FIBROSTATINS, NEW INHIBITORS OF PROLYL HYDROXYLASE

I. TAXONOMY, ISOLATION AND CHARACTERIZATION

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Fibrostatins, potent inhibitors of prolyl hydroxylase, were isolated as orange crystals from the culture broth of strain No. 23924, which was identified as *Streptomyces catenulae* subsp. *griseospora*.

In vitro inhibitory activity (ID_{50} value) of fibrostatins A, B, C, D, E and F against prolyl hydroxylase of chick embryos was 23, 39, 29, 180, 10 and 14 μM, respectively.

The collagen biosynthesis is characterized by several post-translational modifications, one of which is the hydroxylation of certain prolyl residues by prolyl hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate oxygenase, EC 1.14.11.2); this activity is enhanced in tissues of various experimental and pathological fibroses[1~6].

We have reported[7,8] the isolation and biological activities of P-1894B, an inhibitor of prolyl hydroxylase, produced by *Streptomyces albogriseolus* subsp. No. 1894. In further screening for prolyl hydroxylase inhibitor, we found new inhibitors† produced by *Streptomyces catenulae* subsp. *griseospora* No. 23924 and have named them fibrostatins. This paper describes the taxonomy of strain No. 23924 and the fermentation, isolation, physico-chemical and biological properties of the fibrostatins.

Materials and Methods

Taxonomic Studies

The producing organism, strain No. 23924, was isolated from a soil sample collected in Ishigaki-jima, Okinawa Prefecture, Japan. The media and procedures used for cultural and physiological characterization of strain No. 23924 were those described by SHIRLING and GOTTLIEB[9], and WAKSMAN[10]. Inoculated media were incubated at 28°C for 2 weeks before observation. The color names used in these studies were based on the "Color Harmony Manual"††. The chemical composition of the cell wall was analyzed by using the method of BECKER *et al.*[11].

Media

The following media were used in production studies of the inhibitors. Seed medium; glucose 2.0%, soluble starch 3.0%, raw soybean 1.0%, corn steep liquor 1.0%, Polypeptone 0.5%, sodium chloride 0.5%, calcium carbonate 0.5%, pH 7.0. Fermentation medium; soluble starch 4.0%, defatted soybean meal 2.0%, sodium thiosulfate 0.1%, ferrous sulfate 0.05%, dipotassium hydrogen phosphate 0.05%, potassium chloride 0.03%, pH 6.5.

† P-23924 A-F, Jpn. Kokai 74992 ('84), Apr. 27, 1984, Jpn. Kokai 34185 ('85), Feb. 21, 1985, Jpn. Kokai 83592 ('85), May 11, 1985.

†† Color Harmony Manual, 4th Ed., Container Corporation of America, Chicago, 1958.

Assay of Prolyl Hydroxylase Activity

The enzyme activity was measured by the $^{14}CO_2$-release assay of RHOADS *et al.*[12]. The standard reaction mixture (total volume, 1.5 ml) contained sodium α-[1-^{14}C]ketoglutarate (0.016 μCi) 0.1 μmol, ascorbic acid 1.5 μmol, ferrous ammonium sulfate 0.1 μmol, heat-denatured bovine serum albumin (Sigma Chemical Co.) 4 mg, bovine liver catalase (Boehringer Mannheim) 0.1 mg, $(Pro\text{-}Pro\text{-}Gly)_5 \cdot 4H_2O$ 0.45 mg, enzyme 2 μg and Tris-HCl buffer (pH 7.8) 50 μmol.

Materials

Prolyl hydroxylase was purified from chick embryo extracts using affinity column chromatography as described by TUDERMAN *et al.*[13]. The enzyme showed a single band when examined by polyacrylamide gel electrophoresis. Sodium α-[1-^{14}C]ketoglutarate (12 mCi/mmol) was purchased from the New England Nuclear Corp., Boston, MA and $(Pro\text{-}Pro\text{-}Gly)_5 \cdot 4H_2O$ from the Protein Research Foundation, Osaka, Japan.

