K-259-2, a new inhibitor of Ca\(^{2+}\) and calmodulin-dependent cyclic nucleotide phosphodiesterase, was isolated from the cultured broth of *Micromonospora olivasterospora* K-259. K-259-2 has an anthraquinone moiety in its structure. \(IC_{50}\) values for the effect of K-259-2 against Ca\(^{2+}\) and calmodulin-stimulated activity of the enzyme preparations from bovine brain and heart were 6.6 and 2.9 \(\mu M\), respectively. On the other hand, basal activity (the activity in the presence of ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA) instead of Ca\(^{2+}\)/calmodulin) of the bovine brain enzyme, calmodulin-independent cyclic nucleotide phosphodiesterase from bovine heart, and protein kinase C from rat brain were inhibited by K-259-2 to a lesser extent with \(IC_{50}\) values of 27.4, 40.7 and 45.8 \(\mu M\), respectively.

Evidence has been accumulating to suggest a ubiquitous Ca\(^{2+}\)-binding protein, calmodulin, mediates many of the physiological functions of Ca\(^{2+}\) as a second messenger. Calmodulin, thus, activates a number of enzymes in a Ca\(^{2+}\)-dependent manner\(^1,2\). Among the enzymes regulated by calmodulin is a cyclic nucleotide phosphodiesterase\(^3,4\).

To clarify a molecular mechanism of the cellular functions mediated by calmodulin, it is useful to use its specific inhibitor. Phenothiazines, first described by Weiss et al.\(^5\), indeed have been widely used for this purpose. It has been determined, however, that a great variety of compounds commonly used as calmodulin antagonists have other significant biological actions at comparable concentrations to those that antagonize calmodulin\(^6\). This property may limit the usefulness of these compounds as tools for establishing calmodulin functions particularly in *in vivo* cellular systems.

During the course of our screening work to obtain calmodulin antagonists from microorganisms, we found that a novel metabolite of *Micromonospora olivasterospora* K-259 inhibited the Ca\(^{2+}\) and calmodulin-dependent cyclic nucleotide phosphodiesterase (CaM-PDE). The compound, named K-259-2, was isolated from the cultured broth of the producing microorganism, and its structure 1 was determined as shown in Fig. 1. In this article, we described taxonomic studies of the producing organism, production of K-259-2 and its biochemical properties. Studies on the structural determination will be elaborated upon a subsequent paper\(^7\).
Materials and Methods

Microorganisms and Taxonomy

*Micromonospora olivasterospora* K-259 (KY 11103) isolated from a soil of Nagaizumi-cho, Suntogun, Shizuoka, Japan, was used in the present investigation. The media used were those recommended for characterization of *Streptomyces* species. The color of cultures was determined with color chips of the Color Harmony Manual. Carbohydrate utilization test was performed on agar media of yeast nitrogen base without amino acids (Difco) supplemented with filter-sterilized carbon sources (1%) and 20% volume of 1% K2HPO4 solution. Glycosidase activities were assayed by the method of Kawamoto et al. Spore and mycelial morphologies of the cultures were observed by both light microscopy and scanning electron microscopy. Specimens for electron microscopy were prepared according to the method described previously. Chemical analysis of the whole cells was carried out by the method of Lechevalier and Lechevalier. The cell walls were prepared by the method of Kawamoto et al. Reducing sugars were analyzed by HPLC equipped with Shimadzu reducing sugar analyzer. Menaquinone composition was determined by the method of Tamaoka et al. The acyl type of polysaccharides was analyzed by the glycolate test of Uchida and Aida.

