NOVEL MICROBIAL METABOLITES OF THE PHOSLACTOMYCINS FAMILY INDUCE PRODUCTION OF COLONY-STIMULATING FACTORS BY BONE MARROW STROMAL CELLS

I. TAXONOMY, FERMENTATION AND BIOLOGICAL PROPERTIES

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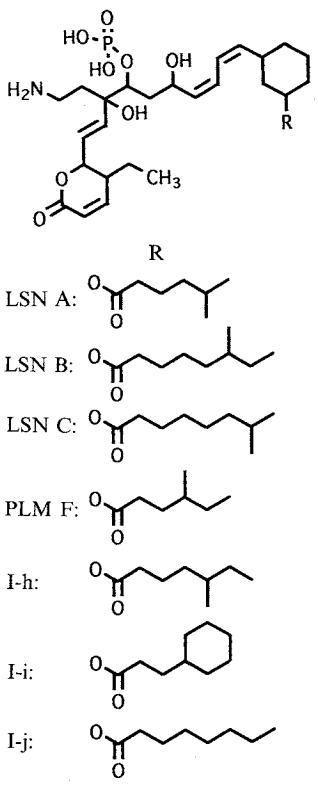
Three metabolites were isolated from the culture broth of an actinomycete strain identified as *Streptomyces platensis* SANK 60191, that induce the production of colony-stimulating factors (CSFs) by stromal cell line KM-102 at ED_{50} concentrations from 40 to 200 ng/ml. The compounds induced quantities of granulocyte CSF (G-CSF) and granulocyte-macrophage CSF (GM-CSF) comparable to those induced by interleukin-1, a strong CSF inducer. These metabolites were called leustroducsins (A, B and C) and were later found to be structurally related to phoslactomycins. This is the first report of CSF inducing activity by members of the phoslactomycin class.

Recent studies have demonstrated the application of colony-stimulating factors (CSFs) in clinical use[1]. These substances have been used to recover the peripheral blood leukocytes in leukopenia patients caused by cancer-chemotherapy, radiotherapy and bone marrow transplantation. When CSFs were administered to patients, restorations of leukocyte counts occurred. However, the *in vivo* role of endogenous CSFs is not precisely understood. In particular, little is known about the regulatory mechanism for CSF production or about the relationship between normal blood cell production and endogenous CSFs.

It is well known that bone marrow stromal cells play an important role in hematopoiesis. Regulation of CSF production by bone marrow stromal cells may be one of the key elements responsible for the control of hematopoiesis *in vivo*[2]. Therefore, substances that affect the regulation of CSF production by stromal cells are of potential interest, so we worked to develop screening methods for CSF inducers.

In the previous paper[3], we described the development of a new screening method for CSF inducers using human bone marrow stromal cell line KM-102. Using this screening method, one strain of actinomycetes, *Streptomyces platensis* SANK 60191, was found to produce novel microbial metabolites that we provisionally named leustroducsins. Structure determination studies revealed that they are congeners of phoslactomycins. In this paper we report the taxonomy of the producing organism, and also describe the fermentation and biological properties of leustroducsins (LSNs), A, B and C (Fig. 1). The isolation, physicochemical properties and structural elucidation of the metabolites are reported in the accompanying paper[4].

Fig. 1. Chemical structures of LSNs and related compounds.



Materials and Methods

Taxonomic Studies

The producing organism, strain SANK 60191, was isolated from a soil sample collected at Ito City, Shizuoka Prefecture, Japan. Methods and media described by the International Streptomyces Project (ISP)[5] and WAKSMAN[6] were used to determine the characteristics of the culture and the physiological properties of the producing organism. For morphological observation, a light microscope and an S-510 scanning electron microscope (Hitachi Co., Ltd.) were used. Cell wall and whole-cell hydrolysates were analyzed by the methods of BECKER *et al.*[7] and HASEGAWA *et al.*[8].

