Lymphostin (LK6-A), a Novel Immunosuppressant from *Streptomyces* sp. KY11783: Taxonomy of the Producing Organism, Fermentation, Isolation and Biological Activities

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In the course of screening for inhibitors of the lymphocyte kinase, Lck ($p56^{lck}$), aiming at novel immunosuppressants, we isolated a novel alkaloid, lymphostin (LK6-A), from the culture broth of *Streptomyces* sp. KY11783. Lymphostin was produced in a fermentation medium supplemented with a highly porous polymer resin, which prevented the degradation of this compound in the culture broth. Lymphostin inhibited the kinase activity of Lck with an IC_{50} value of 0.05 μM, and exhibited potent inhibitory activity against the mixed lymphocyte reaction (MLR) with an IC_{50} value of 0.009 μM.

Immunosuppressants such as cyclosporin A (CsA) and FK506 have been clinically effective in suppressing the rejection of organ transplants. Furthermore, such immunosuppressants have also proved to be effective in the treatment of autoimmune diseases such as rheumatoid arthritis[1,2], uveitis[3,4] and type II diabetes[5,6]. CsA and FK506 form complexes with immunophilins such as cyclophilin and FK506 binding protein (FKBP), respectively, and the drug-immunophilin complexes suppress various T cell-related immune responses, such as the production of interleukin 2 (IL-2), through inhibition of the Ca^{++}/calmodulin-dependent phosphatase, calcineurin[7]. The mechanism of action, however, seems to be related to their side effects, *i.e.* nephrotoxicity, neurotoxicity and diabetogenicity, which limit their clinical usage. Therefore immunosuppressants with different mechanisms of action from that of these drugs are needed.

The lymphocyte kinase, Lck ($p56^{lck}$), is a Src-family tyrosine kinase expressed exclusively in lymphoid cells, and predominantly in thymocytes and peripheral T cells, where it plays important roles in T-cell development and activation[8]. Lck binds to the cytoplasmic domains of the CD4 and CD8 T-cell co-receptors[9], and following T-cell receptor stimulation, phosphorylates T-cell receptor ζ-chains, which then recruit ZAP-70 kinase to promote T-cell activation[10]. A mutant of the human Jurkat T cell line lacking functional Lck fails to respond to anti-T cell receptor antibodies[11], and Lck knockout mice are defective in thymocyte development and do not exhibit the T cell-mediated effectors including allo-skin graft rejection[12~14]. Accordingly, it is considered that Lck inhibitors would be potential immunosuppressants with novel mechanisms of action.

In the course of screening for Lck inhibitors from microorganisms, we found that *Streptomyces* sp. KY11783 produces a novel alkaloid, lymphostin (originally called LK6-A[15]), with potent inhibitory activity against Lck. This paper describes the taxonomy of the producing organism, fermentation, isolation and biological activities. The structure determination of lymphostin is reported in the accompanying paper[16].

Materials and Methods

Microorganism

The lymphostin-producing strain KY11783 was isolated from a soil sample collected in Gunma prefecture, Japan. This strain has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukubashi, Ibaraki, Japan, as *Streptomyces* sp. KY11783 with the accession number FERM BP-5202.

Taxonomical Characterization

Cultural and physiological characteristics of strain KY11783 were determined by the methods of the International *Streptomyces* Project[17] (ISP). Color codes were

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assigned to the substrate and aerial mass pigments according to the Color Harmony Manual[18]. Morphology and spore surface ornamentation of the strain were determined by light and scanning electron microscopy (HITACHI S-570). The temperature ranges for growth of the strain was determined after submerged cultivation for 14 days using ISP No. 5 medium. Analysis of diaminopimelic acid was performed on the hydrolysate of aerial mycelia grown on the solid ISP 4 medium as described previously[19].

