UCE6, a New Antitumor Antibiotic with Topoisomerase I-mediated DNA Cleavage Activity Produced by Actinomycetes: Producing Organism, Fermentation, Isolation and Biological Activity

NOBORU FUJII*,†, FUTOSHI TANAKA†, YOSHINORI YAMASHITA††, TADASHI ASHIZAWA†††, SHIGERU CHIBA†† and HIROFUMI NAKANO††

Kyowa Hakko Kogyo Co. Ltd.,
† Technical Research Laboratories,
1-1 Kyowa-cho, Hofu, Yamaguchi 747, Japan
†† Tokyo Research Laboratories,
3-6-6 Asahimachi, Machida, Tokyo 194, Japan
††† Pharmaceutical Research Laboratories,
1188 Shimotogari, Sunto, Shizuoka 411, Japan

(Received for publication December 20, 1996)

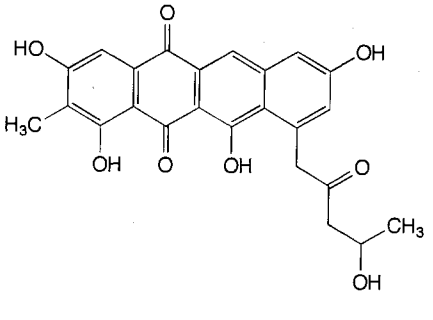
A novel antitumor antibiotic, UCE6 (1,3,8,10,11-pentahydroxy-2-methyl-10-(2-oxo-4-hydroxypentyl)naphthacene-5,12-dione) with topoisomerase I-mediated DNA cleavage activity, was isolated from the culture broth of actinomycetes strain UOE6. Addition of silicone oil antifoam agent, KS69 (2%), to the fermentation enhanced the production of UCE6 by ~3 fold. A total of 1.15 g of UCE6 was recovered as reddish orange crystals from a 100 liter fermentation supplemented with 2% KS69. UCE6 exhibited growth inhibitory activity against HeLa S3, HCT116 and Lu-65 cells comparable to that of camptothecin.

Camptothecin derivatives, CPT-11 and topotecan, showed effectiveness against a broad range of tumor types including pretreated colorectal and cervical cancer[1]. The mechanism of action of these drugs is thought to depend on their ability to interact with topoisomerase I[2~4]. Topoisomerase I catalyzes the passage of a DNA strand by concerted single-strand breaking and rejoining, thereby controlling the topological state of DNA[5]. Camptothecin and its derivatives, which are referred to as topoisomerase I poisons, interrupt the breaking-rejoining cycle of topoisomerase I by stabilizing a covalent topoisomerase I-DNA complex termed "cleavable complex"[6]. Exposure of this cleavable complex to a denaturant leads to the formation of DNA single-strand breaks[6]. A number of studies have shown that the ability to stabilize cleavable complex is responsible for the antitumor activity of these drugs[7,8]. Hence, the identification of new drugs which induce the stabilization of cleavable complex with topoisomerase I is viewed as a promising approach in finding clinically effective antitumor agents.

According to this model, we have screened microbial cultures for their abilities to induce topoisomerase I-mediated DNA cleavage in a purified enzyme assay system. As a result of this screening, we have found UCE6 (Fig. 1), saintopin, saintopin E and UCE1022, which constitute a new chemical family containing a naphthacene-dione structure[9~12]. Among these compounds, UCE6 showed most potent topoisomerase I-mediated activity and cell growth inhibitory activity against HeLa S3 cells *in vitro*[9,12]. Therefore, we have progressed in the further investigation of UCE6 as a representative of a novel class of topoisomerase I-targeting antitumor drugs. Thus far, the production, isolation, physicochemical properties and biological activities of UCE6 were reported in the preliminary communication[9].

In this paper, we report the characterization of the producing strain, increased production of UCE6 through manipulation of culture conditions, a large scale purification method and the growth inhibitory activity of UCE6 against various cell lines.

Fig. 1. Structure of UCE6[18].



Materials and Methods

Materials

Polyvinylpyrrolidone was purchased from Merck. Silicone antifoam agents, KM70, KS66 and KS69 were purchased from Shinetsu Kagaku. Polyoxyalkylene antifoam agents, LG109 and CA-115 were from Asahi Denka Kogyo and NOF Co., respectively.

