GE3, a Novel Hexadepsipeptide Antitumor Antibiotic, Produced by *Streptomyces* sp.

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

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GE3, a novel cyclic hexadepsipeptide antibiotic, was isolated from the culture broth of *Streptomyces* sp. GE3. GE3 was weakly active against some Gram-positive and Gram-negative bacteria and showed potent cytotoxicity against human tumor cell lines. GE3 also exhibited antitumor activity against human pancreatic carcinoma, PSN-1, *in vivo*. GE3B, a linear peptide form of GE3, which was isolated from the same culture broth with GE3, showed no antibiotic and cytotoxic activities, suggesting the necessity of the cyclic structure of GE3 for its biological activities.

In the course of a screening for new antitumor antibiotics, *Streptomyces* sp. GE3 was found to produce a novel compound, GE3. GE3 was shown to be a novel member of cyclic hexadepsipeptide including citropeptin[1], azinothricin[2], A83586C[3], variapeptin[4], L-156,602[5], verucopeptin[6], IC101[7] and aurantimycins[8]. GE3 possesses antibacterial activity and antitumor activity against human tumors both *in vitro* and *in vivo*. Additionally we isolated a linear peptide form of GE3, GE3B, and tested the biological activities of GE3B to examine the relevance of the cyclic structure of GE3 to its biological activities. Almost all compounds of the cyclic hexadepsipeptide class have been found to show potent cytotoxicity, however the mechanisms for cytotoxicity were little described other than the inhibition of DNA and RNA synthesis in cells[6]. The possession of the inhibitory activity on the cell adhesion to extracellular matrix has been also described[7]. In this paper, taxonomy of the producing strains, fermentation, isolation, physico-chemical properties and biological activities of GE3 are described. In addition, the mode of cytotoxicity of GE3 is discussed. Studies on structural determination of GE3 are described in the succeeding paper[9].

Materials and Methods

Microorganism

The producing strain GE3 was isolated from a soil sample collected in Shimane prefecture, Japan. The strain has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukuba-shi, Ibaraki, Japan, as *Streptomyces* sp. GE3 with the accession number FERM BP-4620.

Method for Taxonomical Characterization

Cultural and physiological characteristics of strain GE3 were determined by the methods of the International *Streptomyces* Project[10] (ISP). Color codes were assigned to the pigments of substrate and aerial mycelium according to the Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago). Morphology of the strain was ascertained by light microscope and scanning electron microscope HITACHI S-570. The temperature ranges for growth of the strain was determined after submerged cultivation with ISP No.5 medium for 7 days. Analysis of diaminopimelic acid was performed on the hydrolysate of aerial mycelia grown on the solid medium ISP No. 4.

Culture and Medium Conditions

A loopful of cells from the mature slant was inoculated into 50 ml of the seed medium (pH 7.2 before steriliza-

tion), which was composed of glucose 1%, soluble starch 1%, pancreatic digest of casein (Bacto-Tryptone, Difco) 0.5%, yeast extract (Nihon-Seiyaku) 0.5%, beef extract (Kyokuto) 0.3%, $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05%, in a 300 ml Erlenmeyer flask. The inoculated flask was incubated at 28°C for 4 days on a rotary shaker (200 rpm). Fifteen ml of the above seed culture were added to three 2-liter Erlenmeyer flasks containing 300 ml of the same medium. These flasks were incubated for 2 days on a rotary shaker (200 rpm) at 28°C. Nine hundred ml of the seed culture thus obtained was transferred into 18 liters of a fermentation medium composed of soluble starch 5%, dry yeast 1.5%, KH_2PO_4 0.05%, $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05%, in a 30 liter jar fermenter. The fermenter was operated at 28°C with agitation at 250 rpm and aeration of 18 liters per minute. The fermentation was carried out without particularly regulating the pH value of medium.

Antimicrobial Activity

The *in vitro* antimicrobial activity of GE3 was determined on nutrient agar by a 2-fold serial dilution method. The lowest concentration that inhibited growth of a bacterial strain after 18 hours incubation at 37°C was recorded as the MIC.

