GRISEOLIC ACID, AN INHIBITOR OF CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE PHOSPHODIESTERASE

I. TAXONOMY, ISOLATION AND CHARACTERIZATION

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(Received for publication March 30, 1985)

Griseolic acid, a potent inhibitor of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) phosphodiesterase (EC 3.1.4.17) was isolated from the cultured broth of *Streptomyces griseoaurantiacus* SANK 63479. Griseolic acid has an adenine moiety and two carboxyl groups and possesses the molecular formula $C_{14}H_{13}N_5O_8$. Griseolic acid inhibited cyclic AMP phosphodiesterase competitively with regard to cyclic AMP, the substrate and the resulting *Ki* value was 0.26 μM. Griseolic acid showed the most potent inhibitory effect on rat aorta cyclic AMP phosphodiesterase among the enzymes of several rat organs tested. The plasma level of cyclic AMP was increased when griseolic acid was subcutaneously injected rats.

Cyclic AMP plays an important role in the metabolism of mammalian cells as a second messenger mediating the action of various hormones and is thought to be concerned with the control of many cellular functions[1,2,3]. We reported in a previous paper the isolation of terferol, an inhibitor of cyclic AMP phosphodiesterase, from cultured broth of *Streptomyces showdoensis* SANK 65080[4]. In further screening for cyclic AMP phosphodiesterase inhibitors we found griseolic acid, a new potent inhibitor, in a culture filtrate of *S. griseoaurantiacus* SANK 63479.

This paper deals with the taxonomy of the producing organism, production, isolation, characterization, and biological activities of griseolic acid.

Materials and Methods

Taxonomic Studies

The producing organism, strain SANK 63479 was isolated from a soil sample collected in Kyoto-fu, Japan. Morphological and physiological properties of the organism were determined according to SHIRLING and GOTTLIEB[5], along with several supplementary tests. Observation of the culture was made after incubation at 28°C for 2 weeks, unless otherwise mentioned. Color names were assigned according to "Guide to Color Standard" (a manual published by Nippon Shikisai Kenkyusho, Tokyo). The characteristics of the organism were compared with those of the known species of actinomycetes described in "The Actinomycetes, Vol. 2" by WAKSMAN, the "ISP Report" by SHIRLING and GOTTLIEB"[6], "BERGEY's Manual of Determinative Bacteriology (8th edition)" and other recent references on the taxonomy of the family *Streptomycetaceae*.

Medium

The medium used for the production of the inhibitor was composed of glucose 5.0%, soybean meal 1.0%, Polypepton 0.4%, meat extract 0.4%, yeast extract 0.1%, NaCl 0.25% and $CaCO_3$ 0.5%.

Assay of Cyclic AMP Phosphodiesterase Activity

The assay was performed according to the procedure reported in the previous paper[4]. Each of tissues from rats was homogenized using glass-glass or glass-teflon homogenizers with four volumes of cold 0.17 M Tris-HCl buffer, pH 7.4, containing 5 mM $MgSO_4$. The homogenate was then centrifuged at 100,000×*g* at 0°C for 1 hour. The clear supernatant solution was stored at −20°C and used as a source of cyclic AMP phosphodiesterase. Prior to use, this solution was diluted 100~150 times with 40 mM Tris-HCl buffer (pH 7.5). The reaction mixture (total volume, 0.1 ml) consisted of 40 mM Tris-HCl buffer (pH 7.5), 5 mM $MgSO_4$, 50 μM $CaCl_2$, 20 μg of snake venom (*Crotalus atrox*, Sigma), 0.14 μM [^{14}C]cyclic AMP, test material and the enzyme solution, was incubated at 30°C for twenty minutes. The enzyme preparation from rat brain was used for the screening of cyclic AMP phosphodiesterase inhibitors. The assay of calmodulin-dependent cyclic AMP phosphodiesterase from bovine brain[7] was performed according to the method of LIN *et al.*[8].

Results and Discussion

Taxonomic Studies

The aerial hyphae of strain SANK 63479 were monopodially branched and terminated in spirals. The mature spore chains were generally long with 10 to 50 or more spores per chain. The spores were oval in shape with smooth surface. The cultural characteristics of the organism on various agar media are shown in Table 1. The growth was very good on both the defined and the organic media and the color was pale yellowish brown to dull reddish orange. The aerial mycelium developed abundantly except nutrient agar, and was brownish white to gray in mass color. The physiological characteristics of the strain indicated positive for hydrolysis of starch, liquefaction of gelatin, reduction of nitrate and coagulation of milk, while negative for peptonization of milk and production of melanoid

Table 1. Cultural characteristics of strain SANK 63479.