Microorganism

The producing microorganism, UOE6, was isolated from a soil sample collected at Dohshi river in Yamanashi Prefecture, Japan.

Characterization of the Producing Strain

Growth characteristics and carbohydrate utilization were determined by the methods of the International Streptomyces Project (ISP)[13]. Morphology of the producing strain was observed using light and scanning electron microscopy (Model S-570, Hitachi Co., Ltd.).

Cell wall composition was analyzed by the method of KAWAMOTO *et al.*[14].

Medium and Culture Condition

The seed medium contained soluble starch 20 g, corn steep liquor 2.5 g, NZ-Case 5 g and $CaCO_3$ 1 g per liter (pH 6.0 prior to sterilization). The modified fermentation medium for tank fermentations contained glucose 50 g, soybean protein 10 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $ZnSO_4 \cdot 7H_2O$ 0.1 g, $CaCO_3$ 5 g and antifoam agent KS69 20 g per liter (pH 7.0 prior to sterilization). Deionized water was used in media preparation, and the pH of all media was adjusted with NaOH.

The effects of carbon sources, nitrogen sources, trace elements and antifoam agents in the fermentation medium were investigated in 250-ml Erlenmeyer flasks (narrow-mouth), using the control fermentation medium composed of soluble starch 50 g, soybean meal 15 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g and $CaCO_3$ 5 g per liter (pH 7.0 prior to sterilization), which contained $CaCO_3$ instead of $Mg_3(PO_4)_2 \cdot 8H_2O$ in the original medium described previously[9]. For production in shake flasks, 2 ml of frozen stock of strain UOE6 (20% polyvinylpyrrolidone) were used to inoculate a 250-ml Erlenmeyer flask containing 30 ml of seed medium. The seed culture was incubated at 28°C on a rotary shaker (200 rpm). After 5 days, a 3 ml aliquot of this culture was transferred to a 250-ml Erlenmeyer flask containing 30 ml of fermentation medium. The production cultures were incubated at 28°C on a rotary shaker (200 rpm) for 7 days.

For production in a 200-liter tank fermentor, 30 ml of first seed culture was transferred into a 2-liter Erlenmeyer flask (narrow-mouth) containing 300 ml of the same medium and incubated at 28°C on a rotary shaker (200 rpm). After 3 days, the seed cultures in six 2-liter Erlenmeyer flasks were combined and transferred into a 30-liter jar fermentor containing 15 liters of the same medium containing 2% KS69 and cultured at 28°C for 7 days under aeration at 15 liters per minute with agitation at 200 rpm. Fifteen liters of the third seed culture were transferred into a 200-liter tank fermentor containing 100 liters of modified fermentation medium and cultured at 28°C for 14 days under aeration at 60 liters per minute with agitation at 180 rpm.

Growth of the organism was evaluated as packed cell volume (PCV) by centrifuging the fermentation broth in a 10 ml graduated conical tube at $1200 \times g$ for 10 minutes. The PCV was recorded as % of total broth volume.

Extraction and Analytical Method

To monitor the production of UCE6, whole broth was combined with an equal volume of ethyl acetate and the amount of UCE6 extracted was determined by high performance liquid chromatography (HPLC). HPLC was performed using a column of YMC-Pack ODS-AM-312 (150×6mm, 5 μm particle size, YMC Co.) at 37°C eluted with 5mM KH_2PO_4-MeOH (2:8). The flow rate was 1 ml per minute, and UV absorption of the eluate was monitored at 310 nm. UCE6 was eluted at a retention time of 9.6 minutes.

Growth Inhibitory Activity

HeLa S3 (human uterine cervix carcinoma) and HCT116 (human colon carcinoma) cells were obtained from the American Type Culture Collection (Rockville, MD.), and Lu-65 cells (human lung large cell carcinoma) were supplied by the National Cancer Center. The cells were precultured for 24 hours in 96-well microplates (Nunc, Roskild, Denmark) containing 0.1 ml of the culture medium at 37°C in a water-saturated atmosphere containing 5% CO_2. Then cells were treated with drugs for 72 hours. The growth inhibitory activities of the drugs were determined by the XTT method[15].

