New Antitumor Substances, FR901463, FR901464 and FR901465†

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Biological Activities

HIDENORI NAKAJIMA*, BUNJI SATO, TAKASHI FUJITA, SHIGEHIRO TAKASE, HIROSHI TERANO and MASAKUNI OKUHARA

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

(Received for publication June 10, 1996)

New antitumor substances, FR901463, FR901464 and FR901465 were isolated from the culture broth of a bacterium of *Pseudomonas* sp. No.2663.

FR901463, FR901464 and FR901465 remarkably enhanced the transcriptional activity of the promoter of SV40 DNA virus. Further, these compounds exhibited potent antitumor activities against murine and human tumor cell lines *in vitro*.

Advances in recombinant DNA technology have enabled us to understand at the molecular level many multiple changes which occurred in the DNA of tumor cells, and to employ various screening system for new antitumor agents[1~6]. Although many studies to explore substances which regulate the activities of oncogene products involved in signal transduction have been pursued by the use of transformants with activated oncogenes[7~9], very few attempts in the area of nuclear oncogene products or DNA binding proteins (transcription factors) which participate in the transcriptional regulation have been carried out. Transcription of genes is the final event which is caused by signals transmitted through various signal transduction pathways.

In addition, it is known that SV40 DNA tumor virus promoter contains many recognition sites for major cellular transcription factors and can be activated in tumor cells[10]. They can also strongly drive a down stream reporter gene. Thus, we introduced the promoter of SV40 DNA tumor virus upstream of a CAT reporter gene to detect activities of certain transcription factors expressed in the tumor cells in order to screen for specific transcriptional regulators. If an agent selectively regulates the activity of transcription factors, it would be a new class of antitumor agent.

In the course of searching for transcriptional regulators from soil microorganisms, FR901463, FR901464 and FR901465 were isolated from a bacterium *Pseudomonas* sp. No. 2663. This paper describes the taxonomy of the producing strain, isolation, physico-chemical properties and biological activities of these compounds.

Materials and Methods

Microorganism and Taxonomy

The producing organism of a complex of new antitumor substances (FR901463, FR901464 and FR901465) was isolated from a soil sample obtained from Mie Prefecture, Japan. The morphological, cultural and physiological characterization was carried out by the method described in BERGY's Manual of Systematic Bacteriology, 9th edition, (Volume 1)[11].

Culture and Medium Conditions

A loopful of bacterial strain No. 2663 grown on an agar slant was inoculated into sixty 500-ml Erlenmeyer flasks containing 160 ml of a seed medium consisting of polypeptone 1%, yeast extract 0.5%, and NaCl 0.5%, and incubated in a rotary shaker at 30°C for 24 hours at 220 rpm. The entire culture was transferred to twenty 30-liter jar fermenters by 400 liters of production medium containing soluble starch 1%, glycerin 1%, glucose 0.5%, defatted soybean meal 1%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2%, $MgSO_4 \cdot 7H_2O$ 0.006%, $CaCO_3$ 0.2%, Adecanol LG-109 (Asahi denka) 0.05% and silicon KM-70 (Shin-Etsu Kagaku) 0.05%. Cultivation was carried out at 25°C for 2 days at 200 rpm, an aeration volume of 20 liters/minute and one atom inner pressure.

† FR901463, FR901464 and FR901465 is identical to compound WB2663A, B and C, respectively, in Eur. Pat. Appl. 0 591 534 A1, Jun 12, 1992[15].

Assay for FR901463, FR901464 and FR901465 Production

The amount of FR901463, FR901464 and FR901465 in the fermentation broth was determined by HPLC using YMC AM-303, S-5, A-120 column (4.6 mm inner diameter × 250 mm length, YMC Co., Ltd.) at 210 nm with a mobile phase of 70% aqueous methanol for FR901463, or 40% aqueous acetonitrile for FR901464 and FR901465 at a flow rate of 1 ml/minute. Retention times of FR901463, FR901464 and FR901465 were 7.9, 12.2 and 8.8 minutes, respectively.

Plasmids

pSV2-CAT and pMAM-neo were purchased from Stratagene and Clonetech, respectively. Plasmids DNAs were purified by the use of columns (QIAGEN GmbH) from JM109 harboring the plasmids. For transfection into cells, plasmid DNAs were prepared at a concentration of 0.1 μg/μl in 0.1 mM EDTA solution.

Transfection of Plasmids into MCF-7 Cells

MCF-7 human mammary adenocarcinoma cells were used as the recipient for the reporter gene. MCF-7 cells and their transformed clones were maintained in the culture medium, DULBECCO's modified Eagle medium (DMEM) (Flow Laboratories, North Ryde, Australia) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), penicillin (50 units/ml)-streptomycin (50 μg/ml) (Flow Laboratories), 4 μg/ml insulin (Sigma) and 10 nM estradiol (Sigma) in 5% CO_2-95% air atmosphere at 37°C.

