

KS-501 AND KS-502, NEW INHIBITORS OF  $\text{Ca}^{2+}$  AND CALMODULIN-DEPENDENT CYCLIC-NUCLEOTIDE PHOSPHODIESTERASE FROM *SPOROTHRIX* SP.

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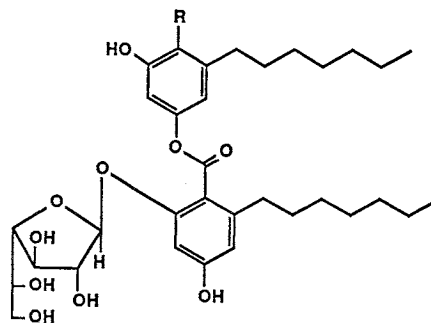
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New inhibitors of  $\text{Ca}^{2+}$  and calmodulin-dependent cyclic-nucleotide phosphodiesterase, KS-501 and KS-502 were isolated from a fungus, *Sporothrix* sp. KAC-1985. Inhibitory concentration causing 50% inhibition ( $\text{IC}_{50}$ ) values of KS-501 and KS-502 for bovine brain enzyme were 1.8 and 4.3  $\mu\text{M}$ , respectively. The compounds exhibited no or weak inhibition for calmodulin-independent cyclic-nucleotide phosphodiesterases and protein kinase C.

The role of  $\text{Ca}^{2+}$  is widely accepted as a second messenger in various cell responses such as contraction of muscle, release of neurotransmitters and chemical mediators, secretion of hormones, cell division and proliferation, and cell motility<sup>1)</sup>. A number of lines of evidence have been accumulating to show that many of the intracellular  $\text{Ca}^{2+}$  functions are mediated by calmodulin (CaM), a ubiquitous  $\text{Ca}^{2+}$ -binding protein. CaM has an ability to activate a variety of enzymes in a  $\text{Ca}^{2+}$ -dependent manner<sup>2,3)</sup>. Among them is  $\text{Ca}^{2+}$  and calmodulin-dependent cyclic-nucleotide phosphodiesterase (CaM-PDE)<sup>4,5)</sup>.

During the course of our screening work to obtain CaM inhibitors from microorganisms, we found that two novel metabolites of *Sporothrix* sp. KAC-1985 inhibited CaM-PDE. The compounds, designated as KS-501 and KS-502<sup>6-8)</sup>, were isolated from the cultured broth, and their structures were determined to be **1** and **2**, respectively (Fig. 1). In this article, we describe production, isolation and some biological properties of KS-501 and KS-502. Structural elucidation studies will be reported in a separate paper.

Fig. 1. Structures of KS-501 and KS-502.



KS-501 (**1**)    R=H  
KS-502 (**2**)    R=COOH

### Materials and Methods

#### Fermentation

A 50-ml culture tube containing 10 ml of a seed medium composed of glucose 1.0%, peptone 0.5%, dry yeast (Ebios, Asahi Brewery) 0.5%, vegetable juice 20%, and  $\text{CaCO}_3$  0.3% (pH 6.0 before sterilization) was inoculated with the mycelia of the organism grown on an agar slant. The composition of the agar slant medium was as follows: Glucose 0.1%,  $\text{KH}_2\text{PO}_4$  0.1%,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.02%, KCl 0.02%,  $\text{NaNO}_3$  0.2%, yeast extract 0.02%, and agar 1.5% (pH 6.5 before sterilization). The inoculated tube was incubated for 7 days on a reciprocating shaker (300 rpm) at 25°C. A 4.5-ml of

