MS-681a, b, c and d, New Inhibitors of Myosin Light Chain Kinase from *Myrothecium* sp. KY6568

I. Characterization of Producing Strain and Production, Isolation and Biological Activities

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Novel compounds MS-681a, b, c and d were isolated from the culture broth of a fungal strain KY6568. The strain was identified as *Myrothecium* sp. from its morphological characteristics. MS-681a, b, c and d inhibited the activity of purified smooth muscle myosin light chain kinase with IC_{50} values of 0.11, 0.29, 0.095 and 0.26 μM, respectively. Cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase and protein kinase C were not inhibited at 100 μM by MS-681 compounds.

Myosin light chain kinase (MLCK) is highly expressed in smooth muscle cells and regulates smooth muscle contraction[1,2]. 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. The expression of MLCK is also detected in non-muscle cells[3,4]. Although the physiological roles of MLCK in non-muscle cells are not well investigated, it is suggested that MLCK activity is necessary for the secretion of transmitter from neurons, the catecholamine release from chromaffin cells and the virus release from infected host cells[5~7]. Therefore, MLCK inhibitors would be good tools to investigate the role of MLCK in non-muscle cells and would be potential pharmaceuticals modifying biological processes regulated by MLCK.

During the course of our screening work, we found that *Myrothecium* sp. KY6568 produced MLCK inhibitors, designated as MS-681a, b, c and d. In this paper, we describe characterization of the producing strain and production, isolation and biological activities of MS-681 compounds. The structural determination studies will appear in the following paper[8].

Materials and Methods

Materials

MLCK and myosin light chain (MLC) from chicken gizzard smooth muscle and calmodulin from bovine brain were isolated as described[9]. Cyclic GMP-dependent protein kinase from porcine lung and protein kinase C from rat brain were prepared as described[10]. Peptide substrate for MLCK (KKRPQRATSNVFS-NH_2) was purchased from Peninsula Lab. Inc., U.S.A. The catalytic subunit of cyclic AMP-dependent protein kinase (from bovine heart), the mixture of alamethicines, and spermidine were obtained from Sigma Chemical Co. All other reagents were of HPLC or analytical grade.

Isolation of the Producing Strain

The fungal strain KY6568 was isolated from a soil sample collected in Kanagawa, Japan by soil dilution technique.

Fermentation

A 50-ml culture tube containing 10 ml of a seed medium composed of V8 vegetable juice (Campbell) 20% and dextrin 3.0% (pH 6.5 before sterilization) was inoculated with a loopful of spores of *Myrothecium* sp. KY6568 grown on an agar slant consisting of malt extract (DIFCO) 2%, glucose 2%, peptone (Kyokuto) 0.1% and agar (Ina Syokuhin Kogyo) 2% (pH 6.5 before sterilization). The inoculated tube was incubated for 4 days at 25°C. A 5-ml portion of the culture was transferred into a 300-ml Erlenmeyer flask containing

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50 ml of the seed medium, and the flask was incubated for 2 days at 25°C 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 sucrose 5.0%, Pharmamedia (Traders Protein Southern Cotton Oil Co.) 1.5%, beef extract (Kyokuto) 0.5%, corn steep liquor (Nihon Shokuhin Kako) 0.5%, and $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05% (pH 6.0 before sterilization). Fermentation was carried out at 25°C on a rotary shaker.

The fermentation was monitored as follows. Two portions of culture broth (5 ml × 2) were sampled each at different times. One portion was used for the measurement of pH and microorganism growth, and the other was for the measurement of the activity. After measuring pH of the broth, culture supernatant and mycelia were separated by centrifugation at 3000 rpm. The supernatant was discarded, and the mycelia was boiled with 20 ml of 3.5% perchloric acid for 15 minutes to extract nucleic acid. The absorbance at 260 nm of the crude nucleic acid extract was used for monitoring the microorganism growth. For measurement of activity, culture supernatant and mycelia were separated as above and the mycelia was extracted with 5 ml of methanol. The methanol extract of the mycelia and the supernatant were added to the assay mixture.

