PEPTICINNAMINS, NEW FARNESYL-PROTEIN TRANSFERASE INHIBITORS PRODUCED BY AN ACTINOMYCETE

I. PRODUCING STRAIN, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

SATOSHI ŌMURA*, DIDIER VAN DER PYL†, JUNJI INOKOSHI, YOKO TAKAHASHI and HIDEO TAKESHIMA

Research Center for Biological Function, The Kitasato Institute, Shirokane 5-9-1, Minato-ku, Tokyo 108 (Japan)

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Pepticinnamins A, B, C, D, E and F, a family of farnesyl-protein transferase (FPT) inhibitors were isolated from the fermentation broth of *Streptomyces* sp. OH-4652. These inhibitors were purified from whole broth by extraction with chloroform, followed by silica gel column chromatography, Sephadex LH-20 chromatography and reverse phase HPLC. Among these, pepticinnamin C showed the most potent inhibition ($IC_{50} \sim 100$ nM).

ras proteins are localized in the inner side of the plasma membrane and has been considered that the proteins are involved in signal transduction processes[1,2]. The interaction of *ras* proteins with the plasma membrane requires post-translational modification of their carboxy-terminus[3~7]. All *ras* proteins share a sequence, known as a CAAX box, which consists of a conserved cysteine (C), two aliphatic amino acids (AA) and a carboxy-terminal residue (X). Genetic studies have demonstrated that *ras* oncogenes require an intact CAAX box, and therefore proper post-translational processing, to induce malignant transformation[8~10]. Recent studies have unveiled the biochemical nature of the post-translational modifications within the CAAX motif of *ras* proteins. They include; (i) farnesylation of the conserved cysteine residue (Cys^{186} in mammalian H-*ras*) by farnesyl-protein transferase (FPT); (ii) cleavage of the three carboxy-terminal amino acid residues (AAX) and (iii) methylation of the resulting carboxy-terminal farnesyl cysteine. Inhibition of such a isoprenylation would alter membrane localization and transforming activity of *ras* oncogene[11]. These findings have raised the possibility that available FPT inhibitor could block neoplastic transformation induced by *ras* oncogenes. During our screening for FPT inhibitors from microbial origins, we discovered pepticinnamins as fermentation products of *Streptomyces* strain OH-4652.

Materials and Methods

Materials

Tritiated farnesyl pyrophosphate (555 GBq/mmol) and En^{3}Hance spray were purchased from New England Nuclear. Cold farnesyl pyrophosphate was synthesized in Rhône-Poulenc Rorer laboratories. Recombinant Ha-*ras* p21 protein was produced in a bacterial expression system and purified as previously described[12]. The tetrapeptide CVLS corresponding to the carboxy-terminal sequence of Ha-*ras* was synthesized with an Applied Biosystems model 431 and purified by HPLC in Rhône-Poulenc laboratories. THP-1 cells were purchased from ATCC (TIB 202).

† Present address: Rhône-Poulenc Rorer, Centre de Recherche de Vitry, 13 quai Jules Guesde, 94403 Vitry Sur Seine, France.

Partial Purification of Farnesyl-protein Transferase

THP-1 culture cells were washed with ice cold phosphate buffered saline (PBS) and incubated at 4°C for 30 minutes in lysis buffer (10 mM Hepes pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 0.5 μM pepstatin, 1 μM leupeptin, 10 mM sodium pyrophosphate and 0.1 mM sodium orthovanadate). The lysate was centrifuged at 10,000 × g at 4°C for 15 minutes after addition of NaF (100 mM final) and the supernatant was further centrifuged at 100,000 × g for 45 minutes at 4°C. The supernatant (S100) was then submitted to fractionation by ammonium sulfate. The 30~60%-saturated ammonium sulfate fraction was dissolved in 30 ml of 50 mM Tris-HCl (pH 7.5) buffer containing 1 mM DTT and 0.02 mM $ZnCl_2$ and then dialysed for 15 hours against 4 liters of the same buffer and then 10 liters of fresh buffer for 20 hours at 4°C. The dialysed material (10.4 U/mg, 1 unit corresponding to the amount of enzyme allowing to form 1 pmol of farnesyl p21 per 0.5 hour under our standard condition) was aliquoted and stored at −70°C, showing *Km* value of 5 μM for p21 protein and 0.5 μM for farnesyl pyropyosphate (FPP) substrate.