Results and Discussion

Taxonomic Studies

Morphological characteristics: On inorganic salts - starch agar strain No. 23924 produces aerial mycelium in dense clusters with straight or flexible short spore-bearing hyphae with occasionally hooks or open loops (Fig. 1). It therefore belongs to the Section Rectus Flexibilis (RF)[14]. The mature spore chains are generally short with 5 to 10 spores per chain. The spores are ellipsoidal to cylindrical ($0.4 \sim 0.7 \times 0.7 \sim 1.2$ μm) and their surfaces are smooth (Fig. 2).

Cultural characteristics: The cultural characteristics on various media are shown in Table 1. This strain gives the most characteristic appearance on inorganic salts - starch agar; the aerial mycelium is gray.

Physiological characteristics: The physiological characteristics and utilization of carbohydrates are shown in Tables 2 and 3, respectively. Strain No. 23924 contains L,L-diaminopimelic acid in the cell wall (Type I). Melanoid pigment is not formed on either tyrosine agar or peptone - yeast extract iron agar.

The above results indicate that strain No. 23924 is a member of the genus *Streptomyces* of the

Fig. 1. Spore-bearing hyphae of strain No. 23924 developing on the surface of inorganic salts - starch agar.
Scale line, 1 μm.

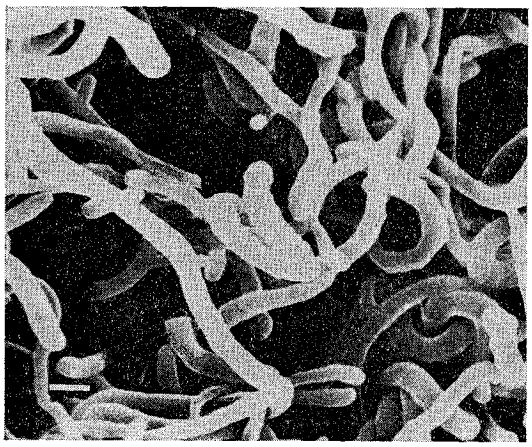


Fig. 2. Morphology of spores of strain No. 23924.
Magnification, $\times$13,000.

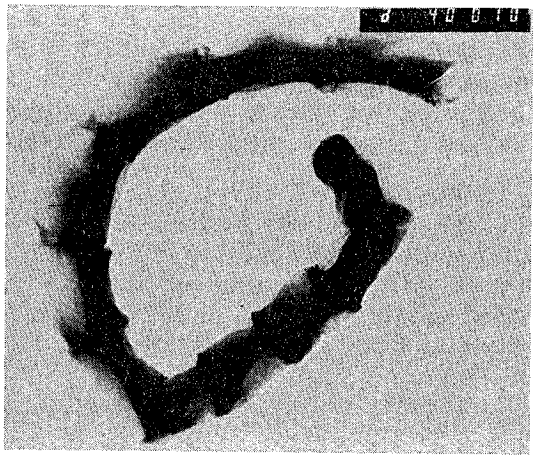


Table 1. Cultural characteristics of strain No. 23924.

Medium	Growth	Aerial mycelium	Reverse side of substrate mycelium	Soluble pigments
Sucrose - nitrate agar	Poor, colorless	Poor, beige (3ge)	Gray (near grays, 3dc, natural to 3fe, silver gray)	None
Glucose - asparagine agar	Poor, colorless	None	Colorless	None
Glycerol - asparagine agar	No growth	None	None	None
Inorganic salts	Abundant, golden brown (3pi)	Abundant, powdery, gray (near grays, 3ih, beige gray)	Dark brown (4nl)	Nude tan (4gc)
Tyrosine agar	Poor, colorless	Poor, gray (near grays, 3ba, pearl)	Light apricot (4ea)	None
Nutrient agar	Moderate, colorless	None	Colorless	None
Yeast malt agar	Moderate, oak brown (4pi)	Poor, gray (near grays, 3fe, silver gray)	Dark luggage tan (4pg)	Luggage tan (4ne)
Oatmeal agar	Moderate, light beige (3ec)	Poor beige (3ge)	Light mustard tan (2ie) to mustard tan (2lg)	None

Table 2. Physiological characteristics of strain No. 23924.