Fermentation

A loopful of vegetative mycelia of strain KY11783 grown on a HICKEY-TRESNER's agar slant was inoculated into 10 ml of seed medium composed of 1% glucose, 1% soluble starch, 0.5% Bacto-tryptone, 0.3% beef extract, 0.5% yeast extract and 0.05% $Mg_3(PO_4)_2 \cdot 8H_2O$ (pH 7.2 prior to sterlization) in a test tube. The seed culture was incubated at 28°C with vigorous shaking for 3 days, and then a 6-ml portion of the culture was transferred to a 2-liter Erlenmeyer flask containing 300 ml of the seed medium, and the flask was cultured at 28°C on a rotary shaker for 3 days. The second seed culture (0.9 liter) was inoculated into a 30-liter jar fermenter containing 18 liters of medium composed of 4% soluble starch, 1% soybean meal, 0.5% corn steep liquor, 0.5% dry yeast, 0.5% KH_2PO_4, 0.001% $ZnSO_4 \cdot 7H_2O$, 0.0001% $CoCl_2 \cdot 6H_2O$, 0.0001% $NiSO_4$, 0.05% $Mg_3(PO_4)_2 \cdot 8H_2O$ and 10% (v/v) highly porous polymer resin, Diaion HP-20 (pH 7.0 prior to sterlization). The fermentation was carried out at 28°C with aeration at 18 liters of air per minute and agitation at 150 rpm for 6 days.

Culture growth was evaluated by centrifuging the fermentation broth in 10-ml graduated conical tubes at $1200 \times g$ for 10 minutes. The packed cell volumn (PCV) was recorded as a % of the total broth volume. The production of lymphostin was analysed by HPLC. For this measurement, the culture broth (2 ml) was extracted with an equal volume of ethyl acetate. After the ethyl acetate extract had been concentrated to dryness, the residue was dissolved in DMSO and then subjected to HPLC analysis.

Analysis of Lymphostin by HPLC

HPLC was performed on a column of CAPCELL PAK C_{18} SG120 (4.6 × 150 mm; Shiseido Co., Ltd.) at 25°C with an eluate of 30% MeCN containing 5 mM ammonium acetate. The flow rate was 1 ml per minute, and UV absorption of the effluent was monitored at 256 nm. The retention time of lymphostin was 7.0 minutes.

Enzyme Assay

Lck was partially purified from bovine thymus tissue by sequential chromatography on columns of DEAE-cellulose, heparin-agarose and butyl-agarose, according to the method of CUSHMAN *et al.*[20]. The activity of Lck was assayed using a peptide substrate $(Tyr\text{-}Ala\text{-}Glu)_7$[21] (suggested by Dr. HIDEYUKI SAYA) and [γ-^{32}P] ATP in the presence of various concentrations of the test compounds. The peptide (0.5 mg/ml) was phosphorylated in a reaction mixture comprising 12 mM MOPS (pH 7.5), 12 mM $MgCl_2$, 60 μM Na_3VO_4, partially purified Lck (156 μg/ml), 50 μM [γ-^{32}P] ATP (1000 ~ 2000 cpm/pmol) and 1% DMSO, as a carrier for the compounds, in a final volume of 25 μl. After 60-minute preincubation at 30°C without ATP, the reaction was started by the addition of [γ-^{32}P] ATP at 30°C and stopped by the addition of 25 μl of 10% trichloroacetic acid after 15 minutes. The acid-precipitable materials were collected on a nitrocellulose membrane filter and washed with 10% trichloroacetic acid. The radioactivity on the filter was measured in a toluene scintillation fluid using a scintillation counter.

Mixed Lymphocyte Reaction (MLR)

MLRs were performed as described previously by WEBB *et al.*[22]. Lymph node responder cells (1.5×10^5 cells) from B10. BR mice (Nihon SLC), mitomycin C-treated spleen stimulator cells (5×10^5 cells) from AKR mice (Nihon SLC) and diluted test compound solutions in RPMI 1640 medium were combined in the wells of a 96-well microtiter plate. The cells were incubated at 37°C for 72 hours under a humidified atmosphere of 5% CO_2. The culture was pulsed with 1 μCi per well of [^{3}H] thymidine 18 hours before harvesting onto fiber filter-mats, followed by counting with a scintillation counter.

Antiproliferative Activity

NFS-60 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and G-CSF at 37°C under a humidified atmosphere of 5% CO_2. The cells were spread onto a 96-well microtiter plate in the presence of various concentrations of the test compounds and then incubated for 24 hours. The culture was pulsed with 1 μCi per well of [^{3}H] thymidine 4 hours before harvesting onto fiber filter-mats, followed by counting with a scintillation counter.