Yeast extract - malt extract agar (ISP 2)	G:	Very good, dull reddish orange
	AM:	Abundant, gray
	SP:	None
Oatmeal agar (ISP 3)	G:	Very good, dull orange
	AM:	Abundant, gray
	SP:	None
Inorganic salts - starch agar (ISP 4)	G:	Very good, dull orange
	AM:	Abundant, brownish white
	SP:	None
Glycerol - asparagine agar (ISP 5)	G:	Very good, dull red
	AM:	Abundant, light brownish white
	SP:	None
Tyrosine agar (ISP 7)	G:	Very good, dull red
	AM:	Abundant, brownish white
	SP:	None
Sucrose - nitrate agar	G:	Good, dull reddish orange
	AM:	Abundant, light brownish white
	SP:	Pale purple (slight)
Glucose - asparagine agar	G:	Good, dull red
	AM:	Abundant, gray
	SP:	Pale purple (slight)
Nutrient agar (Difco)	G:	Good, pale yellowish brown
	AM:	Scarce, brownish white
	SP:	None

G: Growth, AM: aerial mycelium, SP: soluble pigment.

pigment. The strain utilized D-glucose, L-arabinose, D-fructose, L-rhamnose, galactose, mannose and D-mannitol, weakly utilized D-xylose and inositol, but not sucrose and raffinose.

These characteristics suggest that strain SANK 63479 belongs to the genus *Streptomyces*. Among known species of *Streptomyces*, *S. griseoaurantiacus* was selected as a closely related one. These two strains resembled each other in their morphological, cultural and physiological characteristics on various media.

From these results, it was concluded that strain SANK 63479 belonged to *S. griseoaurantiacus* and was designated as *S. griseoaurantiacus* (Krasil'nikov and Yuan) Pridham SANK 63479. It was deposited in the culture collection of Northern Regional Research Laboratories of the U.S. Department of Agriculture, Peolia, under accession number NRRL 12314.

Production and Isolation

The strain of *S. griseoaurantiacus* SANK 63479 was cultured in 30-liter jar fermentors at 28°C for two days with aeration (15 liters/minute) and agitation (200 rpm) to produce the inhibitor. An example of the time course of fermentation is shown in Fig. 1. The cultured filtrate (28 liters) was run through a Diaion HP 20 (Mitsubishi Chemical Industry) column, and run through effluent obtained was chromatographed on a Dowex 1X4 (Cl^-) column with 0.6 M sodium chloride, successively. The active fraction was adjusted to pH 2.5 with 1 N HCl, then chromatographed on a HP 20 column with 60% aqueous acetone. The active fraction was concentrated under reduced pressure and lyophilized to crude powder (250 mg). The powder was dissolved in a small amount of distilled water, and then chromatographed on a Sephadex LH-20 column with distilled water. The active eluate was concentrated under reduced pressure, and stored at 4°C overnight to produce a colorless crystalline griseolic acid (31 mg). The preparation showed a single spot on TLC (Merck Silica Gel 60 plate) with solvent systems butanol - acetic acid - water (4: 1: 1, Rf 0.04) and propanol - water (7: 3, Rf 0.36).

Physico-chemical Properties

The molecula formula of griseolic acid was determined as $C_{14}H_{13}N_5O_8$ by elemental analysis

Fig. 1. Time course of griseolic acid production by *Streptomyces griseoaurantiacus* SANK 63479.
The amount of mycelium is expressed as packed cell volume (ml) per 10 ml of the cultured broth after centrifugation at 3,000 rpm for 15 minutes.
● Potency, ▲ mycelium, ○ pH.

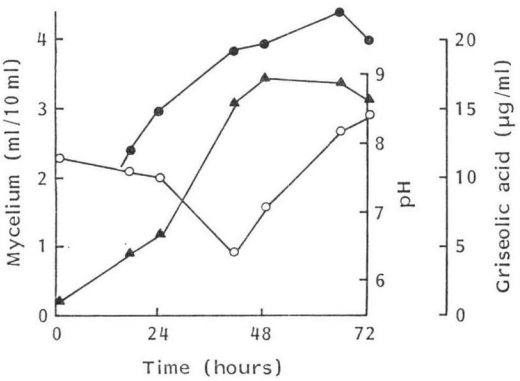


Fig. 2. UV absorption spectra of griseolic acid.

