TAN-1511 A, B and C, Microbial Lipopeptides with G-CSF and GM-CSF Inducing Activity

MASAYUKI TAKIZAWA, TSUNEAKI HIDA[†], TAKASHI HORIGUCHI, AKIKO HIRAMOTO[††], SETSUO HARADA[†††] and SEIICHI TANIDA*

Discovery Research Laboratories[†] I and II,
[††]Pharmaceutical Research Laboratories II and
[†††]Research on Research, Takeda Chemical Industries Ltd.,
2-17-85, Juso-honmachi, Yodogawa-ku, Osaka 532, Japan

(Received for publication February 20, 1995)

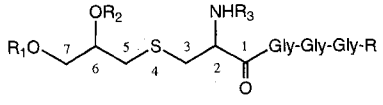
The microbial lipopeptides, TAN-1511 A, B and C, were isolated from the culture broth of *Streptosporangium amethystogenes* subsp. *fukuiense* AL-23456. Their structures were elucidated on the basis of their reactions and spectroscopic analyses. These lipopeptides were mixtures of molecules having different lengths of fatty acids.

The metabolites stimulated the proliferation of bone marrow cells from BALB/c female mice at very low concentrations (concentration giving 30% increase: A and B, 0.313 ng/ml; C, 1.25 ng/ml). We confirmed that chemically synthesized TAN-1511 A analogue [(2*R*,6*R*)-2-tetradecanoylamino-6,7-bis(hexadecanoyloxy)-4-thiaheptanoyl-Gly-Gly-Gly-Glu-Thr-Thr-OH] stimulated the proliferation of bone marrow cells in a manner similar to that of natural TAN-1511 A. This analogue induced the secretion of both granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF), and potentiated the generation of Gr-1 positive cells in the bone marrow cell culture. Moreover, it effected the G-CSF mediated restoration of granulocytopoiesis in a murine leukopenia model.

Leukocytes are indispensable to the host defense system, as they are potentially destructive to invading microorganisms[1]. Cytopenia of these cells results in severe infection and it is often caused by anticancer regimens using antitumor drugs and ionizing radiation[2,3]. Myelopoiesis is regulated by hematopoietic cytokines that originate mainly from lymphocytes and bone marrow stromal cells such as macrophages, fibroblasts and endothelial cells[4,5]. Thus, substances that potentiate the production of these cytokines should protect against microbial infection in patients with leukopenia caused by cancer therapy.

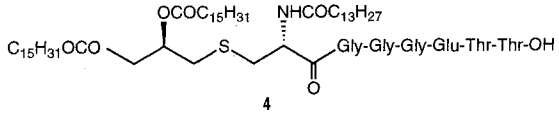
In our search for microbial products that induce granulocytopoietic cytokines, three metabolites, TAN-1511 A (**1**), B (**2**) and C (**3**), which can promote the proliferation of bone marrow cells, were found in the culture broth of *Streptosporangium amethystogenes* subsp. *fukuiense* AL-23456[6]. Degradation reaction and spectral analyses revealed that each component is a mixture of lipopeptides which are 2-amino-6,7-dihydroxy-4-thiaheptanoic acid derivatives with long chain fatty acids of different lengths on the hydroxyl and amino groups, and a peptide including a Gly-Gly-Gly sequence bound to the carboxyl group (Fig. 1). Since **1**~**3** were obtained from a culture broth of the actinomycete at very low yields and are mixtures of molecules with fatty acids of different lengths, we synthesized large amounts of an analogue (**4**, Fig. 1) of **1** for further investigation[7]. This paper describes the production, isolation and structural determination of these metabolites, as well as the granulocytopoietic activity of **4** *in vitro* and *in vivo*.

Fig. 1. Structures of TAN-1511 A (**1**), B (**2**), C (**3**) and synthetic TAN-1511 A analogue (**4**).



	R
TAN-1511 A (**1**)	L-Glu-L-Thr-L-Thr-OH
B (**2**)	L-Asp-L-Ala-Gly-L-Thr-OH
C (**3**)	L-Asp-L-Pro-L-Leu-OH

R_1, R_2 = Fatty Acid Residues (C_{16}-C_{19})
R_3 = Fatty Acid Residues (C_{13}-C_{15})



4

This article is a special contribution in honour of Professor SATOSHI ŌMURA's 60th birthday.

Materials and Methods

General

UV spectra were taken on a Hitachi 320 spectrophotometer. IR spectra were measured using a Horiba FT-200 IR spectrophotometer in KBr pellets. The ^1H NMR spectra were recorded on a Bruker AM-500 spectrometer at a probe temperature of 30°C. DQF-COSY and ROESY spectra were recorded with 512×2048 data points and acquired in the phase sensitive mode using the TPPI procedure. FAB-MS spectra were measured using a JEOL HX110 double-focusing mass spectrometer/DA5000 data system. Automated Edman degradation was performed on an Applied Biosystems 477A protein sequencer equipped with a 120A phenylthiohydantoin (PTH)-amino acid analyzer.

Reagents

RPMI-1640 medium and fetal calf serum (FCS) were obtained from Bio Whittaker Inc., U.S.A. The serum was inactivated by heating at 56°C for 30 minutes prior to use. Other materials and their sources were as follows: FITC-conjugated anti-mouse Gr-1 antibody (Pharmingen, U.S.A.); cyclophosphamide (Endoxan, Shionogi & Co., Ltd., Japan); QuickPrep micro mRNA purification kit (Pharmacia P-L Biochemicals, Sweden); Quantikine human G-CSF ELISA kit (R&D Systems, U.S.A.); mouse GM-CSF ELISA kit (Endogen, U.S.A.).

Fermentation

A loopful of strain AL-23456 grown on mature slant culture was inoculated into a 2-liter Sakaguchi flask containing 500 ml of a sterile seed medium. The flask was placed on a reciprocal shaker at 28°C for 48 hours. The seed medium consisted of soluble starch 3%, glucose 2%, soybean flour 1%, peptone 0.5%, corn steep liquor 0.3%, yeast extract 0.1%, NaCl 0.3% and $CaCO_3$ 0.5%. Seed culture (500 ml) was transferred to a 200-liter fermenter containing 120 liters of the same medium and the fermentation was carried out at 28°C for 48 hours with aeration of 120 liters/minute and agitation of 120 rpm. One hundred liters of the seed culture was transferred to a 6000-liter fermenter containing 3600 liters of a production medium consisting of soluble starch 3%, potato starch 3%, glucose 0.5%, soybean meal 3.5%, yeast extract 0.5%, peptone 0.3%, sodium glutamate 0.3% and $CaCO_3$ 0.7%. The fermentation was carried out at 28°C with aeration of 2160 liters/minute and agitation of 180 rpm.

