Schizostatin, a Novel Squalene Synthase Inhibitor Produced by the Mushroom, *Schizophyllum commune*

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Biological Activities

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In the process of screening for squalene synthase inhibitors from microbial fermentation products we have isolated a novel compound, named schizostatin (Fig. 1), from the culture broth of the mushroom, *Schizophyllum commune* SANK 17785. Schizostatin inhibited rat liver microsomal squalene synthase dose dependently and the IC_{50} value was 0.84 μM. The inhibition was competitive with respect to farnesylpyrophosphate with a *Ki* value of 0.45 μM.

High levels of cholesterol in blood are considered to be one of the most important risk factors for atherosclerosis and coronary heart diseases, and lowering elevated serum cholesterol levels is a precautionary measure against these diseases. *De novo* cholesterol biosynthesis inhibitors, such as pravastatin[1], an inhibitor of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, are effective cholesterol lowering agents. The isoprenoid pathway produces not only sterols but also other essential non-sterol products, such as dolichol, ubiquinone and prenylated proteins.[2] Squalene synthase (SQS, EC 2.5.1.21) occupies a key branch point in the pathway, catalyzing the first committed step of sterols.[3] Selective SQS inhibitors, therefore, are expected to reduce sterols without lowering essential non-sterol products. Several investigators have reported the discovery of SQS inhibitors from microbial fermentation products, and others have synthesized farnesylpyrophosphate (FPP) mimic SQS inhibitors. Among these, squalestatins/zaragozic acids and lipophillic 1,1-bis-phosphonates are potent SQS inhibitors and orally active cholesterol lowering agents *in vivo*[4~6]. In the process of screening for SQS inhibitors from microbial fermentation products, we have isolated a novel compound, named schizostatin (Fig. 1), from the culture broth of the mushroom, *Schizophyllum commune*

SANK17785[7]. Here we report on the fermentation, isolation, physico-chemical properties and biological activities of schizostatin. Full details of the structure elucidation and total synthesis of schizostatin and its *Z*-isomer are described in the following paper[8].

Fig. 1. Structures of schizostatin, chemically synthesized *Z*-isomer and FPP.

Schizostatin

Z-isomer

FPP
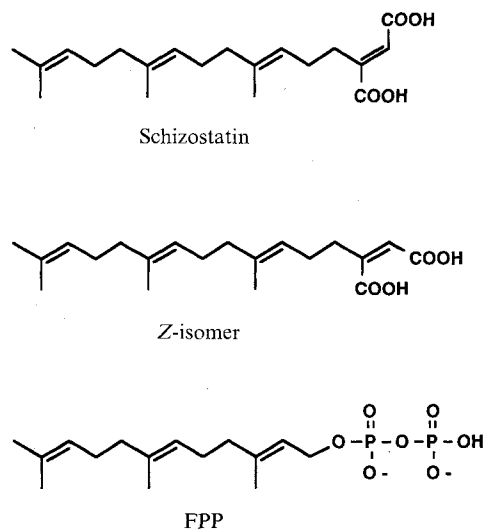
Materials and Methods

Materials

[4-^{14}C]-isopentenyl pyrophosphate (IPP, $56\,\mu$Ci/ μmol) and [1-^{14}C]sodium acetate ($58\,\mu$Ci/μmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.)

Fermentation

The mycelia of *Schizophyllum commune* SANK 17785 grown on potato dextrose agar (PDA) medium were inoculated into 80 ml of the seed medium composed of 5% glycerol, 0.5% malt extract (Difco), 0.5% yeast extract (Difco) and 5% fresh potato (pH 6.4 before sterilization) in a 500-ml Erlenmeyer flask. The seed culture was incubated for 72 hours at 26°C on a rotary shaker operating at 220 rpm (5 cm stroke). Two 25 ml portions of the seed culture were transferred to two 2-liter Erlenmeyer flasks each containing 300 ml of the same medium, and the cultures were incubated for 48 hours at 26°C on a rotary shaker operating at 220 rpm (5 cm stroke). Two 300 ml portions of the second seed culture were then transferred to two 30-liter jar fermentors each containing 15 liters of the same medium with 0.01% CB-422 antifoam (Nippon Oils & Fats Co., Ltd.). During fermentation the temperature was controlled at 26°C, the agitation rate was 250 rpm and the air flow was 1 v/v/ minute. Antifoam was added to the broth on demand.

