MS-444, a New Inhibitor of Myosin Light Chain Kinase from *Micromonospora* sp. KY7123

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A novel compound MS-444 was isolated from the culture broth of a bacterial strain KY7123. The strain was identified as *Micromonospora* sp. from its morphological and cultural characteristics. The compound inhibited the activity of purified smooth muscle myosin light chain kinase with an IC_{50} value of 10 μM. The production, isolation, physico-chemical properties and biological activities of MS-444 were described in this paper.

It is widely accepted that myosin light chain kinase (MLCK) is a regulatory enzyme in smooth muscle contraction[1,2]. Contractile stimuli, such as neurotransmitters and hormones, increase cytoplasmic Ca^{2+} concentration and activate calmodulin, a Ca^{2+} binding protein, in smooth muscle cells. The Ca^{2+}-bound form of calmodulin activates MLCK, which catalyzes transfer of γ-phosphate of ATP to Ser-19 of 20-kDa myosin light chain. Phosphorylated myosin interacts with actin to generate contractile force. Since the contractile property of smooth muscle cells is a major determinant of vascular tone and diameter of bronchial tubes, MLCK inhibitors would be potential vasodilators and bronchodilators.

During the course of our screening work, we found that *Micromonospora* sp. KY7123 produced an MLCK inhibitor, designated as MS-444. In this paper, we will describe characterization of the producing strain and production, isolation, physico-chemical properties and biological activities of MS-444. The structural determination studies will be in the following paper[3].

Materials and Methods

Characterization of the Producing Strain

Strain KY7123 was isolated from a soil sample collected in Okinawa, Japan. Except for carbon utilization tests, the methods of SHIRLING and GOTTLIEB[4] were employed for cultural and physiological characterization of the producing strain. The basal medium for carbon utilization tests contained $MgSO_4 \cdot 7H_2O$ 0.005%, NaCl 0.005%, $(NH_4)_2SO_4$ 0.1%, $CaCO_3$ 0.1%, K_2HPO_4 0.005%, $FeSO_4 \cdot 7H_2O$ 0.0001%, $MnCl_2 \cdot 4H_2O$ 0.0001%, $ZnSO_4 \cdot 7H_2O$ 0.0001% and agar 2% (pH 7.2). The cultural and physiological characterization, except for a gelatin liquefaction test, were done after incubation for 2 weeks at 28°C. Liquefaction of gelatin was determined after incubation for 2 weeks at 20°C. The temperature range for growth of the strain was determined after submerged cultivation for 1 week. For analysis of amino acid components in whole-cell hydrolysate of strain KY7123, the method of KAWAMOTO *et al.*[5] was employed.

Fermentation

A 50-ml culture tube containing 10 ml of a seed medium composed of glucose 1.0%, soluble starch 1.0%, beef extract 0.3%, yeast extract 0.5%, Bactotryptone (Difco) 0.5%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, and $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05% (pH 7.1 before sterilization) was inoculated with the mycelia of *Micromonospora* sp. KY7123 grown on a HICKEY-TRESNER's agar slant. The inoculated tube was incubated for 5 days at 28°C with vigorous shaking. A 5-ml portion of the culture was transferred into a 300-ml Erlenmeyer flask containing 50 ml of the seed medium, and the flask was incubated for 2 days on a rotary shaker. A 5-ml portion of the second seed culture was transferred into a 300-ml Erlenmeyer flask containing 50 ml of a fermentation medium composed of glucose 2.5%, corn steep liquor 1.5%, soluble vegetable protein 1.0%, cotton seed oil 0.5%, $CoCl_2 \cdot 6H_2O$ 10 μg/ml and $Mg_3(PO_4)_2 \cdot 7H_2O$ 0.05% (pH 7.0 before sterilization). Fermentation was carried out at 28°C on a rotary shaker. The growth of the microorganism was monitored during fermentation by the measurement of packed cell volume (PCV).

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Table 1. Cultural characteristics of strain KY7123.

Medium	Growth	Color	Soluble pigment
Yeast extract - malt extract agar (ISP No. 2)	Moderate	Dark brown (4pn)	Dark brown
Oatmeal agar (ISP No. 3)	Moderate	Orange rust (4pe)	Pale brown
Inorganic salts-starch agar (ISP No. 4)	Good	Sepia brown (3pn)	Pale brown
Glycerol - asparagine agar (ISP No. 5)	Moderate	Bright melon yellow (3ia)	None
Tyrosine agar (ISP No. 7)	Moderate	Bright melon yellow (3ia)	None
Sucrose - nitrate agar	Poor	Cork tan (4ie)	Pale brown
Glucose - asparagine agar	Moderate	Dark luggage tan (4pg)	Pale brown
Nutrient agar	Poor	Tile red (5ne)	Pale brown

Color names and numbers used in this table are based on Color Harmony Manual (Container Corporation of America).