Results and Discussion

Characterization of the Producing Strain

Cultural characteristics of strain UOE6 on various descriptive media are presented in Table 1. Strain UOE6

Table 1. Cultural characteristics of strain UOE6.

Medium	Growth degree	Color tone of:	
		Substrate mycelium	Soluble pigment
Glucose-asparagine agar	Somewhat good	Light tan (3gc)	Pale brown
Glycerol-asparagine agar (ISP 5)	Somewhat poor	Light apricot (4ea)	None
Sucrose-nitrate agar	Moderate	Light apricot (4ea)	Slight, Pale brown
Inorganic salts-starch agar (ISP 4)	Moderate	Pastel orange (4ic)	Pale brown
Tyrosine agar (ISP 7)	Moderate	Dusty orange (4lc)	None
Nutrient agar	Poor	Light tan (3gc)	Slight, Pale brown
Yeast extract-malt extract agar (ISP 2)	Good	Pastel orange (4ic)	Slight, Pale brown
Oatmeal agar (ISP 3)	Somewhat good	Light orange (4ia)	Slight, Pale brown
Sporulation agar (ATCC 5)	Good	Dusty orange (4lc)	Pale brown
N-Z Amine with soluble starch and glucose (ATCC172)	Good	Light orange (4ia)	Slight, Pale brown
BENNETT's medium (ATCC 174)	Good	Light orange (4ia)	Slight, Pale brown
BENNETT's modified agar medium (ATCC 185)	Good	Dusty orange (4lc)	Pale brown

Color names and codes used in this table were based on the Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago).

Table 2. Physiological properties of strain UOE6.

Temperature range for growth	15~35°C
Liquefaction of gelatin	−
Hydrolysis of starch	+
Coagulation of milk	−
Peptonization of milk	−
Utilization of carbon source	
L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
Sucrose	+
L-Rhamnose	+
Raffinose	+
D-Mannitol	+
Inositol	−

+: Positive; −: negative.

grew moderately to vigorously in various synthetic and complex media but poorly to somewhat poorly in nutrient agar and glycerol-asparagine agar media. In test agar media, strain UOE6 formed branched substrate hyphae having septal walls. The color of the substrate hyphae was orange to brown. In some media, strain UOE6 produced a soluble brown pigment. The formation of aerial hyphae, spores, sporangium and sclerotium as well as fragmentation of substrate hyphae were not observed.

The physiological properties of strain UOE6 are summarized in Table 2. The temperature range for growth was 15~35°C. Starch hydrolysis was positive, while gelatin liquefaction, milk coagulation and peptonization could not be detected. L-arabinose, D-xylose, D-glucose, D-fructose, sucrose, L-rhamnose, raffinose and D-mannitol were utilized as sole carbon sources, but not inositol. Analysis of the whole-cell hydrolysate revealed that UOE6 contained amino acids including alanine, glutamic acid, *meso*-3-hydroxy-diaminopimelic acid and glycine, and sugars including xylose and arabinose in the cell wall, indicating that UOE6 belongs to cell wall type II and whole cell sugar pattern D[16].

The cultural, morphological and physiological characteristics suggest that strain UOE6 belongs to the family Micromonosporaceae. However, since no spores were observed, the taxonomic position of the strain UOE6 is uncertain. The strain has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan with the accession number FERM BP-3877.

Fermentation

Strain UOE6 grew in liquid media containing various carbon and nitrogen sources. However, the maximum antibiotic titer depended on the carbon and nitrogen sources. As a result of a systematic study of carbon and nitrogen sources, glucose or lactose were found optimum (Table 3A), and the optimum concentration of glucose for production was 5% (Table 3B). In addition, the use of soybean flour, soybean meal and soybean protein as nitrogen sources increased the production of UCE6, while other nitrogen sources tested resulted in no productivity (Table 4A). The optimum concentration of soybean protein for production was 1.0% (Table 4B).

In a preliminary experiment, addition of a trace element dry mixture[17] to fermentation media stimulated the titer of UCE6 (data not shown). Therefore, the effect of trace metals on UCE6 production was examined. As shown in Table 5A, the production of UCE6 was stimulated by zinc sulfate. The optimum concentration

Table 3. Effects of carbon sources on the production of UCE6.