The growth of mycelia, concentration of glycerol, pH and the production of schizostatin were monitored daily. The growth of mycelia was estimated from the packed cell volume (PCV) obtained after centrifugation of 10-ml of culture broth in a conical tube at $600 \times g$ for 15 minutes. The concentration of glycerol in the medium was analyzed by HPLC. The whole broth was centrifuged and $5\,\mu$l of the clear supernatant was loaded on to a Wako Pack T130E column (Wako Pure Chemical Co., 7.8 mm × 300 mm) at 60°C with water as a mobile phase and flow rate at 0.8 ml/minute. The eluent was monitored with a Waters 410 differential refractometer.

The production of schizostatin was also analyzed by HPLC. The culture broth was extracted with acetone. The extracts were chromatographed on a C-18 column (Waters, Radial-PAK cartridge 8NVC18, 8 mm × 10 cm, $4\,\mu$m packing) using 0.3% triethylamine phosphoric acid buffer (pH 3.2)-CH$_3$CN (35:65) as the isocratic mobile phase at 30°C and the flow rate was 2.0 ml/ minute. The eluent was monitored at 210 nm with a diode array detector.

Determination of Physico-chemical Properties

UV spectra were measured on a Shimadzu UV-256FW spectrophotometer. The IR spectrum was recorded on a JASCO FT/IR-8300 infrared spectrophotometer. FAB-MS and FAB-MS-MS (CAD) spectra were obtained on a JEOL JMS-SX/SX102A four-sector tandem mass spectrometer (BEBE configuration) operating at 10 KV

acceleration potential. Parent ions were produced by bombardment with a beam of Xe atoms (6 KeV), and focused into the collision cell in the third field-free region between MS-1 and MS-2 by the first-stage mass spectrometer (MS-1) at a mass resolving power of approximately 1500 (10% valley). Collisionally activated dissociation (CAD) was accomplished *via* the introduction of argon into the collision cell, which was floated at 5 KV, at pressure sufficient to give approximately 80% attenuation of the parent ion beam.

Preparation of Rat Liver Microsomes

We used rat liver microsomes as the enzyme source for the SQS assay. Pravastatin sodium was administered orally to 6-week old male Wistar Imamichi rats (n = 5) at a dose of 500 mg/kg/day for 6 days in order to elevate SQS activity. Microsomes were prepared from the livers of the rats.

Preparation of Radio Active FPP

We used [4-^{14}C]-FPP as the substrate for the SQS assay. [4-^{14}C]-FPP was synthesized from [4-^{14}C]-IPP and [cold]-geranyl pyrophosphate (GPP) by partially purified rat liver FPP synthase as described by RILLING[9]. GPP was synthesized by the pyrophosphorylation of geraniol as described by DAVISSON *et al.*[10]. Rat liver FPP synthase was partially purified by the method of RILLING[9].

Assays of Squalene Synthase Activity

Assay Method 1

SQS activity was determined by a modified method of AGNEW[3]. One hundred μl of assay mixture contained 50 mM potassium phosphate (pH 7.4), 10 mM NaF, 10 mM MgCl$_2$, 1 mM NADPH, 50 mM ascorbate, 20 units/ml ascorbate oxidase, $10\,\mu$M [4-^{14}C]-FPP and $60\,\mu$g/ml rat liver microsomal protein. SQS inhibitors were added as solutions in 100% MeOH or DMSO. The final concentration of MeOH or DMSO in all assays was 5%. After incubation at 37°C for 20 minutes, the reaction was terminated by the addition of $100\,\mu$l 40% KOH-95% EtOH, 1:1 (v/v), and saponified at 65°C for 30 minutes. Then the mixture was cooled and extracted with 2 ml of hexane using a vortex mixer. One ml of the resultant hexane layer was added to 10 ml of scintillation fluid and radioactivity was determined through liquid scintillation counting. This assay method was used for the determination of the inhibitory activities of the compounds.