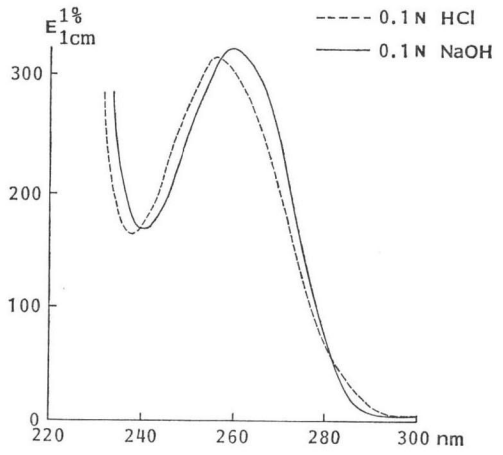


Fig. 3. IR absorption spectrum of griseolic acid (KBr pellet).

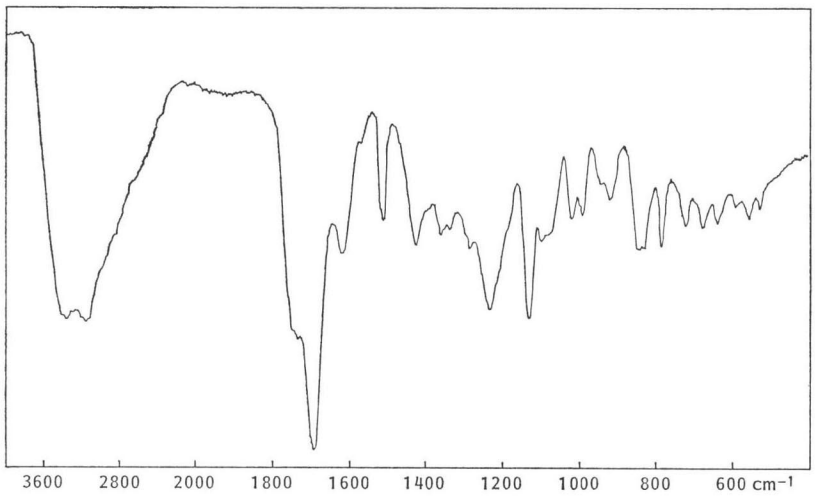


Fig. 4. ^{1}H NMR spectrum of griseolic acid (DMSO-d_6).

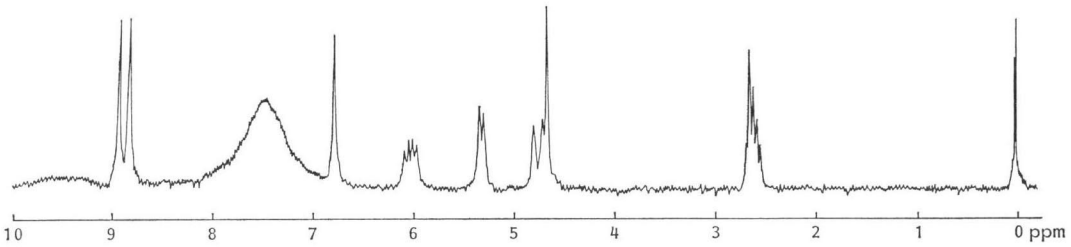
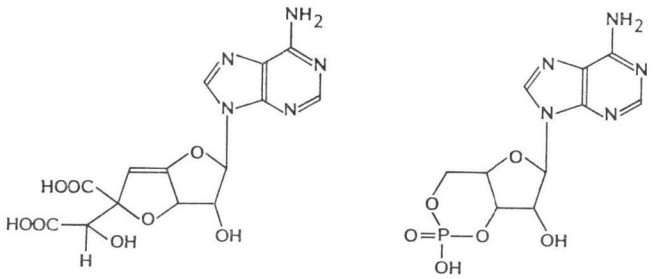


Fig. 5. Structure of griseolic acid.



(Found: C 43.68, H 3.72, N 18.09, Calcd: C 44.33, H 3.43, N 18.47) and by mass spectrum. Griseolic acid decomposed at 220°C, and showed optical rotation ($[\alpha]_D^{20}$ +6.9° (*c* 0.1, DMSO)). Griseolic acid has UV absorption maximum at 260 nm in 0.1 N sodium hydroxide solution, at 256 nm in 0.1 N hydrochloric acid solution (Fig. 2). The IR spectrum of griseolic acid is shown in Fig. 3. The ^{1}H NMR at 60 MHz spectrum is presented in Fig. 4. These spectra showed that griseolic acid was composed of an adenyl group, carboxyl groups and other components. As reported in the next paper, the structure of griseolic acid was determined by further studies of chemical degradation and X-ray analyses. The structure of griseolic acid is shown in Fig. 5.

