KS-505a, A NOVEL INHIBITOR OF BOVINE BRAIN Ca^{2+} AND CALMODULIN-DEPENDENT CYCLIC-NUCLEOTIDE PHOSPHODIESTERASE FROM *Streptomyces argenteolus*

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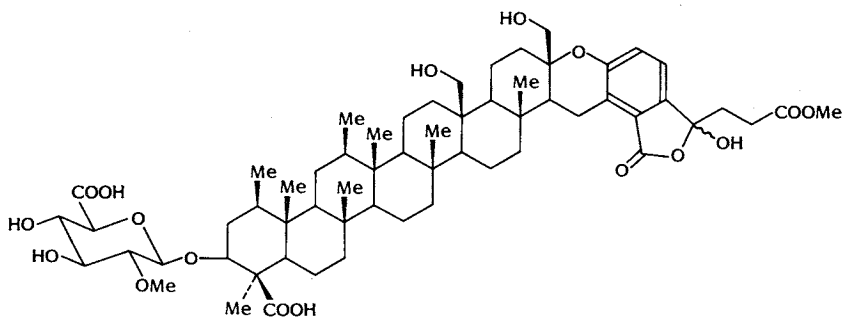
A novel compound, KS-505a was isolated from the culture broth of a strain identified as *Streptomyces argenteolus* A-2. The compound inhibited bovine brain Ca^{2+} and calmodulin-dependent cyclic-nucleotide phosphodiesterase with an IC_{50} value (the concentration causing 50% inhibition) of 0.065 μM. The compound around that concentration had little or no effect on heart calmodulin-dependent and -independent cyclic-nucleotide phosphodiesterases, and protein kinase C.

The role of 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[1]. Evidence has been accumulating to show that many functions of intracellular Ca^{2+} are mediated by calmodulin (CaM), a ubiquitous Ca^{2+}-binding protein. CaM has an ability to activate a variety of enzymes in a Ca^{2+}-dependent manner[2,3]. Among them is Ca^{2+} and CaM-dependent cyclic-nucleotide phosphodiesterase (CaM-PDE)[4,5].

During the course of our screening work to obtain CaM inhibitors from microorganisms, we found that a novel metabolite of *Streptomyces argenteolus* A-2 inhibited CaM-PDE. The compound, designated as KS-505a, was isolated from the cultured broth, and its quite new structure was determined (Fig. 1). In this article, we describe production, isolation and some biological properties of KS-505a. Structural elucidation studies will be reported in a separate paper.

Fig. 1. Structure of KS-505a.



1

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Materials and Methods

Microorganism and Taxonomy

Streptomyces argenteolus A-2 (FERM BP-2065) was isolated from a soil sample collected near Lake Shinji, Yamanashi, Japan. Most of the taxonomic studies of strain A-2 were carried out in accordance with the methods adopted by the International Streptomyces Project (ISP)[6]. Additional media recommended by WAKSMAN[7] were also used. The colors of cultures were determined by comparing them with the color chips from the Color Harmony Manual, 4th Ed.†.

Fermentation

A 50-ml culture tube containing 10 ml of a seed medium composed of glucose 1.0%, soluble starch 1.0%, Bacto-Tryptone (Difco) 0.5%, yeast extract (Daigo Eiyo Chemicals) 0.5%, beef extract (Kyokuto Pharmaceutical Industry Ltd.) 0.3%, and $CaCO_3$ 0.2% (pH 7.2 before sterilization) was inoculated with the mycelia of the strain grown on an agar slant. The inoculated tube was incubated for 7 days on a reciprocating shaker (300 rpm) at 28°C. 4 ml of the culture was transferred into a 300-ml Erlenmeyer flask containing 40 ml of the seed medium and the flask was incubated for 2 days on a rotary shaker (200 rpm) at 28°C. 36 ml of the second culture was further transferred into a 2-liter Erlenmeyer flask containing 360 ml of the seed medium and the flask was incubated for 2 days on a rotary shaker (200 rpm) at 28°C. Five flasks of the third culture were inoculated to a 30-liter jar fermenter containing 18 liters of a fermentation medium composed of glucose 0.5%, maltose 4.0%, 3-(*N*-morpholino)propanesulfonic acid 1%, $MgSO_4 \cdot 7H_2O$ 0.05%, soybean meal 1.5%, Pharmamedia 1.5%, and $CaCO_3$ 0.5% (pH 7.0 before sterilization). Fermentation was carried out for 5 days at 28°C with agitation at 300 rpm, and aeration of 18 liters/minute. During isolation processes KS-505a was detected on a silica gel TLC plate (Merck, Art. No. 5715) developed with $CHCl_3$ - MeOH - EtOH - H_2O (10 : 4 : 4 : 2) and visualized under UV-light.

