VOL. 57 NO. 3, MAR. 2004 THE JOURNAL OF ANTIBIOTICS pp. 173 — 179

Pladienolides, New Substances from Culture of *Streptomyces platensis* Mer-11107

I. Taxonomy, Fermentation, Isolation and Screening

TAKASHI SAKAI*, TOMOHIRO SAMESHIMA†, MOTOKO MATSUFUJI†, NAOTO KAWAMURA†, KAZUYUKI DOBASHI† and YOSHIHARU MIZUI

Tsukuba Research Laboratories, Eisai Co., Ltd.,
5-1-3, Tokodai, Tsukuba, Ibaraki 300-2635, Japan
† Bioresource Laboratories, Mercian Corp.,
1808 Nakaizumi, Iwata, Shizuoka 438-0078, Japan

(Received for publication December 9, 2003)

Seven new macrolides having a 12-membered ring, which we termed pladienolides, were isolated from the fermentation broth of *Streptomyces platensis* Mer-11107. Six of the seven pladienolides inhibited hypoxia-induced reporter gene expression controlled by human VEGF promoter with IC_{50} values of 0.0018~2.89 μM. They also demonstrated growth-inhibitory activity against U251 human glioma cells *in vitro*. Pladienolides are highly potent inhibitors of both hypoxia signals and cancer cell proliferation, and thus may be useful as antitumor agents.

Since many types of human tumors are under severe hypoxia and set up a series of cascading events for survival, a signaling pathway to adapt to a hypoxic environment is regarded as potent target for antitumor drugs. We therefore genetically engineered U251 human glioma cells as stable host and a vector in which a placental alkaline phosphatase (PLAP) reporter gene is under the control of a VEGF promoter.

In the course of screening, we discovered several unique 12-membered macrolides, designated as pladienolides (Fig. 1) which inhibit hypoxia-induced PLAP expression from the culture broth of *Streptomyces platensis* Mer-11107.

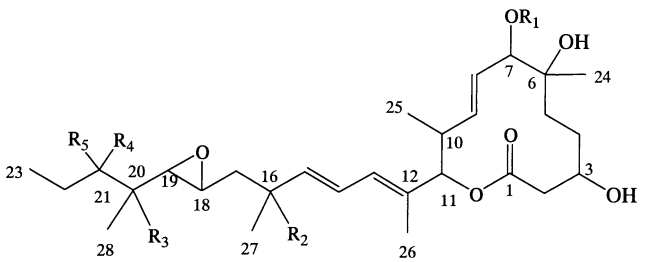
In this paper, we describe the taxonomy of the producing strain, and the fermentation, isolation and biological activities of pladienolides. The antitumor activities of pladienolides are reported in a subsequent paper.

Materials and Methods

Microorganisms

Strain Mer-11107 was isolated from a soil sample collected in Kanagawa, Japan, and was deposited at the Bioconsortia Program Laboratory National Institute of Advanced Industrial Science and Technology in Japan with accession number FERM P-18144.

Fig. 1. Structures of pladienolides.



	R_1	R_2	R_3	R_4, R_5
pladienolide A (**1**)	H	H	H	H, OH
pladienolide B (**2**)	Ac	H	H	H, OH
pladienolide C (**3**)	Ac	H	H	=O
pladienolide D (**4**)	Ac	OH	H	H, OH
pladienolide E (**5**)	Ac	H	OH	H, OH
pladienolide F (**6**)	H	OH	H	H, OH
pladienolide G (**7**)	H	H	OH	H, OH

* Corresponding author: t3-sakai@hhc.eisai.co.jp

Streptomyces lydicus IFO 13058^T, *S. sioyaensis* IFO 12820^T, *S. platensis* IFO 12901^T and *S. libani* subsp. *libani* IFO13452^T were purchased from the Institute for Fermentation, Osaka (IFO) for comparison.

Taxonomic Studies

The morphological characteristics of the spores and mycelia grown on inorganic salt-starch agar for 20 days at 28°C were observed using a scanning electron microscope (JEOL, Model JSM-T300). Its cultural and physiological characteristics were determined by the methods of SHIRLING and GOTTLIEB[1] and of WAKSMAN[2]. Cultures were incubated for 14 to 21 days at 28°C. Carbon utilization was investigated using the procedure of PRIDHAM and GOTTLIEB[3]. The color of the surface of colony was indicated according to the symbols described in the Color Harmony Manual[4]. Detection of the isomer of diaminopimelic acid was performed according to the procedure of STANECK, J. L. *et al.*[5]. The DNA homologies between strains were determined by the method of EZAKI, T. *et al.*[6].