Isolation of **1**, **2** and **3**

The broth filtrate (3,600 liters) was extracted with EtOAc (1,200 liters) at pH 2.0 and the organic layer was extracted with 2% aqueous $NaHCO_3$ (300 liters). The aqueous layer was extracted with EtOAc (2×160 liters) at pH 2.0, and the organic layers were combined and extracted with 2% aqueous $NaHCO_3$ (140 liters) again. The aqueous layer (155 liters) was concentrated to a volume of 100 liters and eluted through a column of Diaion SP-207 (40 liters) with 4% aqueous $NaHCO_3$ (40 liters). The flow-through fraction and the eluate were extracted with EtOAc (2×72 liters) at pH 2.0 and the organic layers combined were concentrated to a small volume (3.5 liters). The solution was applied on a column of silica gel (250 g) and active substances were eluted with mixtures of EtOAc, *iso*-PrOH and water (4:2:1, 1.5 liters, and 2:2:1, 1.5 liters). The active fraction was concentrated and the residue was suspended in 0.05 M HCl (250 ml), then extracted with EtOAc (3×250 ml). The organic layers were combined, washed with water and concentrated to give a crude powder (1.65 g). The powder was eluted through a column of Sephadex LH-20 (3.0 liters) with MeOH, to give TAN-1511 complex as a brown powder (383 mg).

The powder was eluted through a column of Toyopearl HW-40F (200 ml) with a mixture of EtOAc, *iso*-PrOH and water (10:5:2.5), and 10 ml fractions were collected. Fractions 10 to 13 were combined and concentrated to give an oily residue containing **3** (150 mg). Fractions 19 to 27 and 28 to 35 were concentrated to give powders (60 mg and 45 mg) containing **1**, and a mixture of **1** and **2**, respectively.

The powder (60 mg) containing **1** was suspended in a small volume of MeOH and the precipitates (34 mg) were further purified by preparative HPLC using columns of silica gel (System I: column, YMC-Pack SH-043 SIL; solvent, $CHCl_3$-MeOH-water (6:3:0.5); flow rate, 2.5 ml/minute) and aminopropyl silica gel (System II: column, YMC-Pack SH-643-15 NH_2; solvent, 85% MeOH-50 mM phosphate buffer (pH 5.2); flow rate, 5.0 ml/minute), successively. The pure fraction was concentrated and extracted with *iso*-BuOH at pH 2.0 to yield **1** as a white powder (9.3 mg). A portion of the powder (10 mg) containing **1** and **2** was eluted through a column of HW-40S (40 ml) with *iso*-BuOH. The fraction containing **2** was further purified by preparative HPLC using System II, twice. The pure fraction was concentrated and extracted with *iso*-BuOH at pH 2.0 to give a powder, which was then suspended in a small volume of cold MeOH. The precipitates were collected to give **2** as a white powder (3.0 mg).

The oily residue containing **3** was purified by preparative HPLC using Systems I and II, successively. The pure fraction was concentrated and extracted with *iso*-BuOH at pH 2.0 to give **3** as a white powder (10 mg).

Fatty Acid Analysis of TAN-1511 Complex

TAN-1511 complex (4.6 mg) was hydrolyzed with 6 M HCl (1.0 ml) for 12 hours at 110°C. The reaction mixture was concentrated and the residue was suspended in water (4.0 ml). The suspension was extracted with EtOAc (2×4.0 ml) and the organic layers were combined and concentrated to give an oily residue, in which the fatty acids were analyzed after derivatization into 2-nitrophenylhydrazides[8]. A suspension of TAN-1511 complex (20 mg) and K_2CO_3 (10 mg) in MeOH (10 ml) was stirred

for 2 hours at room temperature. The reaction mixture was concentrated and the residue was suspended in water and extracted with EtOAc (2 × 10 ml) at pH 2.0. The organic layers were combined and concentrated to give an oily residue, in which the fatty acids were analyzed as described above after hydrolysis with NaOH. The aqueous layer was extracted with *iso*-BuOH (2 × 10 ml) and the combined organic layers were concentrated to give a powder (10 mg). This powder was hydrolyzed with 6 M HCl and processed as described above to analyze the fatty acids.

Decomposition of 3

A solution of **3** (400 μg) in DMSO (40 μl) was mixed with 300 μl of MeOH and 300 μl of 1 M NaOH and the mixture was allowed to stand for 2 hours. The mixture was neutralized with 1 M HCl and an aliquot (100 μl) was transferred to a glass tube, then dried under a stream of nitrogen gas. The residue was dissolved in 500 μl of 0.02 M HCl and hydrolyzed under reduced pressure for 2 hours at 108°C. The hydrolysate was dried under a stream of nitrogen gas, and redissolved in 60 μl of MeOH and 2 μl of 2 M HCl. The solution was analyzed using the FAB-MS spectrometer and the amino acid sequencer.

Determination of the Amino Acid Configuration

Hydrolysates of **1**, **2** and **3** (6 M HCl for 14 hours at 105°C) were analyzed after derivatization with *o*-phthalaldehyde in the presence of *N*-acetyl-L-cysteine[9] (column, YMC R ODS-5). L-Proline in the hydrolysate of TAN-1511 C was detected by HPLC using Chiralpak WE (solvent, 0.25 mM $CuSO_4$; flow rate, 1.0 ml/minute; detection, UV 250 nm; temperature, 50°C).

Cell Preparation and Culture Method

Bone marrow cells were obtained from the femora of female BALB/c mice aged 8 ~ 10 weeks (Japan Charles River, Japan). After removing the femora aseptically, the marrow was flushed and suspended in RPMI-1640 containing 10% FCS and 50 μM 2-mercaptoethanol. The suspension was passed through cotton gauze and centrifuged. The cells then were resuspended in the same medium at an appropriate concentration. Splenocytes and peritoneal macrophages were prepared as described[10]. Bone marrow cells in 0.1 ml of culture medium were incubated with samples at 37°C for 3 days in an atmosphere of 5% CO_2 in air. The proliferation of the cells were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetry[11].

Isolation of Cellular RNA and Reverse Transcription

Cells (1.0×10^6 cells/ml) in 10 ml of RPMI-1640 medium were incubated with samples at 37°C in an atmosphere of 5% CO_2 in air. After incubation with **4**, the cells were harvested to prepare RNAs. RNA with a poly A-tail was purified using a commercial mRNA purification kit.