the culture was transferred into a 300-ml Erlenmeyer flask containing 45 ml of the seed medium and the flask was incubated for 53 hours on a rotary shaker (200 rpm) at 25°C. Forty five ml of the second culture was further transferred into a 2-liter Erlenmeyer flask containing 450 ml of the seed medium and the flask was incubated for 22 hours on a rotary shaker (200 rpm) at 25°C. Four flasks of the third culture were transferred to a 30-liter jar fermenter containing 18 liters of fermentation medium composed of glucose 1.0%, peptone 0.5%, Ebios 0.5%, vegetable juice 20%, apple juice 20%, and CaCO<sub>3</sub> 0.5% (pH 6.0 before sterilization). Fermentation was carried out for 192 hours at 25°C with agitation at 300 rpm, aeration of 18 liters/minute and continuous feeding of 30 ml/hour water. The growth was monitored by total nucleic acid content. Total nucleic acid was extracted from mycelia by boiling in 0.5 M perchloric acid for 15 minutes and the absorbance at 260 nm of the extract was measured. Production of KS-501 and KS-502 in mycelia was determined by HPLC after extraction of the compounds from the culture broth as follows. To the culture broth was added the same volume of MeOH. After centrifugal separation, the MeOH extract was adsorbed on a SEP-PAK C18 cartridge (Waters Associates), and the cartridge was washed with 50% MeOH and then eluted with MeOH. The eluate was injected to a Unisil Q C8 column (4.6 × 250 mm, 5 μm, Gasukuro Kogyo Co., Ltd.) and developed at 40°C with 70% acetonitril containing 0.07% TFA at a flow rate of 1.0 ml/minute monitoring absorbance at 254 nm. Detection of KS-501 and KS-502 in isolation procedures was carried out on a TLC plates Silica gel 60 F<sub>254</sub> (Merck, Art. No. 5715) developed with CHCl<sub>3</sub> - MeOH - EtOH - water (10 : 4 : 4 : 2) and visualized under UV-light.

#### Enzyme Assay

Bovine brain CaM-PDE activity was measured as described in a previous paper<sup>9)</sup> with indicated concentrations of KS-501 and KS-502. The reaction mixture contained in a final volume of 0.5 ml, imidazole-HCl buffer 80 mM (pH 6.9), MgSO<sub>4</sub> 3 mM, dithiothreitol 0.3 mM, NaCl 100 mM, cAMP 1.2 mM, CaCl<sub>2</sub> 50 μM, bovine brain CaM-PDE 26 mU/ml and CaM 4 U/ml. The basal activity was determined using a large amount of the enzyme (18.6 mU/ml) to magnify the activity in the presence of 3 mM ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA) instead of Ca<sup>2+</sup>/CaM. Bovine heart CaM-PDE activity in the presence of Ca<sup>2+</sup>/CaM was assayed at the enzyme concentration of 40 mU/ml. The activity of bovine heart calmodulin-independent cyclic-nucleotide phosphodiesterase (CaM-independent PDE) was determined in the presence of EGTA at enzyme concentration of 25 mU/ml. The assay condition of protein kinase C was described previously<sup>9)</sup>.

#### Microorganisms and Materials

*Sporothrix* sp. KAC-1985 (FERM BP 1278) was isolated from a fallen leaf collected in Yamakita-cho, Ashigarakami-gun, Kanagawa Prefecture, Japan. Bovine brain CaM-PDE and CaM were prepared according to the method of KAKIUCHI *et al.*<sup>10)</sup> with some modifications<sup>9)</sup>. Protein kinase C was prepared from rat brain according to the method of KIKKAWA *et al.*<sup>11)</sup> with some modifications as described in a previous paper<sup>9)</sup>. Bovine heart CaM-PDE and CaM-independent PDE, cAMP and 5'-nucleotidase (*Crotalus atrox* venom) were purchased from Sigma Chemical Company. All other reagents were commercially available and reagent grade.

## Results

### Fermentation

Time course of KS-501 and KS-502 production by *Sporothrix* sp. KAC-1985 in a 30-liter jar fermenter is shown in Fig. 2. Both compounds were accumulated mainly in mycelia. The amount of KS-502 increased during the logarithmic phase of cell growth, reached maximum after 3-day cultivation and rapidly decreased with concomitant lysis of cells. The production of KS-501 was initiated in 3-day cultivation and increased even after lysis of cells.