Biological Activity

The concentration of griseolic acid required for half-maximal inhibition (IC_{50}) in our assay system toward cyclic AMP phosphodiesterases from various rat tissues is shown in Table 2.

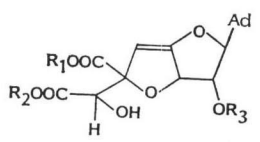
The type of inhibition is shown by the Dixon plots (1/V to inhibitor concentration) in Fig. 6. Inhibition by theophylline and papaverine, well-known cyclic AMP phosphodiesterase inhibitors, are known to be competitive and non-competitive inhibitors, respectively[9]. Inhibition by griseolic acid was found to be competitive with a *Ki* value of 0.26 μM. Griseolic acid has an adenyl group and two carboxyl groups. So it was presumed that these features in the molecule might cause the inhibitory effect. In order to clarify the role of the carboxyl groups, the activity of the inhibitor was examined after treatment with diazomethane to give esterification of the carboxyl groups. Griseolic acid (mono- and diester) had no significant inhibitory effect on cyclic AMP phosphodiesterase (Table 3). The presence of two carboxyl groups in griseolic acid were therefore necessary to inhibit the reaction of cyclic AMP phosphodiesterase. The role of adenyl group is still unclear but the inhibitory effect on the enzyme with regard to cyclic guanosine monophosphate (cyclic GMP) is weaker than with regard to cyclic AMP (Table 2). Therefore an adenyl group may have an important role in the inhibitory activity of the inhbitor. Fig. 7 shows the inhibitory effects of griseolic acid on calmodulin-dependent cyclic AMP phosphodiesterase from bovine brain with or without calmodulin. IC_{50} values of griseolic acid with or without calmodulin were 4.2 μM and 2.7 μM, respectively. Trifluoroperazine, a calmodulin antagonist, showed inhibitory effect on the enzyme only in the presence of calmodulin, and the IC_{50} value was 12.0 μM. These data showed that griseolic acid is a cyclic AMP phosphodiesterase inhibitor, but not a calmodulin antagonist.

The subcutaneous injection of griseolic acid into rats increased the plasma level of cyclic

Table 2. Inhibition by griseolic acid of cyclic nucleotide phosphodiesterases in various rat tissues.

Tissues	IC_{50} (μM)			
	Substrate (μM)			
	Cyclic AMP		Cyclic GMP	
	0.14	100	0.14	100
Brain	0.16	0.80	0.63	3.5
Aorta	0.031	0.51	0.24	3.4
Platelet	0.041	8.0	16.0	50.0
Kidney	0.12	0.58	0.71	1.1

Table 3. IC_{50} values of griseolic acid derivatives. Enzyme from rat brain was used for the assay of inhibitory activities.



	R_1	R_2	R_3	IC_{50} (μM)
Griseolic acid	H	H	H	0.16
Monoester	H	CH_3	H	13.0
Diester	CH_3	CH_3	H	>50.0
Trimethoxy	CH_3	CH_3	CH_3	>50.0
Monomethoxy	H	H	CH_3	1.48

Fig. 6. Dixon plot analysis of the effects of griseolic acid on cyclic AMP phosphodiesterase from rat brain.

Cyclic AMP △: 0.63 μM, ○: 0.84 μM, ■: 1.27 μM, ▲: 2.14 μM, ●: 4.14 μM.

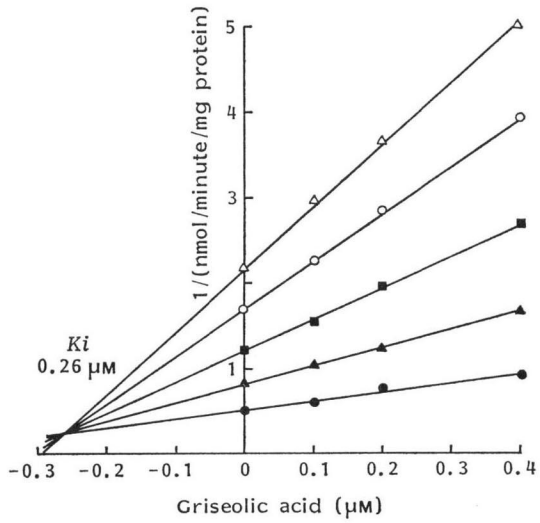


Fig. 7. Inhibition of calmodulin-dependent cyclic AMP phosphodiesterase by griseolic acid and trifluoroperazine with or without calmodulin.