Farnesyl-protein Transferase Assay

Standard reaction mixture contained the following components in a final volume of 60 μl: 13 μg of partially purified FPT from cytosol of human cells THP-1, 1.3 μM of recombinant p21 protein, 0.03 μM of [^{3}H] FPP, 50 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$ and 4 mM DTT. The reaction was initiated by addition of enzyme and incubated for 30 minutes at 37°C. The reaction was stopped by addition of 0.5 ml of 1% SDS in methanol and 0.5 ml of 30% TCA. After vortexing, the tubes were left 60 minutes on ice. The mixture was then filtered on Whatman GF/C filter, washed with 5 ml of 6% TCA using a Skatron cell harvester. Dried filter was finally counted in a liquid scintillation counter. Blank value was determined in parallel incubation containing no p21 substrate; this blank value was subtracted before calculating percent inhibition. IC_{50} values were determined graphically from plots of percent inhibition *versus* log concentration of inhibitors. All data are the average of duplicate determinations.

Taxonomic Studies of Producing Strain

The isomer of diaminopimelic acid (DAP) was determined by the method of TAKAHASHI *et al*[13]. To investigate the cultural characteristics and physiological properties, the International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[14] and media recommended by WAKSMAN[15] were used.

Cultures were observed after two weeks incubation at 27°C. Color Harmony Manual, 4th Ed., 1958 (Containner Corporation of America, Chicago, USA) was used for color names and hue numbers. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium[16] containing 1% carbon source at 27°C. The morphological properties were observed with a scanning electron microscope (Model S-430, Hitachi Co., Ltd.).

Measurement of Cell Growth

Culture of Vero cells, an established cell line from kidney cells of African green monkey and measurement of cell growth were carried out by the method described previously[17]. Briefly, the cells were routinely grown in a humidified incubator (95% air/5% CO_2) at 37°C in a 1-liter flask containing 200 ml of EAGLE's minimum essential medium (GIBCO Co.) supplemented with benzylpenicillin (50 U/ml), streptomycin (50 μg/ml) and calf serum (GIBCO Co.) at 5%. Cells grown on each well of 96-well microplates were washed twice with 100 μl of calcium- and magnesium-free phosphate buffered saline and stained with 50 μl of the staining solution (methylrosaniline 0.5%, NaCl 0.85%, formamide 5% and ethanol 50%) for 20 minutes. The staining solution was removed and the cells were washed with water. The absorbance at 540 nm was measured by microplate photometer (Titertek Co.).

Antimicrobial Activity Test

The antimicrobial spectra of the test materials were determined using 8 mm paper discs (Toyo Seisakusho Co.). Bacteria were grown on Mueller-Hinton agar medium (Difco) and fungi or yeasts were grown on potato-broth agar medium. Antimicrobial activity was observed after 24 hours incubation at 37°C for bacteria or longer incubation at 27°C for fungi or yeasts.

Results

Taxonomy of Producing Strain OH-4652

The strain OH-4652 was isolated from a soil collected from a stock farm at Nasu-Machi, Tochigi prefecture, Japan. The vegetative mycelia grew abundantly on both synthetic and complex media, and did not show fragmentation into coccoid forms or bacillary elements. The aerial mycelia grew abundantly on yeast extract-malt extract agar and inorganic salts-starch agar. The aerial mycelium branched monopodially with sporophores forming spiral spore chains with more than 20 spores per chain. The spores were cylindrical in shape, $1.1 \times 0.6\,\mu m$ in size and had a smooth surface (Fig. 1). Whirls, sclerotic granules, sporangia and flagellated spores were not observed. The DAP-isomer in cell wall of strain OH-4652 was determined to be LL-type.

The cultural characteristics and the physiological properties are shown in Tables 1 and 2. The vegetative mycelia showed ivory color on various media. The aerial mass color showed white or gray color. Melanoid pigment was produced in tyrosine agar. The utilization of carbon sources is shown in Table 3.

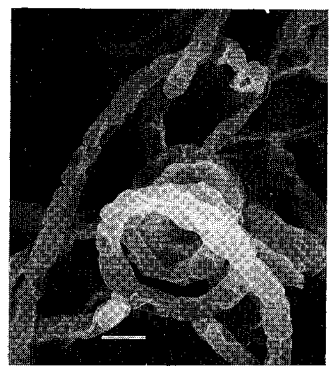
Based on the taxonomic properties described above, strain OH-4652 is considered to the genus *Streptomyces* and to belong to the white or gray series according to the classification by WILLIAMS *et al*[18]. The strain was deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. OH-4652 and the accession No. is FERM P-12740.

