NEW ANTITUMOR SUBSTANCES, BE-12406A AND BE-12406B, PRODUCED BY A STREPTOMYCETE

I. TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

KATSUHISA KOJIRI, HIROHARU ARAKAWA, FUMIO SATOH†, KENJI KAWAMURA†, AKIRA OKURA, HIROYUKI SUDA* and MASANORI OKANISHI

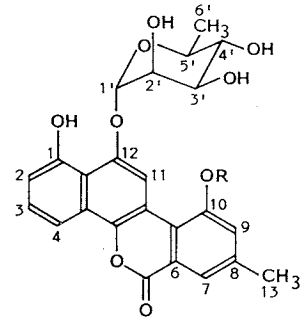
Exploratory Research Laboratories, Banyu Pharmaceutical Co., Ltd.,
2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan
†Okazaki Research Laboratories, Banyu Pharmaceutical Co., Ltd.,
3-9-1 Kamimutsuna, Okazaki 444, Japan

(Received for publication April 25, 1991)

New antitumor substances, designated BE-12406A and BE-12406B, were isolated from the culture broth of a streptomycete, strain BA12406. The active principles were extracted from mycelium by methanol and successively purified by silica gel column chromatography and preparative TLC. BE-12406A and BE-12406B inhibited the growth of vincristine-resistant or doxorubicin-resistant P388 murine leukemia cell lines as well as their parent sensitive cell line. In *in vivo* experiments, BE-12406A inhibited the growth of S-180 murine ascites tumor.

In the course of our screening program for new antitumor substances, a strain BA12406 isolated from a soil sample collected in Tokuyama village, Gifu Prefecture, Japan, was found to produce active principles. This strain was classified as *Streptomyces rutgersensis* subsp. *castelarensis*. The active principles were purified by their activities to inhibit the growth of P388 murine leukemia cell lines. After extraction of the mycelium of the producing organism, these antitumor substances were purified by silica gel column chromatography and preparative TLC. BE-12406A and BE-12406B inhibited the growth of doxorubicin-resistant or vincristine-resistant P388 murine leukemia cell lines as well as their parent sensitive cell line. This paper describes the taxonomy of the producing organism and the isolation, physico-chemical properties and biological properties of BE-12406A and BE-12406B. The structure elucidation studies of these compounds are described in an accompanying paper[1]. The structures of BE-12406A and BE-12406B are shown in Fig. 1.

Fig. 1. The structures of BE-12406A and BE-12406B.



BE-12406A R = CH_3
BE-12406B R = H

Results

Taxonomy of the Producing Organism

Strain BA12406 was isolated from a soil sample collected in Tokuyama village, Gifu Prefecture, Japan. For the taxonomic characterization of strain BA12406, the methods and media recommended by the International Streptomyces Project (ISP)[2] were principally used.

The vegetative mycelia of strain BA12406 grew well on both synthetic and complex media, and no

Table 1. Cultural characteristics of strain BA12406.

Agar medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Yeast extract-malt extract agar (ISP-2)	Good	Abundant Grayish yellow brown Powdery	Brown	None
Oatmeal agar (ISP-3)	Good	Abundant Grayish yellow brown Powdery	Olive gray	None
Inorganic salts-starch agar (ISP-4)	Good	Abundant Grayish yellow brown Powdery	Pale brown	None
Glycerol-asparagine agar (ISP-5)	Good	Abundant Grayish yellow brown Powdery	Brown	Yellowish brown (faint)
Tyrosine agar (ISP-7)	Good	Abundant Grayish yellow brown Powdery	Brown	Reddish orange (faint)
Nutrient agar	Good	None	Pale yellowish white	None
Sucrose-nitrate agar	Poor	None	Pale yellowish white	None
Glucose-asparagine agar	Good	None	Pale yellowish white	None

fragmentation was observed. The aerial mycelia grew poorly on nutrient agar, sucrose-nitrate agar and glucose-asparagine agar but grew abundantly on other media. The mature sporophores were *Spira* to *Retinaculiaperti* type and had more than 20 spores per chain. The spores were short-cylindrical ($0.2\sim0.5\times0.3\sim0.6\,\mu m$) with smooth surfaces. Special morphological organs such as whirls, sclerotia, sporangia were not observed. The cultural characteristics and the physiological properties of strain BA12406 are shown in Tables 1, 2 and 3. The colors of substrate and aerial mycelium were brown to pale yellowish white and grayish yellow brown, respectively. The strain utilized most of the carbon sources, but did not utilize inositol and sucrose. Presence of L,L-diaminopimelic acid and glycine in the whole cell hydrolysate indicated that the strain had type I cell walls.