### Isolation and Purification of KS-501 and KS-502

For isolation of KS-501 and KS-502, fermentation was terminated in 8 days and in 3 days, re-

spectively (Fig. 2). Purification procedures of these compounds were outlined in Figs. 3 and 4. The compounds were extracted from the mycelial cake with methanol. The methanol extract was diluted with the same volume of water, and was applied to a Diaion HP-20 (Mitsubishi Chemical Industries Limited) column. The column was washed with 50% methanol and eluted with methanol. The eluate was concentrated *in vacuo* and adjusted to pH 2.0 with HCl, and the compounds were extracted with ethyl acetate. The extract was subjected to silica gel column chromatography. KS-501 was eluted with 10% methanol in  $\text{CHCl}_3$ , and KS-502 with 20% methanol in  $\text{CHCl}_3$ . Fractions containing each compound were combined separately and concentrated *in vacuo*. Final purification of both compounds were achieved by Sephadex LH-20

Fig. 2. Time course of KS-501 and KS-502 production in a 30-liter jar fermenter.

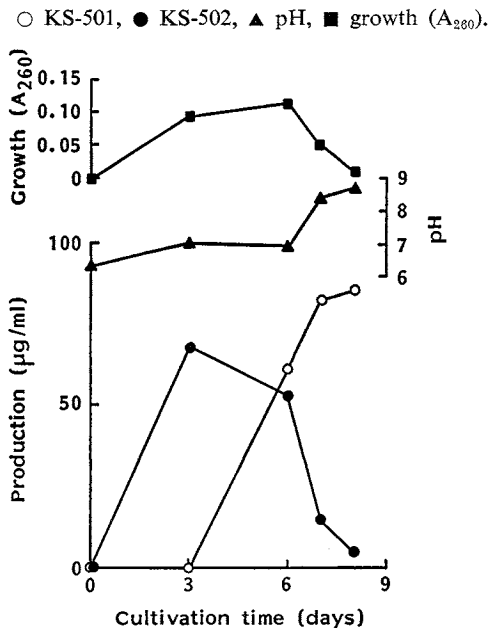
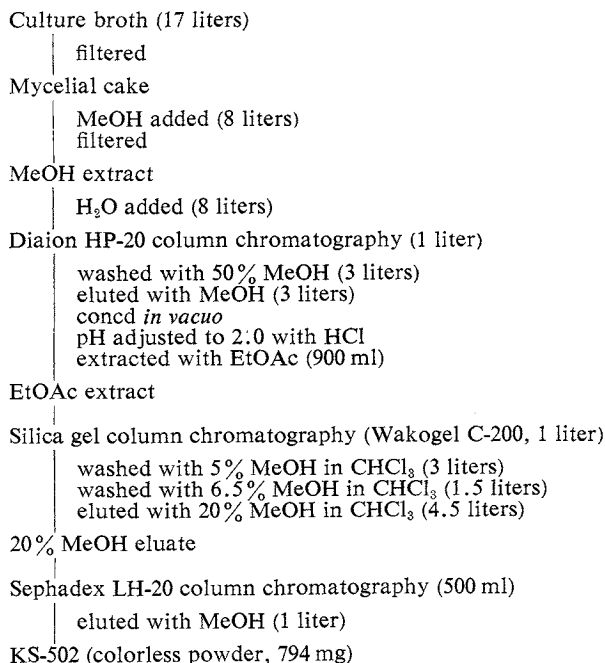


Fig. 3. Purification of KS-501.