Fermentation

A loopful amount from a culture of strain SANK 60191 was inoculated into a 500-ml Erlenmeyer flask that contained 100 ml of medium consisting of soluble starch 3%, pressed yeast 1%, soybean meal 0.7%, fish meal 0.5%, corn steep liquor (C.S.L.) 0.2%, meat extracts 0.1%, $CaCO_3$ 0.1%. The medium was adjusted to pH 7.0 before sterilization. The inoculated flasks were incubated on a rotary shaker (220 rpm) at 28°C for 72 hours. Then a 150-ml aliquot of this seed culture was added to a 30-liter fermenter containing 15 liters of the medium. Fermentation was carried out at 28°C for 72 hours with an air-flow rate of 15 liters/minute and an agitation rate of 150 rpm. The contents of LSNs were determined by HPLC assay. Packed cell volume was determined after centrifugation of 5 g of the culture broth at 3,000 rpm for 10 minutes.

Cell Lines, Screening Method and Bioassays for CSFs

HeLa, WI-38 and WI-38 VA13 were purchased from Dainippon Pharmaceutical Co. Ltd. Primary human endothelial cells (HUVEC) and primary human skin fibroblasts (FB) were purchased from Toyobo Co. Ltd. Other cell lines, screening methods and bioassays for CSFs have been described in a accompanying paper[3].

Cytotoxicity Assay

Cytotoxicity assay was carried out against various kinds of cells. In all cases, cells were cultured in ISCOVE's medium (Boehringer Mannheim) containing 5% fetal bovine serum (FBS) and test sample solutions were added to the wells and incubated at 37°C for 24 hours with 5% CO_2 under high humidity condition. The cytotoxicity of the test samples was determined by crystal violet staining of viable cells[9].

Neutralization of CSF Activities by Specific Antibodies

Neutralization of CSF activities was carried out using antibodies against granulocyte CSF (G-CSF: polyclonal rabbit anti-human G-CSF, code BL-GCP, Genzyme; U.S.A.) and granulocyte-macrophage CSF (GM-CSF: monoclonal mouse anti-human GM-CSF, code ZM-213, Genzyme). The neutralizing antibodies were diluted to give a final concentration of 10 μg/ml. These antibodies are highly specific for each CSF and show no effects on cell growth induced other cytokines (TF-1 cell growth supported by human interleukin-3 (IL-3) or human leukemia inhibitory factor NFS-60 cell growth supported by murine IL-3).

ELISA for CSFs

The production of CSFs in cultures was also determined by commercial ELISA kits for G-CSF (Oncogene science; U.S.A.) and GM-CSF (Genzyme).

Cytokines and Chemicals

Leptomycin-B was a generous gift of Dr. H. GUNJI, Fermentation Research Laboratories, Sankyo Co. Ltd. Human recombinant GM-CSF and IL-1β were purchased from Genzyme (Boston, U.S.A.). Human recombinant G-CSF was used (Sankyo, Japan).

Results and Discussion

Taxonomy of Strain SANK 60191

Strain SANK 60191 grew well on both organic and synthetic media. It formed spiral spore chains in monopodial branching on the aerial mycelium with 10 to 50 or more spores per chain (Plate 1). Most spores were oval and $0.5\sim0.6\times0.6\sim1.3$ mm in size with smooth surface (Plate 2). The cultural characteristics on various agar media at 28°C for 14 days are presented in Table 1. Physiological properties of the strain are summarized in Table 2. The vegetative mycelium was pale yellowish brown to yellowish gray. Melanoid pigment or other soluble pigments were not produced. Temperature range for growth was from $9\sim35$°C with the optimum in a range of $20\sim26$°C. Utilization of carbon sources are shown in Table 3.