Reverse transcription was performed in a reaction mixture (100 μl) containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM each 4 dNTPs, 1 U/μl RNase inhibitor, 2.5 U/μl reverse transcriptase, 2.5 μM oligo[d(T)16] and 1 μg RNA. The mixture was incubated at 42°C for 60 minutes, at 99°C for 5 minutes, then at 5°C for 5 minutes. The samples were extracted with phenol-chloroform and precipitated with ethanol.

PCR Amplification and Analysis

Specific cDNAs (0.5 μg) obtained from the reverse transcription were amplified in a reaction mixture (100 μl) containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μM each 4 dNTPs, 0.025 U/μl Taq polymerase, 0.001% gelatin and 1 μM cytokine-specific primers as listed in Table 1. The mixture underwent PCR cycles in a Thermal cycler 470 (Perkin-Elmer Cooperation, U.S.A.) with denaturation for 1 minute at 94°C, annealing at 60°C for 1 minute, and extension for 1 minute at 72°C. The number of PCR cycles was selected for each cytokine product in the cells so that the level of most of the ethidium bromide-stained, amplified DNA products were between barely detectable and below saturation.

The amplified products (9 μl) were resolved by electrophoresis in an agarose gel at 100 volts (V) for 25 minutes in TBE buffer consisting of 89 mM Tris-borate (pH 8.9) and 2 mM EDTA. The gels were stained with ethidium bromide (0.5 μg/ml) and washed thoroughly with distilled water. DNA bands were visualized under UV light and photographed. The data from the negatives were processed by PHOTO CD (Eastman Kodak Co., New York, U.S.A.) and analyzed using the NIH image program (Ver. 1.44 by WAYNE RASBAND). The mRNA levels of β-actin were not affected by the addition of **4** (data not shown) and were therefore used to standardize the gene expression of the inducible cytokine gene products. The ratio of the densitometric values of cytokine PCR to β-actin products was used to assess the relative changes in the levels of cytokine mRNA in response to **4**. No significant change was observed in the expression of any of the cytokines tested during 24 hours of culture in the absence of **4** (data not shown). Data

Table 1. Primer sequneces and PCR cycles.

Cytokine	Product size (bp)	Upstream primer (5′)	Downstream primer (3′)	Cycle
G-CSF	333	5′-GGAAATACCCGATAGAGCCT-3′	5′-AGTGCACTATGGTCAGGACG-3′	52
GM-CSF	337	5′-CGCATAGGTGGTAACTTGTG-3′	5′-GGCTGTCTATGAAATCCGCA-3′	55
β-Actin	1128	5′-ATGGATGACGATATCGCTGC-3′	5′-CTAGAAGCACTTGCGGTGCA-3′	55

shown in each figure represent the results from triplicate experiments.

Assay of CSF Secretion

Cells in 0.1 ml of culture medium were incubated with samples at 37°C in an atmosphere of 5% CO_2 in air. The secretion of G-CSF and GM-CSF in the culture was determined using commercial ELISA kits. The amount of G-CSF was estimated as that of human G-CSF.

Flow Cytometry

Bone marrow cells and splenocytes were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse Gr-1 antibody. Thereafter, the cells were analyzed using a flow cytometer (FACScan, Becton-Dickinson, U.S.A.).

Peripheral Leukocyte Counts

Blood was collected from the hearts of mice anesthetized with ether. The leukocytes were counted using an automatic counter (Sysmex E-2500, Toa Medical Electronics Co., Japan).

Results

Production and Isolation

1, **2** and **3** were produced by cultivating *Streptosporangium amethystogenes* subsp. *fukuiense* AL-23456. The amount of the metabolites in the culture reached a maximum (1.02 μg/ml which was estimated as **1**) at 114 hours. They were acidic and fat-soluble, and they were isolated from the broth filtrate as follows.

The broth filtrate was extracted with ethyl acetate at pH 2.0, and the organic layer was extracted with aqueous sodium hydrogen carbonate. The aqueous extract was applied to a column of Diaion SP-207 and eluted with aqueous sodium hydrogen carbonate. The flow-through fraction and the eluate were combined and extracted with ethyl acetate at pH 2.0. After concentration, the organic layer was eluted through a silica gel column with a mixture of ethyl acetate, *iso*-propyl alcohol and water. The active fraction was concentrated to give a crude powder, which was eluted through a column of Sephadex LH-20 with methanol, to give TAN-1511 complex as a brown powder.

The powder was purified using columns of Toyopearl HW-40F and HPLC with silica gel and aminopropyl columns to obtain component **1**, **2** and **3** as a white powder.

Characterization

Each TAN-1511 component was soluble in dimethyl sulfoxide or *iso*-butyl alcohol, sparingly soluble in methanol or ethyl acetate, and insoluble in water or hexane. They reacted positively to phosphomolybdic acid, iodine and sulfuric acid, but negatively to Greig-Leaback, Sakaguchi and Dragendorff reagents.

The physico-chemical properties of the components are summarized in Table 2, along with their chromatographic behavior on TLC and HPLC. Their UV spectra showed only an end absorption. Their IR spectra had strong absorption bands around 2900 cm^{-1} arising from long chain alkyl groups, around 1740 cm^{-1} arising from ester groups, and around 1650 and 1540 cm^{-1} arising from amide groups. In the FAB-MS spectrum of **1**, several molecular ion peaks, $(M+Na)^+$, were observed, such as *m/z* 1449, 1447, 1435, 1433, 1421, 1419, 1407, and 1405, indicating that this compound is a mixture of molecules with long chain alkyl groups of different lengths. The presence of one sulfur atom in **1** was confirmed by elemental analysis (Found, 2.3%).

Structural Determination

Hydrolysis of the components with 6 M hydrochloric acid gave glycine (3 mol), glutamic acid and threonine (2 mol) from **1**, glycine (4 mol), aspartic acid, alanine and

Table 2. Physico-chemical properties of TAN-1511 A (**1**), B (**2**) and C (**3**).

		1	2	3
Appearance		White powder	White powder	White powder
UV (MeOH)		End	End	End
IR KBr (νcm^{-1})		2925, 2850, 1740, 1655, 1540	2920, 2850, 1735, 1655, 1540	2925, 2855, 1740, 1650, 1540
FAB-MS (*m/z*)		1449, 1447, 1435, 1433, 1421, 1419, 1407, 1405	1462, 1460, 1448, 1446, 1434, 1432, 1420, 1418	1443, 1441, 1429, 1427, 1415, 1413, 1401, 1399
Amino Acid		Thr (2), Glu, Gly (3)	Asp, Thr, Gly (4), Ala	Asp, Pro, Gly (3), Leu
C-Terminal		Thr	Thr	Leu
TLC (Rf)[a]	Solvent I	0.32	0.28	0.48
	Solvent II	0.27	0.23	0.34
HPLC (t_R)[b] (minute)		14.6	15.8	15.4

[a] Silica gel 60 F_{254} (Merck). Solvent I; $CHCl_3$ - MeOH - H_2O (6 : 3 : 0.5).
Solvent II; EtOAc - *iso*-PrOH - H_2O (10 : 5 : 2.5).