Fermentation

To a 50-ml test tube was added 15 ml of a seed medium composed of glucose 1.0%, soluble starch 1.0%, beef extract 0.3%, yeast extract 0.5%, Bacto-tryptone 0.5% and CaCO3 0.2% (pH 7.2 before sterilization) and inoculated with a loopful amount of spores of the microorganism grown on a surface of an agar slant. The composition of the agar slant medium (Hickey-Tresner) consisted of soluble starch 1.0%, N-Z amine type A 0.2%, beef extract 0.1%, yeast extract 0.1% and agar 2.0% (pH 7.2 before sterilization). The inoculated tube was incubated for 72 hours on a reciprocating shaker (300 rpm) at 28°C. A 10%-inoculation was made from the above vegetative medium to 300-ml Erlenmeyer flasks containing 40 ml of the same medium. This second stage vegetative medium was incubated for 24 hours on a rotary shaker (200 rpm) at 28°C. Four ml of this seed culture was transferred into a 300-ml flask containing 40 ml of production medium composed of glucose 0.5%, soluble starch 3.0%, corn steep liquor 0.5%, yeast extract 1.0%, Pharmamedia 1.0% and CaCO3 0.3% (pH 6.0 after sterilization). This production medium was incubated for 6 days on a rotary shaker (200 rpm) at 28°C. For inoculation of 18 liters of production medium in 30-liter jar fermentors, a 30 ml-aliquot of the second stage vegetative medium was transferred into 2-liter Erlenmeyer flasks containing 300 ml of the same seed medium. This third stage vegetative medium was incubated for 48 to 72 hours on a rotary shaker (200 rpm) at 28°C; 5.0% inoculum was routinely used for inoculation of the production medium. The fermentor was operated for 7 days at 28°C with agitation at 300 rpm and aeration of 18 liters/minute. The growth was monitored by packed cell volume (PCV). The K-259-2 was produced both in broth filtrate and mycelia, and its production was traced by measuring an inhibitory activity of the CaM-PDE. The amount of K-259-2 was also determined spectrophotometrically using pure K-259-2 as a standard on TLC plate (Silica gel 60, Merck, 5631) developed with CHCl3-MeOH - EtOH - H2O (10:4:4:2). The plate was scanned at wavelength of 430 nm with a Shimadzu Dual-wavelength TLC Scanner CS900.

Enzyme Assay

To prepare samples for the assays, culture filtrate was boiled for 10 minutes and packed cells was extracted with the same volume of methanol as that of supernatant of cultured broth. CaM-PDE and its basal (in the presence of ethylene bis(oxyethyleneimino)tetraacetic acid (EGTA) instead of Ca2+/calmodulin) activities, calmodulin-independent PDE activity, and protein kinase C activity were measured as described previously.

Materials

Bovine brain CaM-PDE and calmodulin were prepared according to the method of KAKIUCHI et al. with some modifications. Protein kinase C was prepared from rat brain according to the

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method of Kikkawa et al. with some modifications as described in a previous paper. Bovine heart CaM-PDE and calmodulin-independent PDE, cAMP, and 5'-nucleotidase (Crotalus atrox venom) were purchased from Sigma Chemical Co. All other reagents were commercially available and reagent grade.

**Results**

**Taxonomy**

No true aerial mycelium was produced by strain K-259. The vegetative mycelium developed, branched, septated, and was approximately 0.3 μm in diameter. Spores were born singly from the vegetative mycelium; usually on short sporophores which were sometimes branched. The matured spores were 0.7 to 0.9 μm in diameter, nonmotile, spherical to oval, and blunt-spiny (Figs. 2 and 3). Strain K-259 belongs to type II cell wall, sugar pattern D, and glycolyl type as shown in Table 1. Cultural characteristics of strain K-259 on various agar media are summarized in Table 2. Growth was moderate to good on most of the organic media tested. The color of colonies was beige to orange and it turned to black during spore formation. The organism also produced grayish yellow to light brown-colored soluble pigments. Strain K-259 utilized d-glucose, l-arabinose, d-xylose, l-rhamnose,

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Table 1. Components in the cell wall and the whole cell of *Micromonospora olivasterospora* K-259.

<table>
<thead>
<tr>
<th>Cell wall</th>
<th>3-Hydroxy and meso-diaminopimelic acid, glycine, glutamic acid, alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid</td>
<td></td>
</tr>
<tr>
<td>Sugar (μg/mg)</td>
<td>Arabinose (1.40), galactose (24.59), glucosamine (28.72), glucose (40.16), xylose (4.96)</td>
</tr>
<tr>
<td>Acyl residue</td>
<td>Glycolic acid</td>
</tr>
<tr>
<td>Whole cell</td>
<td></td>
</tr>
<tr>
<td>Sugar</td>
<td>Arabinose, xylose, galactose, glucose, ribose, glucosamine</td>
</tr>
<tr>
<td>Menaquinone (% content)</td>
<td>MK-10 (H₄) (46%), MK-10 (H₆) (40%), MK-10 (H₈) (14%)</td>
</tr>
</tbody>
</table>
Table 2. Culture characteristics of *Micromonospora olivasterospora* K-259.