MCF-7 cells were seeded onto 60 mm Petri dishes at the density of 2×10^5 cells/dish in 5 ml of the culture medium and incubated for overnight. Transfection was carried out according to the calcium phosphate precipitation method modified by CHEN and OKAYAMA[12]. The following reactions were performed in the serum tubes. Buffer A (0.1 M HEPES, pH 6.95 and 0.5 M $CaCl_2$) (250 μl) was diluted with distilled water (50 μl), and then the DNA solution (100 μl) containing pSV2-CAT and pMAM-neo was mixed with the diluted Buffer A. The mixture was incubated for 10 minutes at room temperature. After the incubation, Buffer B (0.05 M HEPES, pH 6.95, 0.28 M NaCl, 75 mM NaH_2PO_4 and 75 mM Na_2HPO_4) (100 μl) was added, vortexed and incubated for 30 minutes at room temperature. The resultant solution (500 μl) was added dropwise to the plates of cells without swirling and incubated for 24 hours at 37°C under 3% CO_2. The medium was removed, and the cells were rinsed twice with the growth medium, refed, and incubated for 24 hours. The cells were split in a ratio of 1:40 into 100 mm Petri dish (10 ml of the culture medium). After 24 hours incubation, G418 selection was started at 300 μg/ml of geneticin (Sigma) and hereafter the cells were refed twice a week. At 20 days after the transfection, G418 resistant foci were cloned into 24 well multiwell plates and then the level of CAT protein in individual clones were determined.

CAT Assay

Protein levels of CAT in G418 resistant clones were quantified by CAT ELISA (Boehringer Mannheim GmbH) according to the manufacturer's instructions. One of the clones, M-8, showed a high level production of CAT protein, was seeded onto 24 well multi plates at the density of 1×10^5 cells/well in 1 ml of the culture medium and incubated for 24 hours. The medium was replaced with 1 ml of DMEM supplemented with 0.1% DCCS-FBS (Dextran coated charcoal stripped-FBS) and then incubated for 48 hours for starvation. The medium was removed and CAT was induced in M-8 cells by adding 1 ml of the culture medium containing test sample for 15 hours. The cells were collected in microcentrifuge tubes, centrifuged and then resuspended in 50 μl of CAT buffer (0.25 M Tris, pH 7.8). The cells were disrupted by subjecting to three times of freezing and thawing. After removal of cell debris, 5 μl of ^{14}C-chloramphenicol solution (925 Bq/ml, Amersham), 20 μl of acetyl-CoA solution (4 mM, Sigma) and 75 μl of CAT buffer were added to cell lysates and then the reaction mixtures were incubated at 37°C for 1 hour. CAT reaction was terminated by adding 1 ml of ethyl acetate and vortexed vigorously for 30 seconds. The organic layer was collected in a new microcentrifuge tube, and the ethyl acetate was evaporated *in vacuo*. The dried sample was suspended in 30 μl of ethyl acetate, spotted onto the TLC sheet (Empore) and developed in dichloromethane : methanol (95 : 5) solution. After drying the sheet, it was exposed to film (X-Omat RP, Kodak) for 18 hours at room temperature. To quantify the reaction products, the exposed film was used as a template to locate the labeled reactant and products. The spots corresponding to 3-acetyl and 1,3-diacetyl chloramphenicol were cut from the sheet for counting of radio activity.

In Vitro Cytotoxicity Test

The cytotoxic activity of FR901463, FR901464 and FR901465 *in vitro* was determined as follows. Concentration of the compound required for 50% inhibition of cell growth (IC_{50}; ng/ml) was examined by plotting the logarithms of the concentration *vs.* the growth rate (percentage of control) of the treated cells. Human lung adenocarcinoma A549 cells, human mammary adenocarcinoma MCF-7 cells or human colon adenocarcinoma HCT116 cells (4×10^4 cells/ml), and murine leukemia P388 cells or murine bone marrow cells (1×10^5 cells/ml) were treated in suspension in 100 μl of DMEM supplemented with 10% FBS and penicillin (50 units/ml)-streptomycin (50 μg/ml) in 5% CO_2-95% air atmosphere at 37°C. The cytotoxity was colorimetrically determined at 550 nm (and 660 nm as a reference) according to MTT method described by MOSMANN[13].