Fermentation

A seed medium (100 ml) containing glucose 0.1%, starch 2.4%, peptone 0.3%, meat extract 0.5% and $CaCO_3$ 0.4% prepared in tap water (pH was adjusted to 7.0 with 6 N NaOH) was poured into each of four 500-ml Erlenmeyer flasks and sterilized at 121°C for 20 minutes. A loopful of OH-4652 from a mature slant, grown on a agar medium containing glycerol 1%, calcium malate 0.5%, NH_4Cl 0.05%, K_2HPO_4 0.05%, yeast extract 0.1% and agar 1% (pH was adjusted to 7.0 as described above) for 2 weeks at 27°C, was used to inoculate each of the flasks. The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) at 27°C for 4 days. The resulting seed culture was used to inoculate (2% inoculum) 20 liters of sterile fermentation medium which consisted of 2% glucose, 0.5% L-asparagine, 0.01% $MgSO_4 \cdot 7H_2O$, 0.07% KH_2PO_4 and 0.2% yeast extract (pH was adjusted to 7.0 as described above prior to sterilization), prepared in tap water into a 30-liter stainless steel jar-fermentor. The fermentation was carried out for 4 days at 27°C employing aeration at 20 liters/minutes and agitation at 200 rpm.

Fig. 1. Scanning electron micrograph of spore chains of strain OH-4652 grown on glycerol asparagine agar for 14 days.

Bar represents 1.0 μm.



Isolation

An equal volume of chloroform was added to 18 liters of culture broth while mixing. The mixture was agitated for 30 minutes and was centrifuged with a Sharples type-centrifuge. The organic solvent layer was concentrated to dryness *in vacuo*. The

Table 1. Cultural characteristics of strain OH-4652.

Medium		Cultural characteristics
Yeast extract - malt extract agar[a]	G:	Good, camel (3ie)
	R:	Light amber (3ic)
	AM:	Abundant, covert gray (2fe) to pearl (2ba)
	SP:	None
Oatmeal agar[a]	G:	Poor, light ivory (2ca)
	R:	Bamboo (2gc)
	AM:	Poor, pearl (2ba)
	SP:	None
Inorganic salts - starch agar[a]	G:	Good, colorless
	R:	Light wheat (2ea)
	AM:	Abundant, silver gray (2fe) to white (a)
	SP:	None
Glycerol - asparagine agar[a]	G:	Good, olive gray (1ig)
	R:	Light olive gray (1li)
	AM:	Abundant, alabaster tint (13ba)
	SP:	None
Glucose - asparagine agar	G:	Good, dark covert gray (2ih)
	R:	Covert brown (2li)
	AM:	Abundant, alabaster tint (13ba)
	SP:	None
Peptone - yest extract - iron agar[a]	G:	Poor, light mustard tan (2ie)
	R:	Light mustard tan (2ie)
	AM:	None
	SP:	None
Tyrosine agar[a]	G:	Good pearl (2ba)
	R:	Dark brown (2pn)
	AM:	Abundant, alabaster tint (13ba)
	SP:	Covert brown (2nl)
Sucrose - nitrate agar[b]	G:	Poor, ivory (2db)
	R:	Ivory (2db)
	AM:	None
	SP:	None
Glucose - nitrate agar[b]	G:	Moderate, ivory (2db)
	R:	Ivory (2db)
	AM:	Poor, ivory (2db)
	SP:	None
Glycerol - calcium malate agar[b]	G:	Moderate, alabaster tint (13ba)
	R:	Alabaster tint (13ba)
	AM:	Poor, white (a)
	SP:	None
Glucose - peptone agar[b]	G:	Good, bamboo (2gc)
	R:	Light wheat (2ea)
	AM:	Abundant, alabaster tint (13ba)
	SP:	None
Nutrient agar[b]	G:	Poor, light ivory (2ca)
	R:	Bamboo (2gc)
	AM:	Very poor, white (a)
	SP:	None

[a] Medium recommended by International Streptomyces Project.
[b] Medium recommended by S. A. WAKSMAN.
G, growth of vegetative mycelium; R, reverse; AM, aerial mycelium; SP, soluble pigment.