Fermentation

A sample of slant culture of *Streptomyces platensis* Mer-11107 was used to inoculate a 500 ml Erlenmeyer flask containing 50 ml of seed medium composed of glycerol 2.0%, glucose 2.0%, soybean meal 2.0%, yeast extract 0.5%, sodium chloride 0.25%, calcium carbonate 0.32% and 0.2% of metal salt solution containing 0.25% $CuSO_4 \cdot 5H_2O$, 0.25% $MnCl_2 \cdot 4H_2O$ and 0.25% $ZnSO_4 \cdot 7H_2O$ (adjusted to pH 7.4 before sterilization). The seed flasks were incubated at 28°C for 72 hours on a rotary shaker. The seed culture (0.6 ml) was transferred to each 500 ml Erlenmeyer flask containing 60 ml of producing medium containing soluble starch 5%, corn steep liquor 0.5%, dry yeast 0.5%, corn gluten meal 0.5% and calcium carbonate 0.1% (adjusted to pH 6.8 before sterilization). The fermentation was carried out for 96 hours at 28°C on a rotary shaker.

Cloning of Human VEGF Promoter and Plasmid Construct

1×10^6 bacteriophage clones from an EMBL3 human genomic library (Clontech, U.S.A.) were screened with a VEGF cDNA fragment. The fragment was amplified by PCR using primers (forward primer 5′-ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG-3′ and reverse primer 5′-CTG GCC TTG GTG AGG TTT GTA CCG CAT AA-3′, 340-bp fragment). After plaque hybridization, six independent clones containing VEGF genomic DNA were obtained. *EcoR* I fragments from one of the clones were inserted into the *EcoR* I site of pUC18. Sequencing of the genomic DNA inserts was performed with a Dye Terminator Cycle Sequencing kit (Applied Biosystems, U.S.A.) and an ABI Prism 377 DNA Sequencer (Applied Biosystems). A sub-clone, pUC18-VEGF-A, was found to include 5.4 kb genomic DNA from the 5′-flanking region of the human VEGF gene. According to the sequence analysis and the published information by TISCHER *et al.*[7], 2.3 kb of *Kpn* I/*Nhe* I fragment including the VEGF promoter region, was isolated and inserted into the *Kpn* I/*Nhe* I sites of the multicloning sites of a placental alkaline phosphatase (PLAP) reporter plasmid[8], The vector was designated as pVEGF-PLAP1.

Transfection of Plasmid into U251 Cells

U251 glioma cell line was purchased from Riken Cell Bank (Tsukuba, Japan). Transfection was carried out with Lipofectin reagent (Invitrogen Corp., U.S.A.) following the manufacturer's instructions. Approximately 3×10^5 cells were seeded in 60 mm dishes and cultured in DMEM supplemented with 10% FCS. Ten μl of Lipofectin was added to 100 μl of OPTI-MEM (Invitrogen Corp.) and incubated for 30 minutes at room temperature. Next, 2 μg of pVEGF-PLAP1 vector was diluted with 100 μl of OPTI-MEM (Invitrogen Corp.). These reactions were combined and incubated for 15 minutes at room temperature, and then 1.8 ml of serum-free medium was added. The resultant mixture was added to the cells which had been pre-washed with serum-free medium and incubated for 7 hours at 37°C under 5% CO_2. Then, the medium was removed, and the cells were washed twice and refilled with growth medium. After 48 hours, G418 selection was started at 1 mg/ml of geneticin (Sigma, U.S.A.); thereafter, the medium was changed every 3～4 days. When resistant colonies emerged, individual clone was selected and screened for hypoxia-induced PLAP. One stable clone that showed a strong response to hypoxic conditions was selected for screening.

PLAP Assay

VEGF-PLAP stable cells were seeded at 4.0×10^4 cells/well in 96-well plates. After overnight incubation, test compounds at serial dilutions were added to the plates. The plates were incubated under hypoxic conditions (2% O_2), with one plate incubated under normoxic conditions as a control. After 18 hours of incubation, the culture supernatant was drawn from each well and PLAP activity was determined with the use of a chemiluminescent substrate (Lumi-Phos 530, Lumigen, U.S.A.). The samples were heated at 65°C for 20 minutes to inactivate non-

specific alkaline phosphatase contained in the medium from FCS. Ten μl aliquots of the heat-treated samples were then mixed with 50 μl assay buffer (0.28 M Na_2CO_3 - $NaHCO_3$, pH 10.0, containing 8.0 mM $MgSO_4$), followed by addition of 50 μl chemiluminescent substrate. The mixtures were incubated at room temperature for 60 minutes, and steady state chemiluminescence was measured using a microplate luminometer (Perkin Elmer, U.S.A.). The experiments were repeated at least three times.

