# CP-225,917 and CP-263,114, Novel Ras Farnesylation Inhibitors from an Unidentified Fungus

## I. Taxonomy, Fermentation, Isolation, and Biochemical Properties

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(Received for publication March 27, 1996)

During the course of our screening for squalene synthase inhibitors and Ras farnesylation inhibitors, a novel fungal culture was discovered to produce two structurally unique compounds, CP-225,917 and CP-263,114, as well as zaragozic acid A (squalestatin I). The two compounds are characterized by a bicyclo[4.3.1]dec-1,6-diene core plus two extended alkyl chains. CP-225,917 and CP-263,114 inhibit Ras farnesyl transferase from rat brain with $IC_{50}$ values of 6 $\mu$M and 20 $\mu$M, respectively. CP-225,917 inhibits squalene synthase with an $IC_{50}$ value of 43 $\mu$M and CP-263,114 with an $IC_{50}$ of 160 $\mu$M. The producing organism, though not fully classified, exhibits the characteristics of a sterile *Phoma* species.

In the search for squalene synthase (SQS) inhibitors, we discovered a strain of an unidentified fungus which produces two novel compounds, CP-225,917 and CP-263,114 and zaragozic acid A (squalestatin I)[1~4]. CP-225,917 and CP-263,114 also possess protein farnesyl transferase (PFT) inhibitory activity. In this paper characteristics of the producing organism, fermentation conditions, isolation and bioactivity of these compounds are described. Structural elucidation of the novel compounds will be detailed elsewhere[5].

Squalene synthase [E.C. 2.5.1.21] catalyzes the head-to-head condensation of two molecules of farnesyl pyrophosphate (FPP) to form squalene in a two-stage process[6~9]. The first half-reaction yields presqualene pyrophosphate, an intermediate containing cyclopropylcarbinyl pyrophosphate, with concomitant release of a proton and inorganic pyrophosphate. The second half-reaction involves release of a second molecule of inorganic pyrophosphate and rearrangement of cyclopropylcarbinyl cation followed by reduction with NADPH to form squalene. Squalene thus formed represents a product of the first committed step in the formation of cholesterol and related sterols[10]. Inhibition of SQS is considered to be an effective intervention point to control cholesterol biosynthesis without affecting the level of non-sterol compounds derived from FPP such as dolichol, haem a, ubiquinone, and farnesylated proteins[10,11].

Protein farnesyl transferases catalyze the covalent attachment of a farnesyl moiety to the cysteine thiol group of a variety of proteins that possess the *C*-terminal sequence CAAX (C, cys; A, aliphatic amino acid; X, ser or met)[11,12]. Most notable among the proteins in this class are the Ras proteins which require this post-translational modification for their membrane localization, a step critical for their function in signal transduction pathways[11,12]. Therefore, it has been proposed that inhibition of Ras farnesylation can be an effective method to prevent the transformational effects of mutated Ras proteins[13,14]. Like SQS above, PFT utilizes farnesyl pyrosphosphate as a substrate, and several natural products have been reported to possess activities against both enzymes[15].

### Materials and Methods

Analytical HPLC was carried out using Hewlett-Packard 1090 Chem-Station with a photodiode array detector. Preparative HPLC was carried out using Waters 600 E system. Scale-up fermentations were carried out using New Brunswick BioFlo 4 fermenters.

#### Taxonomic Studies

The producing fungus was isolated from twigs of *Juniperus ashei Bachh.* in a juniper-scrub oak forest near Dripping Springs, Texas. For the identification of the fungus, the following media were used; Malt Extract Agar[16], Oatmeal Agar[17], Potato Dextrose Agar[18], Cornmeal Agar[19], V-8 Juice Agar[18], and Potato Carrot Agar[20]. Morphological observations were made under

a Zeiss research microscope.