[b] Column, YMC-Pack A-602 NH_2; solvent, 85% MeOH - 20 mM phosphate buffer (pH 4.8); flow rate, 1.0 ml/minute; detection, UV absorbance at 214 nm.

threonine from **2**, and glycine (3 mol), aspartic acid, proline and leucine from **3**. The stereochemistry of these amino acids (except glycine) was determined to be L by HPLC analyses after derivatization with *o*-phthalaldehyde and *N*-acetyl-L-cysteine[9], or by using a chiral column. Amino acid analysis after decomposition with hydrazine revealed that the *C*-terminals of **1**, **2** and **3** were threonine, threonine and leucine, respectively.

The fatty acids liberated from TAN-1511 complex were analyzed after derivatization into 2-nitrophenylhydrazides as described by MIWA *et al.*[8] (Table 3). The total fatty acids obtained from the 6 M hydrochloric acid hydrolysate gave C_{13} to C_{19} fatty acids. When TAN-1511 complex was treated with potassium carbonate in methanol, C_{16} to C_{19} fatty acids were liberated, and further hydrolysis with 6 M hydrochloric acid gave C_{13}

Table 3. Fatty acids obtained from TAN-1511 complex.

Fatty Acid	%		
	Total	*O*-Acyl	*N*-Acyl
Tridecanoic acid (C_{13})	4	<1	14
Isomyristic acid (C_{14})	12	2	36
Myristic acid (C_{14})	9	2	29
Isopentadecanoic acid (C_{15})	4	—	13
Pentadecanoic acid (C_{15})	4	7	4
Isopalmitic acid (C_{16})	5	8	<1
Palmitic acid (C_{16})	24	28	1
Heptadecanoic acid (C_{17})	6	7	<1
Stearic acid (C_{18})	7	11	<1
Oleic acid (C_{18})	5	7	—
Nonadecanoic acid (C_{19})	5	8	—

—: Not detected.

Table 4. ^{1}H NMR data for TAN-1511 A (**1**), B (**2**) and C (**3**) in DMSO-d_6.

1			**2**			**3**		
Position		δ ppm[a] (*J* in Hz)	Position		δ ppm[a] (*J* in Hz)	Position		δ ppm[a] (*J* in Hz)
ADTA[b]	NH	8.06, d (8.5)	ADTA	NH	8.10, d (8.5)	ADTA	NH	8.06 d (8.5)
	2	4.50, dt (5.0, 8.5)		2	4.50, dt (4.8, 8.5)		2	4.50, dt (5.1, 8.5)
	3	2.66, dd (13.6, 8.5)		3	2.67, dd (13.6, 8.5)		3	2.66, dd (13.6, 8.5)
		2.94, dd (13.6, 5.0)			2.95, dd (13.6, 8.5)			2.94, dd (13.6, 5.1)
	5	2.73, dd (14.0, 7.5)		5	2.73, dd (14.0, 7.4)		5	2.73, dd (14.0, 7.5)
		2.83, dd (14.0, 5.4)			2.83, dd (14.0, 5.0)			2.83, dd (14.0, 5.5)
	6	5.11, m		6	5.11, m		6	5.10, m
	7	4.12, dd (11.9, 6.6)		7	4.12, dd (11.9, 6.7)		7	4.12, dd (11.9, 6.7)
		4.31, br d (11.9)			4.31, dd (11.9, 2.6)			4.31, dd (11.9, 2.5)
Gly-1	NH	8.29, br t (5.4)	Gly-1	NH	8.31, t (5.2)	Gly-1	NH	8.28, t (5.7)
	α	3.70~3.86		α	3.70~3.85		α	3.70~3.85
Gly-2	NH	8.13, t (6.5)[e]	Gly-2	NH	8.15	Gly-2	NH	8.10, t (5.7)
	α	3.70~3.86		α	3.70~3.85		α	3.70~3.85
Gly-3	NH	8.11, t (6.5)[e]	Gly-3	NH	8.15	Gly-3	NH	8.04, t (5.7)
	α	3.70~3.86		α	3.70~3.85		α	3.70~3.85
Glu	NH	8.04, d (8.0)	Asp	NH	8.21, d (7.7)	Asp	NH	8.30, d (7.6)
	α	4.42, dt (5.4, 8.0)		α	4.60, m		α	4.86, dt (6.7, 7.6)
	β	1.78, m		β	2.53[d]		β	2.45, dd (16.9, 7.6)
		1.95, m			2.72, dd (17.3, 6.7)			2.72, dd (16.9, 6.7)
	γ	2.28, 2H, m	Ala	NH	8.05, d (7.0)	Pro	α	4.36, dd (10.6, 2.7)
Thr-1	NH	7.86, d (8.1)		α	4.23, m		β	2.00, 2H, m
	α	4.34, dd (8.1, 4.4)		β	1.26, 3H[e]		γ	1.90, 2H, m
	β	3.99, m	Gly-4	NH	8.15		δ	3.65, 2H, m
	γ	1.11, 3H, d (6.3)		α	3.70~3.85	Leu	NH	7.86, d (7.9)
Thr-2	NH	7.72, d (8.6)	Thr	NH	7.71, d (8.2)		α	4.18, m
	α	4.22, dd (8.6, 2.9)		α	4.18, br d (8.2)		β	1.50, 2H, m
	β	4.16, m		β	4.12, m		γ	1.66, m
	γ	1.08, 3H, d (6.3)		γ	1.06, 3H, d (6.3)		δ	0.88, d (6.6)
Fatty Acids		0.87, *ca.* 12H, m	Fatty Acids		0.87, *ca.* 12H, m		δ'	0.92, d (6.6)
		1.16, *ca.* 2H, m			1.16, *ca.* 2H, m	Fatty Acids		0.87, *ca.* 12H, m
		1.26, *ca.* 60H, br			1.26, *ca.* 62H, br			1.16, *ca.* 2H, m
		1.52, 6H, m			1.52, 6H, m			1.26, *ca.* 60H, br
		2.00, *ca.* 2H, m			2.00, *ca.* 1H, m			1.52, 6H, m
		2.15, 2H, m			2.15, 2H, m			1.90, 1H, m
		2.28, 4H, m			2.29, 4H, m			2.15, 2H, m
		5.34, *ca.* 1H, t (4.7)			5.34, *ca.* 0.5H, t (4.7)			2.28, 4H, m
								5.34, *ca.* 1H, t (4.7)

[a] Chemical shifts are indirectly referenced to internal DSS by measuring of the residual methyl resonance of DMSO (2.53 ppm at 30°C).
[b] ADTA; 2-amino-6,7-dihydroxy-4-thiaheptanoic acid.
[c] These assignments are interchangeable.
[d] Overlaps the solvent signal.
[e] Overlaps the methylene signals of fatty acids.

to C_{15} fatty acids. These findings showed that the fatty acid residues attached through the ester linkages and those through the amide linkage are different and that the former were somewhat longer than the latter.