<table>
<thead>
<tr>
<th>Agar medium</th>
<th>Growth</th>
<th>Reverse side of colony</th>
<th>Soluble pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose - nitrate</td>
<td>Poor</td>
<td>Dark covert gray (2ih)</td>
<td>None</td>
</tr>
<tr>
<td>Glucose - asparagine</td>
<td>Poor</td>
<td>Beige (3ge)</td>
<td>None</td>
</tr>
<tr>
<td>Glycerol - asparagine</td>
<td>Poor</td>
<td>Covert brown (2nl) to beige (3ge)</td>
<td>None</td>
</tr>
<tr>
<td>Salts - starch</td>
<td>Poor</td>
<td>Cream (3ie)</td>
<td>None</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Moderate</td>
<td>Ebony (2po)</td>
<td>Cream (3ie)*</td>
</tr>
<tr>
<td>Nutrient</td>
<td>Moderate</td>
<td>Light amber (3ic) to clove brown (3pl)</td>
<td>Bisque (3ec)*</td>
</tr>
<tr>
<td>Yeast extract - malt extract</td>
<td>Good</td>
<td>Black (p)</td>
<td>Dull gold (2ng)</td>
</tr>
<tr>
<td>Oatmeal</td>
<td>Good</td>
<td>Orange rust (4pe)</td>
<td>Covert tan (2ge)*</td>
</tr>
<tr>
<td>Peptone - yeast extract - iron</td>
<td>Poor</td>
<td>Camel (3ie) to sepia brown (3pn)</td>
<td>None</td>
</tr>
<tr>
<td>Hickey-Tresner</td>
<td>Good</td>
<td>Black (p)</td>
<td>None</td>
</tr>
</tbody>
</table>

* Incubation at 28°C for 3 weeks.
* Aerial mycelium were not formed on all the media.
* Soluble pigment was brighter than each color name.

Table 3. Effect of initial pH on K-259-2 production.

<table>
<thead>
<tr>
<th>Initial pH</th>
<th>Final pH</th>
<th>PCV (%)</th>
<th>K-259-2 production (µg/ml)</th>
<th>Supernatant</th>
<th>Packed cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>7.0*</td>
<td>15*</td>
<td>1.9*</td>
<td>5.1*</td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>7.5</td>
<td>17</td>
<td>5.1</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>6.3</td>
<td>7.8</td>
<td>16</td>
<td>3.4</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>8.3</td>
<td>16</td>
<td>3.4</td>
<td>1.9</td>
<td></td>
</tr>
</tbody>
</table>

* pH was adjusted with 0.1 N NaOH after sterilization.
*b* Fermentation was carried out using a 300-ml Erlenmeyer flask on a rotary shaker (200 rpm) at 28°C for 6 days.
*c* Production of K-259-2 in packed cells was determined by extraction with the same volume of MeOH as that of supernatant. Quantitative analysis was done by TLC method as described in Materials and Methods.
*d* Data obtained from several experiments.

PCV: Packed cell volume.

and sucrose but not adonitol, D-arabinose, D-fructose, gluconate, glycerol, lactose, D-mannitol, D-melezitose, α-D-melibiose, methyl-α-D-glycoside, myo-inositol, raffinose, D-ribose, salicin or D-sorbitol. The strain peptonized and coagulated milk, poorly hydrolyzed cellulose, but not liquified gelatin. The temperature range for growth was 18 to 33°C (optimal 26 to 30°C). The strain showed the activities of α- and β-galactosidase but not those of α-mannosidase and β-xylosidase. Morphological and chemical characteristics of strain K-259 permitted an assignment of the organism to the genus *Micromonospora* Orskov. The strain was then compared with *Micromonospora* species in the Approved list of bacterial names and subsequent species published validly. Strain K-259 closely resembled *Micromonospora olivasterospora* Kawamoto *et al.* 1983 based on the following characteristics: The presence of MK-10 as menaquinone, lack of diagnostic mycelial pigments, and failure to utilize melibiose, raffinose, glycerol, inositol and D-arabinose. Strain K-259 was thus classified as a strain of *Micromonospora olivasterospora* and named *Micromonospora olivasterospora* K-259.