Table 2. Physiological properties of strain BA12406.

Melanin formation	Negative
Hydrolysis of starch	Positive
Coagulation of milk	Negative
Peptonization of milk	Positive
Liquefaction of gelatin	Positive
NaCl tolerance	7%
Temperature range for growth	12~32°C
Optimum temperature	20~28°C

Based on the taxonomic properties described above, strain BA12406 was considered to belong to the genus *Streptomyces*. The characteristics of strain BA12406 were compared with those of the known *Streptomyces* species[3], and *S. rutgersensis* subsp. *castelarensis* was considered to be the most related strain. By direct comparison of strain BA12406 and *S. rutgersensis* subsp. *castelarensis* JCM 4978, good agreement was obtained between these two strains except for NaCl tolerance (Table 3). Therefore, strain BA12406 was identified as a strain of *S. rutgersensis* subsp. *castelarensis*[4]. This strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-10414.

Fermentation of Strain BA12406

Spores of strain BA12406 were inoculated into 100 ml of a medium (pH 6.7) composed of glucose 0.1%, dextrin 2.0%, corn gluten meal 1.0%, fish meal 0.5%, yeast extract 0.1%, NaCl 0.1%, $MgSO_4\cdot7H_2O$ 0.05%, $CaCl_2\cdot2H_2O$ 0.05%, $FeSO_4\cdot7H_2O$ 0.0002%, $CuCl_2\cdot2H_2O$ 0.0004%, $MnCl_2\cdot4H_2O$ 0.0004%,

Table 3. Comparison of taxonomic characteristics of strain BA12406 with *Streptomyces rutgersensis* subsp. *castelarensis*.

	BA12406	*S. rutgersensis* subsp. *castelarensis* (JCM 4978)
Aerial mass color	Grayish yellow brown	Yellowish gray
Color of reverse	Brown to olive gray	Brown
Soluble pigment	None	None
Melanin formation (ISP-7)	Negative	Negative
NaCl tolerance	Growth on 7% NaCl	No growth on 7% NaCl
Hydrolysis of starch	Positive	Positive
Carbon source utilization:		
D-Glucose	+	+
D-Xylose	+	+
L-Arabinose	+	+
L-Rhamnose	+	+
D-Fructose	+	+
D-Galactose	+	+
Raffinose	+	+
D-Mannitol	+	+
Inositol	−	−
Salicin	+	+
Sucrose	−	−

JCM: Japan Collection of Microorganisms, RIKEN (The Institute of Physical and Chemical Research).
+: Utilized.

$CoCl_2 \cdot 6H_2O$ 0.0004%, $ZnSO_4 \cdot 7H_2O$ 0.00008%, $Na_2B_4O_7 \cdot 10H_2O$ 0.00008%, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.00024% and 3-(*N*-morpholino)propanesulfonic acid 0.5% in four 500-ml conical flasks and cultured at 28°C for 72 hours. One ml of the seed culture was dispensed into each of one hundred fifty of 500-ml conical flasks containing 100 ml of the above medium and cultured on a rotary shaker (180 rpm) at 28°C for 120 hours.

Isolation of BE-12406A and BE-12406B

The mycelium was obtained by filtration from the whole broth (*ca.* 15 liters) and washed with 500 ml of water. This mycelium was extracted twice with 4:5 liters of methanol and the extract (*ca.* 9 liters) was concentrated to about 800 ml. The concentrated solution was extracted with 5 liters of ethyl acetate and the extract was evaporated. The residue was extracted with about 250 ml of tetrahydrofuran and remaining insolubles were filtered off. The filtrate was concentrated to give 8.2 g of a crude substance containing both BE-12406A and BE-12406B. This crude product was dissolved in 500 ml of chloroform-tetrahydrofuran (3:2) and the solution was applied to a column of silica gel (6.5 × 37 cm) and eluted with a solvent mixture of chloroform-tetrahydrofuran (1:1). In this chromatography, BE-12406A emerged faster than BE-12406B and this BE-12406A fraction was concentrated to isolate 671 mg of BE-12406A as a pale yellow crystalline powder. The subsequent elution with the same solvent gave a fraction containing both BE-12406A and BE-12406B. This fraction was concentrated to give 280 mg of a BE-12406A/B complex. A part of BE-12406A/B complex (14.4 mg) thus obtained was purified by preparative TLC (20 × 20 cm, Merck) with a mixture of chloroform and methanol (5:1) as a developing solvent. The area corresponding to BE-12406B was scraped from the TLC plate and extracted with methanol. The extract was concentrated and subjected to column chromatography on Sephadex LH-20 (1.5 × 120 cm, Pharmacia) using methanol as the eluent. The BE-12406B containing fractions were concentrated to give 5.1 mg of