Enzyme Assay

The activity of MLCK was measured as described previously[11]. The culture supernatant or 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 24 μM and MLCK 1.5 nM, and incubated for 10 minutes at 28°C. Then the reaction was started by the addition of ATP (final concentration is 400 μM) and the mixture was incubated for 30 minutes. The reaction was terminated by the addition of 10% acetic acid and the reaction mixture was directly analyzed by HPLC as described[11].

Another substrate, MLC was used for the measurement of MLCK activity with purified MS-681 compounds as described[12]. The assay conditions for other enzymes were described previously[9].

Results

Taxonomy of the Producing Strain

On 2% malt extract agar media, the colonies of the fungal strain KY6568 are about 38 mm in diameter after culturing at 25°C for 1 week. The color of the surface of the colony is pale beige. The reverse color is reddish brown at the center and cream at the marginal area. Colonies on potato-glucose agar media are about 58 mm in diameter after culturing at 25°C for 1 week. The colony surface is gray and the reverse is cream. The growth range is 11~30°C, and the optimal temperature for growth is near 25°C.

On 2% malt extract agar media, sporodochia occurring, cupulate, stalked, synnema-like, formed on densely compacted conidiophores arising from a stroma, and bearing a mass of slimy, black green conidia. Conidiophores hyaline, smooth, repeatedly branching with several branches arising form each node, the ultimate branches being the phialides. Phialides compacted in a dense parallel layer, 7.5~17 × 2~3.5 μm, cylindrical with tapering tips and usually undifferentiated collarettes. Conidia enteroblastic, 1-celled, hyaline, pale green or olivaceous brown, fusiform to ellipsoid, 4.5~6 × 2.5~4 μm. The teleomorph has not been observed.

From the characteristics mentioned above, the fungal strain (KY6568) was identified as *Myrothecium* sp.[13] The fungus has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-4634.

Production of MS-681 Compounds

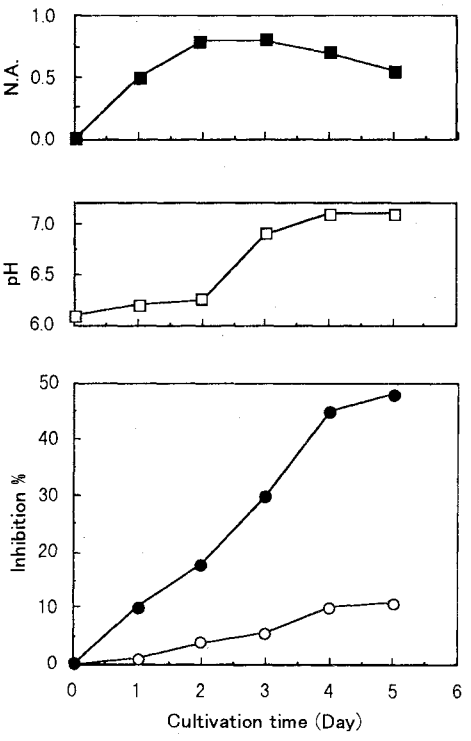
KY6568 was cultured in 300-ml Erlenmeyer flasks containing the fermentation medium described in Materials and Methods. The time course of MLCK inhibitor production is shown in Fig. 1. Active materials were produced mainly in mycelia.

Isolation and Purification of MS-681 Compounds

A purification procedure for MS-681 compounds is outlined in Fig. 2. Culture broth of KY6568 (15 liters) was filtered to obtain the mycelia, and active materials were extracted from the mycelia with methanol (15 liters). The methanol solution was diluted with water (15 liters) and applied to a Diaion HP-20 resin column (2 liters). The column was washed with 50% methanol/water (6 liters), and eluted with methanol (6 liters) and then with methanol containing 0.1% acetic acid (6 liters). The eluate was concentrated *in vacuo* to yield a brown oil (55 g). The oil was applied to an Amberlite XAD-8 resin column (1 liter), and the column was washed with 50% methanol/water containing 0.1% ammonia water (3 liters) and eluted with methanol containing 2% ammonia water (3 liters). The eluate was concentrated *in vacuo* to