Assay Method 2

This assay method was a modification of that of TAIT[11] and was used for multi-screening. Assays were conducted in a total volume of 50 μl in the wells of microtiter plates. The composition of the reaction mixture was the same as in method 1. After incubation at 37°C for 20 minutes, the reaction was terminated by the addition

of 50 μl of isopropanol. Five μl each of the mixtures were spotted onto polyester backed silica gel TLC sheets (20 × 20 cm) using an 8-channel pipette (400 ~ 500 spots per sheet). The sheets were washed in 1% SDS in 0.2 M Tris base (no pH adjustment). After drying, the sheets were exposed to the imaging plates for 18 hours and analyzed with Bio-image analyzer BA 100 (Fuji Film Co. Ltd.).

Assays of Cholesterol Synthesis in Rat Hepatocytes

Rat hepatocytes were freshly isolated using the method of TSUJITA et al.[1]. The hepatocytes (2×10^6) were incubated with 2 mCi of [^{14}C]-acetate in Krebs buffer in the presence of the appropriate concentrations of schizostatin. Incubations were carried out in a shaking water bath at 37°C for 2 hours in O_2-CO_2 (95:5) gas. Cell suspensions were saponified in 20% KOH in 95% EtOH at 80°C for 2 hours. Non-saponifiable substances were extracted with n-hexane, and digitonin precipitable [^{14}C]-sterols were measured through liquid scintillation counting.

Determination of *In Vivo* Hepatic Sterol Synthesis in Mice

This experiment was also done using the method of TSUJITA et al.[1]. Schizostatin was administered orally to male ddY mice (body weight ≈ 20 g, n = 4). After 1 hour [^{14}C]-acetate was injected intraperitoneally. One hour after receiving the acetate, the animals were killed, and the livers were removed and saponified in 20% KOH in 95% EtOH at 80°C for 2 hours. Non-saponifiable substances were extracted with n-hexane, and digitonin precipitable [^{14}C]-sterols were measured through liquid scintillation counting. Data from this experiment were statistically analyzed by Student's t-test. Values were expressed as mean ±S.E.

Assays of Farnesyl-protein Transferase Activity

Farnesyl-protein transferase (FPTase) activity was measured using the method of GOODMAN et al.[12].

Antimicrobial Activity

Antimicrobial activities were determined by a serial 2-fold agar dilution method. Mueller-Hinton agar (Baltimore Biological Laboratory) on which 1 loopful of 10^6 cfu/ml suspension of test microbes was streaked, followed by incubation at 37°C for 20 hours.

Results

Taxonomy of the Producing Organism

Schizophyllum commune SANK 17785 (Fig. 2) was isolated from a contaminated wooden door in 1986. The mycological properties of the strain are as follows. Basidiocarps are side-stalked pileate to palmate, 8 mm in diameter, coriaceous in texture, and composed of clamped hyphae. The surfaces are white to greyish white and furfaceous with rough hairs. Stalks are 0.3 ~ 0.5 cm in length and 1.5 mm in diameter. Lamellae are grayish white, lobed at the marginal area. The basidia are clavate, 15 ~ 20 × 4 ~ 5 μm, with four sterigma on which basidiospores are born. Basidiospores are ellipsoid to elongated in shape, one-celled, smooth, hyaline and 4 ~ 7 × 2 ~ 2.5 μm in size. From the properties stated above, the present fungus was identified as being *Schizophyllum commune* Fr.