Culture broth (17 liters)  
 | filtered  
 Mycelial cake  
 | MeOH added (8 liters)  
 | filtered  
 MeOH extract  
 |  $\text{H}_2\text{O}$  added (8 liters)  
 Diaion HP-20 column chromatography (1 liter)  
 | washed with 50% MeOH (3 liters)  
 | eluted with MeOH (3 liters)  
 | concd *in vacuo*  
 | pH adjusted to 2.0 with HCl  
 | extracted with EtOAc (900 ml)  
 EtOAc layer  
 | concd *in vacuo*  
 Oily material  
 |  
 Silica gel column chromatography (Wakogel C-200, 1 liter)  
 | washed with  $\text{CHCl}_3$  (4 liters)  
 | washed with 10% MeOH in  $\text{CHCl}_3$  (2 liters)  
 | eluted with 10% MeOH in  $\text{CHCl}_3$  (another 2 liters)  
 10% MeOH eluate  
 | concd *in vacuo*  
 Silica gel column chromatography (Wakogel C-300, 100 ml)  
 | eluted with 10% MeOH in  $\text{CHCl}_3$  (400 ml)  
 Sephadex LH-20 column chromatography (200 ml)  
 | eluted with MeOH (400 ml)  
 KS-501 (colorless powder, 852 mg)

Fig. 4. Purification of KS-502.



(Pharmacia Fine Chemicals) columns. KS-501 and KS-502 were obtained as colorless powder (852 and 794 mg, respectively).

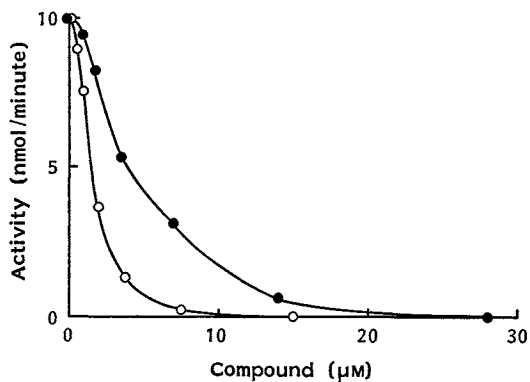
#### Physico-chemical Properties

Physico-chemical properties of KS-501 and KS-502 obtained as colorless powder were summarized in Table 1. Structures of KS-501 and KS-502 were elucidated to be **1** and **2**, respectively (Fig. 1), on the basis of physico-chemical analysis and various spectral data. Details of these studies will be described in a separate paper.

#### Biochemical Properties

Fig. 5 shows effects of KS-501 and KS-502 on bovine brain CaM-PDE. These compounds inhibited the CaM-dependent activity of the enzyme in a concentration-dependent manner. The inhibitory concentration causing 50% inhibition ( $IC_{50}$ ) values of KS-501 and KS-502 were 1.8 and 4.3  $\mu M$ , respectively. Table 2 summarizes the effects of KS-501 and KS-502 on various cyclic-nucleotide phosphodiesterase activities. KS-502 inhibited bovine heart CaM-PDE in the same concentration range as the bovine brain enzyme, whereas KS-501 inhibited the brain enzyme at significantly lower concentration than the heart enzyme.

Fig. 5. Inhibition by KS-501 and KS-502 of bovine brain CaM-PDE.



CaM-PDE activity was measured as described in a previous paper with indicated concentrations of KS-501 (○) and KS-502 (●). Symbols represented means of at least five such experiments made with different protein preparations, and  $IC_{50}$  values means  $\pm$  SE.

KS-501  $IC_{50} = 1.8 \pm 0.08 \mu M$ , KS-502  $IC_{50} = 4.3 \pm 0.09 \mu M$ .

Table 1. Physico-chemical properties of KS-501 and KS-502.