## Fermentation

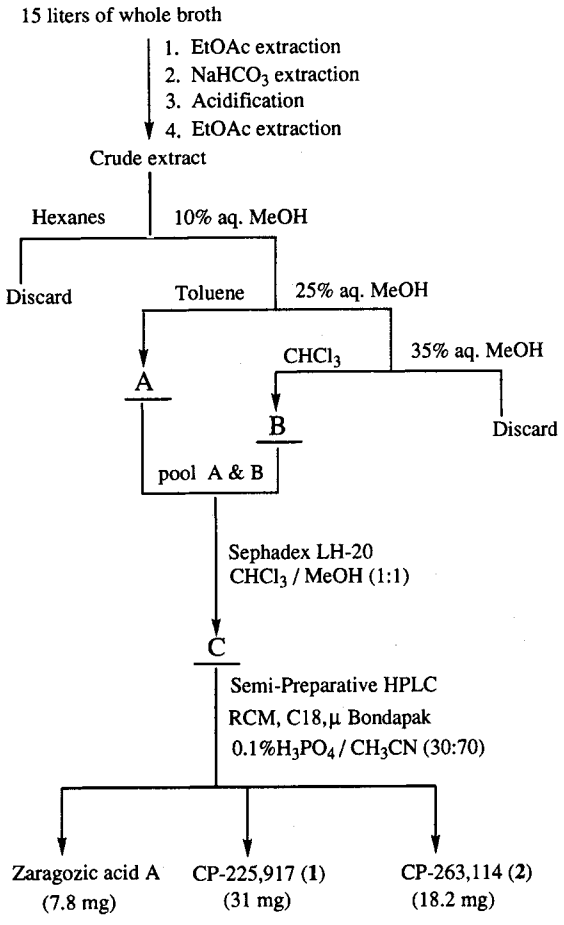
A vial of the fungal culture stored at −80°C was thawed and inoculated into 10 ml seed medium KF[21] in a shake tube. This tube was shaken at 28°C for 72 hours and inoculated into a side arm shake flask containing 60 ml of KF medium. This side arm flask was shaken at 28°C for 72 hours and inoculated into a bench-top fermenter (BioFlo 4) containing 15 liters of sterile SP medium[22]. The fermentation was run for 168 hours at 28°C with aeration of 3 liters per minute and a stirring rate of 350 rpm.

## Isolation

The whole broth (15 liters) was extracted with 15 liters of EtOAc. After the separation of phases, the aqueous layer was acidified to pH 2.8 with conc. HCl and re-extracted with the same volume of EtOAc. The combined EtOAc layers were then extracted with 10 liters of 1% $NaHCO_3$ solution. The $NaHCO_3$ layer was acidified to pH 2.8 with conc. HCl and back-extracted with 10 liters of EtOAc. Evaporation of the solvent gave 1.7 g of crude residue.

The crude residue was dissolved in 100 ml of methanol and was fractionated using the Kupchan scheme[23] (Scheme 1). The toluene and chloroform extracts were combined and purified by a Sephadex LH-20 column (elution with $CHCl_3$ : MeOH = 1 : 1). The SQS inhibitory fractions were combined and purified by HPLC (3× Waters $C_{18}$ Radial Compression Cartridge columns, 0.1% $H_3PO_4$ : $CH_3CN$ = 3 : 7). Fraction 4 provided 7.8 mg of zaragozic acid A as determined by comparison with the authentic sample. Fraction 5 gave 31 mg of CP-225,917 and fraction 6, 18 mg of CP-263,114. The structures of CP-225,917 and CP-263,114 are shown in Fig. 1, and their physico-chemical properties in Table 1.

Scheme 1. Isolation of CP-225,917 (**1**) and CP-263,114 (**2**).