Growth Inhibition Assay

Growth-inhibitory activities against U251 cells were measured using alamarBlue reagent (TREK, U.S.A.) according to the manufacturer's instructions. Briefly, 180 μl of cell suspension (2×10^3 cells) was seeded into each well of 96-well microtiter plates. After overnight incubation at 37°C under 5% CO_2, 20 μl of test compounds at serial dilutions were added to each well of the plates and incubated for 72 hours. Twenty μl of alamarBlue reagent was then added to each well and incubated for 4 hours. The growth-inhibitory activity was determined by measuring fluorescence with excitation wavelength at 530 nm and emission wavelength at 590 nm.

Results

Taxonomy

As morphological characteristics of strain Mer-11107, the aerial mycelium of the cultured strain was extensively branched and the top of the aerial mycelium was spiral. On maturity, it divided and formed spiral spore chains. Each spore chain held about 10 to 20 spores. The size of each elliptical spore was about 0.7×1.0 μm. The surface of the spore was smooth. No sclerotic granules, sporangia or zoospores were observed (Fig. 2).

Cultural characteristics are shown in Table 1. The color of the aerial mycelium was white to gray on ISP media Nos. 2, 3, 4, 5 and 7. No melanoid pigment was observed when grown on peptone - yeast extract - iron agar or tyrosine agar. The carbon source use pattern and the physiological properties are summarized in Table 2. Analysis of whole cell hydrolysates of strain Mer-11107 showed the presence of L, L-diaminopimelic acid.

From the foregoing taxonomic characteristics, strain Mer-11107 was concluded to belong to the genus *Streptomyces*[9]. Compared with the *Streptomyces* species described in the references[9~13], strain Mer-11107 resembled *Streptomyces lydicus*, *S. sioyaensis*, *S. platensis* and *S. libani* subsp. *libani*. Comparisons between these strains are summarized in Table 3.

Since the physiological properties of strain Mer-11107 were similar to those of *S. platensis* and *S. libani* subsp. *libani*, we examined a DNA hybridization test between strain Mer-11107 with *S. platensis* IFO 12901^T and *S. libani* subsp. *libani* IFO 13452^T. Strain Mer-11107 showed 84% homology with *S. platensis* IFO 12901^T and 60% with

Fig. 2. Scanning electron micrograph of spore chains of *Streptomyces platensis* Mer-11107.

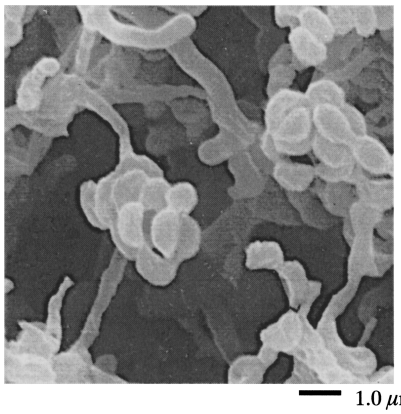


Table 1. Cultural characteristics of strain Mer-11107.

Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Yeast extract-malt extract agar	Good	Light gray [d]	Light melon yellow [3ea]	None
Oatmeal agar	Moderate	Gray [g]	Nude tan [4gc] or Putty [1 1/2 ec]	None
Inorganic salts-starch agar	Good	Gray [e]	Fawn [4ig] or Gray [g]	None
Glycerol asparagine agar	Good	White [a]	Pearl pink [3ca]	None
Peptone-yeast extract-iron agar	Poor	None	Light melon yellow [3ea]	None
Tyrosine agar	Good	White [a]	Pearl pink [3ca]	None

Observation after incubation at 28°C for 14 days.

Table 2. Physiological properties of strain Mer-11107.