Antimicrobial Test

Antimicrobial activities of FR901463, FR901464 and FR901465 were determined by a serial broth dilution method in Nutrient broth for Gram-positive and Gram-

negative bacteria and in Sabouraud media for fungi and yeast. The inoculum size was adjusted to 5×10^5 cfu/ml for bacteria and 1×10^6 cfu/ml for fungi and yeast. Minimum inhibitory concentration is expressed in terms of μg/ml after 18 hours at 37°C for bacteria and 48 hours incubation at 28°C for fungi and yeast.

Acute Toxicity Test

BDF_1 mice (female, 7 weeks old) were purchased from Charles River Japan Inc., Atsugi, Japan. The acute toxicity was determined in mice by a single intraperitoneal injection of graded doses of FR901463, FR901464 and FR901465 prepared in the saline containing 10% polyoxyethylated hydrogenated castor oil (HCO60 solution).

Analytical Measurement

Optical rotation was measured with a Jasco DIP polarimeter using a 10-cm microcell. UV and IR spectra were obtained with 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 Brucker AM400wb spectrometer controlled with an ASPECT 3000 computer.

Results

Taxonomy of the Producing Organism

Morphological observation of strain No. 2663 was made by the optical microscope and the scanning electron microscope with cells grown on nutrient agar at 30°C for 24 hours (Table 1 and Fig. 1). Strain No. 2663 was a Gram-negative, mobile bacilli, and formed a pink soluble pigment on a glucose-peptone agar medium. The cells were $0.8 \sim 1.0 \times 1.5 \sim 3.0\,\mu$m in size.

Physiological characteristics are listed in Table 2. The growth temperature range was from 13 to 35°C. Strain No. 2663 was positive for oxidase and catalase, and was oxidative in the O-F test. This strain was positive for the decomposition of gelatin, casein and Tween 80, but negative for the decomposition of starch. Acid formation was observed from D-glucose, D-fructose, D-mannitol, sucrose and lactose. This strain utilized D-glucose, D-mannose and D-mannitol but did not utilize L-

Fig. 1. Electron micrograph of strain No. 2663.
Scale: 5 μm.

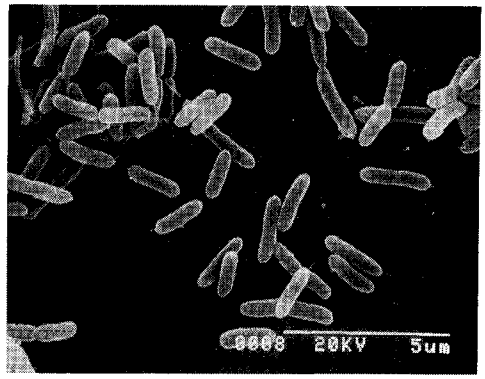


Table 1. Morphological characteristics of strain No. 2663.

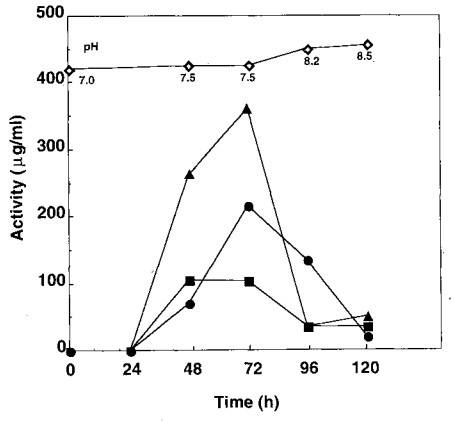
Gram strain	Negative
Color tone of colony	Grayish yellow
Cell morphology	Bacillus
Cell size	$0.8 \sim 1.0 \times 1.5 \sim 3.0$ mm
Mobility	Positive
Sporulation	Negative

Table 2. Physiological characteristics of strain No. 2663.

Growth temperature	13~35°C	Acid production from	
Growth in air	Positive	D-Glucose	Positive
Growth on MacConkey's agar medium	Positive	D-Xylose	Negative
Catalase	Positive	D-Fructose	Positive
Oxidase	Positive	D-Mannitol	Positive
O-F test	Oxidation	Maltose	Negative
Utilization of citric acid	Positive	Sucrose	Positive
Reduction of nitrate	Negative	Lactose	Positive
Indole production	Negative	Salicin	Negative
H_2S production (SIM)	Negative	Utilization of	
Esculin hydrolysis	Negative	D-Glucose	Positive
Starch hydrolysis	Negative	L-Arabinose	Negative
ONPG teat	Negative	D-Mannose	Positive
DNase	Negative	D-Mannitol	Positive
Tween 80 hydrolysis	Positive	*N*-Acetyl-D-glucosamine	Positive
Gelatin hydrolysis	Positive	Maltose	Negative
Casein hydrolysis	Positive	Gluconic acid	Positive
Lysine decarboxylase	Positive (weak)	Capric acid	Positive
Arginine dihydrolase	Negative	Adipic acid	Positive
Ornithine decarboxylase	Negative	Malic acid	Positive
		Citric acid	Positive
		Phenyl acetate	Positive

Fig. 2. Time course of FR901463, FR901464 and FR901465 production.