MS-444 was detected on a silica gel TLC plate developed with $CHCl_3$ - acetone (95 : 5).

Enzyme Assay

The activity of MLCK was measured as described previously[6]. The culture supernatant or a methanol solution of partially purified materials (10 μl) was added to the reaction mixture containing, in a final volume of 0.25 ml, Tris-HCl 25 mM (pH 7.5), $MgCl_2$ 4 mM, $CaCl_2$ 0.2 mM, calmodulin 2.6 nM, peptide substrate for MLCK (KKRPQRATSNVFS-NH_2) 24 μM, MLCK 1.5 nM, and ATP 400 μM, and incubated for 30 minutes at 28°C. The reaction mixture was directly analyzed by HPLC as described[7].

Materials

MLCK was isolated from chicken gizzard smooth muscle as described[8]. Peptide substrate for MLCK was purchased from Peninsula Lab. Inc., U.S.A. All other reagents were of HPLC or analytical grade.

Table 2. Physiological characteristics of strain KY7123.

Temperature for growth	9 ~ 40° C
Optimum temperature	25 ~ 30° C
Liquefaction of gelatin	+
Hydrolysis of starch	+
Coagulation of milk	+
Peptonization of milk	+
Formation of Melanin on:	
Peptone-yeast extract-iron agar (ISP No. 6)	−
Tyrosine agar (ISP No. 7)	−
Utilization of:	
D-Glucose	+
L-Arabinose	+
D-Xylose	+
D-Fructose	−
Sucrose	+
Inositol	−
L-Rhamnose	−
Raffinose	+
D-Mannitol	−

+, Positive; − , negative.

Results

Characterization of the Producing Strain

Vegetative hyphae of strain KY7123 were well developed, branched, and did not fragmented into bacillary or coccoid elements. No aerial mycelium was formed. Single spherical spores were born on the substrate mycelium. The spores, with smooth or warty surface, were non-motile. The formation of sporangia or synnemata was not observed. Cell wall hydrolysate of the strain contained glycine and *meso*-diaminopimeric acid. Based on taxonomic characteristics described above, strain KY7123 is considered to belong to the genus *Micromonospora*. The cultural characteristics of the producing strain are summerized in Table 1. The color of substrate mycelium was orange to brown. Brownish pigment was produced on some agar media. The physiological characteristics of KY7123 are listed in Table 2. The strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as *Micromonospora* sp. KY7123 with the accession No. FERM-BP 3623.

Production of MS-444

KY7123 was cultured in 300-ml Erlenmeyer flasks containing the fermentation medium described in Materials and Methods. The time course of MS-444 production is shown in Fig. 1. Active materials were produced in both culture supernatant and mycelia. However, MS-444 was accumulated mainly in mycelia. We did not purify active materials recovered in the supernatant because of instability of the activity. The amount of MS-444 in mycelia started to increase 4 days after inoculation and reached a maximum in 6 days.

Isolation and Purification of MS-444

A purification procedure for MS-444 is outlined in Fig. 2. Culture broth of KY7123 (15 liters) was centrifuged to obtain mycelia, and active materials were extracted from the mycelia with methanol. The methanol solution was concentrated *in vacuo*, and extracted with ethyl acetate. The ethyl acetate layer was concentrated *in vacuo* to yield a brown oil. MS-444 was purified by repeated silica gel column chromatographies with different elution solvents: chloroform - methanol (99 : 1) once and chloroform - acetone (95 : 5) twice. MS-444 was crystallized in the solution collected after the last chromatography to obtain yellow needles (87.5 mg).

Physico-chemical Properties

The physico-chemical properties of MS-444 are summarized in Table 3. A UV absorption spectrum and the positive color reaction with ferric chloride indicated the presence of phenolic group(s). The structure of MS-444 (Fig. 3) were determined to be 5,8-dihydroxy-3-methyl-4(9*H*)-naphtho[2,3-c]furanone from the physico-chemical properties and the spectral data, which will be described in the following paper[3].

Fig. 1. Time course of the production of MLCK inhibitors by *Micromonospora* sp. KY7123.

Inhibition % of MLCK activity in the presence of culture supernatant (○) and cell extract (●), pH of the culture broth (■), and packed cell volume (▲) were indicated.