Table 2. Physiological properties of strain OH-4652.

Melanin formation	+
Tyrosinase reaction	+
H_2S production	−
Liquefaction of gelatin (21~23°C)	−
Peptonization of milk (37°C)	−
Coagulation of milk (37°C)	−
Cellulolytic activity	−
Hydrolysis of starch	+
Nitrate reduction	−
Temperature range for growth	10~37°C

+, Active; −, not active.

Table 3. Utilization of carbon sources by strain OH-4652.

D-Glucose	+
D-Fructose	±
L-Rhamnose	+
D-Mannitol	+
L-Arabinose	+
I-Inositol	−
Raffinose	+
D-Xylose	+
Sucrose	+
Melibiose	±

+, Utilized; ±, weakly utilized; −, not utilized.

resulting oily material (1.6 g) was washed with *n*-hexane, dissolved in a small amount of a solvent mixture (chloroform - methanol, 100 : 6.5) and applied on a column of silica gel (40 g, Kiesel gel 60, Merck Co.) prepared with the same solvent mixture. The active substances (770 mg) eluted from the column with the same solvent were taken to dryness, dissolved in ethanol and subjected to a column chromatography on

Table 4. Physico-chemical properties of pepticinnamins.

	A	B	C
Appearance	White powder	White powder	White powder
HRFAB-MS *m/z*			
Calcd:	926.3743 $(M+H)^+$	926.3743 $(M+H)^+$	924.3587 $(M+H)^+$
Found:	926.3749	926.3802	924.3602
Molecular formula	$C_{49}H_{56}N_5O_{11}Cl$	$C_{49}H_{56}N_5O_{11}Cl$	$C_{49}H_{54}N_5O_{11}Cl$
MP	136~138°C	134~137°C	125~127°C
$[\alpha]_D^{26}$ (MeOH)	−98.9° (*c* 0.3)	−124.2° (*c* 0.35)	−151.9° (*c* 0.8)
UV λ_{max}^{EtOH} nm	205, 225 (sh), 282 (sh)	204, 225 (sh), 280 (sh)	204, 225 (sh), 280 (sh)
IR cm^{-1} (KBr)	3380, 3250, 2950, 2920, 1740, 1670, 1630	3380, 3250, 2950, 2920, 1740, 1670, 1630	3380, 3250, 2950, 2920, 1740, 1670, 1630

	D	E	F
Appearance	White powder	White powder	White powder
HRFAB-MS *m/z*			
Calcd:	896.3846 $(M+Na)^+$	908.3637 $(M+H)^+$	942.3248 $(M+H)^+$
Found:	896.3864	908.3601	942.3246
Molecular formula	$C_{49}H_{55}N_5O_{10}$	$C_{49}H_{54}N_5O_{10}Cl$	$C_{49}H_{53}N_5O_{10}Cl_2$
MP	137~139°C	143~146°C	143~146°C
$[\alpha]_D^{26}$ (MeOH)	−212.3° (*c* 0.3)	−207° (*c* 0.8)	−193.5° (*c* 0.7)
UV λ_{max}^{EtOH} nm	203, 225 (sh), 281	205, 225 (sh), 282	203, 225 (sh), 282
IR cm^{-1} (KBr)	3380, 3250, 2950, 2920, 1740, 1670, 1630	3380, 3250, 2950, 2920, 1740, 1670, 1630	3380, 3250, 2950, 2920, 1740, 1670, 1630

Fig. 2. An elution profile of pepticinnamins on a reverse phase HPLC.

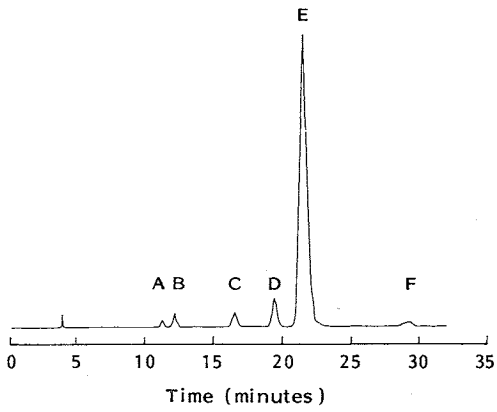


Table 5. FPT inhibition by pepticinnamins and CVLS[a].

