KS-619-1, A NEW INHIBITOR OF Ca^{2+} AND CALMODULIN-DEPENDENT CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM *STREPTOMYCES CALIFORNICUS*

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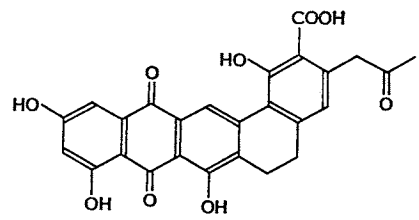
KS-619-1, a new inhibitor of Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase, was isolated from the cultured broth of *Streptomyces californicus*. KS-619-1 has an anthraquinone moiety. IC_{50} values for the effect of KS-619-1 on Ca^{2+} and calmodulin-stimulated activity of bovine brain and heart enzymes were 2.0 and 1.5 μM, 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 KS-619-1 to a lesser extent with IC_{50} values; 12.3, 25.9 and 151 μM, respectively.

The calcium ion plays a crucial role as a second messenger in various biological events[1]. Evidence has been accumulating to suggest many of the physiological functions of Ca^{2+} may be mediated by Ca-receptor proteins such as calmodulin (CaM). CaM indeed activates a number of enzymes in a Ca^{2+}-dependent manner[2,3]. Among enzymes regulated by CaM is a cyclic nucleotide phosphodiesterase[4,5].

In a previous paper[6], we showed that K-259-2 was isolated from the cultured broth of *Micromonospora olivasterospora* as a novel inhibitor of Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase (CaM-PDE). In this article, we describe the fermentation, isolation, physico-chemical properties and biochemical properties of another new potent CaM-PDE inhibitor from *Streptomyces californicus*. The compound, named KS-619-1, was active in the micromolar range, and its structure was determined to be **1** (Fig. 1), which also contained the anthraquinone moiety like K-259-2. Subsequent paper[7] will elucidate studies on the structural determination.

Fig. 1. The structure of KS-619-1 (**1**).



Materials and Methods

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 $CaCO_3$ 0.2% (pH 7.2 before sterilization) and inoculated with a loopful of spores of the microorganism, *Streptomyces californicus* KY 619 (ATCC 3312), 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 24 hours on a reciprocating shaker (300 rpm) at 28°C. An 10%-inoculum 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 glycerol 3.0%, beef extract 3.0%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.03% and $CaCO_3$ 0.3% (pH 6.5 after sterilization). This production medium was incubated for 5 days on a rotary shaker (200 rpm) at 28°C. For inoculation of 18 liters of the 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 24 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 4 days at 28°C with agitation at 300 rpm and aeration of 18 liters/minute. The growth was monitored by packed cell volume (PCV). KS-619-1 was produced in broth filtrate, and its production was traced by measuring the inhibitory activity of CaM-PDE. The amount of KS-619-1 was determined spectrophotometrically using pure KS-619-1 as a standard on TLC plate (Silica gel 60, Merck, 5628) developed with BuOH - EtOH - $CHCl_3$ - NH_4OH (4 : 5 : 2 : 3). The plate was scanned at the wavelength of 460 nm with a Shimadzu Dual-wavelength TLC Scanner CS900.

Enzyme Assay

CaM-PDE and its basal (in the presence of ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA) instead of Ca^{2+}/CaM) activities, CaM-independent PDE activity, and protein kinase C activity were measured as described previously[8].

Materials

Bovine brain CaM-PDE and CaM were prepared according to the method of KAKIUCHI *et al.*[9] with some modifications[8]. Protein kinase C was prepared from rat brain according to the method of KIKKAWA *et al.*[10] with some modifications as described in a previous paper[8]. Bovine heart CaM-PDE and CaM-independent PDE, cAMP, and 5'-nucleotidase (*Crotalus atrox* venom) were purchased from Sigma Chemical Co. All other reagents were reagent grade and commercially available.

Results

Production of KS-619-1 by Fermentation

Fig. 2 shows a time course of KS-619-1 production by the producing microorganism in 30-liter jar fermentor. The production of KS-619-1 gradually increased during growth phase and was rapidly increased on day 4 after the cell growth reached the maximum. The effect of carbon sources and natural nitrogen sources on the production of KS-619-1 were investigated (Table 1-A and -B). The additions of glycerol as a carbon source and beef extract as a nitrogen source were the most influencial to obtain the best titers for the production of KS-619-1. Numerous attempts to increase the biosynthesis of KS-619-1 were conducted, and the resultant defined medium and the optimum conditions for the production were described under Materials and Methods.

Fig. 2. The biosynthesis of KS-619-1 by *Streptomyces californicus*.

The production indicates the amount of KS-619-1 in broth filtrate.

● KS-619-1, ■ packed cell volume (PCV), ▲ pH.