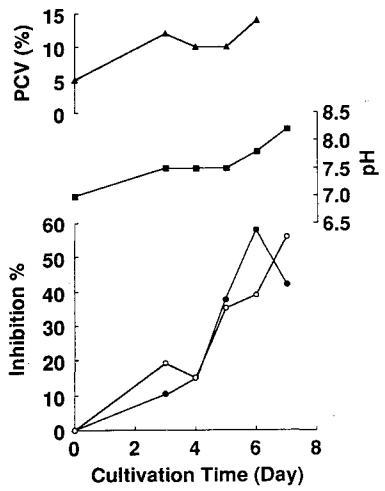


Fig. 2. Purification procedure for MS-444.

Culture broth (15 liters)
| centrifuged
Mycelia
| extracted with MeOH (5.5 liters)
| centrifuged
Supernatant
| concentrated in vacuo
| extracted with EtOAc
EtOAc extract
| Silica gel column (Wakogel C-200, 1000 ml)
| eluted with $CHCl_3$-MeOH (99:1) 3000 ml
Eluate
| concentrated in vacuo
| Silica gel column (Wakogel C-200, 500 ml)
| eluted with $CHCl_3$-acetone (95:5) 1200 ml
Active fractions (elution volume: 530-1200 ml)
| concentrated in vacuo
| Silica gel column (Wakogel C-200, 200 ml)
| eluted with $CHCl_3$-acetone (95:5) 350 ml
Active fractions (elution volume: 270-330 ml)
| crystallized in $CHCl_3$-acetone (95:5)
MS-444 (87.5 mg)

Table 3. Physico-chemical properties of MS-444.

Appearance		yellow needles
Color Reaction (positive)		50%H_2SO_4, I_2, $FeCl_3$, anisaldehyde
Rf Value		0.27 ($Si60F_{254}$, $CHCl_3$-acetone = 95:5)
		0.41 ($Si60F_{254}$, EtOAc-hexane = 1:1)
		0.36 ($RP18F_{254}s$, 60% acetone/H_2O)
Solubility		
	Soluble	acetone, AcOEt, THF, DMSO, DMF, pyridine, dioxane
	Slightly soluble	$CHCl_3$, MeOH, n-BuOH, MeCN, AcOH, hexane, benzene
	Insoluble	CCl_4, H_2O, 0.1N HCl

Fig. 3. Structure of MS-444.

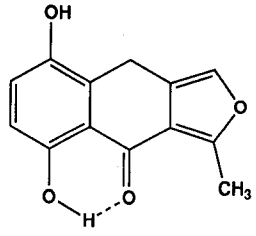
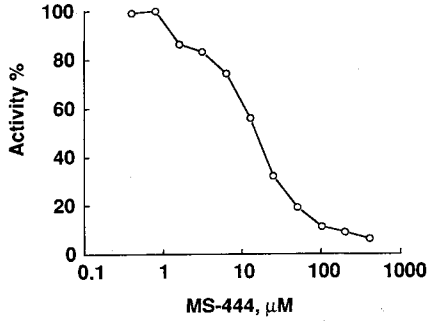


Fig. 4. Effect of MS-444 on MLCK activity.



Data are means of three separate experiments.

Biological Activities

MS-444 inhibited Ca^{2+} and calmodulin-dependent activity of smooth muscle MLCK in a concentration-dependent manner (Fig. 4). The concentration of MS-444 needed to inhibit the enzyme activity by 50% (IC_{50}) was 10 μM.

MS-444 at 100 μg/ml showed no antimicrobial activity against *Staphylococcus aureus* subsp. *aureus* ATCC 6538P, *Enterococcus hirae* ATCC 10541, *Bacillus subtilis* No. 10707, *Escherichia coli* ATCC 26, *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 10031, *Proteus vulgaris* ATCC 6897, *Shigella sonnei* ATCC 9290, *Salmonella choleraesuis* subsp. *choleraesuis* ATCC 9992, *Pseudomonas aeruginosa* BMH No. 1, and *Candida albicans* ATCC 10231.

Discussion

We have isolated a new compound, MS-444, from the culture broth of *Micromonospora* sp. KY7123. There are no reports of isolation of the compounds possessing the 4(9*H*)-naphtho[2,3-c]furanone moiety from natural sources††.

MS-444 inhibited the activity of purified MLCK with an IC_{50} value of 10 μM. The potency of MS-444 was moderate compared with other MLCK inhibitors reported by others and us[6,7,9~13]. We are now working on pharmacological studies of MS-444 to see if it will be a new lead for pharmaceutical drugs.

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†† During revision of our paper, Koyama *et al.* published the isolation of 5-hydroxy-3-methyl-naphtho[2,3-c]furan-4(9*H*)-one from a plant, *Aloe ferox*[14].