The ^1H NMR data for **1** and **2** are shown in Table 4. The assignments of protons arising from threonine, glutamic acid and glycines, and the other two partial structures, $-CH_2-CH(OR_2)-CH_2(OR_1)$ and $-CH_2-CH-NH-R_3$, in **1** were readily deducible by COSY. From the chemical shifts, two methylenes (δ 2.66 and 2.94, 2.73 and 2.83) were connected through the sulfur atom, then the presence of a 2-amino-6,7-dihydroxy-4-thiaheptanoic acid (ADTA) residue was deduced. The sequence of the amino acids was revealed to be -Gly-Gly-Gly-Glu-Thr-Thr-OH by combined analysis of the COSY and ROESY spectra (Fig. 2). That is, the amido protons of glutamic acid (δ 8.04), threonine (δ 7.86) and threonine (δ 7.72) gave cross peaks with the α protons of glycine (δ 3.70~3.86), glutamic acid (δ 4.42), and threonine (δ 4.34), respectively. Furthermore, one of the amido protons of glycine (δ 8.29) , the methylene protons of one of the fatty acids (δ 2.15) and the methylene protons of H-5 of the ADTA residue (δ 2.73, 2.83) gave cross peaks with the H-2 proton (δ 4.50), the amido proton (δ 8.06) and the H-3 protons (δ 2.66, 2.94), respectively.

The structure of **2** was also revealed by analyses of the COSY and ROESY spectra. The sequence of the peptide residue was revealed to be -Gly-Gly-Gly-Asp-Ala-Gly-Thr-OH.

After methanolysis of **3** with sodium hydroxide in methanol, measurement of the FAB-MS spectrum of hydrolysate gave a main peak at *m/z* 848 which was attributable to compound **5** (Fig. 3). By partial acidic hydrolysis of this compound, the peak was shifted to *m/z* 523 which was attributable to compound **6**, and amino acid sequencing of the hydrolysate revealed the presence of prolylleucine (**7**). These observations were explained by the cleavage of compound **5** at both sides of the aspartic acid residue, yielding compounds **6** and **7**. Hence, the sequence of the peptide residue in **3** was revealed to be -Gly-Gly-Gly-Asp-Pro-Leu-OH. After **1** and **2** were processed in the same manner, compound **6** was also detected, and amino acid sequencing of the hydrolysates revealed the presence of threonylthreonine and alanylglycylthreonine, respectively.

Biological Activity

TAN-1511 A, B and C stimulated the proliferation of bone marrow cells from BALB/c mice. The minimum effective concentrations (MEC, concentration giving 30% increase) of the metabolites were 0.313, 0.313 and 1.25 ng/ml, respectively (Fig. 4). Chemically synthesized TAN-1511 A analogue (**4**, Fig. 1) also stimulated the proliferation of bone marrow cells in a manner similar to that of natural TAN-1511 A. Thus, the following experiments were performed using this analogue.

Fig. 2. ROESY study of TAN-1511 A (**1**).

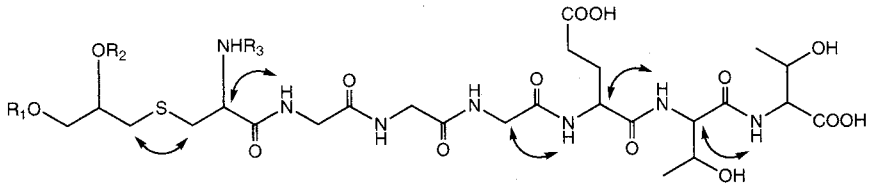
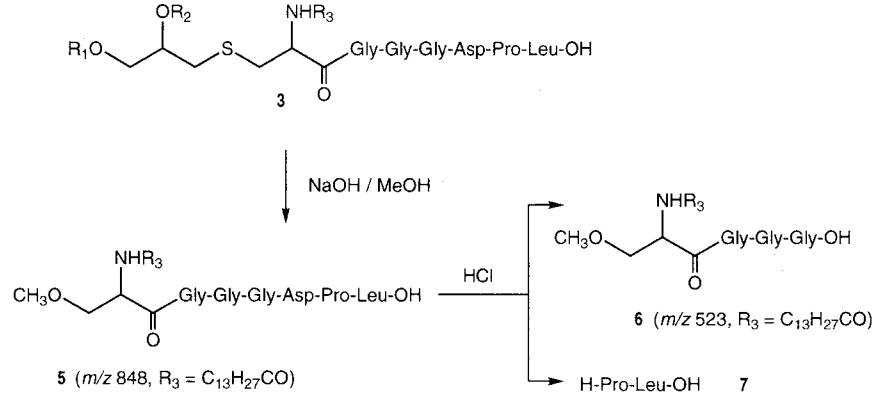


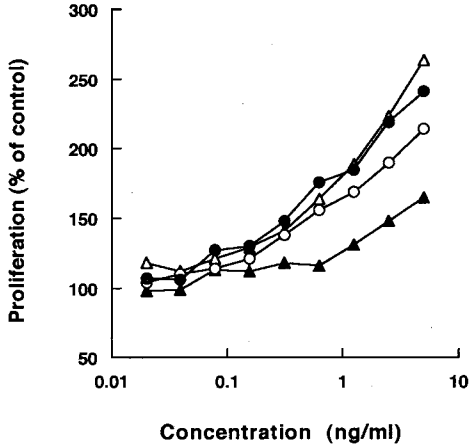
Fig. 3. Decomposition of TAN-1511 C (**3**).