## SQS Inhibition Assays

Rat hepatic microsomes used as the source of SQS activity were prepared as previously described[25]. Solubilized rat hepatic microsomal SQS was prepared by limited trypsinolysis[25]. Inhibition of the catalytic activity of SQS was evaluated by measuring inhibition of the conversion of [1-$^3$H]FPP to [$^3$H]squalene under anaerobic conditions, using an endogenous oxygen consumption system to prevent conversion of nascent squalene to squalene epoxide[25]. SQS activity is expressed as pmoles of squalene formed from FPP per minute of incubation at 37°C per mg microsomal (or solubilized microsomal) protein.

Inhibition of the SQS-catalyzed first half-reaction was evaluated by measuring the rate of proton release from the C-1 position of the prenylating [1-$^3$H]FPP molecule, through equilibration of the expelled tritium with the primary hydroxyl of a fixed volume of added MeOH and then isolating the methanol by distillation and quantitating the specific activity of the distilled MeOH as previously described by AGNEW[26]. SQS first half-reaction activity is expressed as pmoles tritium released from [1-$^3$H]FPP per minute of incubation at 37°C per mg microsomal protein.

Fig. 1. Structures of CP-225,917 (**1**) and CP-263,114 (**2**).

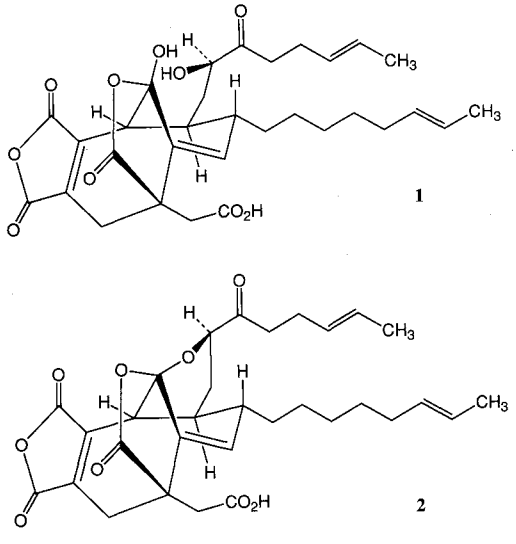


Table 1. Physico-chemical properties of CP-225,917 (**1**) and CP-263,114 (**2**).

| | CP-225,917 (**1**) | CP-263,114 (**2**) |
|---|---|---|
| Appearance | Amorphous solid | Amorphous solid |
| $[\alpha]_D^{25}$ | +23° (*c* 0.50, $CH_2Cl_2$) | −11° (*c* 0.48, $CH_2Cl_2$) |
| UV $\lambda_{max}^{CH_3CN}$ nm (log ε) | 251 (4.96) | 248 (4.95) |
| Molecular weight | | |
| Negative FAB-MS | 569 (M−H) | 551 (M−H) |
| Positive FAB-MS | 571 (M+H) | 553 (M+H) |
| | 593 (M+Na) | 575 (M+Na) |
| HR FAB-MS | Calcd. 571.2542 | Calcd. 553.2437 |
| | Found 571.2532 | Found 553.2427 |
| Molecular formula | $C_{31}H_{38}O_{10}$ | $C_{31}H_{36}O_9$ |

Table 2. Cultural characteristics of the fungus ATCC 74256.

| | Colony diam. in 2 weeks | Texture of colony | Surface color[24] | Reverse color[24] | Sol. pigment[24] |
|---|---|---|---|---|---|
| Malt extract agar | 1.4 cm | Moderately raised, floccose, smooth | Deep mouse gray (LI) | Light mouse gray to deep mouse gray (LI) | Raw sienna to Antique brown (III) |
| Cornmeal agar | 3.1 cm | Thin to slightly raised, lowly floccose, smooth | Off-white, pale olive-buff to olive-buff (XL) | Colorless to pinard yellow (IV) | None to cream (XVI) |
| Potato dextrose agar | 1.1 cm | Highly raised, floccose, radiately wrinkled | Ochraceous-tawny (XV) to blackish mouse gray (LI) | Black | Tawny (XV) to razel (XIV) |
| Potato carrot agar | 2.6 cm | Slightly raised, floccose, smooth | White to pale olive-gray (LI) | Colorless to pale olive gray (LI) | None |
| Oatmeal agar | 3.0 cm | Moderately raised, felty, radiately wrinkled | Pale pinkish cinnamon, pinkish buff to cinnamon (XXIX), but antique brown to raw umber (III) toward edge | Amber brown to argus brown (III) | Grenadine pink to bittersweet orange (II) |
| V-8 Juice agar | 2.1 cm | Moderately raised, floccose, smooth | Tawny-olive to saccardo's umber but pale pinkish buff to pinkish buff (XXIV) toward edge | Ochraceous-tawny to cinnamon-brown (XV) | Yellow ocher to warm buff (XV) |