Hydrolysis of starch	Positive (ISP-medium 4)
Liquefaction of gelatin	Positive (weak), glucose - peptone gelatin medium, 24°C, 3 weeks
Coagulation and peptonization of milk	Negative
Formation of melanoid pigment	Negative (ISP-medium 6 and 7)
Reduction of nitrate	Negative (ISP-medium 8)
Temperature range for growth	15~35°C, optimum 28~30°C, malt extract - yeast extract agar

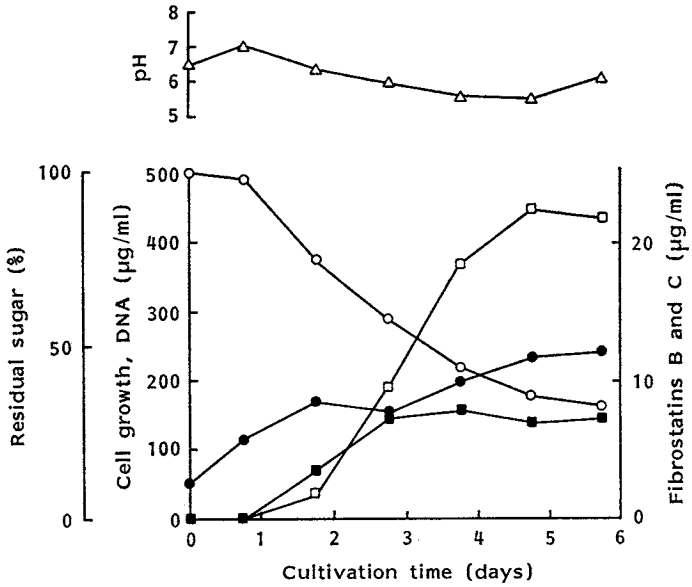
gray color-series with chains of RF-type smooth surface spores and without melanoid pigment formation. Among known species of *Streptomyces*, strain No. 23924 was considered to be similar to *S. catenulae*, and taxonomic characteristics of the strain were compared with those of *S. catenulae* IFO 12848 (ISP 5258) in detail. Both strains have almost the same morphological characteristics, but different capability of utilizing carbohydrates such as xylose, fructose, raffinose and mannitol. Strain No. 23924 did not grow on glycerol - asparagine agar; *S. catenulae* grew well. On inorganic salts - starch agar, the aerial mycelium of the strain was gray; that of *S. catenulae* was greenish gray. From these results, strain No. 23924 was identified as *S. catenulae* subsp. *griseospora* No. 23924. The strain has been deposited in the Institute for Fermentation, Osaka under the accession number IFO 14205.

Table 3. Utilization of carbohydrates of strain No. 23924.

Carbon source	Utilization
L-Arabinose	+
D-Xylose	++
D-Glucose	++
D-Fructose	+
Sucrose	+
meso-Inositol	−
L-Rhamnose	−
Raffinose	−
D-Mannitol	−
No addition	−

The symbols used are; (−) no growth, (+) slight growth, (++) good growth.

Fig. 3. A typical time course of fermentation by strain No. 23924.
○ Residual sugar, ● cell growth, ■ fibrostatin B, □ fibrostatin C, △ pH.



Fermentation

A loopful of spores of strain No. 23924 was inoculated into five 2-liter Sakaguchi flasks containing 400 ml each of the seed medium and incubated for 2 days at 28°C on a reciprocal shaker. The resulting seed culture (2 liters) was transferred into 100 liters of the seed medium in a 200-liter fermentor and incubated for 2 days at 28°C under aeration (120 liters/minute) and agitation (140 rpm). Sixty liters of this culture were transferred into a 2,000-liter fermentor containing 1,200 liters of the fermentation medium and incubated for 6 days at 24°C under aeration (1,200 liters/minute) and agitation (120 rpm). A typical fermentation profile for fibrostatin production is shown in Fig. 3.