Fermentation

Schizostatin was produced by *Schizophyllum commune* SANK 17785 in two 30-liter jar fermentors. Fig. 3 shows the data from a 30-liter fermentation and gives information regarding growth of mycelia, concentration of glycerol, pH and the production of schizostatin. Production of schizostatin began at day 2, and productivity continued to increase after reaching stationary phase. The maximum production was observed at day 5. The fermentation was terminated at day 6 and the

Fig. 2.   *Schizophyllum commune* SANK 17785.

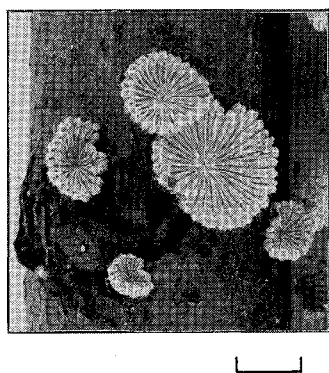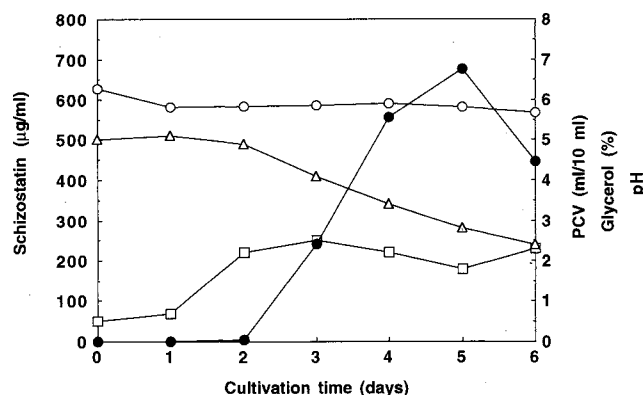Basidiocarps occurring on rotten woods. Bar represents 5 mm.

Fig. 3.   Fermentation profile of schizostatin production.

●: Schizostatin (μg/ml), ○: pH, △: glycerol (%), □: P.C.V. (ml/10 ml).

yield of schizostatin was 447 μg/ml.

Isolation and Purification

The purification procedure of schizostatin is outlined in Fig. 4. The fermentation broth (30 liters) was filtered with celite, and the mycelial cake (4 kg) was extracted twice with 30 liters of 80% acetone. The acetone was evaporated *in vacuo* and the residue was added to the filtrate (30 liters). This mixture was adjusted to pH 3.5 with 6 N HCl and then extracted twice with 50 liters of EtOAc. The combined organic layer was extracted with a 2% NaHCO$_3$ solution. The combined water layer was adjusted to pH 3.5 with conc HCl and then extracted twice with 50 liters of EtOAc. The combined organic layer was washed with saturated NaCl solution, dehydrated with Na$_2$SO$_4$ and concentrated *in vacuo* to give 66 g of an oily substance. The oily substance was purified by preparative HPLC in 11 g aliquots dissolved in 100 ml CH$_3$CN. The preparative reverse phase HPLC of the 6 aliquots was performed on a YMC-pack C-18 column (10 cm × 50 cm, Kurita Industries) using 1.0% triethylamine phosphoric acid buffer (pH 3.2) - CH$_3$CN (30 : 70) as the isocratic mobile phase at room temperature and the flow rate was 200 ml/minute. The effluent was monitored by UV detection at 210 nm. The