Production of K-259-2 by Fermentation

Numerous attempts to increase the biosynthesis of K-259-2 were made, and the resultant defined medium and optimum conditions for the production are described under Materials and Methods.
Among the parameters affecting K-259-2 production in broth filtrate, starting pH has the most influence. As shown in Table 3, the K-259-2 produced in mycelia was released into the supernatant corresponding to an alkalization of starting pH of the producing medium. The time course of K-259-2 production in a 30-liter jar fermentor is shown in Fig. 4. The production of K-259-2, initiated on day 2, was increased through day 7 after the cell growth reached maximum.

Isolation and Purification

Isolation procedure is outlined in Fig. 5. Because of the presence of antibiotic in the culture filtrate and mycelia, the cultured broth (100 liters) was mixed with 20 liters of propyl alcohol stirred vigorously, and centrifuged with a Sharples centrifuge. The supernatant was then applied to a column of Diaion HP-10 (Mitsubishi Chemical Industries Ltd.). The column was washed with water and then 30% methyl alcohol. Adsorbed material was eluted with methyl alcohol. The eluate was concentrated in vacuo to 500 ml, adjusted to pH 2.0, and extracted with ethyl acetate. The ethyl acetate layer was dried over sodium sulfate and then concentrated in vacuo to yield brownish oily material. This oily material was subjected to successive silica gel column chromatographies. Fractions containing crude K-259-2 were pooled and concentrated, and an oil was applied to a Sephadex LH-20 (500 ml, Pharmacia Fine Chemicals) column. The appropriate fractions were pooled and evaporated to dryness. Final purification of the compound was achieved by reverse phase liquid chromatography (Lobar column Lichroprep RP-8, size B, Merck, 11804). Fractions containing K-259-2 were combined and evaporated to yield red powder. The powder thus obtained was crystallized from methyl alcohol - water to yield red crystalline K-259-2 (256 mg).

Physico-chemical Properties

Physico-chemical properties of K-259-2 are summarized in Table 4. K-259-2 was obtained as red crystals, mp 140~145°C (dec). It is soluble in dimethyl sulfoxide and acetic acid, slightly soluble in methyl alcohol and water, and virtually insoluble in chloroform, ethyl acetate, ethyl alcohol and acetone. The Rf values of the compound on silica gel TLC developed in various solvent system and its color reactions are also presented in Table 4. The structure of K-259-2 was determined to be 1 (Fig. 1) on the basis of physico-chemical analyses. Details of these studies will be described in a separate paper.

Biochemical Properties

Fig. 6 shows the effect of various concentrations of K-259-2 on CaM-PDE from bovine brain. K-259-2 inhibited the calmodulin-dependent activity of CaM-PDE in a concentration-dependent man-
Fig. 5. Purification of K-259-2.

Cultured broth (100 liters)
added PrOH (20 liters)
stirred (2 hours)
centrifuged

Supernatant
Diaion HP-10 column chromatography (Mitsubishi Chemical Industries Ltd., 100 liters)
washed with \( \text{H}_2\text{O} \) (10 liters) and then 30% MeOH
eluted with MeOH (30 liters)
concd in vacuo
pH adjusted to 2.0
extracted with EtOAc (1.5 liters)

EtOAc layer
dried over \( \text{Na}_2\text{SO}_4 \)
concd in vacuo

Oily material
1st Silica gel column chromatography (Wakogel C-200, 1 liter)
eluted with BuOH - EtOH - CHCl\(_3\) - NH\(_4\text{OH}\) (4 : 5 : 2 : 4)
concd in vacuo