Enzyme Assay

The activity of bovine brain CaM-PDE was measured as described in a previous paper[8]. 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 $CaCl_2$, varied concentrations of KS-505a, 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 the activity in the presence of 3 mM ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA) instead of Ca^{2+} and CaM. The activity of bovine heart CaM-PDE in the presence of Ca^{2+} and CaM was assayed at the enzyme concentration of 40 mU/ml. The activity of bovine heart CaM-independent cyclic-nucleotide phosphodiesterase (CaM-independent PDE) was determined in the presence of EGTA at enzyme concentration of 25 mU/ml. The assay conditions of protein kinase C were described previously[8].

Materials

Bovine brain CaM-PDE and CaM were prepared accoding 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[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 commercially available and reagent grade.

Results

Taxonomy

The cultural characteristics of strain A-2 on various agar media are summarized in Table 1. The colors of aerial masses belonged to the gray color-series, whereas the reverse side of the growth was colorless to pale yellow (no distinctive pigments). No distinct diffusible pigment was produced except for

† E. JACOBSON *et al.*, Container Corporation of America, Chicago, 1958.

Table 1. Cultural characteristics of *Streptomyces argenteolus* A-2.

Medium	Growth	Color		Diffusable pigment
		Aerial mycelium	Reverse side of colony	
Glucose - asparagine	Moderate	Pearl (3ba)	Yellow tint (1ba) to cream (1½ca)	None
Glycerol - asparagine	Moderate	Pearl (3ba)	Cream (1½ca)	Light yellow
Sucrose - nitrate	Moderate	Pearl (3ba)	Ivory tint (2cb)	None
Salts - starch	Moderate	Pearl (3ba)	Shell (3ca)	None
Tyrosine	Moderate	Pearl (3ba)	Shell (3ca)	None
Nutrient	Moderate	Sand (3cb)	Cream (1½ca) to bamboo (2gc)	None
Yeast extract - malt extract	Good	Sand (3cb)	Clove brown (3ni) to cinnamon (3le)	None
Oatmeal	Moderate	Sand (3cb)	Colorless	None
Peptone - yeast extract - iron	Good	Natural (3dc)	Light amber (3ic)	None
HICKEY-TRESNER's	Moderate	Pearl (3ba)	Bamboo (2gc) to light ivory (2ca)	None

light yellow color in glycerol-asparagine agar. The spore chains consisting of 20 or more spores were born from aerial mycelium, and were spiral. The matured spores were spherical or oval, and 0.7 to 0.9 by 1.0 to 1.1 μm in size, with smooth surfaces. Fragmentation of the substrate mycelium, sclerotium formation, sporangium formation, and flagellated spores were not observed on any of the agar media used in this study. Good growth was observed with such carbohydrates as D-xylose, D-glucose, D-fructose, sucrose, L-rhamnose, or D-mannitol, in PRIDHAM and GOTTLIEB's basal medium, but no growth was obtained with L-arabinose, *i*-inositol, and raffinose. The negative physiological properties of strain A-2 included liquefaction of gelatin, peptonization and coagulation of skim milk, formation of tyrosinase, production of melanoid, and decomposition of cellulose. Starch hydrolysis was positive. The optimal growth was observed between pH 6.5 and pH 7.8, and between 28°C and 35°C. The whole cell hydrolysates contained the LL-isomer of diaminopimelic acid.

The morphological and chemotaxonomic characteristics of strain A-2 place it in the genus *Streptomyces* WAKSMAN and HENRICI. Strain A-2 was compared with the known *Streptomyces* species described in Approved Lists of Bacterial Names[11] and in the lists of validly published names. *Streptomyces argenteolus* ATCC 11009 was closely related to strain A-2 based on the following; grayish aerial mycelium, spiral spore chains, spores with smooth surfaces, no production of melanoid and distinct diffusible pigments, and utilization pattern of carbohydrates except L-arabinose and sucrose. On the basis of these results, strain A-2 is considered to be a strain of *Streptomyces argenteolus* TRESNER *et al.* 1961[12,13].

Fermentation

Time course of KS-505a production by *S. argenteolus* A-2 in a 30-liter jar fermenter is shown in Fig. 2. KS-505a was produced mainly in culture supernatant. The amount of KS-505a significantly increased from 3 days after inoculation, and reached miximum in 5~6 days.

Isolation and Purification of KS-505a

The purification procedure of the compound is outlined in Fig. 3. The culture broth (36 liters) was centrifuged in a Sharples centrifuge. The supernatant was applied to a Diaion HP-20 resin column (2 liters)

Fig. 2. Time course of KS-505a production in a 30-liter jar fermenter.