	KS-501	KS-502
Appearance	Colorless powder	Colorless powder
TLC (Rf)		
CHCl <sub>3</sub> - MeOH (7 : 3) <sup>a</sup>	0.49	0.31
CHCl <sub>3</sub> - MeOH - conc NH <sub>4</sub> OH (19 : 5 : 1) <sup>a</sup>	0.08	0.00
CHCl <sub>3</sub> - MeOH - AcOH (7 : 2 : 1) <sup>a</sup>	0.51	0.42
CHCl <sub>3</sub> - MeOH - EtOH - H <sub>2</sub> O (10 : 4 : 4 : 2) <sup>a</sup>	0.64	0.30
Acetone <sup>a</sup>	0.51	0.12
80% MeOH <sup>b</sup>	0.12	0.34
HPLC, retention time (minutes) <sup>c</sup>	7.88	6.04
Color reaction		
Positive	I <sub>2</sub> , FeCl <sub>3</sub> , anisaldehyde	I <sub>2</sub> , FeCl <sub>3</sub> , anisaldehyde
Negative	Ninhydrin, Rydon-Smith, anilin-phthalate	Ninhydrin, Rydon-Smith, anilin-phthalate
MP (°C)	149~152	119~120
[α] <sub>D</sub> <sup>25</sup> (c 0.3, MeOH)	-53°	-45°
Solubility		
Soluble	CHCl <sub>3</sub> , EtOAc, CH <sub>3</sub> CN, MeOH, BuOH, DMSO, Me <sub>2</sub> CO, pyridine, AcOH, 0.1 N NaOH	MeOH, BuOH, DMSO, Me <sub>2</sub> CO, pyridine, AcOH, 0.1 N NaOH
Slightly soluble		CHCl <sub>3</sub> , EtOAc, CH <sub>3</sub> CN
Insoluble	Hexane, benzene, CCl <sub>4</sub> , H <sub>2</sub> O, 0.1 N HCl	Hexane, benzene, CCl <sub>4</sub> , H <sub>2</sub> O, 0.1 N HCl
UV λ <sub>max</sub> <sup>MeOH</sup> nm (ε)		
Neutral and acidic	254 (6,760), 278 (sh, 5,560)	252 (14,100), 282 (sh, 6,600), 300 (sh, 5,400)
Alkaline	234 (sh, 16,100), 292 (11,500)	234 (sh, 18,900), 298 (24,800)
IR (KBr) cm <sup>-1</sup>	3380, 2960, 2924, 2852, 1722, 1608, 1586, 1466, 1323, 1264, 1168, 1149, 1129, 1064, 996	3450, 2972, 2940, 2872, 1724, 1595, 1463, 1432, 1357, 1343, 1245, 1161, 1135, 1062

<sup>a</sup> HPTLC plates Silica gel 60 F<sub>254</sub> (Merck, Art. No. 5628).

<sup>b</sup> HPTLC plates RP-18 F<sub>254</sub>S (Merck, Art. No. 13724).

<sup>c</sup> Conditions were described in Materials and Methods.

Table 2. Effects of KS-501 and KS-502 on various cyclic-nucleotide phosphodiesterases.

Enzymes	CaM	IC <sub>50</sub> (μM)	
		KS-501	KS-502
Bovine brain CaM-PDE	+	1.8	4.3
	-	116	88
Bovine heart CaM-PDE	+	15	5.9
Bovine heart CaM-independent PDE	-	68	32

+: The activity in the presence of Ca<sup>2+</sup>/CaM.

-: The activity in the presence of EGTA instead of Ca<sup>2+</sup>/CaM.

CaM-independent activities of brain CaM-PDE (activity in the presence of EGTA) and bovine heart CaM-independent PDE were inhibited at higher concentrations of KS-501 and KS-502. Neither of them inhibited protein kinase C at 30  $\mu\text{M}$ . These data represent that KS-501 and KS-502 are novel and potent inhibitors of CaM-PDE. KS-501 exhibited weak antimicrobial activity against *Enterococcus faecium* ATCC 10541, *Staphylococcus aureus* ATCC 6538P, and *Bacillus subtilis* No. 10707 (MIC 100, 12.5, and 12.5  $\mu\text{g}/\text{ml}$ , respectively), and no activity at 100  $\mu\text{g}/\text{ml}$  against *Candida albicans* ATCC 10231, *Pseudomonas aeruginosa* BMH No. 1, *Escherichia coli* ATCC 26, *Proteus vulgaris* HX2 ATCC 6897, *Shigella sonnei* ATCC 9290, *Salmonella typhi* ATCC 9992, and *Klebsiella pneumoniae* ATCC 10031. KS-502 also exhibited weak antimicrobial activity at 12.5  $\mu\text{g}/\text{ml}$  against *S. aureus* ATCC 6538P and *B. subtilis* No. 10707, and no activity at 100  $\mu\text{g}/\text{ml}$  against the others.