Compound	IC_{50} (μM)
A	0.65
B	0.2
C	0.1
D	1.0
E	0.3
F	0.5
CVLS	6.6

[a] H_2N-cysteine-valine-leucine-serine-COOH.

Sephadex LH-20 (1.5 × 100 cm). The column was developed with the same solvent. The fractions containing the desired materials were combined and dried to give a powder (460 mg). The powder was dissolved in 4.6 ml of methanol and an aliquot (0.2 ml) of the solution as applied to a reverse phase HPLC column (Shiseido, C18SG120 type, 20 × 250 mm) and eluted with water - acetonitrile (1 : 1) at the flow rate of 11 ml/minute, detecting at 225 nm. The active compounds were eluted at 11.4, 12.3, 16.7, 19.3, 21.6 and 28.9 minutes of the retention, termed as pepticinnamins A, B, C, D, E and F, respectively. A typical HPLC elution profile of these compounds is shown in Fig. 2, and yields of each compound were 5.51, 10.75, 20.45, 28.37, 230 and 8.69 mg, respectively, from 0.2 ml of the methanol solution.

Physico-chemical Properties

The physico-chemical properties of pepticinnamins are summarized in Table 4. All the compounds

were soluble in methanol, acetone, ethyl acetate and chloroform, insoluble in water and *n*-hexane, and gave positive color reaction using vanilin and iodine, but was negative to ninhydrin.

Biological Activity

The inhibitory activities of pepticinnamins A, B, C, D, E and F in the FPT assay are shown in Table 5. Of these pepticinnamin C was the most potent FPT inhibitor with IC_{50} value of 100 nM, since our peptide control CVLS showed an IC_{50} of 6.6 μM.

Antimicrobial activities and cytotoxicity in Vero cells were tested for OH-4652 E substance, of which producibility is the highest among pepticinnamin family. No antimicrobial activities were shown at the concentration of 1,000 μg/ml against *Xanthomonas oryzae* KB88, *Candida albicans* KF-1, *Saccharomyces sake* KF26, *Mucor racemosus* KF223 (IFO 4581), *Piricularia oryzae* KF180, *Aspergillus niger* KF103 (ATCC 6275), *Staphylococcus aureus* KB34 (FDA 209P), *Bacillus subtilis* KB27 (PCI 219), *Escherichia coli* KB8 (NIHJ), *E. coli* KB176 (NIHJ JC-2), *Pseudomonas aeruginosa* KB105 (P3), *Micrococcus luteus* KB40 (PCI 1001), *Bacteroides fragilis* KB169, *Mycobacterium smegmatis* KB42 (ATCC 607) and *Acholeplasma laidlawii* PG8 KB174.

Also, no cytotoxicity was observed in Vero cells at the concentration of 1,000 μg/ml of pepticinnamin E substance.

Discussion

Recent studies have shown that lovastatin, an inhibitor of HMG-CoA reductase can inhibit the growth of *ras*-transformed mouse fibroblasts. DeClue *et al.*[19], have shown that the primary inhibitory action of lovastatin in cells transformed by a modified *ras* oncogene which lacks a CAAX box does not take place at the level of *ras* farnesylation. Therefore, it is unlikely that HMG-CoA reductase inhibitors can be used as therapeutic agents to block the action of *ras* oncogenes. These observations indicate that drugs capable of inhibiting *ras* oncogene activity by blocking its farnesylation must target FPT. It has been shown that peptides of various length (4-mer to 14-mer) carrying the carboxy-terminal CAAX box sequence of the H-*ras* p21 protein Cys-Val-Leu-Ser-COOH inhibit the incorporation of [^{3}H]FPP into H-*ras* p21, and their relative inhibitory potency ranged from 6 μM to 25 μM in IC_{50}[20,21]. While pepticinnamin C showed 60-fold higher potency than those of synthetic peptides, although both cell toxicity and antimicrobial activity of the compound were extremely low. We also isolated gliotoxin and its acetyl derivative as FPT inhibitors with IC_{50} values of 1.1 and 4.3 μM, respectively[22]. Gliotoxin has been isolated as the metabolites of variety of microorganisms, studied on the various biological activities and reviewed[23]. Thus, it is considered that OH-4652 compounds will become very useful FPT inhibitors. The *in vivo* activity of these compounds is currently under study. The structural elucidation of pepticinnamin E will be published in the following paper[24].

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