● Inhibition %, ■ pH, ▲ packed cell volume (PCV).

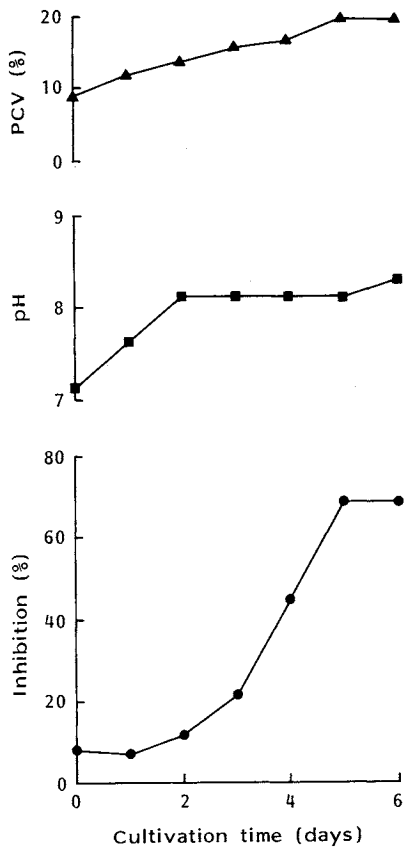

Fig. 3. Purification of KS-505a.

Culture broth (36 liters)
- centrifuged

Supernatant
- Diaion HP-20 column chromatography (2 liters)
  - washed with H_2O, 50% MeOH, and MeOH (6 liters each)
  - eluted with 1% conc NH_4OH-MeOH (10 liters)
- silica gel column chromatography
  - (Wakogel C-200, 500 ml)
  - eluted with solvent A (1.5 liters)
- silica gel column chromatography
  - (Wakogel C-200, 200 ml)
  - eluted with solvent A (600 ml)
- extracted with EtOAc (900 ml) at pH 2

Brown solid (266 mg)
- silica gel column chromatography
  - (Wakogel C-300, 50 ml)
  - eluted with solvent A (150 ml)
- silica gel column chromatography
  - (Lichroprep Si60, size B)
  - eluted with solvent A (1,000 ml) and solvent B (500 ml)

Pale yellow powder (56 mg)
- HPLC (Radial-Pak™ column packed with Nova-Pak (ODS, 4 μm))
  - eluted with MeOH-0.2 M ammonium acetate (pH 7.0) (7:3)

White powder
- dissolved in 1 N HCl
- extracted with EtOAc

KS-505a (white powder, 33 mg)

Solvent A: $CHCl_3$-MeOH-EtOH-H_2O (10:4:4:1)
Solvent B: $CHCl_3$-MeOH-EtOH-H_2O (10:4:4:2)

and washed with water, 50% methanol, and then methanol. The column was eluted with methanol containing 1% conc ammonia water. The eluate was concentrated *in vacuo* to yield a brown oil (2.7 g). The oil was applied to silica gel column chromatography, and the column was eluted with chloroform-methanol-ethanol-water (10:4:4:1, solvent A). The fractions containing KS-505a were collected and concentrated *in vacuo*. This chromatography step was repeated twice. The material thus obtained was suspended in 10% methanol-water, adjusted to pH 2 with 2 N HCl, and then extracted with ethyl acetate. The organic solvent layer was dried and evaporated *in vacuo* to yield a light brown solid. The solid was further purified by repeated silica gel column chromatography with Wakogel C-300 and Lichroprep Si60. The fractions containing KS-505a were collected and concentrated *in vacuo* to yield a pale yellow powder. Subsequent preparative reversed phase HPLC of this material was performed on a Radial-Pak™ column (8 × 100 mm) packed with Nova-Pak (ODS, 4 μm, Waters) using methanol-0.2 M ammonium acetate buffer (pH 7.0) (7:3) as isocratic mobile phase at the flow rate of 3.0 ml/minute. The fractions containing KS-505a were concentrated *in vacuo* and then freeze-dried to obtain a white powder. The

Table 2. Physico-chemical properties of KS-505a.