● FR901463, ▲ FR901464, ■ FR901465.



Activities of FR901463, FR901464 and FR901465 were determined by HPLC using YMC AM-303, S-5, A-120 column.

arabinose and maltose.

According to BERGEY's Manual of Systematic Bacteriology, the strain was identified as *Pseudomonas* sp. No. 2663. The strain was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, under the accession of No. FERM BP-3421.

Fermentation of FR901463, FR901464 and FR901465

Fig. 2 shows a typical example of the time course of FR901463, FR901464 and FR901465 production by *Pseudomonas* sp. No. 2663 in a 30-liter jar fermenter, along with the pH of the medium. The amount of FR901463, 901464 and 901465 in the fermentation broth reached about 200, 360 and 100 μg/ml at 72 hours, respectively.

Isolation and Purification

The fermentation broth (400 liters) was adjusted to pH 7.0 with 6 N-HCl and extracted twice with 600 liters of ethyl acetate. The extracts were combined for concentration under reduced pressure. After dehydration with anhydrous sodium sulfate, silica gel (1.7 liters) was added to the concentrate and dried under reduced pressure. The resulting powder thus obtained, was applied to a silica gel column of 4 liters, which prefilled with *n*-hexane. The material was eluted stepwise with *n*-hexane : ethyl acetate solution (17 liters, 100 : 0, 75 : 25, 50 : 50, and 0 : 100, v/v, respectively). FR901463 was eluted in the *n*-hexane : ethyl acetate (50 : 50) fraction, and FR901464 and FR901465 were eluted in the ethyl acetate fraction, respectively.

First, the method of purification of FR901463 will be described below. The *n*-hexane : ethyl acetate (50 : 50) fraction was concentrated under reduced pressure, and the resulting oily material (21 g) was combined with 50 ml of silica gel and dried under reduced pressure. The resulting powder was placed on a 450 ml silica gel column which pre-filled with *n*-hexane. The column was eluted with 2.5 liters of *n*-hexane and then *n*-hexane : acetone (2.5 liters, 8 : 1, 6 : 1, 4 : 1 and 2 : 1, v/v, respectively), upon which activity was observed in both the *n*-hexane : acetone (4 : 1) and (2 : 1) fractions. The active fractions were combined and concentrated under reduced pressure. The oily substance (8 g) was dissolved in 700 ml of 10% aqueous acetonitrile, and applied on a 200 ml of ODS gel (YMC ODS-AM 120-S50) column which was pre-filled with water for reverse phase chromatography. After developing with 10%, 20%, and 30% aqueous acetonitrile (600 ml each), the column was eluted with 600 ml of 40% aqueous acetonitrile. The active fraction was concentrated under reduced pressure and the acetonitrile removed, after which extraction was made twice with equal volumes of ethyl acetate, and the extracts were combined and concentrated. The crude powder was dissolved in a small amount of dichloromethane, and *n*-hexane was added to obtain colorless needle-shaped crystals of FR901463 (512 mg).

Next, an explanation will be given regarding the method of purification of FR901464. The ethyl acetate fraction previously mentioned was concentrated under reduced pressure, and the resulting oily materials (62.9 g) was dissolved in 1.1 liters of *n*-hexane : chloroform (4 : 7), and placed in 1.2 liters of silica gel which pre-filled with *n*-hexane : chloroform (1 : 1). After developing with 3.6 liters chloroform, the column was eluted with 3.6 liters each of chloroform : acetone mixtures (100 : 1, 50 : 1 and 25 : 1), respectively. The activity of FR901464 was observed in chloroform : acetone (100 : 1 and 50 : 1) fractions, and that of FR 901465 in chloroform : acetone (25 : 1). The active fractions of FR901464 were combined and concentrated under reduced pressure, and the oily substance (7.2 g) was dissolved in 2 liters of 20% aqueous acetonitrile and placed in a 200 ml of ODS gel column which prepacked with 20% aqueous acetonitrile. The column was washed with 600 ml of 25% acetonitrile, and then eluted with 27% aqueous acetonitrile. The active fraction was concentrated under reduced pressure to remove acetonitrile, after which extraction was made twice with equal volumes of ethyl acetate. The extracts

Table 3. Physico-chemical properties of FR901463, FR901464 and FR901465.