### Discussion

In this paper, we demonstrate that novel compounds, KS-501 and KS-502, isolated from *Sporothrix* sp. KAC-1985 are potent inhibitors of  $\text{Ca}^{2+}$ /CaM-dependent phosphodiesterases from bovine brain and heart. YAGINUMA *et al.*<sup>12)</sup> have recently reported *Nodulisporium* sp. M5220 produces phosphodiesterase inhibitors, TPI series of compounds, whose structures are closely related to KS-501 and KS-502. However, it remains uncertain whether those compounds inhibit  $\text{Ca}^{2+}$ /CaM-dependent enzymes or not.

KS-501 and KS-502 inhibited CaM-dependent activities of CaM-PDE's but had low potency against their CaM-independent activities, thereby suggesting that the compounds interacted with CaM to inhibit  $\text{Ca}^{2+}$ /CaM-dependent enzymes. A variety of compounds have been found to inhibit  $\text{Ca}^{2+}$ /CaM-dependent enzymes. These compounds include phenothiazines<sup>13)</sup>, naphthalenesulfonamides<sup>14)</sup>, alkaloids<sup>15,16)</sup>, peptides<sup>17)</sup>, antimycotic agents<sup>18,19)</sup>, and others<sup>20,21)</sup>. The structures of KS-501 and KS-502 are quite different from these CaM inhibitors. Furthermore, in contrast with most of these compounds which inhibited another  $\text{Ca}^{2+}$ -dependent enzyme, protein kinase C, by interacting with phospholipids (an activator of the enzyme), KS-501 and KS-502 had no inhibitory activities on protein kinase C.

Although KS-501 and KS-502 resemble each other in structure, they appear to show different biochemical properties. For instance, KS-502 inhibited CaM-PDE's both from brain and from heart in a similar concentration range, whereas KS-501 inhibited the brain enzyme with the  $\text{IC}_{50}$  value 8 times lower than the heart enzyme (Table 2). The results suggested that KS-501 differentiated the brain enzyme from the heart enzyme. Precise biochemical properties of these compounds and their inhibition mechanisms of CaM-PDE will be reported in the near future.

In conclusion, we found novel and potent inhibitors of CaM-PDE, KS-501 and KS-502, from *Sporothrix* sp. They will be good tools for studying the function of CaM and CaM-PDE.

### References

- 1) RUBIN, R. P.: Historical and biological aspects of calcium action. In *Calcium in Biological Systems*. Ed., R. P. RUBIN *et al.*, pp. 5~11, Plenum Press, New York, 1985
- 2) MANALAN, A. S. & C. B. KLEE: Calmodulin. In *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*. Vol. 18. Eds., P. GREENGARD & G. A. ROBISON, pp. 227~278, Raven Press Books, Ltd., New York, 1984
- 3) LIN, Y. M. & W. Y. CHEUNG:  $\text{Ca}^{2+}$ -dependent cyclic nucleotide phosphodiesterase. In *Calcium and Cell Function*. Vol. 1. Calmodulin. Ed., W. Y. CHEUNG, pp. 79~107, Academic Press, New York, 1980
- 4) KAKIUCHI, S. & R. YAMAZAKI: Calcium dependent phosphodiesterase activity and its activating factor (PAF) from brain. *Biochem. Biophys. Res. Commun.* 41: 1104~1110, 1970
- 5) CHEUNG, W. Y.: Cyclic 3',5'-nucleotide phosphodiesterase: Demonstration of an activator. *Biochem. Biophys. Res. Commun.* 38: 533~538, 1970
- 6) NAKANISHI, S.; K. YAMADA, I. KAWAMOTO, K. ANDO, H. SANO, T. YASUZAWA & H. KASE (Kyowa Hakko):

