**Note**

**Isolation of Myosin Light Chain Kinase Inhibitors from Microorganisms: Dehydroaltenusin, Altenusin, Atrovenetinone, and Cyclooctasulfur**

Satoshi Nakanishi,* Shinichiro Toki, Yutaka Saitoh, Eiji Tsukuda,** Kiyotaka Kawahara, Katsuhiko Ando, and Yuzuru Matsuda

*Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 3–6–6 Asahimachi, Machida-shi, Tokyo 194, Japan*

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Dehydroaltenusin, cyclooctasulfur, atrovenetinone, and altenusin were isolated from the culture broths of *Penicillium verruculosum* IAM-13756, *Streptomyces verticillus* subsp. *tsukushiensis* ATCC-21633, *Penicillium* sp. SPC-16375, and *Penicillium* sp. SPC-16524, respectively, as new myosin light chain kinase (MLCK) inhibitors. These compounds inhibited the calmodulin-dependent activity of MLCK with IC₅₀ values of 0.89, 0.86, 3.7, and 340 µM, respectively. Among them, dehydroaltenusin was the best MLCK inhibitor in terms of potency and selectivity examined in the purified enzyme systems.

Myosin light chain kinase (MLCK) is an enzyme which, upon activation, triggers contraction of smooth muscle.¹,² It is generally accepted that hormonal and neural signals for contraction induce increases in cytoplasmic Ca²⁺ concentrations in smooth muscle cells via receptor-mediated pathways. The rise in cytoplasmic Ca²⁺ is transduced to calmodulin, a ubiquitous Ca²⁺ binding protein, and the Ca²⁺-bound form of calmodulin activates MLCK. The activated MLCK catalyzes transfer of γ-phosphate of ATP to Ser-9 of the 20-kDa myosin light chain. Smooth muscle myosin, when phosphorylated in the light chain, interacts with actin and generates contractile force. Since smooth muscle cells are distributed in arteries and bronchi, inhibitors of MLCK would be potential vasodilators and bronchodilators.

*Penicillium verruculosum* IAM-13756, *Streptomyces verticillus* subsp. *tsukushiensis* ATCC21633, *Penicillium* sp. SPC-16375, and *Penicillium* sp. SPC-16524 were selected from tens of thousands of microorganisms kept in our laboratories or isolated newly from various sources by testing the effects of their culture extracts on the activity of MLCK. The active components in the culture broths of IAM-13756, ATCC21633, SPC-16375, and SPC-16524 were tentatively designated as MS-3, MS-146, MS-323, and MS-341, respectively. These active components were isolated and purified from mycelia (MS-146 and MS-323) or culture supernatants (MS-3 and MS-341) with combination of column chromatography, solvent extraction, and crystallization.

MS-3 (Fig. 1), obtained as a racemic form, was identified as dehydroaltenusin⁴ by spectral measurements (¹H-NMR, ¹³C-NMR, and HR-EIMS). MS-146 was identified as cyclooctasulfur by comparison of EIMS data and the IR chart with an authentic sample purified from commercially available sulfur. MS-323 (Fig. 1), obtained as mixtures of diastereomers, was identified as the methyl acetal of atrovenetinone⁵ by spectral data (¹H-NMR, ¹³C-NMR, and HR-EIMS). Although both enantiomers of atrovenetinone were reported,⁴,⁶ we could not identify the absolute stereochemistry of MS-323 because of limitations of purified samples. MS-323 was convertible from methyl acetal to ethyl acetal⁶ by dissolving and recrystallizing in ethanol. MS-341 (Fig. 1) was identified as altenusin⁷ by spectral measurements (¹H-NMR, ¹³C-NMR, and HR-EIMS). It has been reported that dehydroaltenusin and altenusin can be isolated from some *Alternaria* species, and *Talaromyces flavus*, and that atrovenetinone is from the scleroderris canker fungus, *Gremmeniella abietina*.⁵,⁷,⁹ We showed in this paper that *Penicillium* species were able to produce these metabolites.