	FR901463	FR901464	FR901465
Appearance	Colorless needles	White powder	White powder
MP	102~104°C	65~70°C	86~91°C
$[\alpha]_D^{23}$	−36° (*c* 0.5, CH_2Cl_2)	−12° (*c* 0.5, CH_2Cl_2)	−9° (*c* 0.5, CH_2Cl_2)
Molecular formula	$C_{27}H_{42}ClNO_8$	$C_{27}N_{41}NO_8$	$C_{27}H_{41}NO_9$
Molecular weight			
FAB-MS (*m/z*)	566 $(M+Na)^+$	508 $(M+H)^+$	524 $(M+H)^+$
HRFAB-MS (*m/z*)			
Calcd:		508.2910	524.2860
Found:		508.2929	524.2849
Elemental analysis			
Calcd for $C_{27}H_{24}ClNO_8$:	C 59.60, H 7.78, N 2.57, Cl 6.52		
Found:	C 60.11, H 8.10, N 2.44, Cl 6.86		
UV $\lambda_{max}^{acetonitrile}$ nm (ε)	234 (38,000)	235 (30,500)	235 (29,000)
Color test			
Positive	I_2, $Ce(SO_4)_2$-H_2SO_4, $KMnO_4$	I_2, $Ce(SO_4)_2$-H_2SO_4, $KMnO_4$	I_2, $Ce(SO_4)_2$-H_2SO_4, $KMnO_4$
Negative	$FeCl_3$, ehrlich, ninhydrin	$FeCl_3$, ehrlich, ninhydrin	$FeCl_3$, ehrlich, ninhydrin
Solubility			
Soluble	CH_2Cl_2, $CHCl_3$, acetone, EtOAc	CH_2Cl_2, EtOAc, $CHCl_3$, acetonitrile	CH_2Cl_2, $CHCl_3$, acetonitrile
Slightly soluble		H_2O	H_2O
Insoluble	Hexane, H_2O	Hexane	Hexane
TLC (Rf value)			
System I[a]	0.74	0.50	0.08
System II[b]		0.18	0.81

[a] Plate: Silica gel 60 F_{254} (E. Merck Co.), CH_2Cl_2-acetone = 2 : 3.
[b] Plate: RP-18 WF_{254} (E. Merck Co.), 60% aq. acetonitrile.

were combined and concentrated under reduced pressure to obtain white powder of FR901464 (819 mg).

Further, an explanation will be given regarding the method of purification of FR901465. The chloroform : acetone (25 : 1) fraction as described above was concentrated under reduced pressure, and the oily substance was dissolved in 300 ml of chloroform : hexane (1 : 1), and placed in a 100 ml of silica gel column which pre-filled with *n*-hexane : acetone (10 : 1). After developing with 300 ml each of *n*-hexane : acetone mixtures (5 : 1, 4 : 1, 7 : 2, 3 : 1, 2 : 1), the column was eluted with 300 ml each of *n*-hexane : acetone (1 : 1) and acetone. The eluted fractions were combined and concentrated under reduced pressure, and the oily substance was dissolved in 125 ml of a 20% aqueous acetonitrile and placed in a 200 ml of ODS gel column which was pre-filled with 10% aqueous acetonitrile. After developing with 24% aqueous acetonitrile, the column was eluted with 26% aqueous acetonitrile. The active fraction was concentrated under reduced pressure to remove acetonitrile. The concentrate was extracted twice with an equal volume of ethyl acetate, and the extracts were combined and concentrated to dryness. The crude powder was dissolved in a small amount of dichloromethane, and then *n*-hexane was added to obtain white powder of FR901465 (70 mg).

Physico-chemical Properties

The physico-chemical properties of FR901463, FR901464 and FR901465 are given in Table 3. FR901463, FR901464 and FR901465 are soluble in acetonitrile, chloroform and ethyl acetate, sparingly soluble in water but insoluble in *n*-hexane. They show positive color reactions to iodine vapor and $KMnO_4$, though negative against $FeCl_3$ and ninhydrin. The ^1H, ^{13}C and IR spectra of FR901463 are reproduced in Figs. 3, 4 and 5, respectively. The strong UV absorption at 235 nm are characteristic of a conjugated diene. Their IR bands at 3400, 1735 and 1665 cm^{-1} are indicative of the presence of hydroxyl, ester and a conjugated amide carbonyl.

The determination of the structures were accomplished primarily by a series of 2-D NMR techniques (Fig. 6). Details of the structure determination will be reported in due course.

Biological Activity

Transcriptional Regulatory Activity

The transcriptional regulatory activity of FR901463, FR901464 and FR901465 for the promoter of SV40 DNA tumor virus was determined by using the CAT assay system. The activity of added sample for transcriptional

Fig. 3. 400 MHz ^{1}H NMR spectrum of FR901463 in CD_2Cl_2.