Detection of LL-diaminopimelic acid in the whole-cell hydrolysates of the culture indicated that this strain had type I cell walls. Based on the taxonomic properties described above, the strain SANK 60191 is considered to be a member of the genus *Streptomyces*. A direct comparison of strain SANK 60191 and the type strain of *Streptomyces platensis* ATCC 13865 showed that the only differences on them were noted for cultural characteristics as shown in Table 1. However, those differences noted above are not enough to conclude these two strains as different species, and the strain is identified as *Streptomyces platensis* SANK 60191. The strain SANK 60191 has been deposited in the National Institute of Bioscience and Human-Technology (formerly the Fermentation Research Institute), Agency of Industrial Science and Technology, Ibaraki Prefecture, Japan, with the accession number of FERM BP-3288.

Fermentation of Strain SANK 60191

The time course for fermentations of LSN B in a 30-liter jar fermentor is shown in Fig. 2. Maximum production of 1.0 μg/ml was achieved at 72 hours after inoculation. The time-course of production of LSN A and C was similar to that of LSN B (data not shown).

Plate 1. Light micrograph of strain SANK 60191 (on potato extract-carrot extract agar, 28°C, 7 days).

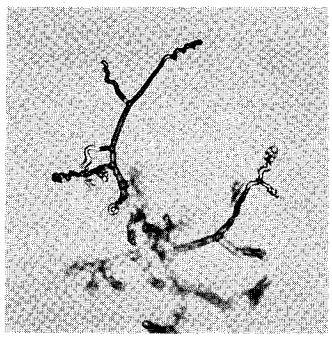


Plate 2. Scanning electron micrograph of strain SANK 60191 (on water agar, 28°C, 7 days).

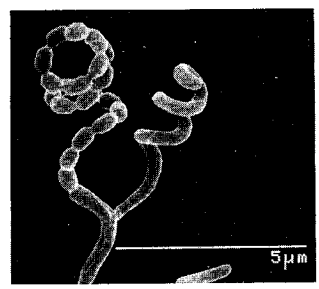


Table 1. Cultural characteristics of strains SANK 60191 and *S. platensis* ATCC 13865.

Medium		SANK 60191	*S. platensis* ATCC 13865
Yeast extract - malt extract agar (ISP 2)	G:	Abundant, flat, pale yellowish orange	Abundant, flat, grayish red brown
	AM:	Abundant, velvety, light brownish white	Abundant, velvety, pale pink
	R:	Pale yellowish brown	Dull reddish orange
	SP:	None	Dull reddish orange
Oatmeal agar (ISP 3)	G:	Good, flat, pale yellowish orange	Good, flat, pale reddish brown
	AM:	Good, velvety, dark brownish gray	Abundant, velvety, pale pink
	R:	Yellowish brown	Dull reddish orange
	SP:	None	Pale brown
Inorganic salts - starch agar (ISP 4)	G:	Good, flat, pale yellowish orange	Abundant, flat, pale pink
	AM:	Good, velvety, brownish white	Abundant, velvety, grownish gray
	R:	Brownish gray	Pale yellowish orange to grayish red brown
	SP:	None	Pale brown
Glycerol - asparagine agar (ISP 5)	G:	Abundant, flat, pale yellowish orange	Abundant, flat, light brown
	AM:	Moderate, velvety, brownish gray	Abundant, velvety, pink
	R:	Pale yellowish brown	Dull orange
	SP:	None	Yellowish brown
Peptone - yeast extract - iron agar (ISP 6)	G:	Good, flat, pale yellowish orange	Abundant, raised, pale yellowish brown
	AM:	Moderate, velvety, white	Poor, white
	R:	Pale yellowish brown	Pale yellowish brown
	SP:	None	Yellowish brown
Tyrosine agar (ISP 7)	G:	Abundant, flat, pale yellowish orange	Abundant, flat, brown purple
	AM:	Good, velvety, light brownish gray	Abundant, velvety, light brownish gray
	R:	Pale yellowish brown	Brown
	SP:	None	Light brown
Sucrose - nitrate agar	G:	Good, flat, pale yellowish orange	Abundant, flat, yellowish brown
	AM:	Good, velvety, light brownish gray	Good, velvety, pale pink
	R:	Pale brown	Grayish yellow brown to dull red
	SP:	None	Pale yellowish brown
Glucose - asparagine agar	G:	Good, flat, pale yellowish orange	Abundant, flat, dull red
	AM:	Moderate, velvety, white	Abundant, velvety, pinkish gray
	R:	Pale brown	Reddish brown
	SP:	None	Pale brown
Nutrient agar (Difco)	G:	Good, flat, pale yellowish orange	Good, flat, yellowish brown
	AM:	Moderate, velvety, white	Moderate, velvety, light brownish gray
	R:	Pale yellowish brown	Pale yellowish brown
	SP:	None	Pale yellowish brown
Potato extract - carrot extract agar	G:	Moderate, flat, yellowish gray	Good, flat, reddish brown
	AM:	Moderate, velvety, brownish gray	Good, velvety, light brownish gray
	R:	Light brownish gray	Light brown
	SP:	None	Light brownish gray
Water agar	G:	Moderate, flat, yellowish gray	Moderate, flat, reddish brown
	AM:	Moderate, velvety, brownish gray	Moderate, velvety, light brown
	R:	Light brownish gray	Dark reddish brown
	SP:	None	None