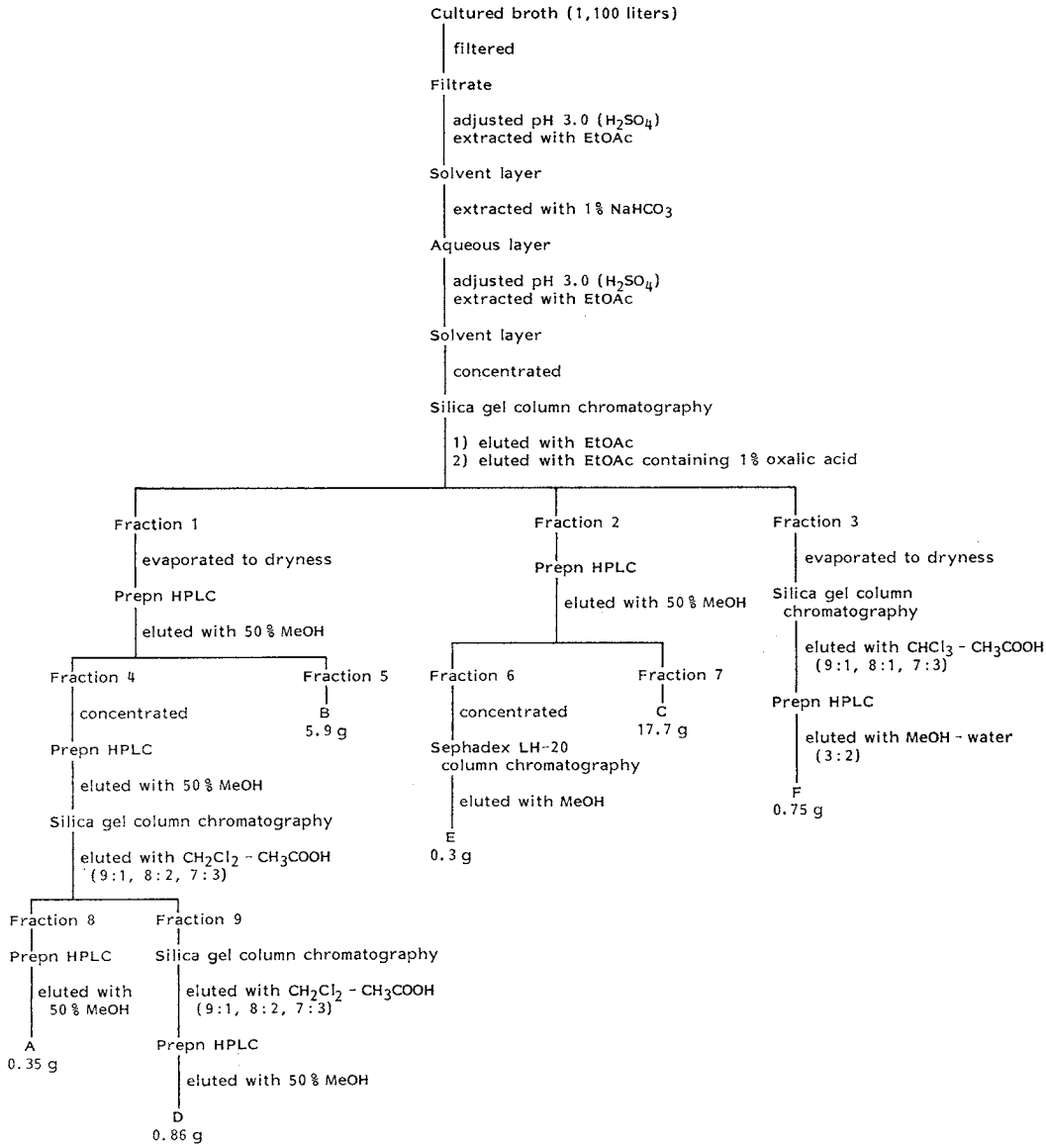
Isolation

A flow diagram for isolating fibrostatin compounds is given in Fig. 4. Fibrostatins A, B, C, D, E and F were isolated from fractions (1~9) by a combination of purification procedures; solvent extraction, silica gel chromatography, preparative reverse-phase high performance liquid chromatography (HPLC) fitted with a Prep-500/C_{18} column, and crystallization. Throughout this purification procedure, the fibrostatins were monitored by thin-layer chromatography (TLC).

Physico-chemical Properties

Fibrostatins were isolated as colored crystals of various shades of orange. They give positive color reactions for ferric chloride, methanolic magnesium acetate and Rydon-Smith reagents, and negative color reaction for ninhydrin and Ehrlich reagents. They are soluble in dimethyl sulfoxide, pyridine, methanol, dioxane, acetic acid, ethyl acetate and *N*,*N*-dimethylformamide, and sparingly soluble or insoluble in water, chloroform and *n*-hexane. Their physico-chemical properties and spectral data are summarized in Table 4. These data strongly suggested that all the fibrostatins have the same naphthoquinone skeleton. Typical UV and IR spectra are shown in Figs. 5 and 6.

Fig. 4. Isolation procedure of fibrostatins.



The structural determination of six inhibitors will be reported in the following paper[15].

Biological Activity

Fibrostatins were found to inhibit prolyl hydroxylase dose-relatedly (Fig. 7). The ID_{50} values of A, B, C, D, E and F were estimated to be about 23, 39, 29, 180, 10 and 14 μM, respectively. They show no antimicrobial activity at a concentration of 100 μg/ml by the agar dilution method against yeasts, fungi and bacteria including mycoplasma. The acute toxicity (LD_{50}) of fibrostatins A, B, C, D, E and F was 100~200, 50~100, 100~200, >400, 50 and 50~100 mg/kg, respectively when administered intraperitoneally to rats; when administered orally the LD_{50} of fibrostatins B and C was more than 1,000 mg/kg.