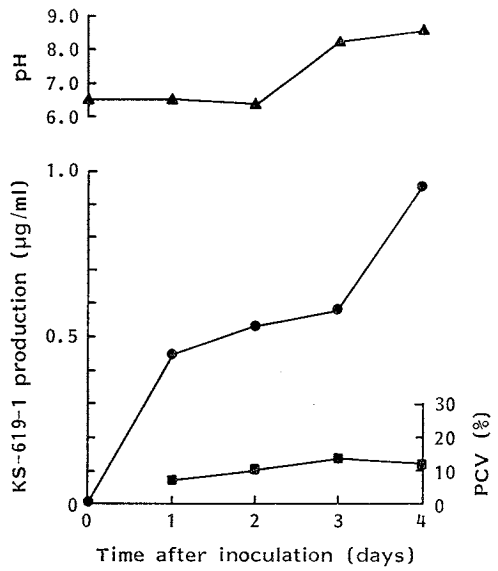


Table 1. Effect of carbon sources and natural nitrogen sources on the production of KS-619-1.

A)

Carbon sources[a] (%)	KS-619-1 production[b] (μg/ml)
Glycerol 3	0.29[c]
Maltose 3	0.02
Lactose 3	0.01
Sucrose 3	0.04
Glycerol 2 and dextrin 2	0.16
Galactose 2 and dextrin 2	0.04

B)

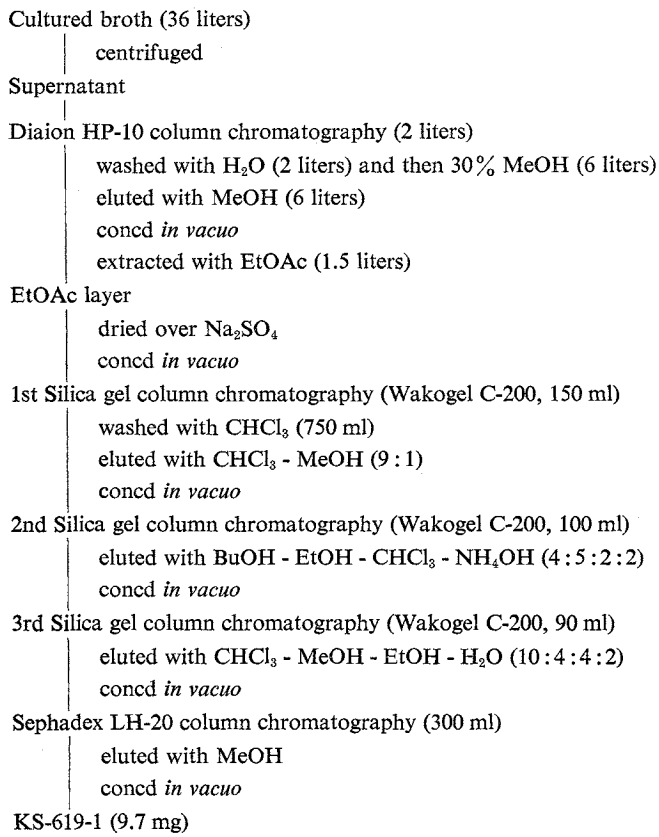
Nitrogen sources[a] (%)	KS-619-1 production[b] (μg/ml)
Beef extract 3	0.29[c]
Meat extract 0.5, Polypepton 0.5 and yeast extract 1	0.14
Dried yeast 3	0.14
Yeast extract 0.5 and casamino acids 0.5	0

[a] Glycerol (3%) or beef extract (3%) in the control medium were replaced by 3% each of various carbon sources (A) or natural nitrogen sources (B), respectively.

[b] Fermentation was carried out using 300-ml Erlenmeyer flask on a rotary shaker (200 rpm) at 28°C for 5 days.

[c] Data obtained from several experiments.

Fig. 3. Purification of KS-619-1.



Isolation and Purification

Isolation procedure is outlined in Fig. 3. The fermentation broth (36 liters) was centrifuged with Sharples centrifuge. The supernatant was then applied onto a column of Diaion HP-10 (Mitsubishi Chemical Industries Ltd.). The column was washed with water and then 30% methyl alcohol successively, and adsorbed material was eluted with methyl alcohol. The eluate was concentrated *in vacuo*,

and extracted with ethyl acetate. The ethyl acetate layer was dried over sodium sulfate and then concentrated *in vacuo* to yield oily brown material (2.0 g). This oily material was applied to silica gel column chromatography. Fractions containing crude KS-619-1 were pooled and concentrated down to an oil (130 mg). The residue was fractionated on second silica gel column chromatography. Fractions containing KS-619-1 was collected and concentrated *in vacuo* to yield oily material (71 mg). The resultant oily material was further applied to third silica gel column. The appropriate fractions were pooled and evaporated to dryness (57 mg). Final purification of the compound was achieved by Sephadex LH-20 (Pharmacia Fine Chemicals) column. Fractions containing KS-619-1 were combined and evaporated to yield a dark red powder (9.7 mg).