### PFT Inhibition Assay

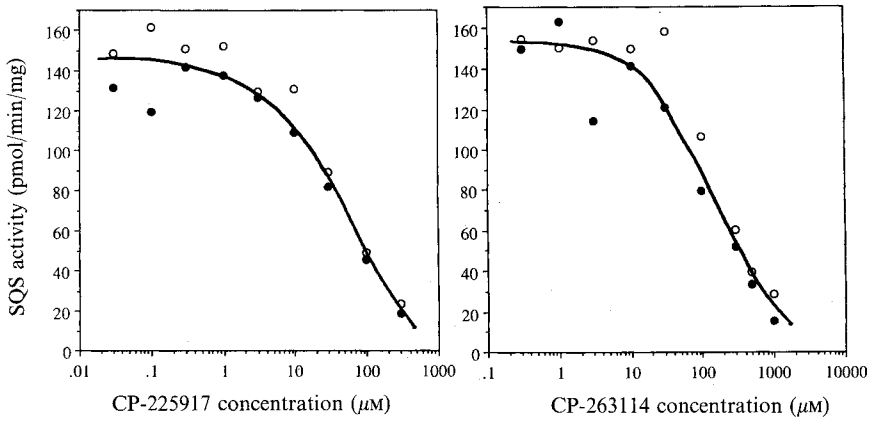
Rat brain cytosol used as the source of PFT activity was prepared as previously described[27]. Inhibition of the catalytic activity of PFT was evaluated by measuring inhibition of the covalent incorporation of the farnesyl moiety of [1-$^3$H]FPP into H-Ras using a procedure that combines room temperature protein precipitation with sample filtration and radioactivity assessment in 96-well format using microplate liquid scintillation counting technology[27]. PFT activity is expressed as pmoles H-Ras farnesylated per minute of incubation at 37°C per mg cytosolic protein.

## Results

### Characteristics of the Producing Organism

The producing organism is characterized by the pink-buff, ochraceous-tawny, olive gray to mouse gray aerial mycelium, and brown gray to black substrate mycelium (Table 2). The growth was moderate at 20°C, moderate to good at 28°C, but none at 37 to 50°C. The hyphae were septate, branched, pale brown to dark brown, and measured 2 to 5 μm diameter. The chlamydospores, borne terminally, laterally, or intercalarily, were catenulate (rarely single); they were globose, subglobose, oval, elliptical to elongated, colored pale brown, brown to dark brown, and measured 6~20×4~12 μm or 7~12 μm diameter. Fruiting bodies or conidia were not found on any of the six media used. Pycnida-like structures, however, were found on a slant culture. The structures were dark brown to brown-black, globose to subglobose, and measured 190~240 μm

Fig. 2. Inhibition of the SQS first half-reaction by CP-225,917 and CP-263,114.