Table 4. Physico-chemical properties of fibrostatins.

	Fibrostatin		
	A	B	C
MP (°C)	186~188	200~202	187~190
Molecular formula	$C_{18}H_{19}NO_7S$	$C_{19}H_{21}NO_8S$	$C_{18}H_{19}NO_8S$
Anal Calcd	C 54.95, H 4.87, N 3.56, S 8.15	C 53.89, H 5.00, N 3.31, S 7.57	C 52.81, H 4.68, N 3.42, S 7.83
Found	C 54.75, H 4.92, N 3.77, S 8.09	C 53.74, H 4.99, N 3.26, S 7.29	C 52.43, H 4.72, N 3.50, S 7.82
MW (MS, *m/z*)	393 (M^+)	423 (M^+)	409 (M^+)
$[\alpha]_D^{23}$ (MeOH)	−91° (*c* 0.50)	−90° (*c* 0.51)	−93° (*c* 0.51)
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	220 (886), 263 (sh, 421), 270 (458), 420 (129)	220 (793), 262 (sh, 392), 268 (428), 308 (214), 420 (108)	221 (877), 260 (sh, 398), 265 (415), 306 (256), 420 (124)
$\lambda_{max}^{0.1N\ HCl-80\%\ MeOH}$ nm ($E_{1cm}^{1\%}$)	221 (914), 264 (sh, 442), 270 (476), 420 (133)	220 (789), 261 (sh, 390), 268 (431), 308 (210), 420 (107)	221 (866), 260 (sh, 403), 266 (414), 306 (250), 420 (119)
$\lambda_{max}^{0.1N\ NaOH-80\%\ MeOH}$ nm ($E_{1cm}^{1\%}$)	216 (505), 238 (681), 277 (248), 540 (131)	216 (482), 237 (748), 284 (215), 540 (128)	216 (500), 236 (791), 286 (213), 540 (142)
IR ν_{max}^{KBr} cm^{-1}	3300, 1710, 1665, 1640, 1620, 1540, 1420, 1335, 1280, 1180	3300, 1725, 1705, 1660, 1630, 1590, 1540, 1420, 1330, 1270, 1170	3400, 1720, 1680, 1635, 1600, 1540, 1420, 1310, 1250, 1180
Rf on TLC*			
(I) $CHCl_3$ - AcOH (8 : 2)	0.39	0.42	0.30
(II) 2-PrOH - AcOH (96 : 4)	0.45	0.48	0.29
(III) MeOH - H_2O (7 : 3)	0.61	0.55	0.69