(A)

Carbon sources (5%)	UCE6 (μg/ml)
Glycerol	<0.1
Glucose	2.68
Fructose	1.33
Maltose	0.56
Sucrose	0.21
Lactose	2.47
Soluble starch	1.44

(B)

Glucose (%)	UCE6 (μg/ml)
0	0.38
2.5	2.57
5.0	2.86
7.5	1.64
10.0	<0.1

Basal fermentation medium: soybean meal 15 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g and $CaCO_3$ 5 g per liter (pH 7.0 prior to sterilization).

Table 4. Effects of nitrogen sources on the production of UCE6.

(A)

Nitrogen sources (1.5%)	UCE6 (μg/ml)
Soybean flour	0.98
Soybean meal	2.35
Soybean protein	2.53
Soy casein	<0.1
Corn steep liquor	<0.1
Malt extract	<0.1
Pharmamedia	<0.1
Dry yeast	<0.1
Yeast extract	<0.1
Beef extract	<0.1
Peptone	<0.1

(B)

Nitrogen sources (%)	UCE6 (μg/ml)
Soybean meal	
0.5	0.66
1.0	1.51
1.5	2.28
2.0	2.93
3.0	0.96
Soybean protein	
0.1	0.38
0.5	2.75
1.0	4.46
1.5	2.63
2.0	1.92

Basal fermentation medium: glucose 50 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g and $CaCO_3$ 5 g per liter (pH 7.0 prior to sterilization).

Table 5. Effects of trace metals on the production of UCE6.

(A)

Metal salts (0.005%)	UCE6 (μg/ml)
None	2.50
$Li_2SO_4 \cdot H_2O$	<0.1
$BaSO_4$	1.61
$CaSO_4 \cdot 2H_2O$	1.43
$CuSO_4 \cdot 5H_2O$	0.34
$CoSO_4 \cdot 7H_2O$	1.24
$FeSO_4 \cdot 7H_2O$	1.18
$Fe_2(SO_4)_3 \cdot nH_2O$	2.08
$MnSO_4 \cdot 5H_2O$	3.15
$NiSO_4 \cdot 7H_2O$	2.68
$ZnSO_4 \cdot 7H_2O$	6.05

(B)

$ZnSO_4 \cdot 7H_2O$ (%)	UCE6 (μg/ml)
0	4.90
0.001	5.89
0.005	8.73
0.01	8.92
0.05	3.69
0.1	0.49

Basal fermentation medium: (A) glucose 50 g, soybean meal 15 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g and $CaCO_3$ 5 g per liter (pH 7.0 prior to sterilization). (B) glucose 50 g, soybean protein 10 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g and $CaCO_3$ 5 g per liter (pH 7.0 prior to sterilization).

Table 6. Effects of antifoam agents on the production of UCE6.

(A)

Antifoam agents (0.1%)	UCE6 (μg/ml)
None	8.2
Glycerol	2.6
Soybean oil	8.9
Polyoxyalkylene antifoam	
LG109	1.2
CA-115	1.2
Silicone antifoam	
KM70	8.5
KS66	10.2
KS69	11.3

(B)

Antifoam agents (%)	UCE6 (μg/ml)
0	8.2
KS66	
0.1	10.2
0.5	14.1
1.0	25.1
2.0	27.5
5.0	25.7
KS69	
0.1	11.3
0.5	16.3
1.0	18.1
2.0	25.8
5.0	20.0

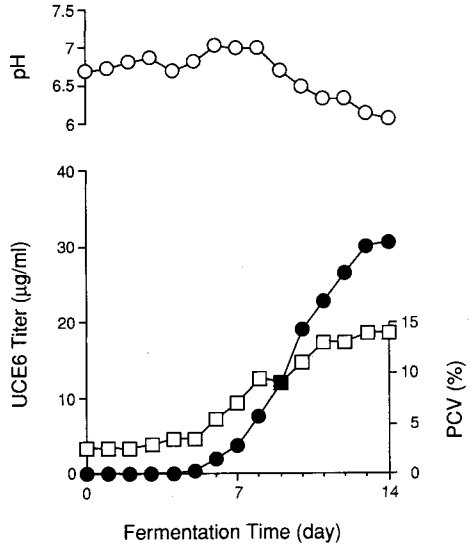
Basal fermentation medium: glucose 50 g, soybean protein 10 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $ZnSO_4 \cdot 7H_2O$ 0.1 g and $CaCO_3$ 5 g per liter (pH 7.0 prior to sterilization).

of $ZnSO_4 \cdot 7H_2O$ for production was 0.01% (Table 5B).