Temperature range for growth (ISP No.2)	12~37ºC
Optimum temperature for growth (ISP No.2)	17~33ºC
Formation of melanoid pigment	
Peptone-yeast extract-iron agar (ISP No.6)	–
Tyrosine agar (ISP No.7)	–
Liquefaction of gelatin	–
Coagulation of milk	–
Peptonization of milk	–
Hydrolysis of starch	+
Reduction of nitrate	–
H_2S production	–
NaCl tolerance (ISP No.2)	≤8%
Carbon source utilization	
L-Arabinose	–
D-Xylose	±
D-Glucose	+
D-Fructose	+
Sucrose	+
Inositol	+
L-Rhamnose	–
Raffinose	+
D-Mannitol	+

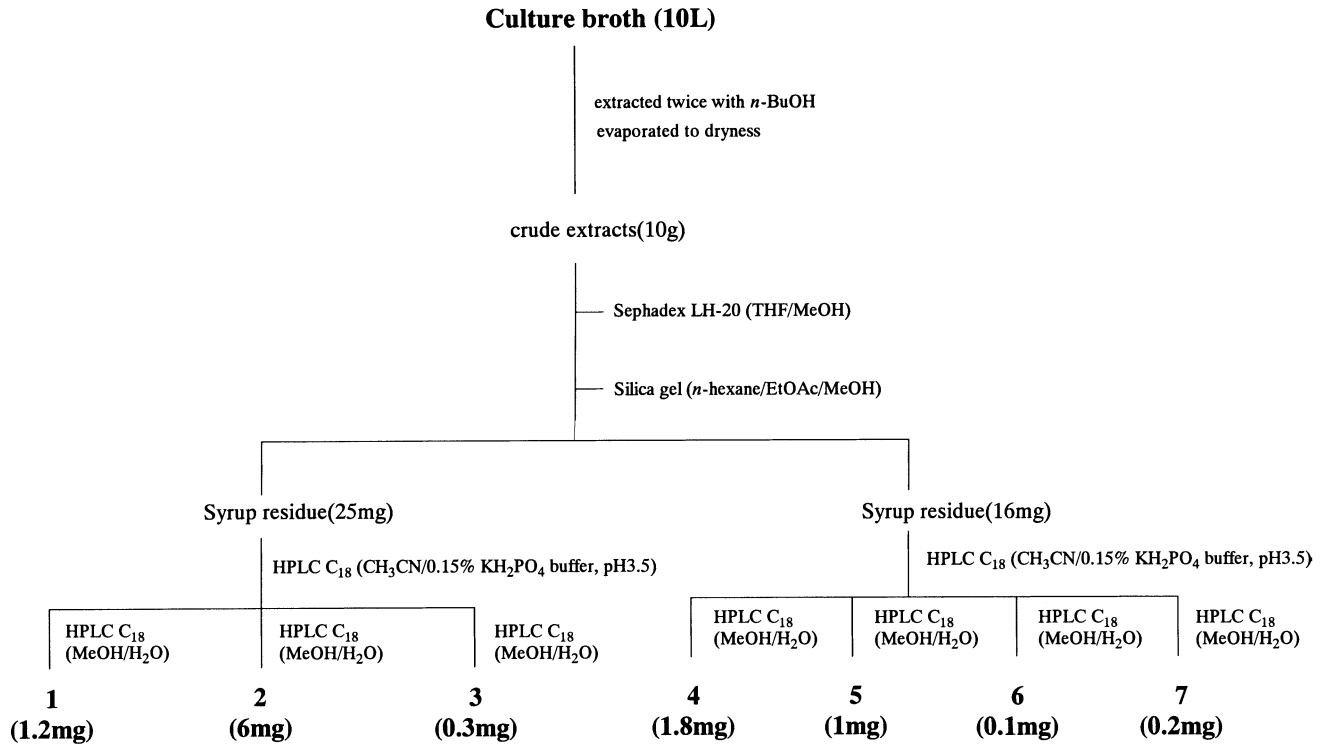
+; positive, ±; slightly positive, –; negative

Table 3. Comparisons among strain Mer-11107, *Streptomyces lydicus* IFO 13058^T, *S. sioyaensis* IFO 12820^T, *S. platensis* IFO 12901^T and *S. libani* subsp. *libani* IFO 13452^T.