Since CSFs directly promote the proliferation of bone marrow cells, we investigated whether **4** induces the production of G-CSF and GM-CSF in cultured bone marrow cells. When bone marrow cells were cultured with **4**, messages of G-CSF and GM-CSF genes were induced within 4 hours (Fig. 5). The amount of corresponding cytokines in the culture supernatant also significantly increased after 24 hours (Table 5). These results imply that G-CSF and GM-CSF contribute to the proliferation of bone marrow cells stimulated by **4**. In fact, cytofluorometric analysis using anti-mouse Gr-1 antibody revealed that **4** increased the population of Gr-1 positive cells in bone marrow cells *in vitro* (Fig. 6). Compound **4** also induced G-CSF and GM-CSF in

Fig. 4. Effect of TAN-1511 A (**1**), B (**2**), C (**3**) and synthetic TAN-1511 A analogue (**4**) on proliferation of murine bone marrow cells.

TAN-1511 A (○), synthetic TAN-1511 A analogue (●), TAN-1511 B (△) and TAN-1511 C (▲).



The proliferation of bone marrow cells from BALB/c mice was determined by means of the MTT assay.

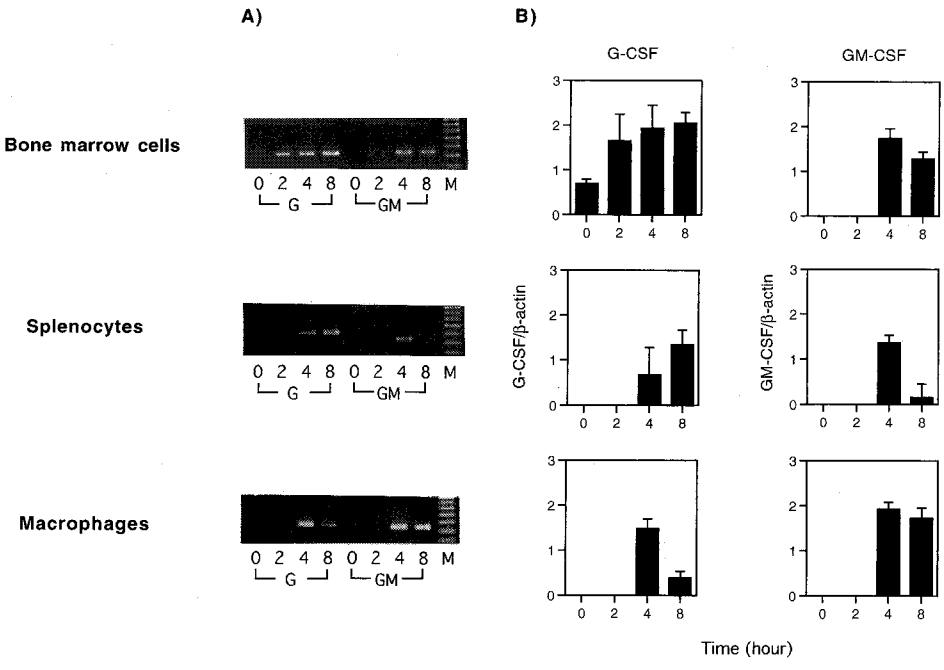
Table 5. Effect of synthetic TAN-1511 A analogue (**4**) on the secretion of G-CSF and GM-CSF from bone marrow cells, splenocytes and peritoneal macrophages prepared from normal mice.

Cell	TAN-1511 A (ng/ml)	CSF (pg/ml)	
		G-CSF	GM-CSF
Bone marrow cells	0	1.6	1.8
	40	2.7	5.6
	200	3.5	6.2
	1000	4.6	8.8
Splenocytes	0	<0.5	2.6
	40	<0.5	12
	200	<0.5	17
	1000	<0.5	30
Peritoneal macrophages	0	<0.5	<0.5
	40	340	10
	200	760	31
	1000	1000	40

Cells (1.6×10^6 cells/ml) from BALB/c mice were incubated with **4** for 24 hours. The amount of each CSF in the culture was determined by means of an ELISA.

Fig. 5. Effect of synthetic TAN-1511 A analogue (**4**) on mRNA expression of G-CSF and GM-CSF in bone marrow cells, splenocytes and peritoneal macrophages.

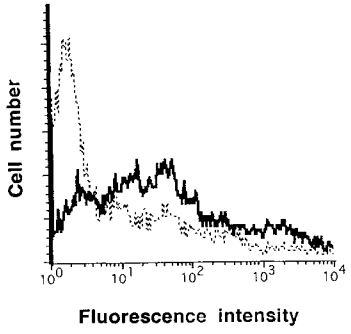
Cells (1.0×10^6 cells/ml) from BALB/c mice were incubated with **4** for various periods. (A) Representative results of agarose gel electrophoresis. (B) The relative expression levels of each cytokines.



The ratio of the densitometric value of the cytokine PCR to β-actin products was used to assess the relative changes in the levels of cytokine mRNA abundance in response to **4**.

Fig. 6. Effect of synthetic TAN-1511 A analogue (**4**) on the Gr-1 positive cell population in cultured bone marrow cells.

Control (---) and synthetic TAN-1511 A analogue (—).



Bone marrow cells from BALB/c mice were incubated with **4** (10 ng/ml) for 5 days. After cultivation, the cells were stained with FITC-conjugated anti-mouse Gr-1 antibody and analyzed using a FACScan.

Table 6. Effect of synthetic TAN-1511 A analogue (**4**) on peripheral leukocyte counts of mice treated with cyclophosphamide (CY).

Treatment		Leukocyte counts ($\times 10^2/\mu l$)	% counts
CY	TAN-1511 A Dose (mg/kg/day)		
−	—	50.0±10.4**	100
+	—	11.3±3.1	22.6
+	0.03	17.5±3.5	35.0
+	0.1	23.3±5.4*	46.6
+	1	61.0±12.0***	122

*$P<0.05$; **$P<0.01$; ***$P<0.001$ *versus* CY (Student's *t*-test).