BE-12406B as a pale yellow crystalline powder.

Physico-chemical Properties of BE-12406A and BE-12406B

BE-12406A was obtained as a pale yellow crystalline powder, which was sparingly soluble in water and moderately soluble in methanol. Rf 0.49 (chloroform - methanol (5 : 1), Kieselgel 60, Merck), Rt on HPLC: 8.58 minutes (Capcell Pak C18, Shiseido, 4.6 × 250 mm, MeOH - H_2O (75 : 25), 1 ml/minute), mp: 238 ~ 243°C (dec), FAB-MS: 469 $(M+H)^+$, UV λ_{max}^{MeOH} nm 243, 264, 273, 300, 310, 324, 338, 375. The IR and ^1H NMR spectra of BE-12406A are shown in Figs. 2 and 3, respectively.

BE-12406B was obtained as a pale yellow crystalline powder, which was sparingly soluble in water

Fig. 2. IR spectrum of BE-12406A in KBr.

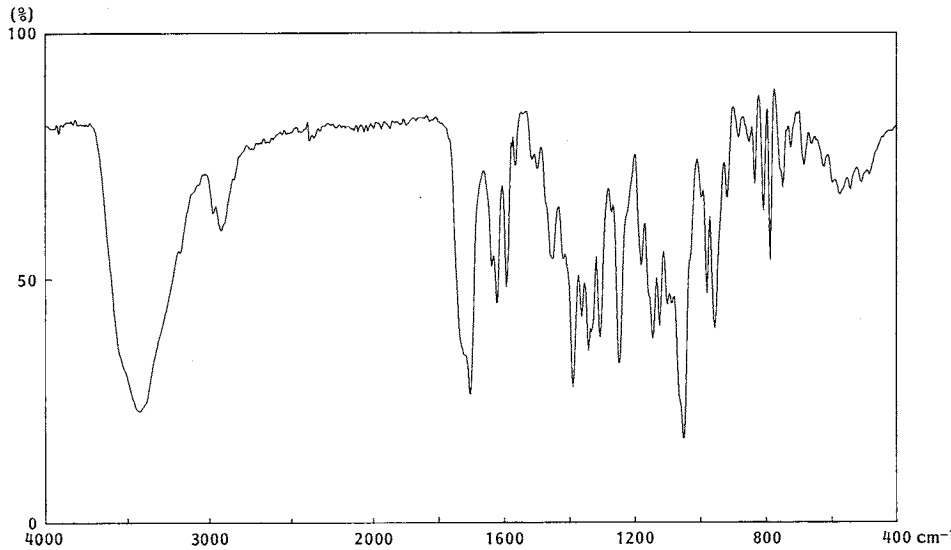


Fig. 3. ^1H NMR spectrum of BE-12406A in DMSO-d_6.