	Fibrostatin		
	D	E	F
MP (°C)	205~207	174~176	191~195
Molecular formula	$C_{18}H_{19}NO_8S$	$C_{18}H_{19}NO_8S$	$C_{19}H_{21}NO_9S$
Anal Calcd	C 52.81, H 4.68, N 3.42, S 7.83	C 52.81, H 4.68, N 3.42, S 7.83	C 51.93, H 4.82, N 3.19, S 7.30
Found	C 52.58, H 4.61, N 3.55, S 7.84	C 52.18, H 4.58, N 3.19, S 7.55	C 51.67, H 4.75, N 3.45, S 7.30
MW (MS, *m/z*)	409 (M^+)	410 $(M+1)^+$	439 (M^+)
$[\alpha]_D^{23}$ (MeOH)	−62° (*c* 0.51)	−86° (*c* 0.50)	−91° (*c* 0.51)
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	220 (764), 271 (450), 306 (209), 420 (102)	221 (851), 263 (sh, 393), 271 (435), 420 (128)	222 (866), 262 (sh, 379), 268 (381), 308 (205), 420 (91)
$\lambda_{max}^{0.1N\ HCl-80\%\ MeOH}$ nm ($E_{1cm}^{1\%}$)	220 (762), 271 (456), 306 (207), 420 (99)	221 (869), 264 (sh, 411), 271 (445), 420 (127)	221 (852), 262 (sh, 374), 267 (383), 307 (200), 415 (107)
$\lambda_{max}^{0.1N\ NaOH-80\%\ MeOH}$ nm ($E_{1cm}^{1\%}$)	216 (438), 234 (576), 299 (531), 540 (89)	216 (478), 238 (633), 287 (231), 540 (122)	222 (sh, 548), 236 (730), 270 (257), 542 (115)
IR ν_{max}^{KBr} cm^{-1}	3400, 1720, 1670, 1635, 1610, 1540, 1440, 1340, 1300, 1260, 1220	3300, 1725, 1660, 1640, 1610, 1540, 1455, 1420, 1325, 1300, 1240, 1180	3300, 1720, 1680, 1660, 1630, 1600, 1530, 1420, 1335, 1310, 1260, 1190
Rf on TLC*			
(I) $CHCl_3$ - AcOH (8 : 2)	0.19	0.12	0.22
(II) 2-PrOH - AcOH (96 : 4)	0.57	0.45	0.39
(III) MeOH - H_2O (7 : 3)	0.60	0.75	0.72

* Silica gel 60 F_{254} plate (Merck, Art No. 5729) in I or II and reverse-phase HPTLC plate (Merck, RP-8) in III were used. Detection was carried out by UV light (254 nm).

Fig. 5. UV spectrum of fibrostatin C.