Physico-chemical Properties

Physico-chemical properties of KS-619-1 are summarized in Table 2. KS-619-1 was obtained as orange powder, mp 198~200°C (dec). It is soluble in dimethyl sulfoxide and acetic acid,

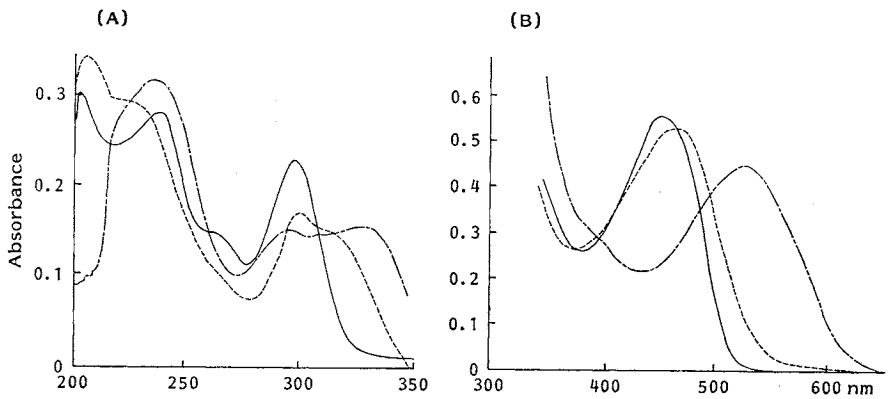
Table 2. Physico-chemical properties of KS-619-1.

Appearance	Dark red powder
MP (°C, dec)	198~200
TLC* (Rf)	
$CHCl_3$ - MeOH - EtOH - H_2O (10 : 4 : 4 : 2)	0.48
BuOH - EtOH - $CHCl_3$ - NH_4OH (4 : 5 : 2 : 3)	0.33
Color reaction	
Positive	$FeCl_3$, I_2, anisaldehyde
Solubility	
Soluble	AcOH, DMSO
Slightly soluble	MeOH, EtOH, PrOH, BuOH, $(CH_3)_2CO$, EtOAc, CH_3CN, pyridine, THF
Insoluble	$CHCl_3$, H_2O
Absorption λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	
Neutral	225 (816), 302 (587), 317 (542), 470 (339)
Acidic	240 (761), 265 (491), 300 (681), 345 (236), 450~475 (330)
Alkaline	242 (877), 298 (540), 330 (721), 390~410 (250), 515 (340)

* Silica gel 60 (Merck, 5631).

Fig. 4. Absorption spectra of KS-619-1.

KS-619-1 (0.04 mg/ml) was dissolved into MeOH (- - -), MeOH+0.1 N HCl (——), and MeOH+0.1 N NaOH (— · —). (A) UV spectra (2.8 μg/ml of KS-619-1), (B) visible spectra (15 μg/ml of KS-619-1).



slightly soluble in the lower alcohols, and virtually insoluble in chloroform and water. Its absorption spectra (Fig. 4) in UV and visible regions shift with pH. The Rf values of the compound on silica gel TLC developed in various solvent systems and its color reactions are also presented in Table 2. The structure of KS-619-1 was determined to be **1** (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 the effect of various concentration of KS-619-1 on the activity of CaM-PDE from bovine brain. KS-619-1 inhibited the stimulated activity by Ca^{2+}/CaM in a concentration-dependent manner, whereas it showed a less degree of inhibitory effect on its basal activity (the activity in the presence of EGTA instead of Ca^{2+}/CaM); IC_{50} values (the concentration producing 50% inhibition) under present assay conditions were 2.0 and 12.3 μM, respectively. Table 3 summarizes the effect of KS-619-1 on the activity of several cyclic nucleotide phosphodiesterases. KS-619-1 also inhibited CaM-PDE from bovine heart in the same concentration range (IC_{50} value, 1.5 μM) as the case of the bovine brain enzyme. In addition, CaM-independent PDE from bovine heart was only weakly inhibited by KS-619-1; the IC_{50} value was 25.9 μM. The inhibitory activity of KS-619-1 for protein kinase C (IC_{50} value, 151 μM) was much less than that for CaM-PDE. Taken together, the results presented here demonstrate that KS-619-1 is a novel potent inhibitor of CaM-PDE.

Fig. 5. Inhibition by KS-619-1 of bovine brain Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase.