MS-3, MS-146, MS-323, and MS-341 inhibited calmodulin-dependent activity of MLCK in a concentration-dependent manner. The IC₅₀ values of these compounds are summarized in Table. The authentic cyclooctasulfur inhibited MLCK with an IC₅₀ the same as that of MS-146. The IC₅₀ values of MS-3 and MS-146 were comparable under our assay conditions with those of wortmannin¹⁰ and KT926,¹¹ which are the strongest inhibitors of MLCK as far as we know. Since MLCK is a calmodulin-dependent protein kinase, the effects of the MLCK inhibitors on

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* To whom all correspondence should be addressed.

Abbreviation: MLCK, myosin light chain kinase.
other calmodulin-dependent enzymes and protein kinases were also examined. MS-3 inhibited calmodulin-dependent phosphodiesterase and protein kinase C at concentrations more than ten times higher than those needed to inhibit MLCK, and had no effect on cAMP- and cGMP-dependent protein kinases (Table). MS-146 inhibited protein kinase C as it did MLCK, but not three other enzymes examined. MS-323 inhibited all the enzyme activities tested in a similar concentration range. Since MS-341 inhibited MLCK at high concentrations, we did not examine its effect on other enzymes. Taken together, MS-3 was the best MLCK inhibitor in terms of potency and selectivity examined in the purified enzyme systems among the four.

The inhibition mechanism of MS-3 and MS-146 was examined by the measurement of MLCK activity with different concentrations of ATP, the peptide substrate, or calmodulin in the reaction mixture (Fig. 2a–c). The potency and efficacy of MS-3 to inhibit MLCK were hardly affected by ATP and the peptide substrate. On the contrary, high concentrations of MS-3 were needed to inhibit MLCK in the presence of a high concentration of calmodulin. In addition, MS-3 did not inhibit trypsin-activated, calmodulin-independent activity of MLCK at lower than 10 μM, and slightly inhibited it at more than 10 μM (Fig. 2d). The inhibitory profile of MS-146 on MLCK was essentially the same as that of MS-3 (data not shown). These data suggest that MS-3 and MS-146 inhibit the calmodulin-dependent activity of MLCK via blocking the interaction of calmodulin and the enzyme. However, an interesting point is that the concentrations of MS-3 needed to inhibit calmodulin-dependent phosphodiesterase were more than ten times higher than those needed to inhibit the calmodulin-dependent activity of MLCK, and that MS-146 did not inhibit the activity of phosphodiesterase at all. Since it has been reported that conventional calmodulin antagonists, such as calmidazolium and W-7, inhibit calmodulin-dependent enzymes at comparable concentrations under our assay conditions \(^\text{[12]}\) and others, \(^\text{[13,14]}\) the inhibition mechanism of MS-3 and MS-146 could be different from those of conventional calmodulin antagonists. It is possible to speculate that MS-3 and MS-146 bind to MLCK, rather than calmodulin, to inhibit the activation of the enzyme and not to inhibit the activation of calmodulin-dependent phosphodiesterase.

Further studies will be necessary to identify the binding site(s) of the inhibitors.

It has been reported that cyclooctasulfur suppressed the histamine release from mast cells. \(^\text{[15]}\) Since it is proposed that MLCK and/or protein kinase C are involved in histamine release from mast cells, our data that cyclooctasulfur inhibits purified MLCK and protein kinase C may provide another line of evidence to support the involvement of MLCK and/or protein kinase C in histamine release from mast cells.

We have already reported that we isolated four MLCK inhibitors, MS-54 (wortmannin), MS-347a, MS-282a, and MS-282b, from the culture of three different microorganisms. \(^\text{[16,17]}\) Four additional compounds described in this paper were found in the same series of screening studies aimed to look for new MLCK inhibitors. All these MS-series of compounds showed diversity in their chemical structure, inhibitory potency, and inhibitory mechanism. We believe that our screening system is efficient enough to detect various types of MLCK inhibitors in culture broth of microorganisms and to isolate them from the broth.