Cytotoxicity

Cytotoxicity against HeLa S3 cells, A431 cells, Saos-2 cells, and Balb/3T3 cells was measured by the MTT (3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, St.Louis, MO)-staining method described in the previous paper[11]. Briefly, 100 μl of a 4×10^3 cells/ml suspension of cells was pipetted into each well of a 96-well microtiter plate. This plate was incubated in the CO_2 gas incubator at 37°C for 20 hours. Then 50 μl of test compound, which was appropriately diluted with medium, was added to each well followed by incubation in the CO_2 gas incubator at 37°C for additional 72 hours. After removing the culture supernatant, 50 μl of 1 mg/ml of MTT, which was dissolved in the medium, was added to each well. Then 5 hours later, the pigment was extracted with 150 μl of DMSO and the $A_{550\,nm}$ was measured. By comparing the absorbance of the treated cells and untreated cells, the IC_{50} was calculated.

Antitumor Effect on PSN-1 Human Pancreatic Carcinoma-transplanted Nude Mouse

The antitumor effect on a nude mouse having PSN-1 human pancreatic carcinoma transplanted thereinto was examined as described previously[12]. Namely, a PSN-1 human pancreatic carcinoma was excised from a mouse for subculture and a tumor section (8 mm^3, $2 \times 2 \times 2$ mm) was subcutaneously transplanted into the abdomen of BALB/c-nu/nu mouse (nude mouse) by using a trocar. Eleven days after the transplantation, the growth of the tumor was confirmed. Then test drug was intraperiotoneally administered once on the same days. The antitumor effect was determined in the following manner. The major axis and the minor axis of tumor are measured with calipers and the tumor volume was calculated in accordance with the formula ((major axis) $\times$ (minor axis)^2/2). The antitumor effect is expressed in terms of the ratio (T/C) of the tumor volume of the tested group (T) to the tumor volume of the control group (C) to which no drug was administered.

Results

Characterization of the Producing Microorganisms

The cultural characteristics of strain GE3 on various agar media are shown in Table 1. The vegetative mycelia grew well or moderately on various agar media. Strain GE3 produced gold or bright yellow soluble pigment on ISP No. 2, No. 3, No. 4 and nutrient agar media after 14 days (Table 1). No fragmentation of substrate mycelia

Table 1. Cultural characteristics of strain GE3.

Medium	Amount of growth	Color of:		
		Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract - malt extract agar (ISP No. 2)	Good	White	Light brown	Brite gold
Oatmeal agar (ISP No. 3)	Good	White	Beige	Brite yellow
Inorganic salt - starch agar (ISP No. 4)	Good	Pale yellow	Light gold	Brite yellow
Glycerol - asparagine agar (ISP No. 5)	Good	Pale yellow	Light olive gray	None
Tyrosine agar (ISP No. 7)	Good	White	Light ivory	None
Sucrose - nitrate agar	Moderate	White	Light olive gray	None
Glucose - asparagine agar	Moderate	White	Light wheat	None
Nutrient agar	Moderate	White	Light wheat	Gold

was observed, and sclerotia, sporangia, or flagellated spores were not formed. The color of aerial mycelia showed white and pale yellow on the tested agar media. The aerial mycelium grew well and formed moderately short and simple branches arranged in spiral chains of 10 or more short-rod spores of 0.8 to 1.0 μm by 0.9 to 1.1 μm. As shown in Fig. 1, the surface of the spore was smooth. The physiological characteristics of strain GE3 is shown in Table 2. Analysis of whole-cell hydrolysates of the strain revealed that the cell walls contained L,L-diaminopimelic acid. These taxonomic observations indicate that the strain GE3 belongs to the genus *Streptomyces*.

Isolation and Purification

The isolation scheme is shown in Fig. 2. The culture broth obtained as described in 'materials and methods' was divided into culture filtrate and mycelial cake by filtration. The mycelial cake was extracted with acetone. After concentrating to remove acetone, water was added to dilute residual acetone. Then it was combined with the culture filtrate and passed through a column packed with a polystyrene adsorbent Diaion HP-20 (2 liters, Mitsubishi chemical industries), on which the active component was adsorbed. After eluting the impurities with deionized water, 33% aqueous methanol and 66%

Fig. 1. Scanning electron micrograph of strain GE3.
Bar represents 1.0 μm.

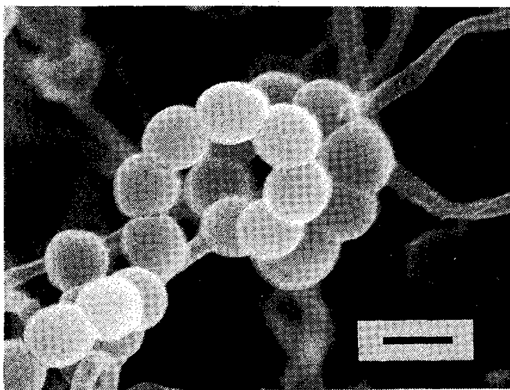

Table 2. Physiological properties of strain GE3.