Rat liver microsomes (75 μg microsomal protein; 160 pmol/minute/mg) were incubated for 30 minutes at 37°C in 225 μl PMED buffer [50 mM $K_xPO_4$ (pH 7.4), 5 mM $MgCl_2$, 1.0 mM EDTA, and 5.0 mM dithiothreitol] containing 5.1 μM [$^3$H]FPP (sp. act. 270 dpm/pmol), 258 μM $NADP^+$, 2.1 mM glucose-6-phosphate, 0.95 U glucose-6-phosphate dehydrogenase, 9.4 mM NaF, 50 mM sodium ascorbate, 1.5 U ascorbate oxidase, 4% DMSO, and the indicated concentrations of CP-225,917 or CP-263,114. Following incubation, reactions were terminated by addition of 120 μl of 10 M NaOH. Aliquots, 115 μl, were used for assessing squalene formation (overall reaction; ●) while 230 μl aliquots were used for assessing tritium release (first half-reaction; ○).

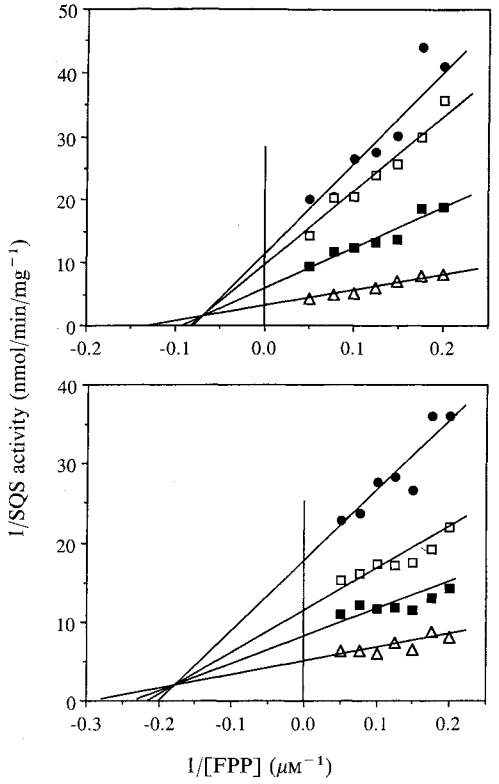
diameter, with long beaks which measured 130 ~ 660 × 95 ~ 125 μm. No conidia were formed within the pycnidia-like structures.

Efforts to induce spore production by using different media, exposing the strain to fluorescent or black light, or incubating the strain for up to two months failed. The pycnidia-like structures at the time of isolation did not produce spores of any type. Thus, the strain was considered to be an unidentified fungus. It has been deposited at the American Type Culture Collection under accession number ATCC 74256.

## Biochemical Properties

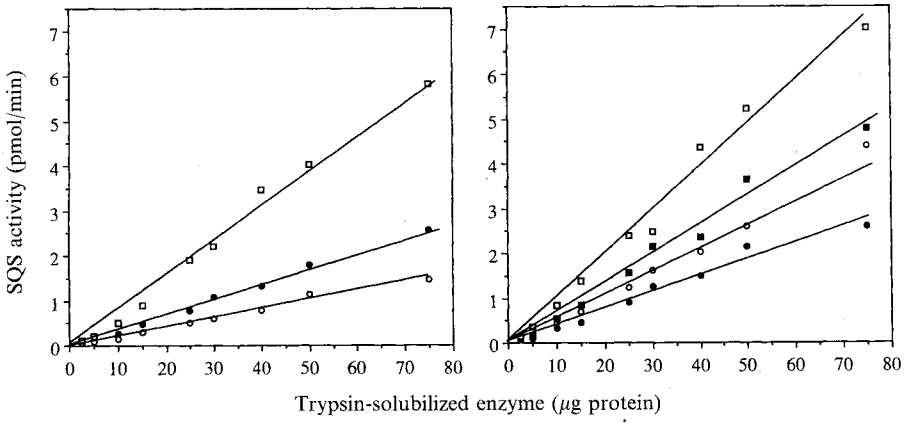
In the presence of 5.1 μM FPP (Km FPP concentration)[25] and 25 μg of rat liver microsomal protein, the two novel compounds, CP-225,917 and CP-263,114, inhibited SQS activity with $IC_{50}$ values of 43 μM and 160 μM, respectively (Fig. 2). Inhibition was considerably weaker than that previously noted for zaragozic acid A[1~4]. CP-225,917 and CP-263,114, both inhibited the enzymatic first half-reaction since inhibition of proton release (first half-reaction) by both compounds was equivalent to inhibition of squalene formation (Fig. 2)[28]. Inhibition by both compounds was of the mixed non-competitive type with respect to the substrate, FPP (Fig. 3), suggesting that these compounds can bind with different affinities to both the free enzyme and the enzyme-FPP complex. Both CP-225,917 and CP-263,114, however, are reversible inhibitors of enzymatic activity, since plots of remaining enzymatic activity versus enzyme