Vigorous foam formation was observed during the UOE6 fermentation. Usually, form formation was prevented by adding antifoam agents to the fermentation, therefore, the effect of antifoam agents (0.1%) on UCE6 production was examined. As shown in Table 6A, the production of UCE6 was: (1) repressed by addition of glycerol and polyoxyalkylene antifoam agents (LG109, CA-115); (2) not affected by addition of soybean oil and silicone emulsion antifoam agent (KM70); (3) enhanced by addition of silicone oil antifoam agents (KS66, KS69). The optimum concentration of KS66 or KS69 for production was 2% (Table 6B), and the production of UCE6 in the KS66 or KS69-supplemented cultures was 3.1~3.4 fold higher than that of the non-supplemented culture. Furthermore, the use of KS69 resulted in continuous antifoam activity during the cultivation. Thus, we developed media, the modified fermentation medium (described in Materials and Methods), for improve production of UCE6, and the fermentation titer was increased ~18 fold as compared with the control fermentation medium (described in Materials and Methods). Based on these results, the modified fermentation medium was used for production in

Fig. 2. Time course of the UCE6 fermentation in a 200-liter tank fermentor.

pH (○), PCV (□), UCE6 production (●).



200-liter tank fermentations.

Fig. 2 represents the time course of the UCE6 fermentation in a 200-liter tank fermentor using the optimum conditions. The amount of UCE6 increased during logarithmic phase and reached 30.5 μg/ml after 14 days cultivation.

Isolation

UCE6 was isolated from the culture broth of a 200-liter tank fermentor by the following improved procedure. The fermentation beer (100 liters) was extracted with 40 liters of 1-propanol. After filtration using filter aid (Radiolite #600, Showa Kagaku Co., Ltd., Japan) (10 kg), the 1-propanol extract was applied to a Diaion HP-20 column (10 liters) (Mitsubishi Chemical Industries Limited). The column was washed with water - MeOH (2 : 8) and eluted with MeOH. The active eluate was concentrated under reduced pressure to give residue. The resulting residue was collected and dried *in vacuo* to afford 1.64 g of crude UCE6. Crude UCE6 residue was suspended in MeOH (0.25 g-UCE6/liter), and ammonia solution was added to dissolve the residue. After filtration, the crude UCE6 solution was concentrated under reduced pressure to 1 g-UCE6/liter. UCE6 was crystallized at 4°C for 2 days, and the resulting reddish orange crystals were collected, washed in ice-cold MeOH and dried *in vacuo* at 30°C to afford 1.15 g of UCE6.

Biological Activities

UCE6 exhibited growth inhibitory activity against HeLa S3, HCT116 and Lu-65 cells in the nM range which was comparable to that of camptothecin. The concentrations inhibiting 50% of cell growth (IC_{50}) are shown in Table 7.

Table 7. Growth inhibitory activities against human carcinoma cell lines.

Cell line	IC_{50} (nM)	
	UCE6	Camptothecin
HeLa S3	18	16
HCT116	4.9	3.2
Lu-65	21	6.6

HeLa S3 cells (2×10^3 cells/well) and HCT116 or Lu-65 cells (3×10^3 cells/well) were cultured on day 0, and treated with the compounds from day 1 to day 4 for 72 hours. On day 4, the growth inhibitory activity was determined by the XTT method.

Acknowledgments

We thank CHIKA OKITSU for skillful technical assistance.

