# EPIDERSTATIN, A NEW INHIBITOR OF THE MITOGENIC ACTIVITY INDUCED BY EPIDERMAL GROWTH FACTOR

## I. TAXONOMY, FERMENTATION, ISOLATION AND CHARACTERIZATION

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Inhibitors of mitogenic activity induced by epidermal growth factor (EGF) were screened from culture broths of soil microorganisms. A strain of actinomycetes has been found to produce a new glutarimide antibiotic named epiderstatin which inhibits the incorporation of [$^3$H]thymidine into quiescent animal cells stimulated by EGF. Taxonomic studies have revealed that the producing strain belongs to a subspecies of *Streptomyces pulveraceus*, thus the name, *Streptomyces pulveraceus* subsp. *epiderstagenes* was given to this strain. The molecular formula ($C_{15}H_{20}N_2O_4$) and UV profile ($\lambda_{max}$ 295 nm) of the antibiotic are distinct from other known antibiotics. It inhibited the incorporation of [$^3$H]thymidine into quiescent cells stronger than into growing cells.

Recently, there have been a number of arguments concerning the relationship between growth factors and oncogenesis[1,2]. Specific inhibitors of growth factors should be useful to elucidate the mechanism of action and relationship between growth factors and oncogenesis. In addition, they are expected to have a new type of antitumor activity. We have chosen epidermal growth factor (EGF) as a screening target, because EGF is one of the best characterized growth factors[3]. EGF is a homologous protein to transforming growth factor alpha (TGF-$\alpha$) and both growth factors share the same receptor[4]. Therefore, the inhibitor of EGF is expected as a growth inhibitor of tumor cells.

During the screening, we found that a new inhibitor named epiderstatin was produced by a streptomycete, strain RK-441. Taxonomy, fermentation of the producing strain and isolation, physico-chemical properties, and biological activity of epiderstatin are described in this paper.

## Materials and Methods

### Taxonomic Studies

Methods and media recommended by International Streptomyces Project (ISP)[5] were used to examine the taxonomic characterization of strain RK-441. Morphology on ISP media was observed after incubation at 28°C for 14 days. The Color Harmony Manual (4th Ed., 1958, Container Corporation of America, Chicago, Illinois) was used to identify the color of mycelial and soluble pigments. A scanning electron microscope (SEM) was used to study morphology of the spore chains. Whole-cell sugars were identified by the method of LECHEVALIER and LECHEVALIER[6] and diaminopimelic acid (DAP) isomers were analyzed by the method of BECKER *et al.*[7].

### Bacterial Strain

The strain RK-441 has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the accession No. FERM-P10306. *Streptomyces bobili* JCM 4012 and JCM 4627 were obtained from Japan Collection of Microorganisms (JCM), RIKEN Institute, and *Streptomyces pulveraceus* IFO 3855 and IFO 3857 from Institute for Fermentation, Osaka (IFO).

Fermentation

The composition of the seed medium is as follows; glucose 2%, soluble starch 1%, meat extract 0.1%, dried yeast 0.4%, soybean meal 2.5%, NaCl 0.2% and $K_2HPO_4$ 0.005%. The medium was adjusted to pH 7.0 prior to sterilization. The seed culture was incubated on a rotary shaker at 250 rpm for 48 hours in 500 ml cylindrical flasks containing 70 ml of the seed medium. Then 180 ml of the culture was transferred to 30-liter jar fermenters charged with 18 liters of the same medium containing 0.01% of DF 40P antifoam. The fermenters were agitated at 250 rpm, aerated at 1.0 vol/vol/minute and temperature was maintained at 28°C.

The fermentation was monitored by antifungal activity against *Pyricularia oryzae* IFO 5994 and inhibitory activity of incorporation of [$^3$H]thymidine into quiescent Balb/MK cells[8], a mouse epidermal keratinocyte. Samples for this assay were prepared as follows: A filtrate of a culture specimen was extracted twice with same volume of ethyl acetate. The solvent layer was evaporated under reduced pressure and then the residue was dissolved in equal volume of 50% MeOH to the initial broth.