G; Growth, AM; aerial mycelium, R; reverse, SP; soluble pigment.

Biological Properties of LSNs

CSF Inducing Activities

Media conditioned by KM-102 cells in the presence of LSNs at various concentrations were assayed both for G- and GM-CSF. Fig. 3 shows that growth of factor dependent cell lines was significantly

Table 2. Physiological properties of strains SANK 60191 and *S. platensis* ATCC 13865.

	SANK 60191	*S. platensis* ATCC 13865
Hydrolysis of starch	Positive	Positive
Liquefaction of gelatin	Negative	Negative
Reduction of nitrate	Negative	Negative
Coagulation of milk	Negative	Negative
Peptonization of milk	Negative	Negative
Temperature range for growth (medium 1)	9～35°C	9～36.5°C
Optimum temperature range for growth (medium 1)	20～26°C	26～32°C
Sodium chloride torelance (medium 1)	10%	7%
Decomposition of casein	Negative	Negative
Decomposition of tyrosine	Positive	Positive
Decomposition of xanthine	Positive	Positive
Production of melanoid pigment		
(medium 2)	Negative	Negative
(medium 3)	Negative	Negative
(medium 4)	Negative	Negative

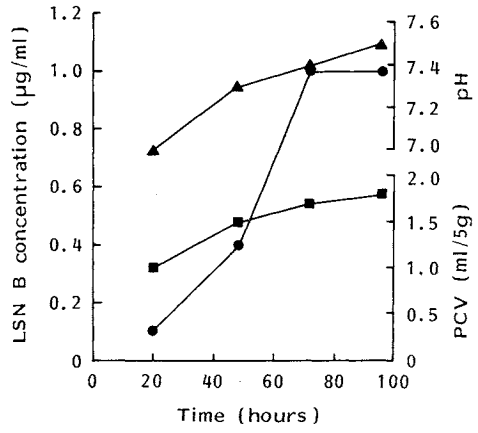
Medium 1: Yeast extract-malt extract agar (ISP 2).
Medium 2: Tryptone-yeast extract broth (ISP 1).
Medium 3: Peptone-yeast extract-iron agar (ISP 6).
Medium 4: Tyrosine agar (ISP 7).

Table 3. Carbon utilization of strains SANK 60191 and *S. platensis* ATCC 13865.

	SANK 60191	*S. platensis* ATCC 13865
D-Glucose	+	+
L-Arabinose	−	−
D-Xylose	−	−
Inositol	+	+
D-Mannitol	+	+
D-Fructose	+	±
L-Rhamnose	−	−
Sucrose	+	+
Raffinose	+	+
Control	−	−

Fig. 2. Fermentation of strain SANK 60191.