major peaks were collected at maximum purity. Each was then reinjected into the column and recollected. The combined 9.5-liter of active fraction was concentrated *in vacuo* and then extracted with 15 liters of EtOAc. The combined organic solvent layer was washed with saturated NaCl solution, dehydrated with Na$_2$SO$_4$ and concentrated *in vacuo* to yield 9.5 g of schizostatin.
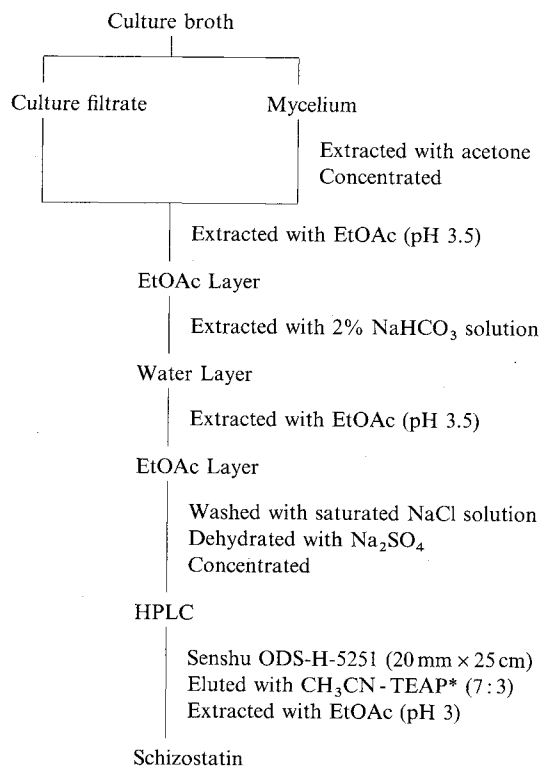
Physico-chemical Properties

The physico-chemical properties of schizostatin are summarized in Table 1. Schizostatin is soluble in methanol, ethanol, ethylacetate, chloroform and alkaline water, but insoluble in *n*-hexane and water. The UV spectrum of schizostatin showed maximum absorption at 214 nm in methanol. The IR spectrum of schizostatin in KBr showed an absorption band at 1713 cm^{-1} suggesting the existence of a carbonyl group. The molecular formula of schizostatin was determined to be C$_{20}$H$_{30}$O$_4$ by FAB-MS ((M + H)$^+$ m/z 335, (M − H)$^-$ m/z 333), EI-MS (M$^+$. m/z 334) and HRFAB-MS ((M − H)$^-$: found, 333.2053; calcd for C$_{20}$H$_{29}$O$_4$, 333.2066). The survey based on this data revealed that no known microbial metabolites shared the physico-chemical properties of schizostatin. Therefore, it was concluded that schizostatin was a new compound.

Biological Activities

Squalene Synthase

Squalene synthase activity was measured in the presence of various concentrations of schizostatin. Schizostatin, in a concentration range from 0.01 μM to 100 μM, inhibited rat liver microsomal squalene synthase dose dependently. The IC$_{50}$ value was 0.84 μM (Fig. 5). In the experiments shown in Table 2, squalene synthase was preincubated with various concentrations of schizostatin at 37°C for 30 minutes, and the concentrations of schizostatin were reduced to one-tenth for enzyme assay. With or without preincubation the inhibition % of SQS by schizostatin was almost the same, indicating that the

Fig. 4. Isolation procedure for schizostatin.