Condition		Mer-11107	*S. lydicus* IFO 13058^T	*S. sioyaensis* IFO 12820^T	*S. platensis* IFO 12901^T	*S. libani* subsp. *libani* IFO 13452^T
Spore mass		Hygroscopic	Not hygroscopic	Hygroscopic	Hygroscopic	Hygroscopic
Carbon utilization	L-Arabinose	–	+	–	–	–
	D-Xylose	±	±	±	–	±
	D-Fructose	+	+	+	+	+
	Sucrose	+	–	+	+	+
Production of	ISP No.2	None	None	Yellow	None	None
soluble pigment	Bennett's agar	None	None	Yellow	None	None
NaCl tolerance (ISP No.2)		≤8%	≤8%	≥10%	≤6%	≥10%
Temperature range for growth (ISP No.2)		12~37°C	12~37°C	12~37°C	12~37°C	12~37°C
Optimum temperature for growth (ISP No.2)		17~33°C	21~33°C	17~33°C	21~33°C	12~33°C

+; positive, ±; slightly positive, –; negative

Fig. 3. Purification of **1**～**7** from *Streptomyces platensis* Mer-11107.



S. libani subsp. *libani* IFO 13452^T. Consequently, strain Mer-11107 was identified as a strain of *Streptomyces platensis*.

Isolation

The isolation procedure for pladienolides is shown in Fig. 3. The fermentation broth (10 liters) was extracted twice with 8 liters of *n*-BuOH. The crude extracts were concentrated *in vacuo* to give approximately 10 g of brown syrup. The residues were then subjected to gel chromatography over Sephadex LH-20 (1,500 ml) with THF - MeOH (1 : 1) as the eluent. The eluent was concentrated *in vacuo* to yield 3 g of yellow syrup. The syrup was then chromatographed on a silica gel column (Wakogel C-200, 50 g) and eluted with EtOAc - MeOH (9 : 1). The first bioactive fraction containing **1**～**3** and the second active fraction containing **4**～**7** were collected, separately.

The first fraction was concentrated to obtain 25 mg of a syrup residue. This residue was subjected to preparative HPLC (YMC-Pack ODS-AM SH-343-5AM, 20 i.d.× 250 mm) using a linear gradient of CH_3CN - 0.15% KH_2PO_4 buffer, pH 3.5 (80 : 20～20 : 80) at a flow rate of 10 ml/minute. Fractionation was guided by UV absorbance at 240 nm to afford three peaks in the order of **1**, **2**, and **3**. After these fractions were concentrated *in vacuo*, the resulting aqueous solutions were subjected to preparative HPLC (YMC-Pack ODS-AM SH-343-5AM, 20 i.d.×250 mm) using a linear gradient of MeOH - H_2O (80 : 20～100 : 0) at a flow rate of 10 ml/minute. Each fractions containing pure pladienolides were freeze-dried respectively, yielding **1** (1.2 mg), **2** (6.0 mg), and **3** (0.3 mg).

The second fraction was combined to give 16 mg of a syrup residue. Using repeated reversed-phase chromatography in analogous manner as mentioned above, the second fraction provided **4** (1.8 mg), **5** (1.0 mg), **6** (0.1 mg), and **7** (0.2 mg).

Fig. 1 shows the chemical structures of pladienolides A (**1**), B (**2**), C (**3**), D (**4**), E (**5**), F (**6**), and G (**7**).

Screening

VEGF-PLAP Inhibitory Activity

Seven pladienolide analogues were evaluated in a PLAP reporter assay (Table 4). The potency was classified into

Table 4. Inhibition of hypoxia-induced VEGF-PLAP secretion and anti-proliferative activity of pladienolides.

compound	Anti-VEGF-PLAP activity IC_{50} (nM)	Anti-proliferative activity IC_{50} (nM)
Pladienolide B(**2**)	1.8	3.5
Pladienolide D(**4**)	5.1	6.0
Pladienolide C(**3**)	7.4	14.7
Pladienolide E(**5**)	65.2	146.8
Pladienolide A(**1**)	451.5	967.5
Pladienolide F(**6**)	2894.2	2595.2
Pladienolide G(**7**)	> 10000.0	> 10000.0

three categories: high, medium and low activity. Pladienolides B (**2**), C (**3**), and D (**4**) included in the high activity category have acetyl groups in the R_1 position. Lack of an acetyl group dramatically decreased the activity by more than two orders of magnitude, as seen in pladienolides A (**1**), F (**6**), and G (**7**). Addition of a hydroxyl group at R_2 or R_3 position of **2** decreased the activity. Since the activity of **4** was fifteen times stronger than that of pladienolide E (**5**), hydroxylation of the R_2 position was more significant than that of the R_3 position. Pladienolide B (**2**) was the strongest, with the order of the strength of VEGF-PLAP inhibitory activity as follows: **2**>**4**>**3**>**5**>**1**>**6**>**7**.