Results

Characterization of the Producing Organism

Strain KY11783 grew well or moderately on seven agar media, but not on sucrose-nitrate agar medium as shown in Table 1. The color of the aerial mycelia of strain KY11783 was white to gray on the agar media. Strain KY11783 produced a brown or light brown soluble pigment on ISP No. 2, No. 3, No. 4, No. 5 and nutrient media after 14 days (Table 1). The aerial mycelia were moderately short with simple branches and formed flexous spore chains of 10 or more. The long chain of spores formed pseudosporangia (Fig. 1). The spores were short rod of 0.5 to 0.7 μm by 0.7 to 1.0 μm and smooth surface. No fragmentation of substrate mycelia was observed, and sclerotia, flagellated spores were not formed in cultures. The physiological characteristics of strain KY11783 are shown in Table 2. Analysis of cell hydrolysates of the strain revealed that the cell walls contained LL-diaminopimelic acid. The predominant menaquinone type was MK-9 (H_6), and there was a significant amount of MK-9 (H_8). These taxonomic observations indicate that strain KY11783 belongs to the genus *Streptomyces*.

Fermentation

Strain KY11783 was cultured in a 30-liter jar fermenter containing the fermentation medium described under Materials and Methods. Fig. 2 shows a typical time course for the production of lymphostin, along with the pH of the medium and the packed cell volume (PCV). The lymphostin production started at 24 hours and reached a maximum at about 49 hours. The pH of the medium fell to 6.6 and then began to rise gradually to 7.2. The cell growth continued to increase and reached 38% at 138 hours. The fermentation medium was supplemented with a highly porous polymer resin, Diaion HP-20, because examination in shake flasks indicated the addition of Diaion HP-20 resin to the production medium resulted in a significant increase in the accumulation of lymphostin. Lymphostin accumulated on the Diaion HP-20 resin, and was only present in the culture supernatant and mycelia in extremely small amounts.

Isolation

Lymphostin was isolated from 25 liters of culture broth by the following procedure. The filtered cake containing Diaion HP-20 was extracted with ethyl acetate twice. The extracts were concentrated to dryness and then subjected to column chromatography on silica gel (7 × 40 cm) with stepwise elution with toluene - ethyl acetate - isopropyl alcohol 3 : 6 : 0 (1.6 liters), 3 : 6 : 1 (1.6 liters), 3 : 6 : 2 (4.8 liters) and 3 : 6 : 3 (1.6 liters) as solvents system. The active fractions containing lymphostin eluted with toluene - ethyl acetate - isopropyl alcohol (3 : 6 : 2) were purified by HPLC on CAPCELL PAK C_{18} SG120 (30 × 250 mm; Shiseido Co., Ltd.) with 30% MeCN containing 5 mM ammonium acetate to give lymphostin as an orange solid (100.2 mg).

Physico-chemical Properties

The physico-chemical properties of lymphostin are summarized in Table 3. Lymphostin shows Rf values of 0.31 in toluene - EtOAc - MeOH (5 : 10 : 1) and 0.43 in $CHCl_3$ - MeOH (15 : 1) on Merck silica gel TLC plates. Lymphostin is soluble in DMSO and in DMSO containing 1% AcOH at a concentration of 2 mg/ml, slightly soluble in $CHCl_3$, acetone, toluene, MeCN and EtOAc, but insoluble in H_2O. Lymphostin is unstable in MeOH and in DMSO containing 2% ammonia water but stable in DMSO and in DMSO containing 1% AcOH at room temperature. The structure of lymphostin is shown in Fig. 3. The details of the structural determination are given in the accompanying paper.

Table 1. Cultural characteristics of strain KY11783.

Medium	Amount of growth	Color of:		Soluble pigment
		Aerial mycelium	Substrate mycelium	
Yeast extract - malt extract agar (ISP No. 2)	Abundant	Gray (f)	Dark brown (4pn)	Brown
Oatmeal agar (ISP No. 3)	Moderate	Gray (f)	Rose beige (4gc)	Brown
Inorganic salt - starch agar (ISP No. 4)	Abundant	Silver gray (3fe)	Dark brown (4pn)	Brown
Glycerol - asparagine agar (ISP No. 5)	Abundant	Silver gray (3fe)	Light tan (3gc)	Light brown
Tyrosine agar (ISP No. 7)	Abundant	White (a)	Light tan (3gc)	None
Sucrose - nitrate agar	Poor	Oxford gray (l)	White (a)	None
Glucose - asparagine agar	Abundant	White (a)	Dark brown (5nl)	None
Nutrient agar	Moderate	White (a)	Light brown (3lg)	Brown

Fig. 1. Scanning electron micrograph of strain KY11783.