Characteristics	GE3
Temperature for growth	5～37°C
Optimum temperature	25～30°C
Liquefaction of gelatin	Negative
Hydrolysis of starch	Positive
Coagulation of milk	Negative
Peptonization of milk	Positive
Formation of melanine	Negative
Utilization of	
D-Glucose	Positive
L-Arabinose	Negative
D-Xylose	Positive
D-Fructose	Positive
L-Rhamnose	Positive
Sucrose	Negative
Raffinose	Negative
D-Mannitole	Positive
Inositol	Negative

Fig. 2. Isolation procedure of GE3 (**1**) and GE3B (**2**).

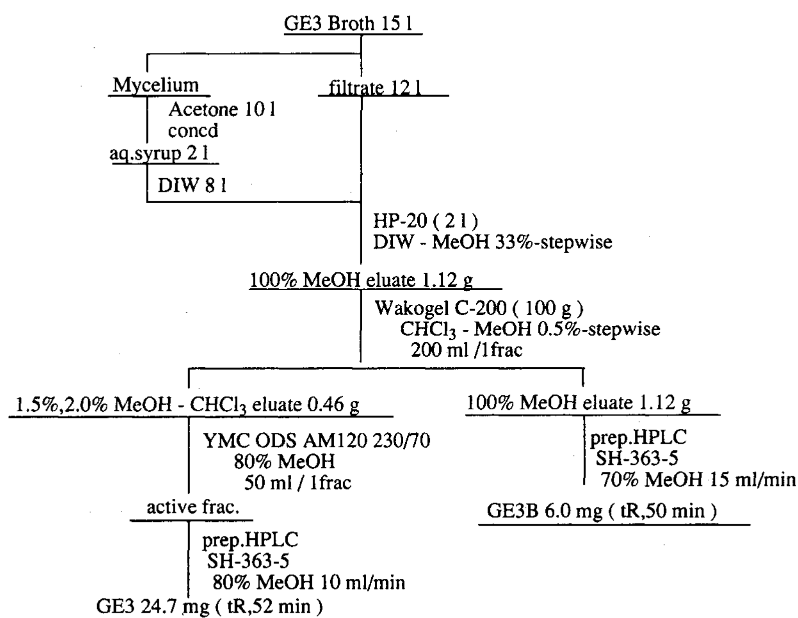

Table 3. Physico-chemical properties of GE3 (**1**) and GE3B (**2**).

	GE3 (**1**)	GE3B (**2**)
Appearance	White powder	White powder
Melting point	213～215°C	168～170°C
Molecular formula	$C_{49}H_{80}N_8O_{14}$	$C_{49}H_{82}N_8O_{15}$
HRFAB-MS *m/z*		
Found	1003.5720 $[M-H]^-$	1021.5849 $[M-H]^-$
Calcd.	1003.5716	1021.5821
Elemental analysis		
Found	C, 57.08; H, 7.81; N, 10.76%	—
Calcd.	C, 57.52; H, 8.08; N, 10.95% ($C_{49}H_{80}N_8O_{14}\cdot H_2O$)	—
Optical rotation	$[\alpha]_D^{25}$ +111.5° (*c* 0.08, $CHCl_3$)	$[\alpha]_D^{24}$ −16.0° (*c* 0.25, MeOH)
UV λ_{max} (MeOH)	237 nm (sh)	230 nm (sh)
IR ν_{max} (KBr)	3419, 2935, 1734, 1645, 1506, 1315, 1217 cm^{-1}	3427, 2958, 1635, 1520, 1406, 1244 cm^{-1}

aqueous methanol, the active substance was eluted with 100% methanol (10 liters). The methanol fraction was concentrated *in vacuo* and subject to a column chromatography on silica gel Wakogel C-200 (Wako pure chemical Industries) developed and fractionated stepwise with chloroform to 5% methanol-chloroform (0.5% stepwise). And then adsorbed substances were eluted with 100% methanol. The active fractions (1.5% and 2.0% methanol-chloroform eluates) were combined and concentrated, applied on reverse phase column packed with ODS-AM 120-230/70 (YMC Inc.) and developed and fractionated with 80% aqueous methanol. The active fractions thus eluted were concentrated and applied to a preparative HPLC using a packed ODS column (SH363-5, 250 mm × 30 mm diameter, YMC Inc.) with 80% aqueous methanol (flow rate 20 ml per minute). After developing, a fraction with retention time of 53 minutes was taken up with the use of the UV absorption at 230 nm as detection. Finally lyophilised, 25 mg of GE3 was obtained as white powder. The isolation procedure for GE3B, which is a minor relative of GE3, is as follows. The fractionation was monitored by the similarity of the UV spectrum with GE3. The 100% eluate from silica gel column (Wakogel C-200) was dried up *in vacuo* and dissolved in 90% methanol, then passed through a small ODS column (ODS-AM 120-230/70) to remove substances which were tightly adsorbed to ODS. The effluent was subjected to a reverse phase preparative HPLC (SH363-5) developing with 75% methanol (flow rate: 15 ml per minutes). By concentrating the fraction with retention time of 50 minutes, 6 mg of GE3B was obtained.