Growth-inhibitory Activity

Low-density conditions were used to evaluate growth inhibitory activity. The cell density was different from that used for the VEGF-PLAP assay. The results are shown in Table 4. Pladienolides also inhibited the growth of U251 cells. The order of the strength of growth inhibition was the same as that for VEGF-PLAP inhibitory activity. Each IC_{50} value of growth inhibition was slightly higher than that VEGF-PLAP, indicating that pladienolides inhibit both cancer cell growth and hypoxia signals.

Discussion

Hypoxia signals are currently thought to be an attractive target for anticancer therapy[14]. Since hypoxia-inducible factor (HIF) is a master regulator of hypoxia signals, many groups have been searching for small molecules that might inhibit the HIF pathway[14,15], and although several compounds have been found to inhibit the HIF pathway[16], they do not appear to be sufficiently potent to act as anticancer agents. Therefore, a novel HIF-pathway inhibitor with high potency is needed to regulate tumors in a hypoxic microenvironment.

To search for HIF pathway inhibitors, we constructed a VEGF-PLAP system and screened fermented microbial broth libraries. After screening approximately 27,000 samples, we isolated seven novel macrolides, which we designated as pladienolides. These seven pladienolide analogues allowed us to elucidate the structure-activity relationships (SARs) between the structures and the inhibition activity against hypoxia-induced VEGF expression and cell proliferation using a U251 cell. Pladienolides also showed strong antitumor effects in human tumor xenograft models in mice. Details of these antitumor effects of pladienolides will be described in a subsequent paper[17].

References

1) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
2) WAKSMAN, S. A.: Classification, identification and description of genera and species. The Actinomycetes

Vol. 2, The Williams and Wilkins Co., Baltimore, 1961
3) 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
4) TAYLER, H. D.; L. KNOCHE & W. C. GRANVILLE (*Ed.*): Color Harmony Manual, Container Corporation of America, Chicago, 1948
5) STANECK, J. L. & G. D. ROBERTS: Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. Appl. Microbiol. 28: 226～231, 1974
6) EZAKI, T.; Y. HASHIMOTO & E. YABUUCHI: Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int. J. Syst. Bacteriol. 39: 224～229, 1989
7) TISCHER, E.; R. MITCHELL, T. HARTMAN, M. SILVIA, D. GOSPODAROWICZ, J. C. FIDDES & J. A. ABRAHAM: The human gene for vascular endothelial growth factor. J. Biol. Chem. 266: 11947～11954, 1991
8) GOTO, M.; K. YAMADA, K. KATAYAMA & I. TANAKA: Inhibitory effect of E3330, a novel quinone derivative able to suppress tumor necrosis factor-α generation, on activation of nuclear factor-κB. Mol. Pharmacol. 49: 860～873, 1996
9) WILLIAMS, S. T.: BERGEY's Manual of Systematic Bacteriology. Vol. 4, The Williams and Willkins Co., Baltimore, 1989
10) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. Int. J. Syst. Bacteriol. 18: 69～189, 1968
11) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. III. Additional Species descriptions from first and second studies. Int. J. Syst. Bacteriol. 18: 279～392, 1968
12) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. Int. J. Syst. Bacteriol. 19: 391～512, 1969
13) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. V. Additional descriptions. Int. J. Syst. Bacteriol. 22: 265～394, 1972
14) PUGH, C. W. & P. J. RATCLIFFE: Regulation of angiogenesis by hypoxia: role of the HIF system. Nat. Med. 9: 677～684, 2003
15) SEMENZA, G. L.: Targeting HIF-1 for cancer therapy. Nat. Rev. Cancer 3, 721～732, 2003
16) RAPISARDA, A.; B. URANCHIMEG, D. A. SCUDIERO, M. SELBY, E. A. SAUSVILLE, R. H. SHOEMAKER & G. MELILLO: Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. Cancer Res. 62: 4316～4324, 2002
17) MIZUI, Y.; T. SAKAI, M. IWATA, T. UENAKA, K. OKAMOTO, H. SHIMIZU, T. YAMORI & K. YOSHIMATSU: Pladienolides, new substances from culture of *Streptomyces platensis* Mer-11107. III. *In vitro* and *in vivo* antitumor activities. J. Antibiotics 57: 188～196, 2004