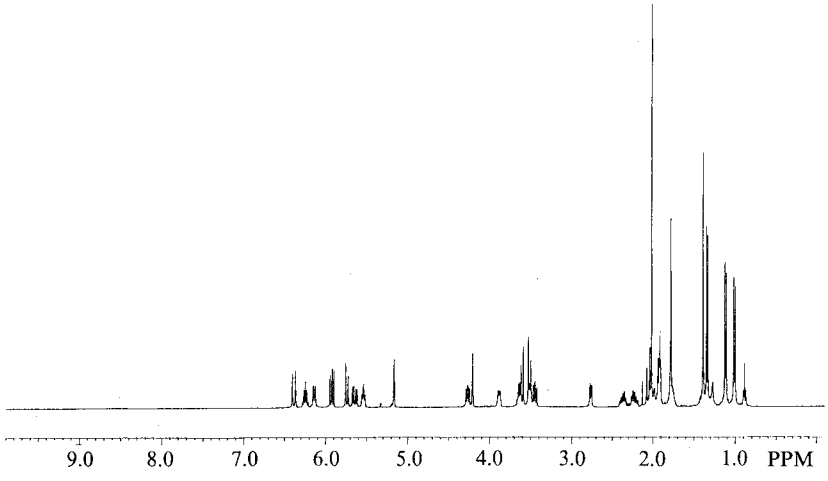


Fig. 4. 100 MHz ^{13}C NMR spectrum of FR901463 in CD_2Cl_2.

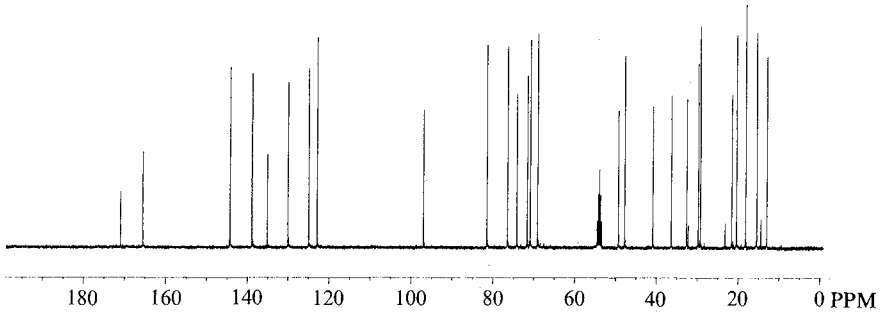
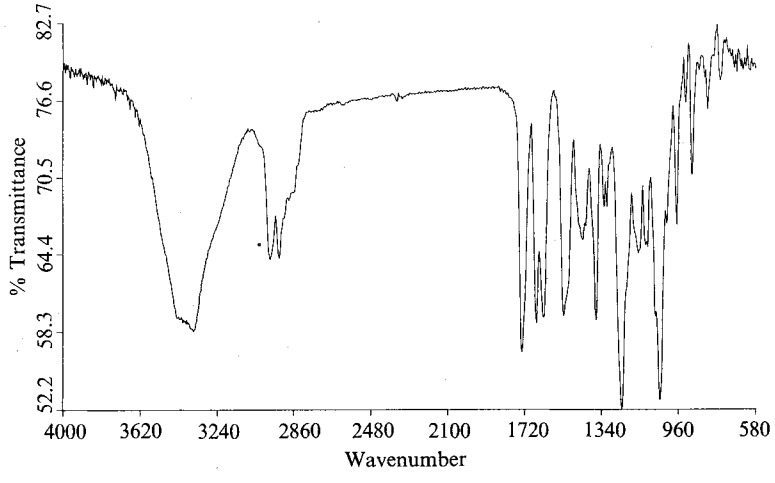


Fig. 5. IR spectrum of FR901463 in KBr.



regulation was measured by counting the formation of 3-acetyl and 1, 3-diacetyl ^{14}C-chloramphenicol. The autoradiography in Fig. 7 shows Rf values of and its acetylated products of ^{14}C-chloramphenicol on the exposed film in CAT assay. FR901463, FR901464 and FR901465 remarkably enhanced the transcriptional activity of SV40 promoter in the assay.