Bar represents 5.0 μm.

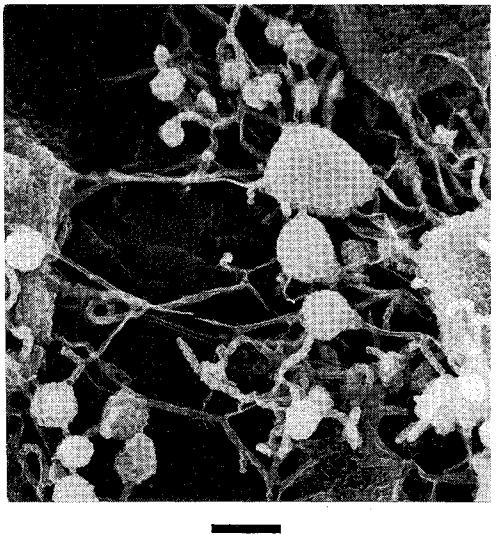


Table 2. Physiological properties of strain KY11783.

Characteristics	KY11783
Temperature for growth	10°C~40°C
Optimum temperature	28°C~30°C
Liquefaction of gelatin	Negative
Hydrolysis of starch	Positive
Coagulation of milk	Negative
Peptonization of milk	Negative
Melanin pigment production	
on peptone-yeast extract-iron agar	Positive
on tyrosine agar	Negative
Utilization of	
D-Glucose	Positive
L-Arabinose	Negative
D-Xylose	Negative
D-Fructose	Weakly positive
L-Rhamnose	Negative
Sucrose	Negative
Raffinose	Negative
D-Mannitole	Negative
Inositol	Negative

Biological Activities

Lymphostin inhibited the protein-tyrosine kinase activity of Lck in a concentration-dependent manner (Fig. 4). Under the conditions given under Materials and Methods, the IC_{50} value for enzyme inhibition was 0.05 μM, which was almost the same as that of staurosporine, a potent protein kinase inhibitor (IC_{50}, 0.1 μM).

The immunosuppressive activity of lymphostin was assessed using the mixed lymphocyte reaction (MLR), which represents T cell activation followed by IL-2 dependent T cell proliferation, and is considered to be an *in vitro* model of allograft rejection. Lymphostin inhibited MLR in a dose-dependent manner (Fig. 5), the IC_{50} value of lymphostin being 0.009 μM, while that of CsA was 0.05 μM. On the other hand, the IC_{50} values for the antiproliferative activities of lymphostin and CsA

Fig. 2. Time course of lymphostin production in a 30-liter jar fermenter.

The production of lymphostin (●), pH of the culture broth (■), and packed cell volume (▲) are presented.

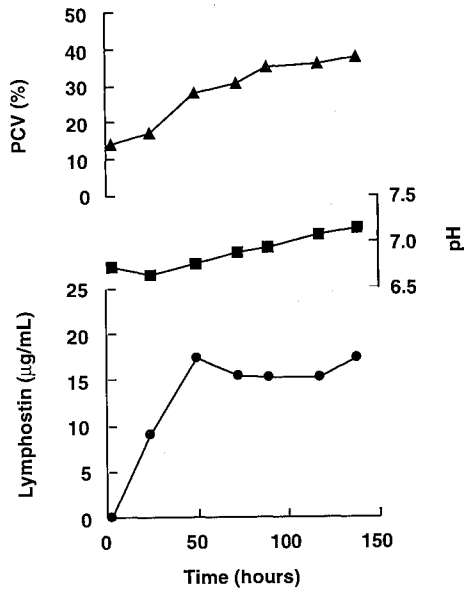


Table 3. Physico-chemical properties of lymphostin.