Structures of GE3 and GE3B

The physico-chemical properties of GE3 are shown in Table 3. On the basis of the analysis of a variety of the spectra, the structures of GE3 and GE3B were determined as shown in Fig. 3. GE3 was a novel cyclic hexadepsipeptide closely related to citropeptin[1], one of the cyclic hexadepsipeptide antibiotics previously reported, which contains *N*-hydroxy-*O*-methyl-serine instead of *N*-hydroxy-alanine of GE3. GE3B had linear peptide structure, possibly converted from GE3 by mono hydrolyzation. Details of structural elucidation studies are described in the succeeding paper[9].

Antimicrobial Activity

The antimicrobial activities of GE3 and GE3B are shown in Table 4. GE3 showed weak antibacterial activity against both Gram-positive bacteria, *Staphylococcus aureus* and *Enterococcus faecium*, and Gram-negative bacteria, *Pseudomonas aeruginosa* with the MIC value ranged from 10.4 μg/ml to 41.6 μg/ml. On the contrary, GE3B, which has the linear peptide structure different from the cyclic peptide GE3, did not show any inhibitory activities against the 10 strains of microorganism used in this study.

In Vitro Cytotoxicity

As shown in Table 5, GE3 exhibited potent cytotoxicity against human tumor cell lines; HeLa S3 cells with IC_{50} value of 6 nM, A431 cells (IC_{50} 16 nM), Saos-2 cells (IC_{50} 3.6 nM), and a normal mouse cell line; Balb/3T3 cells (IC_{50} 7 nM). Likewise the case of antimicrobial activities, GE3B showed no growth inhibitory activity

Fig. 3. Structures of GE3 (1) and GE3B (2).

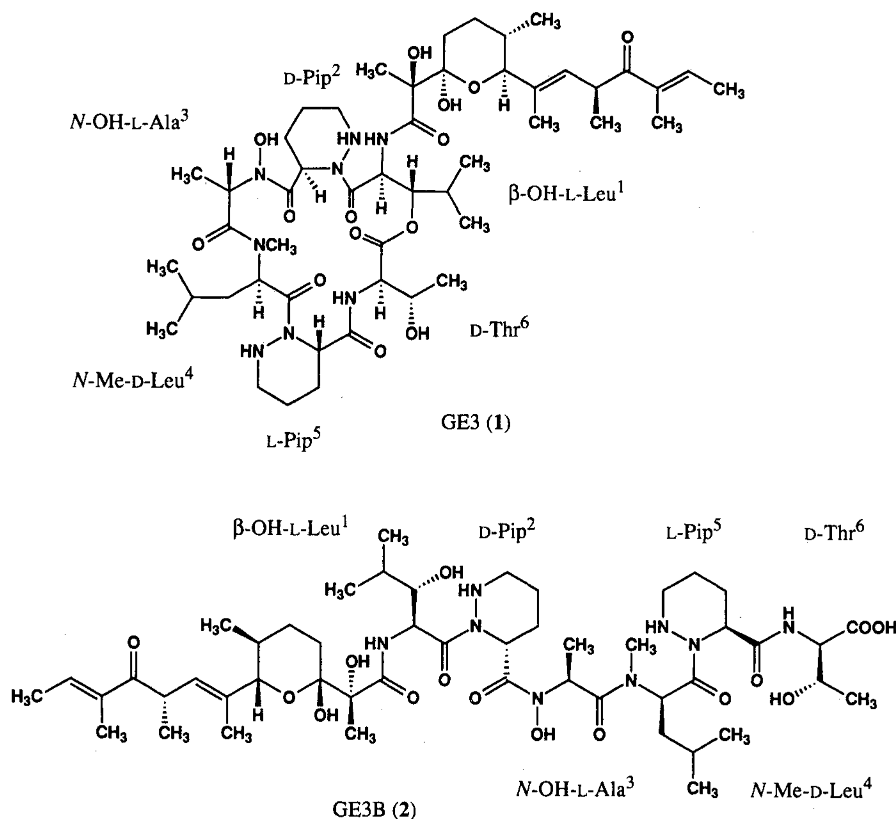

Table 4. Antimicrobial activities of GE3 (1) and GE3B (2).