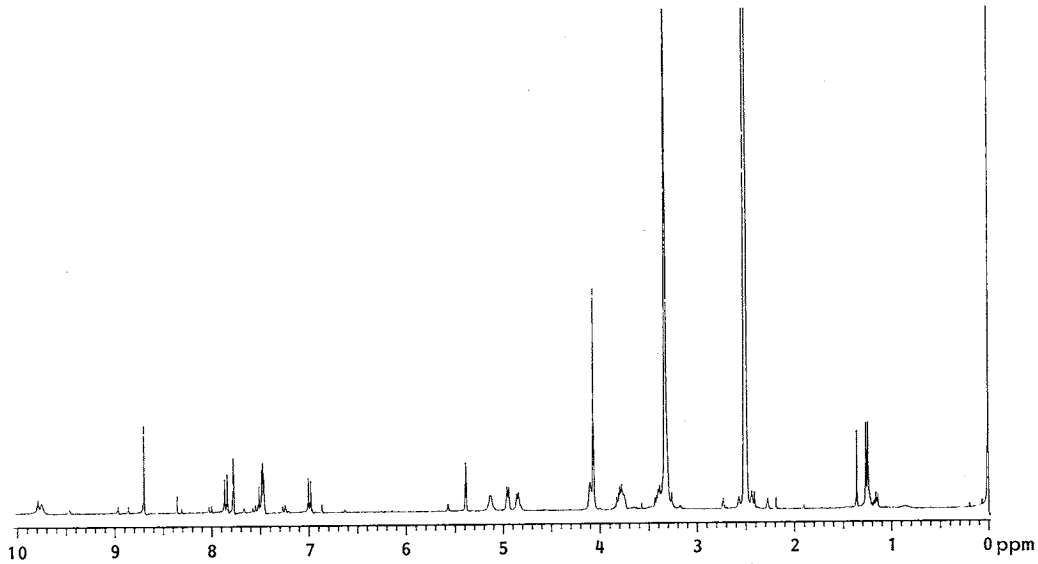


Fig. 4. IR spectrum of BE-12406B in KBr.

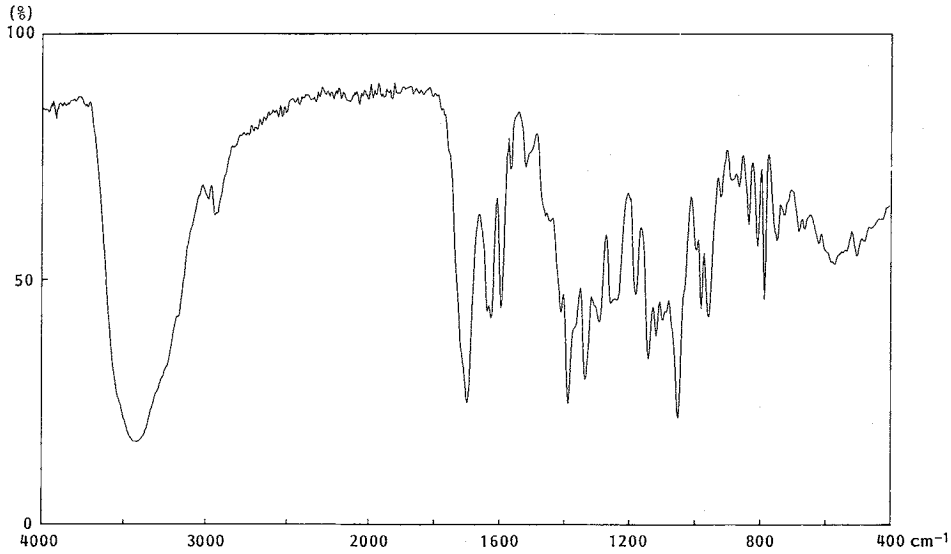
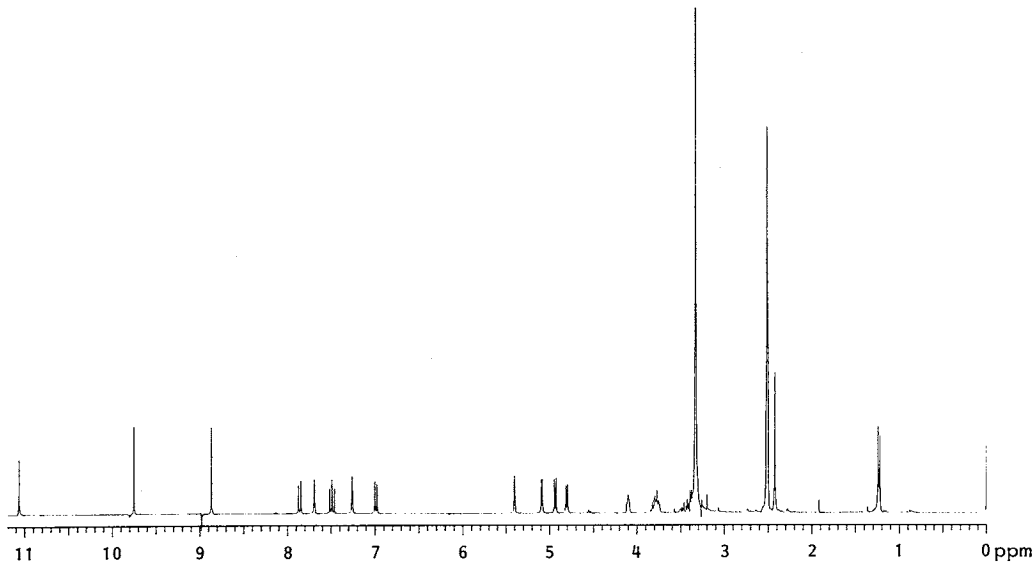