2nd Silica gel column chromatography (Wakogel C-200, 500 ml)
eluted with CHCl\(_3\) - MeOH - EtOH - H\(_2\text{O}\) (10 : 4 : 4 : 2)
concd in vacuo

Oily material
Sephadex LH-20 column chromatography (Pharmacia Fine Chemicals, 500 ml)
eluted with CHCl\(_3\) - MeOH - EtOH - H\(_2\text{O}\) (10 : 4 : 4 : 2)
concd in vacuo

Lobar column chromatography (Lichroprep RP-8, size B, Merck, 11804)
eluted with MeOH - H\(_2\text{O}\) (7 : 3)
concd in vacuo

Crystallized in MeOH - H\(_2\text{O}\)
Red crystalline K-259-2 (256 mg)

<table>
<thead>
<tr>
<th>Table 4. Physico-chemical properties of K-259-2.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Appearance</strong></td>
</tr>
<tr>
<td><strong>MP ( (°C, \text{dec}) )</strong></td>
</tr>
<tr>
<td><strong>Optical rotation ([\alpha])_{D}^{2})</strong></td>
</tr>
<tr>
<td><em><em>TLC</em> (Rf)</em>*</td>
</tr>
<tr>
<td><strong>CHCl(_3) - MeOH - EtOH - H(_2\text{O}) (10 : 4 : 4 : 2)</strong></td>
</tr>
<tr>
<td><strong>BuOH - EtOH - CHCl(_3) - NH(_4\text{OH}) (4 : 5 : 2 : 4)</strong></td>
</tr>
<tr>
<td><strong>Color reaction</strong></td>
</tr>
<tr>
<td><strong>Positive</strong></td>
</tr>
<tr>
<td><strong>Negative</strong></td>
</tr>
<tr>
<td><strong>Solubility</strong></td>
</tr>
<tr>
<td><strong>Soluble</strong></td>
</tr>
<tr>
<td><strong>Slightly soluble</strong></td>
</tr>
<tr>
<td><strong>Insoluble</strong></td>
</tr>
<tr>
<td><strong>Absorption ( \lambda_{\text{max}} \text{nm (E)}_{1\text{cm}} )</strong></td>
</tr>
<tr>
<td><strong>Neutral</strong></td>
</tr>
<tr>
<td><strong>Acidic</strong></td>
</tr>
<tr>
<td><strong>Alkaline</strong></td>
</tr>
</tbody>
</table>

* Silica gel 60 (Merck, 5631).
Table 5. Effect of K-259-2 on cyclic nucleotide phosphodiesterases.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>CaM</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine brain CaM-PDE</td>
<td>+ᵃ</td>
<td>6.6</td>
</tr>
<tr>
<td>Bovine heart CaM-PDE</td>
<td>+ᵇ</td>
<td>27.4</td>
</tr>
<tr>
<td>Bovine heart CaM-independent PDE</td>
<td>+ᶜ</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>-ᵈ</td>
<td>40.7</td>
</tr>
</tbody>
</table>

ᵃ The activity in the presence of 4 µ/ml calmodulin, and 50 µM CaCl₂. The enzyme concentration was 26 µg/ml.
b The activity in the presence of 3 mM ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA), without CaCl₂ and calmodulin. The enzyme concentration was 18.6 µg/ml.
c The activity in the presence of 2.5 µ/ml calmodulin, and 50 µM CaCl₂. The enzyme concentration was 40 µg/ml.
d The activity in the presence of 3 mM EGTA, without CaCl₂ and calmodulin. The enzyme concentration was 25 µg/ml.

CaM: Calmodulin. CaM-PDE: Ca²⁺ and calmodulin-dependent cyclic nucleotide phosphodiesterase.

K-259-2 at 100 µg/ml exhibited no antimicrobial activity against Candida albicans KY 5011, Enterococcus faecalis KY 4280, Pseudomonas aeruginosa KY 4276, Staphylococcus aureus KY 4779, Escherichia coli KY 4271, Bacillus subtilis KY 4773, Proteus vulgaris KY 4277, Shigella sonnei KY 4281, Salmonella typhosa KY 4278 or Klebsiella pneumoniae KY 4275.