- A novel substance KS-501. Jpn. Kokai 222189 ('88), Sept. 16, 1988
- 7) NAKANISHI, S.; K. YAMADA, I. KAWAMOTO, K. ANDO, H. SANO, T. YASUZAWA & H. KASE (Kyowa Hakko): A novel substance KS-502. Jpn. Kokai 222190 ('88), Sept. 16, 1988
  - 8) NAKANISHI, S.; K. YAMADA, I. KAWAMOTO, K. ANDO, H. SANO, T. YASUZAWA & H. KASE (Kyowa Hakko): Novel substances KS-501 and KS-502 and process for their preparation. Eur. Pat. Apr. 282322, Sept. 14, 1988
  - 9) KASE, H.; K. IWAHASHI & Y. MATSUDA: K-252a, a potent inhibitor of protein kinase C from microbial origin. J. Antibiotics 39: 1059~1065, 1986
  - 10) KAKIUCHI, S.; R. YAMAZAKI, Y. TESHIMA, K. UENISHI & E. MIYAMOTO: Multiple cyclic nucleotide phosphodiesterase activities from rat tissues and occurrence of calcium-plus-magnesium-ion-dependent phosphodiesterase and its protein activator. Biochem. J. 146: 109~120, 1975
  - 11) KIKKAWA, U.; Y. TAKAI, R. MINAKUCHI, S. INOHARA & Y. NISHIZUKA: Calcium-activated, phospholipid-dependent protein kinase from rat brain. J. Biol. Chem. 257: 13341~13348, 1982
  - 12) YAGINUMA, S.; M. AWATA, M. TAKADA & K. KINOSHITA (Toyo Jozo): Cyclic adenosine-3',5'-monophosphate phosphodiesterase inhibitors. Jpn. Kokai 215551 ('87), Sept. 22, 1987
  - 13) LEVIN, R. M. & B. WEISS: Selective binding of antipsychotics and other psychoactive agents to the calcium-dependent activator of cyclic nucleotide phosphodiesterase. J. Pharmacol. Exp. Ther. 208: 454~459, 1979
  - 14) TANAKA, T.; T. OHMURA, T. YAMAKADO & H. HIDAKA: Two types of calcium-dependent protein phosphorylations modulated by calmodulin antagonists. Mol. Pharmacol. 22: 408~412, 1982
  - 15) WATANABE, K. & W. L. WEST: Calmodulin, activated cyclic nucleotide phosphodiesterase, microtubules and vinca alkaloids. Fed. Proc. 41: 2292~2299, 1982
  - 16) GIETZEN, K.; A. WUTHRICH & H. BADER: Effects of microtubular inhibitors on plasma membrane dependent  $Ca^{2+}$ -transport ATPase. Mol. Pharmacol. 22: 413~420, 1982
  - 17) DEGRADO, W. F.; F. G. PRENDERGAST, H. W. WOLFE, Jr. & J. A. COX: The design, synthesis, and characterization of tight-binding inhibitors of calmodulin. J. Cell. Biochem. 29: 83~93, 1985
  - 18) GIETZEN, K.; A. WUTHRICH & H. BADER: R-24571, a new powerful inhibitor of red blood cell  $Ca^{2+}$ -transport ATPase and of calmodulin-regulated functions. Biochem. Biophys. Res. Commun. 101: 418~425, 1981
  - 19) VAN BELLE, H.: R-24571, a potent inhibitor of calmodulin-activated enzymes. Cell Calcium 2: 483~494, 1981
  - 20) GIETZEN, K.; P. ADAMCZYK-ENGELMAN, A. WUTHRICH, A. KONSTANTINOVA & H. BADER: Compound 48/80 is a selective and powerful inhibitor of calmodulin-regulated functions. Biochim. Biophys. Acta 736: 109~118, 1983
  - 21) MATSUDA, Y.; S. NAKANISHI, K. NAGASAWA, K. IWAHASHI & H. KASE: The effect of K-252a, a potent microbial inhibitor of protein kinase, on activated cyclic nucleotide phosphodiesterase. Biochem. J. 256: 75~80, 1988