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                    Culture broth
                         |
          |——————————————————————————|
    Culture filtrate              Mycelium
          |                           |
          |                           | Extracted with acetone
          |                           | Concentrated
          |———————————————————————————|
                         |
                         | Extracted with EtOAc (pH 3.5)
                         |
                    EtOAc Layer
                         |
                         | Extracted with 2% NaHCO₃ solution
                         |
                    Water Layer
                         |
                         | Extracted with EtOAc (pH 3.5)
                         |
                    EtOAc Layer
                         |
                         | Washed with saturated NaCl solution
                         | Dehydrated with Na₂SO₄
                         | Concentrated
                         |
                       HPLC
                         |
                         | Senshu ODS-H-5251 (20 mm × 25 cm)
                         | Eluted with CH₃CN-TEAP* (7 : 3)
                         | Extracted with EtOAc (pH 3)
                         |
                    Schizostatin
```

*: Triethylamine phosphate buffer (0.5%, pH 3.2).

Table 1. Physico-chemical properties of schizostatin.

Appearance	White powder
FAB-MS m/z	positive: 335 (M+H)$^+$ Negative: 333 (M-H)$^-$
Molecular weight	334
Molecular formula	C$_{20}$H$_{30}$O$_4$
HRFAB-MS Calcd:	335.2223 for C$_{20}$H$_{31}$O$_4$
Found:	335.2205 (M+H)$^+$
UV λ max (in EtOH)	214.5 nm
IR ν (KBr) cm^{-1}	2965, 2923, 2611, 1713, 1694, 1409, 1264, 905
HPLC retention time	5.2 minutes
	Column; ODS-RadialPAK cartridge 8NVC184 (Waters) 65%CH$_3$CN-H$_2$O containing 0.3%triethylamine-phosphoric acid buffer (pH3.2)

Fig. 5. Inhibition of SQS by schizostatin.
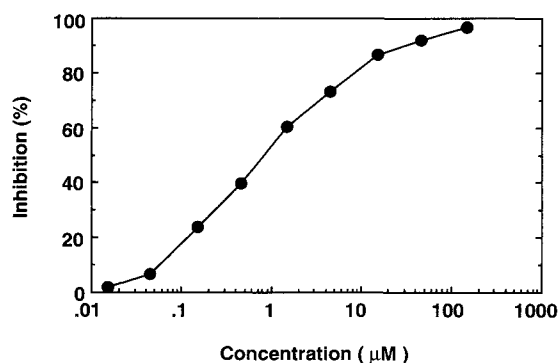
Rat liver microsomal SQS activity was determined as described under "Materials and Methods".

Table 2. Reversibility of the inhibition of SQS by schizo-statin.

Concentration of schizostatin (μM)		Inhibition
At preincubation	At SQS assay	(%)
0	150	96
1500	150	98
0	15	85
150	15	81
0	1.5	54
15	1.5	59
0	0.15	15
1.5	0.15	16

Enzyme assay was carried out as described in "Materials and Methods", except that the enzyme was preincubated with various concentrations of schizostatin at 37°C for 30 minutes, and the concentrations of schizostatin were then reduced to one-tenth of the preincubated level.

inhibition pattern was completely reversible. From kinetic observations, the inhibition was competitive with respect to farnesylpyrophosphate with a Ki value of $0.45 \mu M$ (Fig. 6). The calculated Km value for FPP was $8.6 \mu M$. We synthesized the Z-isomer of schizostatin[7,8] and determined its SQS inhibitory activity at the IC_{50} value of $12 \mu M$. It was 15 times less potent than schizo-statin (Table 4).

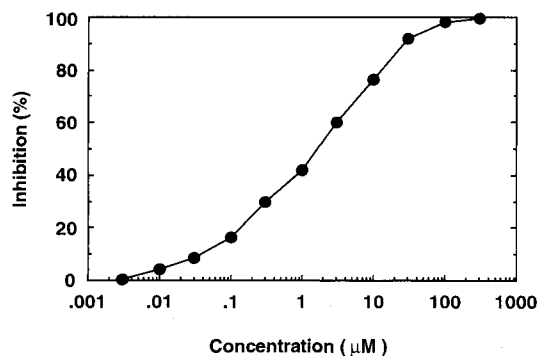## Inhibition of Cholesterol Synthesis in Freshly Isolated Rat Hepatocytes

Schizostatin was shown to inhibit the incorporation of $[^{14}C]$-acetate into cholesterol in freshly isolated rat hepatocytes. Schizostatin gave a dose-dependent de-crease in cholesterol synthesis with an IC_{50} value of $1.2 \mu M$ (Fig. 7).

Fig. 6. Lineweaver-Burk plot for the inhibition of SQS by schizostatin.
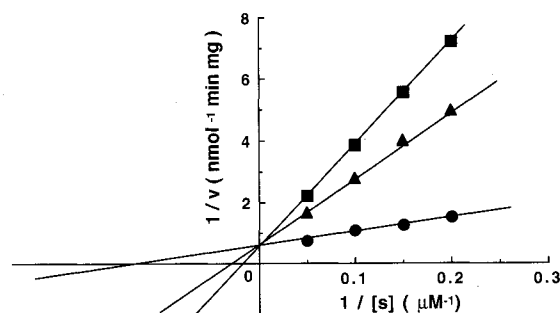