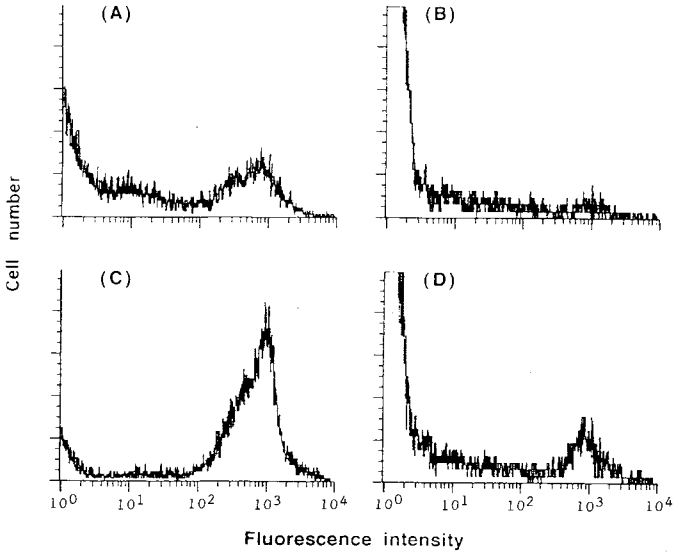
BALB/c mice (4 per group) were intraperitoneally injected with CY at a dose of 200 mg/kg on day 0. Compound **4** was subcutaneously administered daily for five consecutive days (day 1 to 5). On day 6, blood was collected from the mice anesthetized with ether and the leukocytes were counted automatically. Data represent means ±S.D. of values.

cultured splenocytes and peritoneal macrophages (Fig. 5 and Table 5).

Next, we examined the effect of **4** on the number of peripheral leukocytes in mice treated with cyclophosphamide (CY). CY significantly decreased the number of peripheral leukocytes, whereas the following administration of **4** restored it in these mice. The restoration was complete at a dose of 1 mg/kg/administration (Table 6). The increase of peripheral granulocytes was also confirmed as Gr-1 positive cells in bone marrows and spleens from CY-injected mice. As shown in Fig. 7, the number of Gr-1 positive cells in bone marrow cells and splenocytes significantly increased when treated with **4**. These results suggest that **4** augments granulocytopoiesis in the murine leukopenia model. In the *in vitro*

Fig. 7. Effect of synthetic TAN-1511 A analogue (**4**) on the Gr-1 positive cell population in bone marrow cells and splenocytes from leukopenic mice.

Bone marrow cells (A, C) and splenocytes (B, D) from mice treated with CY (A, B) and CY + synthetic TAN-1511 A analogue (C, D).



The treatment schedule was the same as that described in the legend to Table 6, and **4** was administered at a dose of 0.1 mg/kg/day. The cells were stained with FITC-conjugated anti-mouse Gr-1 antibody and analyzed using a FACScan.

Table 7. Effect of synthetic TAN-1511 A analogue (**4**) on the secretion of G-CSF and GM-CSF from bone marrow cells, splenocytes and peritoneal macrophages prepared from leukopenic mice.

Cell	TAN-1511 A (ng/ml)	CSF (pg/ml)	
		G-CSF	GM-CSF
Bone marrow cells	0	<0.5	2.1
	40	77	11
	200	220	32
	1000	250	34
Splenocytes	0	<0.5	3.9
	40	<0.5	14
	200	<0.5	18
	1000	<0.5	30
Peritoneal macrophages	0	<0.5	1.1
	40	22	2.0
	200	170	5.7
	1000	340	9.1

Four days before harvesting the cells, BALB/c mice were intraperitoneally injected with CY at a dose of 200 mg/kg. Cells (1.6×10^6 cells/ml) were incubated with **4** for 24 hours. The amount of each CSF in the culture was determined by means of an ELISA.

experiment, **4** promoted the production of G-CSF in cultured bone marrow cells from normal mice. When the bone marrow cells from the leukopenic mice were cultured for 24 hours with **4**, the amount of G-CSF in the supernatants markedly increased (Table 7). G-CSF

was also significantly induced in cultured peritoneal macrophages from the leukopenic mice, although the amount was lower than that in the culture of peritoneal macrophages from normal mice. These results suggest that G-CSF induced by **4** is the major factor involved in the restoration of granulocyte counts in the murine leukopenia model. Since the amount of GM-CSF also increased significantly in this experiment, the cytokine may also support this granulocytopoiesis to some extent (Table 7).

Discussion

Here we described novel microbial lipopeptides having granulocytopoietic properties. When bone marrow cells from leukopenic mice were incubated with the synthetic TAN-1511 A analogue, a large amount of G-CSF was detected in the culture supernatant. This amount of G-CSF was about 50 times higher than that in the case of normal bone marrow cells. Peritoneal macrophages from the leukopenic mice also maintain sufficient potential to produce G-CSF in the presence of this analogue. These facts suggest that the analogue preferentially activates the production of G-CSF in the bone marrow accessory cells and peripheral macrophages of leukopenic mice. The prophylactic effect of the synthetic analogue was confirmed in an experimental infection model with some gram negative bacteria, where neutrophils were the primary effector cells involved in host resistance (OKONOGI *et al.*, unpublished data). Thus, this compound should be clinically applicable to leukopenia in cancer therapy.

Hematopoietic cytokines cooperatively regulate hematopoiesis *in vivo*. Trials of combined treatments with these cytokines in cancer therapy are increasing and some of them show synergistic effects on hematopoiesis[12,13]. As a result, awareness of multiple cytokine inducers as therapeutic agents has considerably increased[14]. As described here, a synthetic TAN-1511 A analogue induced the production of not only G-CSF but also GM-CSF in leukopenic mice. In addition, messages of M-CSF, interleukin (IL)-1, IL-6 and stem cell factor genes are upregulated by the analogue in bone marrow cells and splenocytes[15]. Thus, it is a multiple cytokine inducer and it should be also applicable to other types of cytopenia, such as thrombocytopenia in cancer therapy. In fact, the synthetic analogue has megakaryocytopoietic activity[15].

The biological properties of microbial lipopeptides and their derivatives have been intensively studied, including for example, mitogenicity to B-lymphocytes and activation of macrophages[16~23]. Their structural similarity to TAN-1511A suggests that some of them also potentiate hematopoiesis. This contention is supported by the recent discovery of a novel lipopeptide WS1279, which is a metabolite of *Streptomyces willmorei* No. 1279, stimulates the proliferation of bone marrow cells from mice and accelerate the recovery of granulocyte counts in mitomycin-induced leukopenic mice[24~26]. Furthermore, we have proven that a synthetic lipopeptide derivative [trisodium (2*R*,6*R*)-2-amino-6,7-bis (hexadecanoyloxy)-4-thiaheptanoyl glycyl glutamyl glutamate] described in the following paper[7] promotes G-CSF dependent granulocytopoiesis in CY-treated leukopenic mice and induces various hematopoietic cytokines, in a manner similar to that of the synthetic TAN-1511 A analogue (data not shown).

Acknowledgments

We thank Drs. H. OKAZAKI, K. KITANO and H. SHIRAFUJI for their continuous interest and encouragement. We also thank Messrs. Y. NAKANO and K. KAWAKAMI for their skillful assistance.