Appearance	Orange powder
Rf value	0.31 ($Si60F_{254}$, toluene-EtOAc-MeOH 5:10:1)
	0.43 ($Si60F_{254}$, $CHCl_3$-MeOH 15:1)
Solubility	
Soluble	DMSO, 1% AcOH/DMSO
Slight soluble	$CHCl_3$, acetone, toluene, MeCN, EtOAc
Insoluble	H_2O
Stability	Unstable in MeOH, 2% ammonia water/DMSO
	Stable in DMSO, 1% AcOH/DMSO

Fig. 3. Structure of lymphostin.

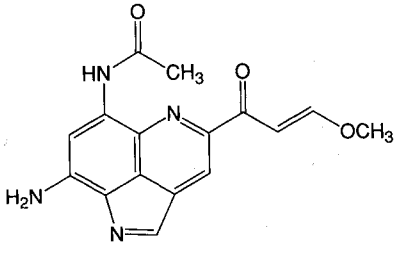


Fig. 4. Inhibition of Lck activity by lymphostin.

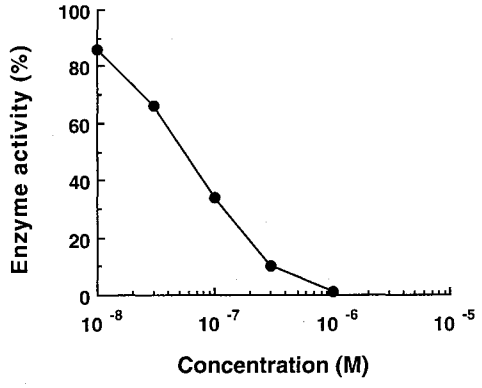
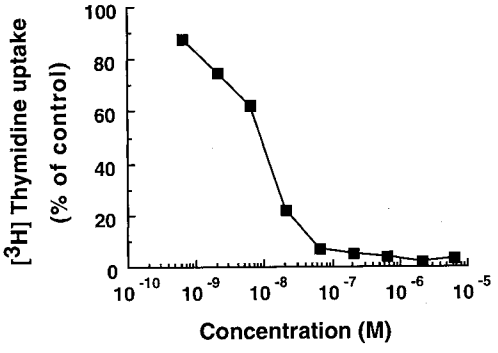


Fig. 5. Effect of lymphostin on the mixed lymphocyte reaction.



against NFS-60, a murine myeloid leukemia cell line, were 0.16 μM and 0.19 μM, respectively (Table 4).

Lymphostin showed no antimicrobial activity at a concentration of 83 μg/ml against *Bacillus subtilis* No. 10707, *Staphylococcus aureus* subsp. *aureus* ATCC6538P, *Enterococcus hirae* ATCC10541, *Escherichia coli* ATCC26, *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC10031, *Pseudomonus aeruginosa* BMH No. 1, *Proteus vulgaris* ATCC6897, *Shigella sonnei* ATCC9290, *Salmonella choleraesuis* subsp. *choleraesuis* ATCC9992, or *Candida albicans* ATCC10231.

Discussion

We have isolated a novel alkaloid, lymphostin, possessing the pyrrolo[4,3,2-*de*]quinoline skeleton from *Streptomyces* sp. KY11783 as an inhibitor of Lck protein-tyrosine kinase. Lymphostin was absorbed on the highly porous polymer resin, Diaion HP-20, in the culture. In fermentation studies, the addition of Diaion HP-20 resin was effective for the production of lymphostin, presumably because degradation of the compound was prevented by absorption on the resin.

Table 4. Effects of lymphostin and CsA on the mixed lymphocyte reaction (MLR) and the proliferation of NFS-60 cells.

Compound	IC_{50}, μM	
	MLR	NFS-60
Lymphostin	0.009	0.16
CsA	0.05	0.19

Lymphostin showed potent inhibitory activity against Lck. Although potent inhibitors of Lck, such as 7,8-dihydroisoquinoline derivatives[23] and non-amine based analogues of lavendustin A[24], have already been reported, their intracellular activities have not been described. Pyrazolopyrimidine derivatives were recently reported to be potent inhibitors of Lck and Fyn[25]. However, the inhibition of MLR is significantly less potent than that of kinase activity.

Lymphostin exhibited more potent inhibitory activity against MLR than the clinically useful immunosuppressant, CsA. Although lymphostin showed antiproliferative activity against NFS-60 cells, it inhibited MLR at a lower dose. It is expected that lymphostin may be a candidate for immunosuppressant with a different mechanism of action from that of CsA and FK506.

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