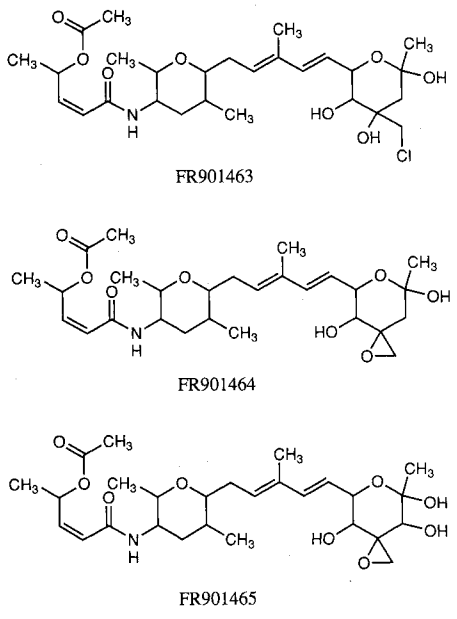
Cytotoxic Activity

The result was shown in Table 4. FR901463, FR901464 and FR901465 were effective against the human solid tumor cells, though weaker, against mouse bone marrow cells.

Antimicrobial Activity

FR901463, FR901464 and FR901465 were devoid of antimicrobial activity when tested *vs.* the following microorganisms at 100 μg/ml; *Escherichia coli* NIHJ Jc-2, *Pseudomonas aeruginosa* ATCC 14970, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* 209P, *Candida albicans*, *Aureobasidium pullulans*, *Aspergillus niger*, *Geotricum candidum* and *Shizosaccharomyces pombe*.

Fig. 6. Structures of FR901463, FR901464 and FR901465.



Acute Toxicity

The LD_{50} was 10 mg/kg for FR901463, 3.2 mg/kg for FR901464 and 0.32 mg/kg for FR901465, respectively.

Discussion

FR901463, FR901464 and FR901465, which exhibited transcriptional regulatory activities, were isolated from the culture broth of *Pseudomonas* sp. No. 2663. From the evidence of physico-chemical data, these compounds were to have novel structures (Fig. 6) which contained a conjugated diene and a conjugated amide carbonyl as their side chain units.

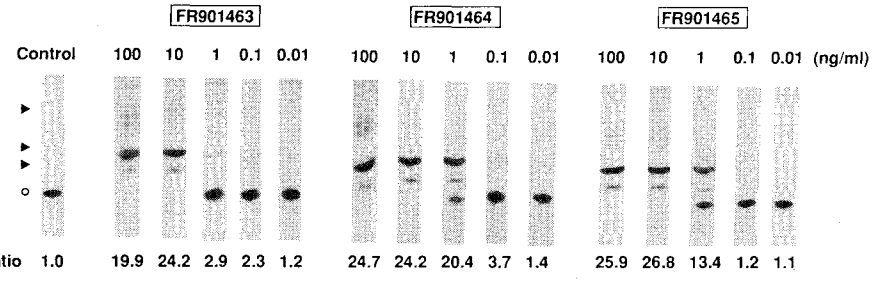
In vitro FR901463, FR901464 and FR901465 remarkably enhanced the transcriptional activity of SV40 DNA virus promoter and further showed potent cytotoxic activities against human tumor cells. On the other hand, known antitumor drugs such as adriamycin, mitomycin or vinblastine did not affect the transcriptional activity, though these compounds have strong cytotoxic activities (data not shown). These results suggest that FR901463, FR901464 and FR901465 may be a new type of antitumor drug having a novel mode of action. The details of the *in vivo* evaluation and the mode of action of these compounds will be published in

Table 4. Antitumor activities of FR901463, FR901464 and FR901465 against tumor cell lines (*in vitro*).

	IC_{50} (ng/ml)		
	FR901463	FR901464	FR901465
MCF-7	0.46	0.91	0.59
A549	0.35	0.66	0.44
HCT116	0.22	0.31	0.34
SW480	0.40	0.51	0.53
P388	0.82	1.69	0.48
BM*	2.03	5.01	1.91

* Mouse bone marrow cells.

Fig. 7. Effects of FR901463, FR901464 and FR901465 on SV40 promoter dependent CAT expression in M-8 cells.



Serum-starved M-8 cells were induced for CAT protein with the culture medium containing varying concentrations of FR901463, FR901464 and FR901465 for 15 hours. CAT protein was extracted by freezing and thawing of treated cells and CAT assay was performed with ^{14}C-chloramphenicol as described in Materials and Methods. Arrow heads show acetylated products of chloramphenicol (1,3-diacetyl product shows the highest Rf values, followed by 3-acetyl and 1-acetyl ones). Open circle shows chloramphenicol. T/C (Treatment/Control) ratio was calculated, using combined radio activities of 1, 3-diacetyl and 3 acetyl converted products in each sample.

another paper[14].

In our screening system, we used MCF-7 human mammary adenocarcinoma as the recipient for the CAT expression plasmid pSV2-CAT, which is one of the most well-characterized tumors in signal transduction. The CAT activity reflects the activities of certain transcriptional factors expressed in the tumor cells and CAT protein is extremely stable when expressed in eukaryotic cells. However, it is possible that the stability of CAT protein may disturb the correct detection of the subtle changes in the transcriptional regulation. Serum starvation of the cells is necessary to exhaust preexisting CAT protein before induction. In our experiments, starvation for 48 hours in DMEM containing DCCS-FBS was revealed to be effective, sustaining high viability of starved cells.