Fig. 3. Kinetics of SQS inhibition by CP-225,917 and CP-263,114.



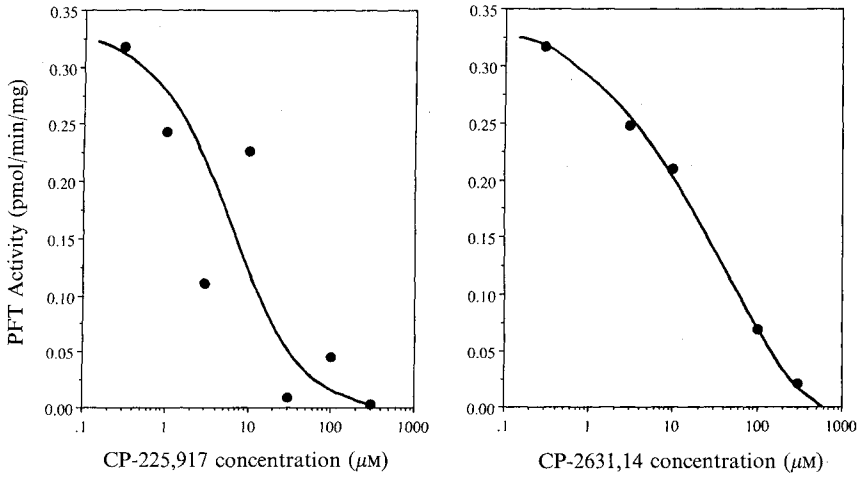

Rat liver microsomes (25 μg microsomal protein, 125 pmol/minute/mg) were incubated for 30 minutes at 37°C in 75 μl of PMED buffer containing substrates and cofactors as described in the legend to Fig. 2, the indicated concentrations of [$^3$H]FPP (sp. act. 253 dpm/pmol), and either 0 μM (△), 25 μM (■), 50 μM (□), or 75 μM (●) CP-225,917 (upper) or 0 μM (△), 150 μM (■), 300 μM (□), or 500 μM (●) CP-263,114 (lower).

Fig. 4. Reversible inhibition of SQS activity by CP-22,5917 and CP-263,114.




Aliquots of a preparation of trypsin-solubilized rat liver SQS (5 mg/ml; 95 pmol/minute/mg) containing the indicated amounts of protein were incubated for 30 minutes at 37°C in 75 μl of PMED buffer containing substrates and cofactors as described in the legend to Fig. 2, and either 0 μM (□), 100 μM (●), or 250 μM (○) CP-225,917 (left) or 0 μM (□), 150 μM (■), 300 μM (○), or 500 μM (●) CP-263,114 (right).

Fig. 5. Concentration-dependent inhibition of PFT by CP-225,917 and CP-263,114.