The enzyme assay was carried out as described in "Materials and Methods", except that the concentration of the substrate was varied as indicated. The analysis was performed under the condition that the enzyme reaction was a linear function of time. The points in the reciprocal plots are the experimentally determined values, while the lines are calculated from the fits of these data to the rate equation for competitive inhibition. The concentration of schizostatin in this experiment was 0 (●), 1.25 (▲) and $2.5 \mu M$ (■).

Fig. 7. Inhibition of cholesterol synthesis in rat hepatocytes.

The assay method for sterol synthesis is described in "Materials and Methods".

Inhibition of Hepatic Sterol Synthesis in Mice by Schizostatin

Schizostatin was shown to inhibit hepatic cholesterol synthesis in mice with an ED_{50} value of about 30 mg/kg (Table 3).

Farnesyl Protein Transferase (FPTase)

Inhibition of FPTase from yeast was not observed at the concentration of $300 \mu M$ of schizostatin. But the Z-isomer was observed to inhibit it 30% at the concen-tration of $300 \mu M$ (Table 4).

Antimicrobial Activities

Schizostatin showed no *in vitro* antimicrobial activity

Table 3. Inhibition of hepatic sterol synthesis in mice by schizostatin.

Schizostatin (mg/kg)	Sterols (dpm/mg liver protein)	Inhibition (%)
0	219.1 ± 23.1	0
10	169.9 ± 21.7 *	23
50	82.6 ± 9.8 **	62
250	49.7 ± 14.4 **	77

Results are described as the mean ± S.E., n = 4.
* P < 0.01, ** P < 0.001.

Table 4. Inhibitory activity of schizostatin and related compounds against SQS and farnesyl-proteintransferase.

	SQS IC$_{50}$ (μM)	PFTase IC$_{50}$ (μM)
Schizostatin	0.84	no inhibition at 300
Z-isomer	12	30% inhibition at 300
Chaetomellic acid A	34% inhibition at 150*	0.055*
Chaetomellic acid B	no inhibition at 150*	0.185*

Enzyme assay was carried out as described in "Materials and Methods".
* Data from reference 14.

at a concentration of 1,000 μg/ml against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Mycobacterium smegmatis, Proteus vulgaris, Proteus mirabilis, Candida albicans* or *Mycoplasma mycoides.*

Discussion

Schizostatin is a novel squalene synthase (SQS) inhibitor isolated from microbial fermentation broth, and is distinct from squalestatins/zaragozic acids that are also microbial products. Squalestatins and zaragozic acids are structurally related to presqualene pyrophosphate, an intermediate of the SQS catalyzed reaction, but schizostatin is a FPP mimic compound which has a farnesyl group and two carboxylic acid moieties in the molecule. Given this structure, the reversible and competitive inhibition of SQS by schizostatin is understandable. Many investigators have previously reported chemically synthesized FPP mimic SQS inhibitors.[6,13] Among these, schizostatin is a modestly potent inhibitor. When administered orally, schizostatin inhibited murine hepatic synthesis of sterols *in vivo*. It is reported that SQS inhibitors are also effective *in vivo* cholesterol lowering agents, such as HMG-CoA reductase inhibitors[4,6]. Schizostatin is also expected to have *in vivo* cholesterol lowering effects. From the comparative study of schizostatin and its Z-isomer (Table 4), the E-isomer schizostatin is a more selective SQS inhibitor than the Z-isomer. It is reported that chaetomellic acids A and B[14], which have a Z-isomer configuration of dicarboxylic acid moieties, have potent inhibitory activity against FPTase but have weak or no inhibitory activity against SQS at the concentration of 150 μM. It is interesting that the E-isomer configuration is suitable for the inhibition of SQS, while on the other hand, the Z-isomer configuration is good for the inhibition of FPTase.

Acknowledgments

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