● LSN B concentration, ■ pH, ▲ packed cell volume (PCV).



stimulated by conditioned media of KM-102 treated with LSNs. The maximum level of tritiated thymidine incorporation was almost the same as that stimulated by conditioned media treated with interleukin-1 (100 units/ml). ED_{50} values of LSN A, B and C were 197, 40 and 53 ng/ml (G-CSF production) and 175, 48 and 47 ng/ml (GM-CSF production), respectively. To confirm the specificity of the bioassay, we tried to neutralize the CSF activities in KM-102 conditioned media with specific antibodies. The proliferative responses of TF-1 cells and NFS-60 cells were inhibited (by about 90 to 95%) with neutralizing anti-GM-CSF antibody and anti-G-CSF antibody, respectively, thus suggesting that the major active substances in KM-102 conditioned media induced by LSNs are G-CSF and GM-CSF.

Since the strain SANK 60191 also produces 4 structurally related compounds[4] (phoslactomycin-F, I-h, I-i and I-j in Fig. 1) previously reported as antifungal antibiotics, we assessed their CSF inducing

Fig. 3. Colony stimulating factor induction by leustroducsins.

(A) G-CSF, (B) GM-CSF, ○ LSN A, ● LSN B, □ LSN C, △ IL-1, ▲ none.

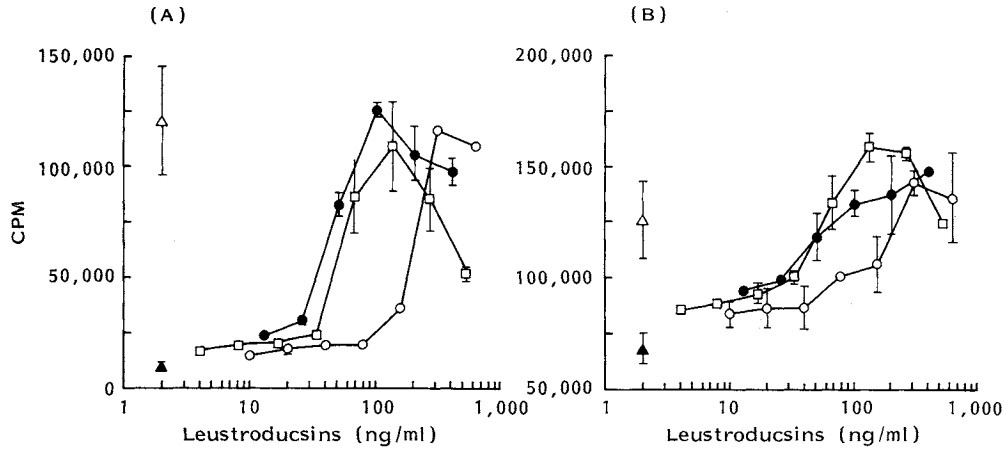


Table 4. Production of colony-stimulating factors by KM-102 cells cultured with leustroducsin B (ELISA).

Leustroducsin B (ng/ml)	G-CSF (pg/ml)	GM-CSF (pg/ml)
200	3,707±138	2,197± 7
100	7,680±542	1,892±25
50	91±91	ND
25	ND	ND
0	ND	ND

ND: Note detected.

Table 5. Cytotoxicity of LSN B against various kinds of cells.

Cell	Cytotoxicity (IC_{50}, ng/ml)
HeLa	59± 10
WI-38	694± 95
WI-38 VA13	193± 13
HUVEC	984± 60
FB	1,706±196
KM-102	93± 5

activities. All 4 compounds induced production of both G- and GM-CSF by stromal cell line KM-102 with ED_{50} concentrations between 100 and 200 ng/ml. The ED_{50} concentration of these compounds correlated positively with hydrophobicity as assessed by reversed phase HPLC[4]. LSN B and C, which are the most hydrophobic of the 7 compounds, produced by SANK 60191 were also the most potent inducers.