References

1) GABIG, T. G. & B. M. BABIOR: The killing of pathogens by phagocytes. Ann. Rev. Med. 32: 313~326, 1981
2) JOYCE, R. A.: Chemical agents which suppress myelopoiesis: agranulocytosis and leukemia. *In* Handbook of Experimental Pharmacology. *Ed.*, J. W. FISHER, pp. 493~515, Springer-Verlag, Berlin, 1992
3) MARKMAN, M. & M. D. ABELOFF: Management of hematologic and infectious complications of intensive induction therapy for small cell carcinoma of lung. Am. J. Med. 74: 741~746, 1983
4) QUESENBERRY, P. J.: Granulocyte-macrophage growth factors. *In* Handbook of Experimental Pharmacology. *Ed.*, J. W. FISHER, pp. 449~474, Springer-Verlag, Berlin, 1992
5) QUESENBERRY, P. J.; I. K. MCNIECE, H. E. MCGRATH, D. S. TEMELES, G. B. BABER & D. H. DEACOM: Stromal regulation of hematopoiesis. Ann. N. Y. Acad. Sci. 554: 117~124, 1989
6) IINUMA, I.; A. YOKOTA & T. KANAMARU: In preparation
7) HIDA, T.; K. HAYASHI, K. YUKISHIGE, S. TANIDA, N. KAWAMURA & S. HARADA: Synthesis and biological activities of TAN-1511 analogues. J. Antibiotics 48: 589~603, 1995
8) MIWA, H. & M. YAMAMOTO: High performance liquid chromatographic analysis of serum long-chain fatty acids by direct derivatization method. J. Chromatogr. 416: 237~245, 1987
9) NIMURA, N. & T. KINOSHITA: *o*-Phthalaldehyde-*N*-acetyl-L-cysteine as a chiral derivatization reagent for liquid chromatographic optical resolution of amino acid enantiomers and its application to conventional amino acid analysis. J. Chromatogr. 352: 169~177, 1986
10) TAKIZAWA, M.; T. HIDA, S. HARADA & S. TANIDA: Augmentation of host defense mechanisms against tumor by sperabillin polymers, new peptidyl biopolymers, in mice. Int. J. Immunopharmac. 16: 67~74, 1994
11) TADA, H.; O. SHIHO, K. KUROSHIMA, M. KOYAMA & K. TSUKAMOTO: An improved colorimetric assay for interleukin 2. J. Immun. Meth. 93: 157~165, 1986
12) WINTON, E. F.; J. SRINIVASIAH, B. K. KIM, C. D. HILLYER, E. A. STROBERT, J. L. ORKIN, R. B. SWENSON, H. M.

McClure, L. A. Myers & R. Saral: Effect of recombinant human interleukin-6 (rhIL-6) and rhIL-3 on hematopoietic regeneration as demonstrated in a non-human primate chemotherapy model. Blood 84: 65~73, 1994

13) Fay, J. W.; H. Lazarus, R. Herzig, R. Saez, D. A. Stevens, R. H. J. Collins, L. A. Piñeiro, B. W. Cooper, J. DiCesare, M. Campion, J. M. Felser, G. Herzig & S. H. Bernstein: Sequential administration of recombinant human interleukin-3 and granulocyte-macrophage colony-stimulating factor after autologous bone marrow transplantation for malignant lymphoma: a phase I/II multicenter study. Blood 84: 2151~2157, 1994

14) Azuma, I.: Development of the cytokine inducer romurtide: experimental studies and clinical application. Trends Pharmacol. Sci. 13: 425~428, 1992

15) Nishi, K.; T. Horiguchi, F. Kawashima & S. Tanida: In preparation

16) Shimizu, T.; Y. Iwamoto, Y. Yanagihara, M. Kurimura & K. Achiwa: Mitogenic activity and the induction of tumor necrosis factor by lipopeptide analogs of the *N*-terminal part of lipoprotein in the outer membrane of *Escherichia coli*. Biol. Pharm. Bull. 17: 980~982, 1994

17) Kurimura, M.; A. Ochiai & K. Achiwa: Synthesis and mitogenic activity of chiral lipopeptide WS1279 and its derivatives. Chem. Pharm. Bull. 41: 1965~1970, 1993

18) Dong, Z.; X. Qi & I. J. Fidler: Tyrosine phosphorylation of mitogen-activated protein kinases is necessary for activation of murine macrophages by natural and synthetic bacterial products. J. Exp. Med. 177: 1071~1077, 1993

19) Dong, Z.; X. Qi, K. Xie & I. J. Fidler: Protein tyrosine kinase inhibitors decrease induction of nitric oxide synthase activity in lipopolysaccharide-responsive and lipopolysaccharide-nonresponsive murine macrophages. J. Immunol. 151: 2717~2724, 1993

20) Boscá, L.; C. Márquez & C. Martínez-A: B cell triggering by bacterial lipopeptide involves both translocation and activation of the membrane-bound form of protein kinase C. J. Immunol. 147: 1463~1469, 1991

21) Baschang, G.: Muramylpeptides and lipopeptides: studies towards immunostimulants. Tetrahedron 45: 6331~6360, 1989

22) Steffens, U.; W. Bessler & S. Hauschildt: B cell activation by synthetic lipopeptide analogues of bacterial lipoprotein bypassing phosphatidylinositol metabolism and proteinkinase C translocation. Mol. Immunol. 26: 897~904, 1989

23) Bessler, W. G.; M. Cox, A. Lex, B. Suhr, K. H. Wiesmller & G. Jung: Synthetic lipopeptide analogs of bacterial lipoprotein are potent polyclonal activators for murine B lymphocytes. J. Immunol. 135: 1900~1905, 1985

24) Tanaka, M.; Y. Hori, H. Ueda, M. Ezaki, T. Goto, M. Hashimoto & M. Okuhara: WS1279, a novel lipopeptide isolated from *Streptomyces willmorei*: fermentation, isolation and physico-chemical properties. J. Antibiotics 46: 858~860, 1993

25) Tanaka, M.; Y. Hori, F. Sakai, H. Ueda, T. Goto & M. Okuhara: WS1279, a novel lipopeptide isolated from *Streptomyces willmorei*: biological activities. J. Antibiotics 46: 1699~1706, 1993

26) Tsuda, Y.; Y. Okada, M. Tanaka, N. Shigematsu, Y. Hori, T. Goto & M. Hashimoto: Structure and synthesis of an immunoactive lipopeptide, WS1279, of microbial origin. Chem. Pharm. Bull. 39: 607~611, 1991