Organisms	MIC (μg/ml)	
	GE3	GE3B
Staphylococcus aureus	10.4	>100
Enterococcus hirae	10.4	>100
Bacillus subtilis	>100	>100
Klebsiella pneumoniae	>100	>100
Escherichia coli	>100	>100
Pseudomonas aeruginosa	41.6	>100
Salmonella choleraesuis	>100	>100
Proteus vulgaris	>100	>100
Shigella sonnei	>100	>100
Candida albicans	>100	>100

against HeLa S3 (IC_{50} >10 μM).

In Vivo Antitumor Activity

In vivo antitumor effect of GE3 against a human tumor was examined in the human xenograft mouse tumor model. When administered by single intraperitoneally injection on day 0, GE3 showed significant antitumor activity against the PSN-1 human pancreatic carcinoma (T/C 0.47) at dose of 2 mg/kg (Table 6). At this dose, however, weight reduction (−18.2%) of mice was observed.

Table 5. Cytotoxicities of GE3 (1) and GE3B (2) against human and murine cells.

Compounds	IC_{50} (μM)			
	HeLa S3	A431	Saos-2L	BALB3T3
GE3	0.006	0.016	0.0036	0.007
GE3B	>10	ND[a]	ND	ND

[a] Not determined.

Table 6. Antitumor activity of GE3 (1) against PSN-1 human pancreatic carcinoma (ip).

Compound	Cell line	Dose (mg/kg) ×frequency	T/C (%)	Mortality
GE3	PSN-1	2.0×1	0.47[a]	0/5

[a] $P<0.01$ against control by Mann-Whiteney's *U*-test.

Discussion

GE3 showed potent cytotoxicity against various human and mouse cell lines with the IC_{50} value ranged

from 6 nM to 16 nM. This activity seemed to be as strong as other cyclic hexadepsipeptide antibiotics[1~8], surveying the data in their reports. Thus strong cytotoxicity may be common property of this class of compounds.

Two compounds, citropeptin[1] and verucopeptin[6], among the cyclic hexadepsipeptide antibiotics were reported active *in vivo* against murine tumors, P388 (lymphotic leukemia) and B16 (melanoma), respectively, and variapeptin[4] and L156,602[13] were inactive against P388. GE3 was active when administered by single intraperitoneally injection but was inactive by consecutive injection (data not shown), just like the case of verucopeptin[6], suggesting treatment schedule might be critical for cyclic hexadepsipeptide antibiotics to exhibit *in vivo* antitumor activity. In all the cases reported on *in vivo* antitumor activity, the toxicity to experimental animals caused narrow active dose range. Thus this toxicity must be reduced for consideration of clinical use of these cyclic hexadepsipeptide antibiotics.

In the present study, we also isolated GE3B, linear peptide form of GE3. This is the first report on the activities of the linear form of cyclic hexadepsipeptide antibiotics. GE3B showed no antimicrobial activities nor cytotoxicities, suggesting the cyclic structure is necessary to both activities of GE3. The cyclic structure might contribute to interaction with its intracellular target.

Nevertheless strong cytotoxicity, the modes of action of cyclic hexadepsipeptide antibiotics were little known. It was proposed that the inhibition of DNA and RNA synthesis[6] and/or the inhibition of the adhesion to extracellular matrix[7] are included in the mode of action. To elucidate the mode of action of GE3, the effect of the drug on cell cycle progression and on the expression of cell cycle-related gene, cyclin A, were analyzed[14]. GE3 inhibited progression of the cell cycle from the G1 to S phase in A431 cells. Additionally, GE3 inhibited the cyclin A gene expression in Saos-2 cells without repression of β-actin gene expression. Thus it seems that GE3 selectively represses the expression of the genes essential for the progression of cell cycle from G1 to S phase. Furthermore, GE3 was shown to prevent the E2F transcriptional factor, the intracellular target of retinoblastoma susceptibility gene product[15], from binding to its recognition sequence. These results strongly suggest that GE3 exhibits cytotoxicity by acting as an inhibitor of E2F transcriptional factor[14].

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