Phosphodiesterase activity was measured as described in a previous paper[8] with various concentrations of KS-619-1 added as indicated. For the assay of Ca^{2+}/CaM-stimulated activity (●), the reaction mixture contained, in a final volume of 0.5 ml, 80 mM imidazole-HCl buffer (pH 6.9), 3 mM $MgSO_4$, 0.3 mM dithiothreitol, 100 mM NaCl, 1.2 mM cAMP, 50 μM $CaCl_2$, 26 mU/ml bovine brain CaM-PDE, and 4 U/ml CaM. The basal activity (○) was determined using a large amount of the enzyme (18.6 mU/ml) to magnify PDE activity in the presence of 3 mM EGTA instead of Ca^{2+}/CaM.

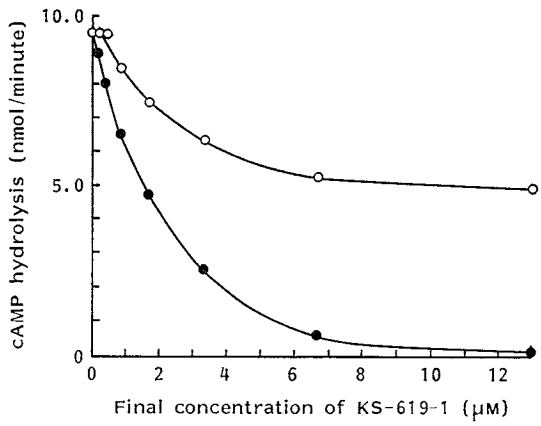


Table 3. Effect of KS-619-1 on cyclic nucleotide phosphodiesterase.

Enzymes		CaM	IC_{50} (μM)
Bovine brain	CaM-PDE	+[a]	2.0
		−[b]	12.3
Bovine heart	CaM-PDE	+[c]	1.5
Bovine heart	CaM-independent PDE	−[d]	25.9

[a] The activity in the presence of 4 U/ml calmodulin, and 50 μM $CaCl_2$. The enzyme concentration was 26 mU/ml.

[b] The activity in the presence of 3 mM ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA), without $CaCl_2$ and calmodulin. The enzyme concentration was 18.6 mU/ml.

[c] The activity in the presence of 2.5 U/ml calmodulin, and 50 μM $CaCl_2$. The enzyme concentration was 40 mU/ml.

[d] The activity in the presence of 3 mM EGTA, without $CaCl_2$ and calmodulin. The enzyme concentration was 25 mU/ml.

CaM: Calmodulin. CaM-PDE: Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase.

KS-619-1 at 100 μg/ml exhibited no antimicrobial activity against *Candida albicans* KY 5011, *Enterococcus faecalis* KY 4280, *Pseudomonas aeruginosa* KY 4276, *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, but it showed only a weak antimicrobial activity at 50 μg/ml against *Staphylococcus aureus* KY 4779.

Discussion

Several CaM-dependent enzymes can be inhibited by a wide range of chemically unrelated substances *in vitro*[11]; these include phenothiazines[12], naphthalensulfonamide compounds (*e.g.*, W-7)[13], alkaloids (*e.g.*, vinblastine)[14,15], local anesthetics[16], and antimycotic agents (*e.g.*, calmidazorium)[17,18].

Because almost all CaM antagonists presently available interact with lipid[19], they also inhibit protein kinase C, which is thought to regulate a variety of cellular responses[20,21], in the similar concentration range[22]. Among these so-called CaM antagonists is adriamycin, an anthracycline-aminoglycoside antitumor antibiotic; it has also an anthraquinone moiety. Adriamycin inhibits weakly CaM-activated protein kinase (IC_{50} value, 50~85 μM[23]) or other CaM functions (30~300 μM[24]) by acting at a hydrophobic region of CaM, and it also antagonizes protein kinase C at the similar concentration[23]. Adriamycin inhibited CaM-PDE activity only weakly under the present assay conditions described above (IC_{50} value, 700 μM, data not shown), whereas the novel anthraquinone metabolite, named KS-619-1, inhibited this activity intensely (IC_{50} value, 2.0 μM). The ability to inhibit the CaM action of adriamycin is therefore much less than that of KS-619-1. Furthermore, it is noteworthy of comment that the IC_{50} value for the effect of KS-619-1 on protein kinase C was 151 μM, which is two orders of magnitude higher than that for CaM-PDE.

Experiments to be described elsewhere have shown that well-known CaM antagonists, if not all, interact with amphipathic (hydrophobic plus anionic) compounds such as acidic phospholipids as discussed above in contrast with KS-619-1, which does not interact. To clarify whether or not the strong inhibitory activity of KS-619-1 as compared with adriamycin can be explained by this non-interacting property with amphipathic compounds, detailed mechanisms by which KS-619-1 acts are now under investigations.

In conclusion, KS-619-1 is a novel potent inhibitor of CaM-PDE and its significant feature will be helpful to unravel the physiological functions of calmodulin in Ca^{2+}-messenger system.

Acknowledgment

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