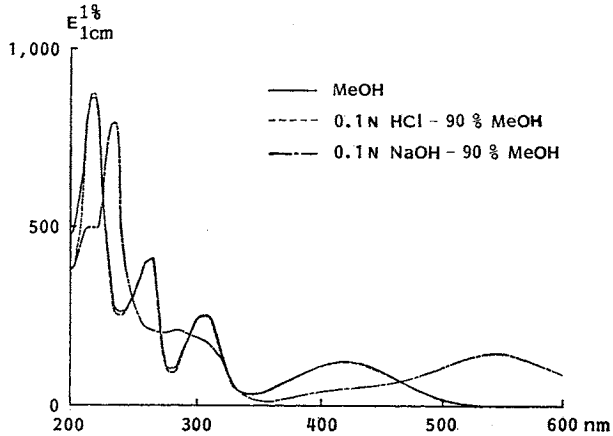


Fig. 6. IR spectrum of fibrostatin C (KBr).

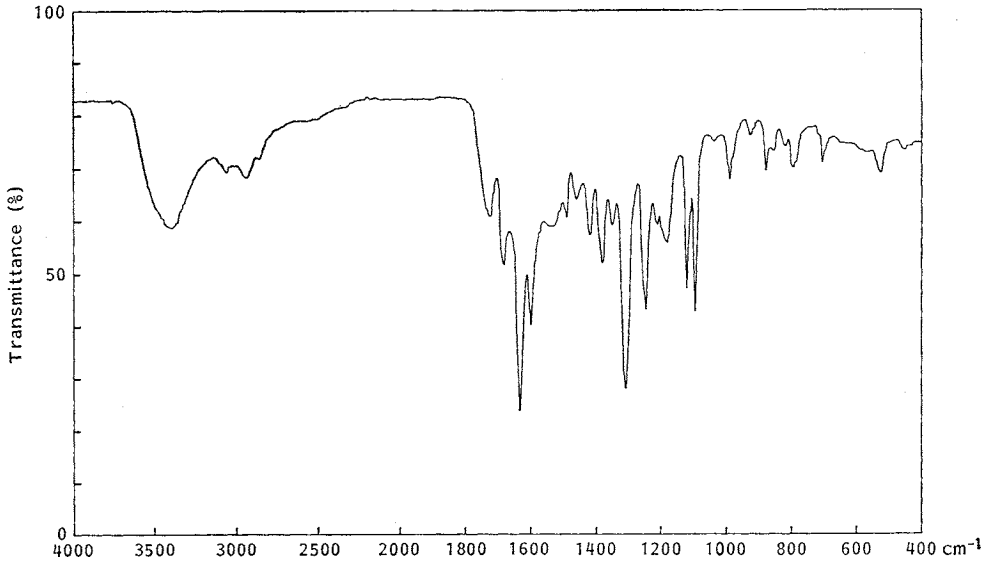
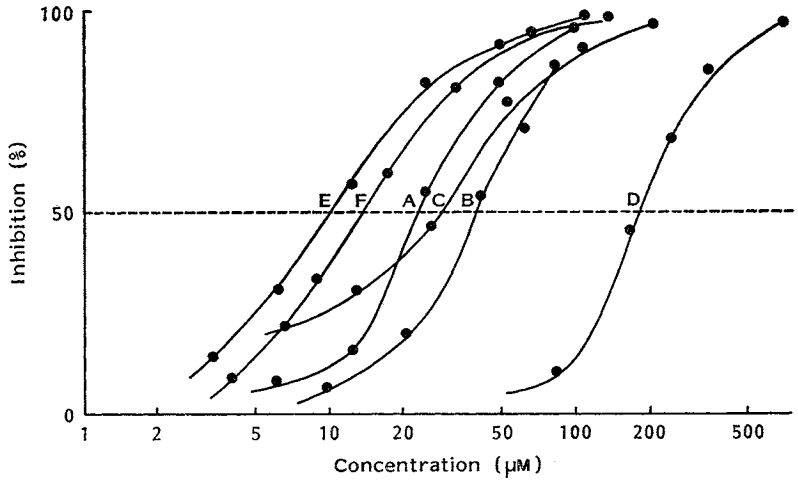


Fig. 7. Inhibition of prolyl hydroxylase activity by fibrostatins.



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