yield a brown oil (30 g). The oil was then applied to an Amberlite XAD-4 resin column (300 ml), and the column was washed with methanol (900 ml) and eluted with methanol containing 0.1% acetic acid (900 ml). The elute was concentrated *in vacuo* to yield a yellow oil (21 g). The oil was applied to a reverse phase silica gel column (YMC ODS-AM #300, 200 ml). The column was eluted with 70% methanol/water containing 0.1% trifluoroacetic acid (TFA), and the fractions containing crude MS-681 compounds were collected, concentrated *in vacuo*, adjusted at pH 12 with *ca.* 10 M ammonia water and extracted with $CHCl_3$. The organic solvent layer was concentrated to yield a yellow solid, consisting of the crude MS-681 components (1.2 g). Subsequent preparative reverse phase HPLC of this material was performed on a YMC-R-ODS-5B column (ODS, 30 × 250 mm, YMC Co., Ltd.) using 72% methanol/water containing 0.1% TFA as isocratic mobile phase at the flow rate of 20 ml/minutes. The fractions containing MS-681a, b, c and d, respectively were collected separately, concentrated *in vacuo*, adjusted at pH 12 with *ca.* 10 M ammonia water and extracted with $CHCl_3$. The organic solvent layers were washed with diluted ammonia water adjusted at pH 12, dried over anhydrous magnesium sulfate, and concentrated to dryness. Again, the same preparative HPLC separation was done to purify each compound further. The purity of the compounds were checked by

Fig. 1. Time course of the production of MLCK inhibitors by *Myrothecium* sp. KY6568.



Inhibition % of MLCK activity by culture supernatant (○) and cell extract (●), pH of the culture broth (□), and crude nucleic acid content (N.A.: ■, absorbance at 260 nm) are indicated.

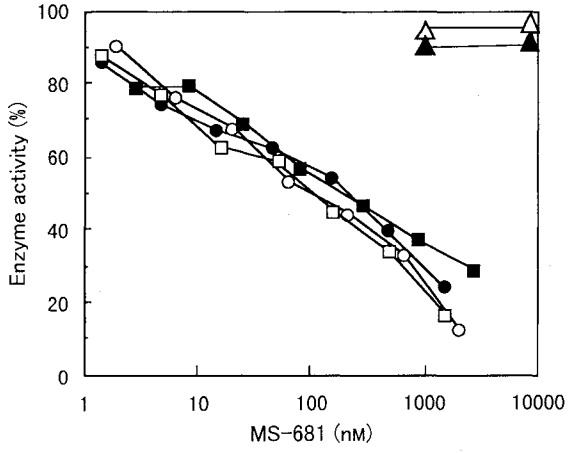
Fig. 2. Purification procedure for MS-681 compounds.

Culture broth (15 liters)
| filtered
Mycelia
| extracted with methanol
| Diaion HP-20 resin column
| eluted with methanol and methanol containing 0.1% acetic acid
Brown oil (55 g).
| Amberlite XAD-8 resin column
| eluted with methanol containing 2% ammonia water
Brown oil (30 g)
| Amberlite XAD-4 resin column
| eluted with methanol containing 0.1% acetic acid
Yellow oil (21 g)
| ODS silica gel column
| eluted with 70% methanol containing 0.1% TFA
Fraction containing crude MS-681s
| concentrated in vacuo
| adjusted at pH 12 with diluted ammonia water
| extracted with $CHCl_3$
Crude MS-681compounds (1.2 g)
| preparative reverse phase HPLC
| eluted with 72% methanol containing 0.1% TFA
Active fraction
| concentrated in vacuo
| adjusted at pH 12 with diluted ammonia water
| extracted with $CHCl_3$
Active fraction
| concentrated in vacuo
| adjusted at pH 12 with diluted ammonia water
| extracted with $CHCl_3$
|
MS-681a (21 mg), b (85 mg), c (22 mg) and d (27 mg)

Table 1. Effects of MS-681 compounds on various protein kinase activities.

Enzyme	IC_{50} (μM)			
	MS-681 a	b	c	d
MLCK				
Peptide as substrate	0.11	0.29	0.095	0.26
MLC as substrate	0.63	1.30	0.58	1.10
cAMP-dependent protein kinase	>100	>100	>100	>100
cGMP-dependent protein kinase	>100	>100	>100	>100
Protein kinase C	>100	>100	>100	>100

Fig. 3. Effects of MS-681 compounds, alamethicine, and spermidine on MLCK activity.