Further, in order to neglect the nonspecific cytotoxic effect due to the contaminating DNA synthesis inhibitors in the samples, CAT induction with serum had been done for 15 hours which was the minimum time to induce detectable level of CAT activity without cell cycle transition of cells to S phase (data not shown).

The complex of No. 2663 products, which was discovered by use of an innovative screening system, affects transcriptional regulation and have antitumor effects.

References

1) LOWE, S. W. & H. E. RULEY: Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes & Dev. 7: 535~545, 1993
2) WAGNER, A. J.; J. M. KOKONTIS & N. HAY: Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce $p21^{waf1/cip1}$. Genes & Dev. 8: 2817~2830, 1996
3) WU, X. & A. J. LEVINE: p53 and E2F-1 cooperate to mediate apoptosis. Proc. Natl. Acad. Sci. U.S.A. 91: 3602~3606, 1994
4) TAN, T. M. C. & R. C. Y. TING: *In vitro* and *in vivo* inhibition of human papillomavirus type 16 *E6* and *E7* genes. Cancer Res. 55: 4599~4605, 1995
5) EASTHAM, J. A.; S. J. HALL, I. SEHGAL, J. WANG, T. L. TIMME, G. YANG, L. CONNELL-CROWLEY, S. J. ELLEDGE, W. ZHANG, J. W. HARPER & T. C. THOMPSON: *In vitro* gene therapy with *p53* or *p21* adenovirus for prostate cancer. Cancer Res. 55: 5151~5155, 1995
6) YANG, Z.; N. D. PERKINS, T. OHNO, E. G. NABEL & G. J. NABEL: The p21 cyclindependent kinase inhibitor suppresses tumorigenicity *in vitro*. Nature Medicine 1: 1052~1056, 1995
7) SHAO, Z.; M. I. DAWSON, X. S. LI, A. K. RISHI, M. S. SHEIKH, Q. HAM, J. V. ORDONEZ, B. SHROOT & J. A. FONTANA: p53 independent G_0/G_1 arrest and apoptosis induced by a novel retinoid in human breast cancer cells. Oncogene 11: 493~504, 1995
8) KOHL, N. E.; S. D. MOSSER, S. J. DESOLMS, E. A. GIULIANI, D. L. POMPLIANO, S. L. GRAHAM, R. L. SMITH, E. M. SCOLNICK, A. OLIFF & J. B. GIBBS: Selective inhibition of *ras*-dependent transformation by a farnesyltransferase inhibitor. Science 260: 1934~1937, 1993
9) UEDA, H.; H. NAKAJIMA, Y. HORI, T. FUJITA, M. NISHIMURA, T. GOTO & M. OKUHARA: FR901228, a novel antitumor bicyclic depsipeptide produced by chromobacterium violaceum No. 968. I. Taxonomy, fermentation, isolation, physico-chemical and biological properties, and antitumor activity. J. Antibiotics 47: 301~310, 1994
10) JONES, N. C.; P. W. J. RIGBY & E. B. ZIFF: Trans-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. Genes & Dev. 2: 267~281, 1988
11) SNEATH, P. H. A.: *Genus Chromobacterium* Bergonzini 1881, 153. In BERGEY's Manual of Systemic Bacteriology 9th Edition Vol. 1., *Eds.*, N. R. KRIEG & J. G. HOLT, pp. 580~582, Williams & Wilkins Co., Baltimore, 1984
12) CHEN, C. & H. OKAYAMA : High-efficiency transformation of mammalian cell by plasmid DNA. Mol. Cell Biol. 7: 2745~2752, 1987
13) MOSMANN, T.: Rapid colorimetric assay for cellular growth and survival; Application to proliferation and cytotoxicity assays. J. Immunol. Methods 65: 55~63, 1983
14) NAKAJIMA, H.; Y. HORI, H. TERANO, M. OKUHARA, T. MANDA, S. MATSUMOTO & K. SHIMOMURA: New antitumor substances, FR901463, FR901464 and FR901465. II. Activities against experimental tumors in mice and mechanism of action. J. Antibiotics 49: 1204~1211, 1996
15) NAKAJIMA, H.; H. HORI, T. GOTO, S. TAKASE, K. VERHAEGHE, H. TERANO & M. OKUHARA (Fujisawa): Substance WB2663, production thereof and use. Eur. Pat. Appl. 0 591 534 A1, Jun 12, 1992