References

1) POTMESIL, M.: Camptothecins: from bench research to hospital wards. Cancer Res. 54: 1431～1439, 1994
2) ANDOH, T.; K. ISHII, Y. SUZUKI, Y. IKEGAMI, Y. KUSUNOKI, Y. TAKEMOTO & K. OKADA: Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. Proc. Natl. Acad. Sci. U.S.A. 84: 5565～5569, 1987
3) KANEDA, N.; H. NAGATA, T. FURUTA & T. YOKOKURA: Metabolism and pharmacokinetics of the camptothecin analog CPT-11 in the mouse. Cancer Res. 50: 1715～1720, 1990
4) KINGSBURY, W. D.; J. C. BOEHM, D. R. JAKAS, K. G. HOLDEN, S. M. HECHT, G. GALLAGHER, M. J. CARANFA, L. F. MCCABE, L. F. FAUCETTE, R. K. JOHNSON & R. P. HERTZBERG: Synthesis of water-soluble (aminoalkyl) camptothecin analogues: inhibition of topoisomerase I and antitumor activity. J. Med. Chem. 34: 98～107, 1991
5) WANG, J. C.: DNA topoisomerases. Annu. Rev. Biochem. 54: 665～695, 1985
6) HSIANG, Y. H.; R. HERTZBERG, S. HECHT & L. F. LIU: Camptothecin induces protein-linked DNA breaks *via* mammalian DNA topoisomerase I. J. Biol. Chem. 260: 14873～14878, 1985
7) JAXEL, C.; K. W. KOHN, M. C. WANI, M. E. WALL & Y. POMMIER: Structure-activity study of the actions of camptothecin derivatives on mammalian topoisomerase I: evidence for a specific receptor site and a relation to antitumor activity. Cancer Res. 49: 1465～1469, 1989
8) HSIANG, Y. H.; L. F. LIU, M. E. WALL, M. C. WANI, A. W. NICHOLAS, G. MANIKUMAR, S. KIRSCHENBAUM, R. SILBER & M. POTMESIL: DNA topoisomerase I-mediated DNA cleavage and cytotoxicity of camptothecin analogues. Cancer Res. 49: 4385～4389, 1989

9) FUJII, N.; Y. YAMASHITA, S. CHIBA, Y. UOSAKI, Y. SAITOH, Y. TUJI & H. NAKANO: UCE6, a new antitumor antibiotic with topoisomerase I mediated DNA cleavage activity, from actinomycetes. J. Antibiotics 46: 1173~1174, 1993
10) YAMASHITA, Y.; S. KAWADA, N. FUJII & H. NAKANO: Induction of mammalian DNA topoisomerase I and II mediated DNA cleavage by saintopin, a new antitumor agent from fungus. Biochemistry 30: 5838~5845, 1991
11) FUJII, N.; Y. YAMASHITA, K. ANDO, T. AGATSUMA, Y. SAITOH, K. GOMI, Y. NISHIIE & H. NAKANO: UCE1022, a new antitumor antibiotic with topoisomerase I mediated DNA cleavage activity, from *Paecilpmyces*. J. Antibiotics 47: 949~951, 1994
12) FUJII, N.; Y. YAMASHITA, T. MIZUKAMI & H. NAKANO: Correlation between the formation of cleavable complex with topoisomerase I and inhibition of the cell growth for saintopin type antibiotics. Mol. Pharmacol. 51: 269~276, 1997
13) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
14) KAWAMOTO, I.; T. OKA & T. NARA: Cell wall composition of *Micromonospora olivoasterospora*, *Micromonospora sagamiensis*, and related organisms. J. Bacteriol. 146: 527~534, 1981
15) SCUDIERO, D.; R. H. SHOEMAKER, K. D. PAULL, A. MONKS, S. TIERNEY, T. H. NOFZIGER, M. J. CURRENS, D. SENIFF & M. R. BOYD: Evaluation of a soluble tetrazolium/formazan assay for the growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res. 48: 4827~4833, 1988
16) LECHEVALIER, M. P. & H. LECHEVALIER: Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Bacteriol. 20: 435~443, 1970
17) HUNTER, S. H.: Inorganic nutrition. Ann. Rev. Microbiol. 26: 313~346, 1972
18) NAKANO, H.; N. FUJII, Y. YAMASHITA, Y. UOSAKI, S. CHIBA, S. KATSUMATA & Y. TUJI (Kyowa Hakko Kogyo Co., Ltd.): Antitumor anthracycline analogue UCE6 is isolated from Actinomycetes fermentation and has good activity. Jpn. Kokai 05339193, Dec. 21, 1993