Rat brain cytosol (30.5 μg cytosolic protein; 0.31 pmol/minute/mg) was incubated for 30 minutes at 37°C in a final volume of 25 μl of PFT assay buffer [50 mM tris HCl (pH 7.5), 5 mM dithiothreitol, and 20 μM $ZnCl_2$] containing 0.5 μM [$^3$H]FPP (sp. act. 12770 dpm/pmol), 5 mM $MgCl_2$, 4 μM H-Ras, 20 mM KCl, 1.2% DMSO and the indicated concentrations of CP-225,917 or CP-263,114. After incubation, reactions were terminated by addition of 200 μl of 10% (v/v) concentrated HCl in EtOH and FPP incorporated into H-Ras was assessed.

concentration for varying concentrations of CP-225,917 and CP-263,114 yielded a series of lines that intersected at the origin (Fig. 4). A similar plot for an irreversible inactivator would yield a series of lines intersecting at varying points along the x-axis whose distance from the origin is a function of the amount of enzyme titrated by the irreversible inactivator[25].

Both CP-225,917 and CP-263,114 inhibited PFT activity. In the presence of 0.5 μM FPP (Km FPP concentration)[27], 4 μM H-Ras, and 30.5 μg rat brain CP-225,917 and CP-263,114 inhibited H-Ras farnesylation by PFT with $IC_{50}$ values of 6 μM and 20 μM, respectively (Fig. 5).

## Discussion

The two novel compounds, CP-225,917 and CP-263,114, have a unique array of features including a bridgehead double bond, a γ-lactol or γ-lactol acetal functions, and a maleic anhydride moiety. Both CP-225,917 and CP-263,114 inhibited SQS activity in a reversible manner by interfering with the enzymatic first half-reaction, the condensation of two molecules of farnesyl pyrophosphate to form presqualene pyrophosphate. SQS inhibition by both compounds, however, was of the mixed noncompetitive type with respect to

the substrate, FPP, indicating that neither CP-225,917 nor CP-263,114 interfere with the SQS-catalyzed reaction through an interaction that can be fully competed against by a single molecule of FPP. Indeed, such a kinetic pattern of inhibition, which suggests that CP-225,917 and CP-263,114 can interact with different affinities with both the free enzyme as well as the enzyme-FPP complex, would imply that interaction of CP-225,917 and CP-263,114 with SQS could potentially occur at either of the two FPP binding sites in the SQS active center, thus allowing CP-225,917 and CP-263,114 to interact with the free enzyme and also with the enzyme containing only one FPP molecule in its active center, or simultaneously at both FPP binding sites in the active center of the free enzyme. Additional studies to determine the nature of the interaction of CP-225,917 and CP-263,114 with the SQS active center are in progress. Both compounds have low $\mu$M $IC_{50}$'s toward H-Ras farnesyl transferase showing 7~8 fold specificity toward PFT over SQS. The relative potency of CP-225,917 and CP-263,114 for inhibition of PFT activity suggests that these compounds are members of an emerging group of natural products with FPT inhibitory activities[29~33]. Additional studies related to their biological effects are being pursued.

Regarding the producing organism, numerous attempts to induce spore production failed, and it has not been possible to identify the strain to the genus or species level. However, the production of pycnidia-like structures and the highly branched, simple septate, and dematiaceous hyphae implies that this organism may represent a sterile member of *Phoma* with its hyphae characteristic of many ascomycetous fungi.

This fungus also yields zaragozic acid A, and the zaragozic acids-producing fungi have been reported to occur in water, bark, stem, dead wood, wood chips, a corticolous lichen, a basidioma, dung and soil[34]. With the exception of *Amauroascus niger* which belongs in the Onyginales, all of the other producers are either ascomycetes or their related anamorphs or sterile fungi with ascomycete affinities. Most of them belong to the Pleosporaceae of the Loculoascomycetes. The fact that the present fungus ATCC 74256 was isolated from a twig parallels the general occurrence of this group of fungi in bark as reported in the literature[34].

Acknowledgments

We thank Dr. BARRY KATZ of Mycosearch for isolating the producing organism, and we also thank Dr. KEN WILSON of Merck Research Laboratories for a gift of zaragozic acid A. The assistance of Ms. T. BOISELLE in fermentation and Ms. A-M. SMITH in typing this manuscript is gratefully acknowledged.

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