### Table Inhibition of Various Enzyme ActivitiesMeasured in the Presence of MS-3, MS-323, MS-146, and MS-341

The activity of each enzyme was measured as described. \(^\text{[10]}\)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC(_50) (μM)</th>
<th>MS-3</th>
<th>MS-323</th>
<th>MS-146</th>
<th>MS-341</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin light chain kinase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calmodulin-activated</td>
<td>0.69</td>
<td>3.7</td>
<td>0.86</td>
<td>340</td>
<td></td>
</tr>
<tr>
<td>Trypsin-activated</td>
<td>&gt;40</td>
<td>8.6</td>
<td>&gt;70</td>
<td>NT*</td>
<td></td>
</tr>
<tr>
<td>Calmodulin-dependent</td>
<td>17</td>
<td>5.6</td>
<td>&gt;70</td>
<td>NT*</td>
<td></td>
</tr>
<tr>
<td>phosphodiesterase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>8</td>
<td>38</td>
<td>1.6</td>
<td>NT*</td>
<td></td>
</tr>
<tr>
<td>cAMP-dependent protein kinase</td>
<td>&gt;40</td>
<td>26</td>
<td>&gt;70</td>
<td>NT*</td>
<td></td>
</tr>
<tr>
<td>cGMP-dependent protein kinase</td>
<td>&gt;40</td>
<td>NT*</td>
<td>&gt;70</td>
<td>NT*</td>
<td></td>
</tr>
</tbody>
</table>

* Not tested.

### Experimental

**Culture.** The microbial strains were cultured in 300-ml Erlenmeyer flasks containing the fermentation media: 0.5% glucose, 4.0% maltose, 1.0% soybean meal, 1.5% soluble vegetable protein, 0.5% yeast extract, 0.05% Mg\(_2\)(PO\(_4\))\(_2\)·8H\(_2\)O, and 0.05% Mg\(_2\)(PO\(_4\))\(_2\)·8H\(_2\)O (pH 6.5 before sterilization) for IAM-13756; 1.0% glucose, 2.0% dextrin, 1.0% corn steep liquor, 1.5% cotton seed flour, 0.5% yeast extract, and 0.05% Mg\(_2\)(PO\(_4\))\(_2\)·8H\(_2\)O (pH 7.0 before sterilization) for ATCC21633; and 2.0% glucose, 2.0% mashed potato powder, 0.5% peptone, 0.5% KH\(_2\)PO\(_4\), and 0.05% Mg\(_2\)(PO\(_4\))\(_2\)·8H\(_2\)O (pH 6.0 before sterilization) for SPC-16375 and SPC-16524.

**Fig. 2.** Effects of a High Concentration of Substrates and Calmodulin and Trypsin-treatment of the Enzyme on Susceptibility of MLCK to Inhibition by MS-3.

Phosphorylation of the peptide substrate was measured in the standard assay mixture or in assay mixture containing a different concentration of ATP, calmodulin, or the peptide substrate (a–c). Phosphorylation of the peptide substrate by trypsin-activated MLCK was measured in the mixture containing 1 mM EGTA instead of CaCl\(_2\), and calmodulin (d). a, 400 μM (O) and 12 μM ATP (●); b, 2.6 nm (O) and 32 nm calmodulin (●); c, 24 μM (O) and 3 μM the peptide substrate (K-MLC 1-23, KBPQPRATSNNFYSGNH\(_3\)) (●); d, calmodulin-activated MLCK (O) and trypsin-activated MLCK (●).
MS-3 (dehydroaurein). EIMS m/z 288 (M\(^+\)). HR-EIMS m/z: 288.0624 (err. − 0.8 ppm for C\(_{18}\)H\(_{20}\)O\(_{4}\)). 1\(^{H}\)-NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.13 (3H, s; 4a-Me), 3.91 (3H, s; 9-OMe), 6.28 (1H, s; 4-H), 6.23 (1H, s; 3-OMe), 6.63 (1H, d, J = 2.6 Hz; 8-H), 6.69 (1H, s; 1-H), 6.73 (1H, d, J = 2.6 Hz; 10-H), 11.29 (1H, s; 7-OH). 1\(^{3}\)-NMR (100 MHz, CDCl\(_3\)) \(\delta\) 29.5 (q; 4a-Me), 56.0 (q; 9-OMe), 79.1 (s; C-4a), 99.9 (s; C-6a), 103.8 (d; C-8), 104.4 (d; C-10), 116.2 (d; C-4), 120.8 (d; C-1), 135.1 (s; C-10a), 146.2 (s; C-3), 153.2 (s; C-10b), 164.8 (s; C-7), 166.4 (s; C-9), 167.4 (s; C-6), 180.8 (s; C-2).