Increased production both of G- and of GM-CSF by LSN B was confirmed by ELISA (Table 4). The results obtained by ELISA were comparable to those obtained by bioassays. The concentrations of both CSFs induced by LSNs are extremely high and are comparable to those induced by IL-1 (10 units/ml IL-1β induced about 600 pg/ml of GM-CSF and 1,000 pg/ml of G-CSF in our assay system). All the above results indicate that LSNs, at low concentrations, enhance the production of biologically active and immunologically responsive colony-stimulating factors by human bone marrow stromal cell line KM-102.

Other Biological Activities

Because phoslactomycins and phospholin, which are structurally related to LSNs, possess antifungal activity and/or cytotoxic activity, we examined antimicrobial and cytotoxic activities of LSNs. LSNs (100 μg/ml) showed no effect against Gram-positive and Gram-negative bacteria, but showed significant antifungal activity against *Trichophyton mentagrophtes* (MIC=0.8 μg/ml). As shown in Table 5, LSN B

showed cytotoxic activity against a series of cell lines. The IC_{50} values against primary cell cultures were higher than those against HeLa or oncogene transformed variant cells. LSN B also possesses cytotoxic activity against KM-102 cells. The IC_{50} value is approximately only two fold higher than the ED_{50} values for CSFs induction, suggesting that the compound may have limited potential as a therapeutic agent. The above results indicate that LSNs possess antifungal and cytotoxic activities similar to those of phoslactomycins, as expected from their structural relation.

LSNs and Structurally Related Substances

LSNs contain an α,β-unsaturated δ-lactone, an amino group, a phosphate ester and a cyclohexyl residue. (Fig. 1) Recently, many compounds structurally related to LSNs (phoslactomycins[10,11], phosphazomycins[12], phospholine[13,14], 2-pyranone derivatives[15] and MA-5000[16]) have been reported as antifungal and/or antitumor antibiotics. The chemical structures of all these compounds differs only at the substituent bound to the cyclohexane ring. However, to our knowledge, activity as cytokine inducers has never been reported for these compounds.

In addition to the above compounds, many compounds that contain an α,β-unsaturated δ-lactone structure have been reported as antitumor antibiotics. These compounds can be assigned to 4 groups according to their structural features (Table 6). We assessed CSF inducing activities of one of these compounds, leptomycin B, which contains an α,β-unsaturated δ-lactone and a conjugated diene but not a phosphate ester, an amino group or a cyclohexyl residue[17]. Interestingly, leptomycin B possesses cytotoxic activity but shows no inducing activities. Therefore, the structures containing an α,β-unsaturated δ-lactone and a conjugated diene are insufficient for induction of CSF production, and additional or other moieties are required for this activity.

The target molecules of leustroducsins' activity remain unknown. In previous studies we showed that LSNs are different from a series of compounds that activate protein kinase C. Other signal transduction modulators (*e.g.*, protein phosphatase inhibitors, A-kinase activators and calcium ionophores) showed only weak inducing activities. Therefore, LSNs do not seem to affect the signal transduction pathway of

Table 6. Compounds containing an α,β-unsaturated δ-lactone structure.

Group	Name of compounds	Structural feature	Activity	Ref
Phoslactomycin	Leustroducsin A~C Phoslactomycin A~F Phosphazomycin A~D 2-Pyranone Derivatives I-a~j Phospholin MA-5000 I~IV	α,β-Unsaturated δ-Lactone Phosphate ester Amino Conjugated diene Cyclohexan ring	CSF inducing Antifungal Antitumor	10~16
Leptomycin	Leptomycin A, B Kazusamycin A, B Anguinomycin A, B	α,β-Unsaturated δ-Lactone Conjugated diene	Antifungal Antitumor Cell cycle arrest	17~22
Fostriecin	Fostriecin PD 113270~1	α,β-Unsaturated δ-Lactone Phosphate ester Conjugated triene	Antifungal Antitumor	23, 24
Sultriecin	Sultriecin	α,β-Unsaturated δ-Lactone Sulfate ester Conjugated triene	Antifungal Antitmor	25

these substances. Further studies are required to reveal the molecular mechanism underlying the induction of CSF by LSNs.

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