Discussion

A great variety of lipid-interacting agents share the ability to inhibit calmodulin actions in vitro. These compounds include antipsychotic agents of the phenothiazine, naphthalensulfonamide compounds (e.g., W-7), alkaloids (e.g., vinblastine), peptides (e.g., β-endorphin), antimycotic agents (e.g., calmidazolium), and a novel class of agents that includes K-259-2. K-259-2 was found to be a potent inhibitor of CaM-PDE from bovine brain and heart, as well as of calmodulin-independent PDE from bovine heart and protein kinase C from rat brain. The IC₅₀ values for K-259-2 were 6.6, 27.4, and 40.7 µM, respectively, for the various enzymes.

Phosphodiesterase activity was measured as described in a previous paper with various concentrations of K-259-2 added as indicated. For the assay of Ca²⁺/calmodulin-dependent activity (●), the reaction mixture contained, in a final volume of 0.5 ml, 80 mM imidazole-HCl buffer (pH 6.9), 3 mM MgSO₄, 0.3 mM dithiothreitol, 100 mM NaCl, 1.2 mM cAMP, 50 µM CaCl₂, 26 µg/ml bovine brain CaM-PDE, and 4 µg/ml calmodulin. The basal activity (○) was determined using a large amount of the enzyme (18.6 µg/ml) to magnify PDE activity in the presence of 3 mM EGTA instead of Ca²⁺/calmodulin.

![Graph showing inhibition by K-259-2 of bovine brain Ca²⁺ and calmodulin-dependent cyclic nucleotide phosphodiesterase.](image-url)

Fig. 6. Inhibition by K-259-2 of bovine brain Ca²⁺ and calmodulin-dependent cyclic nucleotide phosphodiesterase.
zorium), and so on\textsuperscript{21}). While the mode of action of these compounds is complicated, it is indisputable that calmodulin antagonists presently available interact with the hydrophobic region on calmodulin exposed by $\text{Ca}^{2+}$-binding. The inhibition of calmodulin stimulation of various target enzymes can therefore be explained by a competition between compounds and the enzymes for this hydrophobic region\textsuperscript{22}). This interpretation is consistent with the observation that the potency of antagonism increases with increasing hydrophobic character of the antagonists\textsuperscript{22-25}). This lipid-interacting property probably explains the fact that the inhibitory activities of these compounds are by no means restricted to calmodulin-dependent enzyme systems. These agents indeed inhibit other enzymes including protein kinase C, which is thought to regulate a variety of cellular responses\textsuperscript{26-27}). This property may therefore limit their use as tools for establishing calmodulin functions particularly in whole cells and intact tissues.

Among these so-called calmodulin antagonists is adriamycin, an anthracycline-aminoglycoside antitumor antibiotic, which also has an anthraquinone moiety as K-259-2. Adriamycin inhibits calmodulin-activated protein kinase weakly (IC$_{50}$ value, 50$\sim$ 85 $\mu$M\textsuperscript{28}) by acting at a hydrophobic region of calmodulin, and it also antagonizes protein kinase C at similar concentrations\textsuperscript{28}). Furthermore, under the assay conditions described above, adriamycin inhibited the CaM-PDE activity only weakly (IC$_{50}$ value, 700 $\mu$M, data not shown), whereas K-259-2, a novel anthraquinone metabolite form Micromonospora olivasterospora K-259, inhibited the activity intensely (IC$_{50}$ value, 2.9$\sim$ 6.6 $\mu$M).

Experiments to be described elsewhere have shown that many well-known calmodulin antagonists, if not all, interact with amphipathic (hydrophobic plus anionic) compounds such as acidic phospholipids, but not K-259-2.

To clarify whether or not the strong inhibitory activity of K-259-2 as compared with that of adriamycin can be explained by its non-interacting property with amphipathic compounds, detailed mechanisms by which K-259-2 acts are under investigation. In conclusion, K-259-2 is a novel potent inhibitor of CaM-PDE and its advantageous characteristics will be helpful in unraveling the physiological functions of calmodulin in $\text{Ca}^{2+}$-messenger system.

Acknowledgment

We acknowledge with thanks the expert assistance of Mrs. Miyoko Funayama-Kojima.

References