Effect of MS-681a (○) MS-681b (●) MS-681c (□) and MS-681d (■), alamethicines (△), and spermidine (▲) on MLCK activity are indicated. Data are means of three experiments. Enzyme activity in the presence of compound is shown as % of vehicle treated enzyme activity.

Table 2. The antimicrobial activities of the crude mixture of MS-681 compounds.

The microorganisms	MIC (μg/ml)
Candida albicans ATCC 10231	>100
Enterococcus faecium ATCC 10541	25
Pseudomonas aeruginosa BMH No. 1	>100
Staphylococcus aureus subsp. *aureus* ATCC6538P	25
Escherichia coli ATCC26	>100
Bacillus subtilis No. 10707	3.1
Proteus vulgaris ATCC 6897	>100
Shigella sonnei ATCC 9290	>100
Salmonella typhosa ATCC 9992	>100
Klebsiella pneumoniae subsp. *pneumoniae* ATCC 10031	>100

analytical HPLC (YMC-ODS-AM 4.6×250 mm, 70% MeOH/0.1% TFA/H_2O). MS-681a (21 mg), b (85 mg), c (22 mg) and d (27 mg) were obtained as pale yellow solids.

Biological Activities

MS-681 compounds inhibited the activity of chicken gizzard MLCK in concentration-dependent manners (Fig. 3). Although 1 μM MS-681 compounds inhibited MLCK activity to 20~40% of the vehicle treated MLCK activity, alamethicines, structural analogues of the peptide moiety of MS-681 compounds, and spermidine[8] had no effect on MLCK activity even at 10 μM (Fig. 3). The concentrations of MS-681a, b, c and d needed to inhibit the several protein kinase activities by 50% (IC_{50}) were summarized in Table 1.

The antibacterial spectrum of crude MS-681 (a mixture of MS-681 a, b, c, and d before HPLC separation) are listed in Table 2. MS-681 compounds have weak antibacterial activities against Gram-positive bacteria.

Discussion

We have isolated novel compounds, MS-681a, b, c and d from the culture broth of *Myrothecium* sp. KY6568. MS-681 compounds are bimolecular compounds consisted of a peptide moiety and a polyamine moiety[8]. The peptide moiety has acetylated amino-terminus, carboxyl-terminus bonded to an amino alcohol equivalent and α,α-dialkylated amino acids such as α-aminoisobutyric acid and isovaline. Although these structural properties are also seen in a class of peptide antibiotics generally known as peptaibols, there are no reports on peptaibols that have polyamine at their carboxyl-terminal amino alcohol.

Peptaibols are fungal metabolites as are MS-681 compounds. However, the producing strain of MS-681 compounds is *Myrothecium* sp., whereas that of well characterized peptaibols are *Trichoderma* sp.[14] and its

related strains[15]. There are no papers that reported the production of peptaibol-like compounds by *Myrothecium* sp. as far as we know.

MS-681a, b, c and d inhibited the activity of MLCK with IC_{50} values of 0.11, 0.29, 0.095 and 0.26 μM, respectively. The inhibitory potency of MS-681 compounds were relatively high compared with other MLCK inhibitors reported by others and us[11,12,16~21]. MS-681 compounds also inhibited MLCK activity measured with MLC as substrate, although the IC_{50} values obtained with MLC are higher than those obtained with the peptide substrate. MS-681 compounds are selective MLCK inhibitors among several protein kinases that we investigated. A mixture of alamethicines, members of peptaibol, and spermidine had no effect on MLCK activity even at 10 μM, suggesting that both peptide moiety and polyamine moiety are required in one molecule to inhibit MLCK. We are now studying the precise mechanism of MS-681 compounds to inhibit MLCK.

Peptaibols are known to perturb cellular membrane permeability by forming voltage-gated ion channels[22]. It is suggested that this membrane-modifying property of peptaibol causes many biological activities such as weak antibiotic activity against Gram-positive bacteria and stimulation of catecholamine-secretion from chromaffin cells[23,24]. MS-681 compounds also showed weak antibiotic activity against Gram-positive bacteria, but other biological activities of MS-681 compounds are now under investigation.

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