Appearance	White powder
TLC, Rf	
$CHCl_3$-MeOH-EtOH-H_2O (10:4:4:2)[a]	0.23
$CHCl_3$-MeOH-EtOH-H_2O-AcOH (10:4:4:1:1)[a]	0.44
$CHCl_3$-MeOH-EtOH-H_2O-conc NH_4OH (10:4:4:1:1)[a]	0.07
70% MeOH[b]	0.38
Color reaction	
Positive	I_2, H_2SO_4, anisaldehyde, bromocresol green
Negative	Ninhydrin, $FeCl_3$, dinitrophenylhydrazine, Rydon-Smith, Dragendorff, anilin-phthalate
Melting point	Indefinite (gradually decomposed)
$[\alpha]_D^{25}$[c]	−63.5° (*c* 0.1, MeOH)
Solubility	
Soluble	MeOH, DMSO, EtOAc, alkaline water
Slightly soluble	Hexane, $CHCl_3$, acidic water
UV absorption, λ_{max} nm (log ε)	221 (4.47), 308 (3.63)
IR (KBr, cm^{-1})	3450, 2950, 1730, 1715, 1460, 1385, 1270, 1240, 1120,1045

[a] HPTLC plate silica gel $60F_{254}$ (Merck, Art. No. 5628).
[b] HPTLC plate RP-$18F_{254}$s (Merck, Art. No. 13724).
[c] Measured immediately after dissolving in MeOH.

powder was dissolved in 1 N HCl, and extracted with ethyl acetate. The organic solvent layer was washed with saturated sodium chloride solution, dried over anhydrous magnesium sulfate, and concentrated to dryness. KS-505a was obtained as white powder (33 mg).

Table 3. Effects of KS-505a on various cyclic-nucleotide phosphodiesterases.

Enzyme	CaM	IC_{50} (μM)
Bovine brain CaM-PDE	+[a]	0.065
	−[b]	>10
Bovine heart CaM-PDE	+[a]	1.4
Bovine heart CaM-independent PDE	−[b]	>10

[a] In the presence of Ca^{2+} and CaM.
[b] In the presence of EGTA instead of Ca^{2+} and CaM.

Physico-chemical Properties

Physico-chemical properties of KS-505a are summarized in Table 2. The structure of KS-505a was elucidated to be **1** (Fig. 1), which equilibrated in solution to its chemically equivalent *keto*-carboxylic acid structure, on the basis of physico-chemical analysis and various spectral data. Details of structural elucidation studies will be described in a separate paper.

Biochemical Properties

KS-505a inhibited CaM-dependent activity of brain CaM-PDE in a dose-dependent manner with an IC_{50} value of 0.065 μM. The IC_{50} values of the compound for various phosphodiesterase activities are summarized in Table 3. Among four CaM-dependent and -independent activities examined, KS-505a inhibited CaM-dependent activity of brain CaM-PDE most strongly. KS-505a at 10 μM had no effect on the activity of protein kinase C.

KS-505a had no antimicrobial activity at 100 μg/ml against *Staphylococcus aureus* ATCC 6538P, *Streptococcus faecium* ATCC 10541, *Bacillus subtilis* No. 10707, *Escherichia coli* ATCC 26, *Klebsiella pneumoniae* ATCC 10031, *Proteus vulgaris* ATCC 6897, *Shigella sonnei* ATCC 9290, *Salmonella typhi* ATCC 9992, *Pseudomonas aeruginosa* BMH No. 1, and *Candida albicans* ATCC 10231.

Discussion

In this paper, we demonstrate that a novel compound, KS-505a, isolated from *Streptomyces argenteolus* is a potent inhibitor of bovine brain CaM-PDE. The structure of KS-505a is found to be quite novel by searching Chemical Abstracts database. There has been no compound reported containing the following structural moieties of KS-505a; 2-*O*-methylglucuronic acid and decacyclic isoprenoid skeleton, and only a few compounds containing tautomeric aromatic γ-hydroxy-γ-lactone have been reported.

KS-505a inhibited CaM-dependent activity of brain CaM-PDE, but not its basal activity, suggesting that the compound binds to CaM or CaM binding site of the enzyme to inhibit the CaM-dependent activity. A variety of compounds have been found to inhibit CaM-dependent enzymes. These compounds include phenothiazines[14], naphthalenesulfonamides[15], alkaloids[16,17], peptides[18], antimycotic agents[19,20], and others[21~24]. KS-505a is more potent than calmidazorium (IC_{50} for brain CaM-PDE was 0.12 μM under our conditions), which was the most potent inhibitor among them.

KS-505a also inhibited bovine heart CaM-PDE. However, its IC_{50} value for heart enzyme was much higher than that for brain enzyme. Several investigators reported that bovine brain and heart enzymes are different molecules[25]. The brain isozymes possess two distinct subunit molecular weights of 61 kdaltons and 63 kdaltons, whereas the heart isozyme possesses a molecular weight of 59 kdaltons. The selectivity of KS-505a for brain enzyme suggests that the compound distinguishes the structural differences between the brain enzyme and the heart enzyme.

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