Fig. 5. ^{1}H NMR spectrum of BE-12406B in DMSO-d_6.



and moderately soluble in methanol. Rf 0.30 (chloroform - methanol (5 : 1), Kieselgel 60, Merck), Rt on HPLC: 6.48 minutes (Capcell Pak C18, Shiseido, 4.6 × 250 mm, MeOH - H_2O (75 : 25), 1 ml/minute), mp: 230 ~ 235°C (dec), FAB-MS: 455 $(M+H)^+$, UV λ_{max}^{MeOH} nm 244, 264, 273, 325, 340, 375. The IR and ^{1}H NMR spectra of BE-12406B are shown in Figs. 4 and 5, respectively.

Biological Activities of BE-12406A and BE-12406B

In the *in vitro* antitumor assay using P388 tumor cell lines[5], BE-12406A or BE-12406B was first dissolved in dimethyl sulfoxide (DMSO) and the solution was serially diluted with a cell culture medium containing 20% of DMSO (20% DMSO - RPMI-1640 medium). Then, 2 μl of each dilution was added to 200 μl of a cell culture medium (10% fetal calf serum - RPMI-1640 medium) containing 2.5×10^4 tumor

cells and the mixture was incubated under 5% CO_2 at 37°C for 72 hours. The viable cells were then counted with a Coulter counter. The results were compared with the control data. The concentrations of BE-12406A and BE-12406B required to inhibit 50% of the growth (IC_{50}) of the P388/S cell line were 0.8 and 7 μM, respectively. The IC_{50} values of BE-12406A and BE-12406B for P388/VCR were 0.2 and 7 μM, respectively. The P388/S cell line is one of the mouse leukemia cell lines sensitive to antitumor agents and the P388/VCR cell line is a subline of P388 leukemia which has acquired resistance to the antitumor agent vincristine. Furthermore, BE-12406A and BE-12406B inhibited the growth of the P388/ADR cell line, which had acquired resistance to doxorubicin, with IC_{50} values of 0.9 and 11 μM, respectively.

Table 4. Effect of BE-12406A on S-180 ascites tumor.

Substance	Dosage, ip (mg/kg/injection)	MST (day)	T/C (%)
BE-12406A	40	>30.0	>300
	20	>30.0	>300
	10	15.0	150
	5	13.0	130
	2.5	12.0	120
Control group	—	10.0	100

Female ICR mice were injected (ip) with 10^6 S-180 tumor cells on day 0. BE-12406A was administered (ip) on days 1, 4, 7, 9 and 13.

BE-12406A showed an antitumor effect on transplanted mouse S-180 tumor cells (ascites type). In this assay 10^6 S-180 tumor cells were injected intraperitoneally into ICR mice and the 5% DMSO-phosphate buffered saline solution of BE-12406A in a dilution series was also injected intraperitoneally. The results are summarized in Table 4. Acute toxicity of BE-12406A was tested at 100 mg/kg (female ICR) and no death was found on day 5 after a single ip injection.

Discussion

It is well known that many currently available antitumor drugs are not always fully effective in various kinds of tumors, which are encountered clinically and resistance of tumor cells to these drugs has also been known and studied. Therefore, we are continuing the screening of new leads to find more effective antitumor drugs. BE-12406A and BE-12406B were found in the course of our screening. BE-12406A and BE-12406B inhibited the growth of vincristine-resistant or doxorubicin-resistant P388 cell lines as well as their parent sensitive cell line. The structural novelty of BE-12406A and BE-12406B is the position of their sugar moiety, which is attached to position 12 of benzonaphthopyranone chromophore, in contrast to other benzonaphthopyranone antibiotics such as gilvocarcins[6~10], ravidomycin[11,12] or chrysomycin[13~16]. BE-12406A and BE-12406B described in this report may be new leads to antitumor agents.

