive compound during germination of bacterial spores.

FR901537 was found to have a potent inhibitory activity against aromatase from human placenta or rat ovary. The inhibitory activity of FR901537 was 5~30 times more potent than that of AG. Studies on the kinetics of inhibition revealed that FR901537 shows a competitive manner of inhibition, and its manner is the same as that of AG (data not shown). Furthermore, FR901537 did not inhibit 11β-hydroxylase. These results suggest that FR901537 may be a specific aromatase inhibitor and a promising drug in the treatment of cancer patients with breast cancer.

References

1) Thompson, Jr. E. A. & P. K. Siiteri: The involvement of human placental microsomal cytochrome P-450 in aromatization. J. Biol. Chem. 249: 5373~5378, 1974
2) Nimrod, A. & K. J. Ryan: Aromatization of androgens by human abdominal and breast fat tissue. J. Clin. Endocrinol. Metab. 40: 367~372, 1975
3) Judd, H. L.; G. E. Judd, W. E. Lucas & S. S. C. Yen: Endocrine function of the postmenopausal ovary: concentration of androgens and estrogens in ovarian and

peripheral vein blood. J. Clin. Endocrinol. Metab. 39: 1020~1024, 1974

4) LONGCOPE, C.; J. H. PRATT, S. H. SCHNEIDER & S. E. FINEBERG: Aromatization of androgens by muscle and adipose tissue *in vivo*. J. Clin. Endocrinol. Metab. 46: 146~152, 1978

5) O'NEIL, J. S. & W. R. MILLER: Aromatase activity in breast adipose tissue from women with benign and malignant breast disease. Br. J. Cancer 56: 601~604, 1987

6) CASH, R.; A. J. BROUGH, M. N. P. COHEN & P. S. SATOH: Aminoglutethimide as an inhibitor of adrenal steroidogenesis. Mechanism of action and therapeutic trial. J. Clin. Endocrinol. Metab. 27: 1239~1248, 1967

7) SANTEN, R. J.; A. LIPTON & J. KENDALL: Successful medical adrenalectomy with aminoglutethimide: role of altered drug metabolism. J. Am. Med. Ass. 230 1661~1665, 1974

8) BANTING, L.; P. J. NICHOLLS, M. A. SHAW & H. J. SMITH: Recent developments in aromatase inhibition as a potential treatment for oestrogen-dependent breast cancer. Prog. Med. Chem. 26: 253, 1989

9) SANTEN, R. J.; A. MANNI, H. HARVEY & C. REDMOND: Endocrine treatment of breast cancer in women. Endocrine Rev. 11: 221~265, 1990

10) SANTEN, R. J.: Clinical use of aromatase inhibitors in human breast carcinoma. J. Steroid Biochem. Molec. Biol. 40: 247~253, 1991

11) HORI, Y.; Y. YAMAGISHI, N. SHIGEMATU, K. SAKAMOTO, H. TERANO & M. OKUHARA (Fujisawa): A new compound FR901537, production thereof and use thereof. Eur. Pat. Appl. 9,226,620, Dec. 21, 1992

12) SNEATH, P. H. A.; N. S. MAIR, M. E. SHARPE & J. G. HOLT (*Ed.*): Bergey's manual of systematic bacteriology, Vol. 2. The Williams & Wilkins Company, Baltimore. 1989

13) COWAN, S. T. & K. J. STEEL: Manual for the identification of medical bacteria, 2nd ed. Cambridge University Press, England, 1974

14) STEELE, R. E.; L. B. MELLOR, W. K. SAWYER, J. W. WASVARY & L. J. BROWNE: *In vitro* and *in vivo* studies demonstrating potent and selective estrogen inhibition with the nonsteroidal aromatase inhibitor CGS 16949A. Steroids 50: 147~161, 1987

15) KREKELS, M. D. W. G.; W. WOUTERS & R. D. COSTER: Aromatase inhibition by R76713: a kinetic analysis in rat ovarian homogenates. Steroids 55: 69~73, 1990

16) BOSSCHE, H. V.; G. WILLEMSENS, W. COOLS & D. BELLENS: Effects of etomidate on steroid biosynthesis in subcellular fractions of bovine adrenals. Biochem. Pharmac. 33: 3861~3868, 1984