MS-323 (atoventenone). SIMS (NBA) m/z: 341 (M + H–MeOH\(^+\)). HR-EIMS m/z: 340.0906 (err. − 3.9 ppm for C\(_{18}\)H\(_{19}\)O\(_{5}\)). Molecular formula C\(_{18}\)H\(_{20}\)O\(_{4}\). 1\(^{H}\)-NMR (500 MHz, CDCl\(_3\)) major diastereomer: \(\delta\) 1.28 (3H, s; 8-Me), 1.47 (3H, d, J = 6.4 Hz; 9-Me), 1.62 (3H, s; 8-Me), 2.76 (3H, d, J = 0.9 Hz; 1-Me), 3.41 (3H, s; 5-MeO), 4.58 (1H, br; 5-OH), 4.64 (1H, q, J = 6.7 Hz; 9-H), 6.73 (1H, br, q, J = 0.9 Hz), 12.47 (1H, s; 3-OH), 12.89 (1H, s; 7-OH); minor diastereomer: \(\delta\) 1.30 (3H, s; 8-Me), 1.46 (3H, d, J = 6.7 Hz; 9-Me), 1.51 (3H, s; 8-Me), 2.75 (3H, d, J = 0.9 Hz; 1-Me), 3.41 (3H, s; 5-MeO), 4.58 (1H, br; 5-OH), 4.66 (1H, q, J = 6.7 Hz; 9-H), 6.73 (1H, br, q, J = 0.9 Hz), 12.47 (1H, s; 3-OH), 12.91 (1H, s; 7-OH). 1\(^{3}\)-NMR (125 MHz, CDCl\(_3\)) major diastereomer: \(\delta\) 14.7 (q; 9-Me), 20.5 (q; 8-Me), 24.2 (q; 1-Me), 25.5 (q; 8-Me), 43.3 (s; C-8), 50.9 (s; 5-MeO), 90.2 (s; C-5), 91.7 (d; C-9), 102.2 (s; C-6a), 105.2 (s; C-3a), 110.1 (s; C-10b), 118.1 (d; C-2), 118.6 (s; C-7a), 137.8 (s; C-3b), 149.4 (s; C-1), 165.4 (s; C-7), 166.3 (s; C-3, C-10a), 192.5 (s; C-4*, 194.3 (s; C-6*).

MS-341 (alterenin). EIMS m/z: 290 (M\(^+\)). 1\(^{H}\)-NMR (400 MHz, DMSO-d\(_6\)) \(\delta\) 1.86 (3H, s; 2'-Me), ~ 3.35 (OH), 3.76 (3H, s; 5-MeO), 6.10 (1H, d, J = 7.2 Hz; 6-H), 6.42 (1H, s; 6-H), 6.43 (1H, d, J = 2.7 Hz; 4-H), 6.54 (1H, s; 3-H), 8.57 (1H, br, s; 5-OH), 8.61 (1H, br, s; 4-OH), ~ 11.6 (1H, br; OH). 1\(^{3}\)-NMR (100 MHz, DMSO-d\(_6\)) \(\delta\) 18.8 (q; 2'-Me), 55.3 (q; 5-MeO), 99.6 (d; C-4), 108.8 (s; C-2), 108.9 (d; C-6), 115.9 (d; C-6), 116.6 (d; C-3), 124.9 (s; C-2), 132.4 (s; C-7), 142.1 (s; C-5), 143.9 (s; C-4*), 145.0 (s; C-1), 161.6 (s; C-3), 162.0 (s; C-5), 171.6 (s; 2-CO\(_2\)) H.

References