Acknowledgments

The authors thank Prof. TAKASHI TSURUO of the Tokyo University for generously providing us with the P388/S, P388/VCR and P388/ADR cell lines.

References

1) NAKAJIMA, S.; K. KOJIRI, H. SUDA & M. OKANISHI: New antitumor substances, BE-12406A and BE-12406B, produced by a streptomycete. II. Structure determination. J. Antibiotics 44: 1061~1064, 1991
2) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
3) BUCHANAN, R. E. & N. E. GIBBONS (*Ed.*): BERGEY'S Manual of Determinative Bacteriology. 8th Ed., Williams & Wilkins Co., 1974
4) CERCOS, A. P.: Nuevas propiedades de la canfomicina. Rve. Invest. Agr. 8: 263~283, 1954
5) TSURUO, T.: Reversal of acquired resistance to vinka alkaloids and anthracycline antibiotics. Cancer Treat. Rep. 67: 889~894, 1983
6) NAKANO, H.; Y. MATSUDA, K. ITO, S. OHKUBO, M. MORIMOTO & F. TOMITA: Gilvocarcins, new antitumor antibiotics

1. Taxonomy, fermentation, isolation and biological activities. J. Antibiotics 34: 266~270, 1981
7) TAKAHASHI, K.; M. YOSHIDA, F. TOMITA & K. SHIRAHATA: Gilvocarcins, new antitumor antibiotics. 2. Structural elucidation. J. Antibiotics 34: 271~275, 1981
8) MORIMOTO, M.; S. OKUBO, F. TOMITA & H. MARUMO: Gilvocarcins, new antitumor antibiotics. 3. Antitumor activity. J. Antibiotics 34: 701~707, 1981
9) BALITZ, D. M.; F. A. O'HERRON, J. BUSH, D. M. VYAS, D. E. NETTLETON, R. E. GRULICH, W. T. BRADNER, T. W. DOYLE, E. ARNOLD & J. CLARDY: Antitumor agents from *Streptomyces anandii*: Gilvocarcins V, M and E. J. Antibiotics 34: 1544~1555, 1981
10) WEI, T. T.; J. A. CHAN, P. P. ROLLER, U. WEISS, R. M. STROSHANE, R. J. WHITE & K. M. BYRNE: Detection of gilvocarcin antitumor complex by a biochemical induction assay (BIA). J. Antibiotics 35: 529~532, 1982
11) SEHGAL, S. N.; H. CZERKAWSKI, A. KUDELSKI, K. PANDEV, R. SAUCIER & C. VÉZINA: Ravidomycin (AY-25,545), a new antitumor antibiotic. J. Antibiotics 36: 355~361, 1983
12) NARITA, T.; M. MATSUMOTO, K. MOGI, K. KURITA, R. KAWAHARA & T. NAKASHIMA: Deacetylravidomycin *N*-oxide, a new antibiotic. Taxonomy and fermentation of the producing organism and isolation, structure and biological properties of the antibiotic. J. Antibiotics 42: 347~356, 1989
13) WEI, T. T.; K. M. BYRNE, D. WARNICK-PICKLE & M. GREENSTEIN: Studies on the mechanism of action of gilvocarcin V and chrysomycin A. J. Antibiotics 35: 545~548, 1982
14) WEISS, U.; K. YOSHIHIRA, R. J. HIGHET, R. J. WHITE & T. T. WEI: The chemistry of the antibiotics chrysomycin A and B. Antitumor activity of chrysomycin A. J. Antibiotics 35: 1194~1201, 1982
15) CARTER, G. T.; A. A. FANTINI, J. C. JAMES, D. B. BORDERS & R. J. WHITE: Biosynthesis of chrysomycins A and B. Origin of the chromophore. J. Antibiotics 38: 242~248, 1985
16) MATSON, J. A.; W. C. ROSE, J. A. BUSH, R. MYLLYMAKI, W. T. BRADNER & T. W. DOYLE: Antitumor activity of chrysomycins M and V. J. Antibiotics 42: 1446~1448, 1989