The results are expressed as % of inhibition. The values are shown by symbols, ○, griseolic acid with 10 units calmodulin; ●, without calmodulin; △, trifluoroperazine with calmodulin; ▲, without calmodulin.

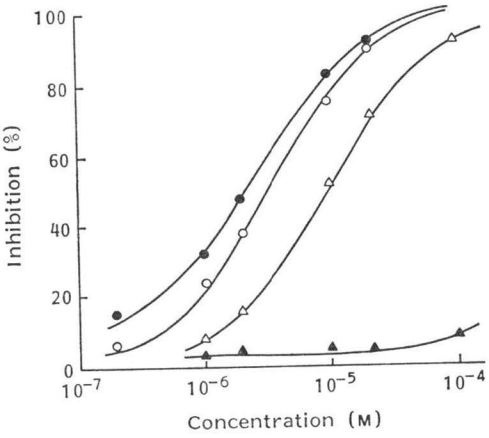
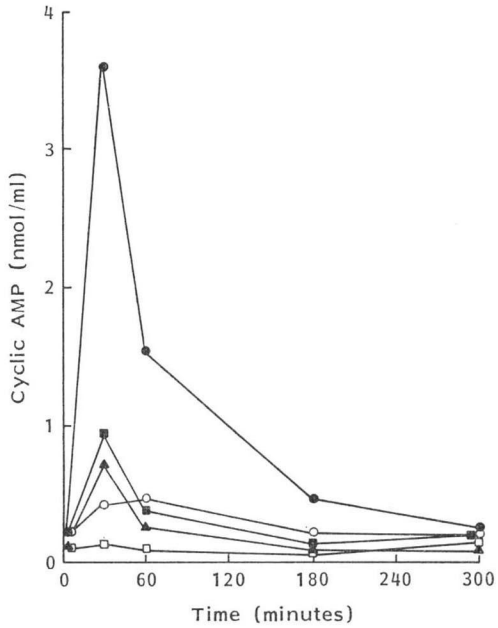


Fig. 8. Increases in plasma cyclic AMP level by isoproterenol in rats pretreated with or without the inhibitor.

Isoproterenol was injected sc at 0 time. The inhibitor were injected sc 20 minutes before that of isoproterenol. The mean values (n=4) are shown by symbols, ●, isoproterenol 0.1 mg/kg, griseolic acid 10 mg/kg; ○, griseolic acid 10 mg/kg; ■, isoproterenol 0.1 mg/kg, theophylline 10 mg/kg; □, theophylline 10 mg/kg; ▲, isoproterenol 0.1 mg/kg.



AMP, but theophylline did not. The injection of isoproterenol after that of griseolic acid resulted in a greater increase in plasma cyclic AMP level than with isoproterenol alone, whereas theophylline injected into rats elicited a much smaller increase (Fig. 8). Theophylline have not shown a significant effect on cyclic AMP level in plasma because its inhibitory effect on cyclic AMP phosphodiesterase is weaker than griseolic acid.

Griseolic acid has a unique structure resembling cyclic AMP with an adenyl group in the molecule, and showed higher inhibitory activity toward cyclic AMP phosphodiesterase than toward cyclic GMP phosphodiesterase. A modification of an adenyl group of griseolic acid to a guanyl group have the possibility that the guanyl derivative may have higher inhibitory activity toward cyclic GMP phosphodiesterase than toward cyclic AMP phosphodiesterase, and may show other type of biological activities.

Mammalian tissues possess several types of cyclic AMP phosphodiesterase. For example, it has been reported that cyclic AMP phosphodiesterases from rat brain and from human kidney composed of at least three different type of cyclic nucleotide phosphodiesterases, respectively[10,11]. Griseolic acid showed the most potent inhibitory effect on cyclic AMP phosphodiesterase from rat aorta and showed the weakest inhibitory effect on that from rat brain. These data show an inhibitory specificity of griseolic acid toward cyclic AMP phosphodiesterases in tissues.

Griseolic acid elevated the plasma cyclic AMP level of the rats when injected subcutaneously before that of isoproterenol. It seemed likely that the plasma level of cyclic AMP increases due to the inhibition of cyclic AMP phosphodiesterase activity by griseolic acid and the stimulation of ad-

enylate cyclase [EC 4.6.1.1.] by isoproterenol in tissues and to the increment of cyclic AMP excretion from mammalian cells.

The LD_{50} value of griseolic acid was 100~200 mg/kg on rats when administered po.

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