Isolation

Culture broth (72 liters) was filtered with the aid of Celite. The filtrate was extracted with the same volume of ethyl acetate. The organic layer was evaporated under reduced pressure. The oily material was applied onto the silica gel column (7.4 i.d. × 40 cm) equilibrated with chloroform - methanol (96 : 4). It was developed with the same solvent system and active fractions were pooled. After evaporation *in vacuo*, the crude active material was applied onto the second silica gel column (2.4 i.d. × 56 cm) equilibrated with chloroform - methanol (92 : 8). Active fractions were eluted with the same solvent system and collected. They were concentrated *in vacuo* and purified by Sephadex LH-20 column chromatography (2.6 i.d. × 90 cm, solvent; 80% methanol). Preparative HPLC was carried out by using a reverse phase column (Senshu Pak N5171, 20 i.d. × 250 mm, monitored by UV at 220 nm) with 50% methanol as a solvent. Finally, preparative HPLC was repeated by using the same column with 45% methanol. The eluate obtained was concentrated and lyophilized to yield 9.2 mg of pure epiderstatin as an amorphous powder.

Instrumental Analyses

Optical rotation was determined on a Perkin-Elmer 241MC polarimeter. The mp was taken on a Yanagimoto micro melting point apparatus. UV and IR spectra were measured on Hitachi 220A spectrophotometer and Shimadzu IR27G recording IR spectrophotometer, respectively. Hitachi M-80 mass spectrometer was used to determine the molecular weight. $^1$H and $^{13}$C NMR spectra were obtained by using Jeol GSX-500 and Jeol FX-100 fourier transform (FT)-NMR spectrometers, respectively. For NMR measurement, $CDCl_3$ was used as a solvent and TMS was used as an internal standard.

Biological Activity

Balb/MK cells were cultured in minimal essential medium (MEM) containing a calcium concentration of 0.05 mM supplemented with 5 ng/ml of EGF (receptor grade, Collaborative Research Inc., Bedford, MA., U.S.A.) and 10% fetal bovine serum (FBS, Gibco Laboratories, Grand Island, NY., U.S.A.). At confluent growth stage, culture medium of the cell was changed to fresh low calcium MEM medium without EGF and FBS. Mitogenic activity of EGF can be measured by the incorporation of [$^3$H]thymidine (ICN Radiochemicals, Irvine, CA, U.S.A.) into quiescent Balb/MK cells 17 hours after addition of EGF[9]. The incorporation of [$^3$H]thymidine into Balb/MK cells was measured in the presence or absence of epiderstatin.

Antimicrobial activity was determined by the conventional agar dilution method. Potato - sucrose agar plates and nutrient agar plates containing a serial dilution of epiderstatin were used for measurement of antifungal activity and antibacterial activity, respectively.

## Results and Discussion

Taxonomic Studies

The producing strain, RK-441 (FERM-P10306), was isolated from a soil sample collected in Yamanashi

Prefecture, Japan. The strain was cultured on various ISP media and the characteristics are summarized in Table 1.

Melanoid and/or reddish soluble pigments were produced on ISP media No. 2, No. 5, No.7, tyrosine agar, starch-yeast extract agar and nutrient agar media. Strain RK-441 utilized D-glucose, D-fructose, D-xylose, L-rhamnose and raffinose, and the characteristic reddish soluble pigment was observed when the strain was cultured on the agar plates containing these sugars. The strain did not utilize L-arabinose, sucrose, inositol and D-mannitol.

Strain RK-441 had spiral spore chains (Fig. 1A). The spore surface ornamentation was smooth, and its shape was cylindrical and averages $1.0 \times 0.6\,\mu m$ in size (Fig. 1B). The whole cell sugar composition of strain RK-441 was found to be galactose, glucose and ribose, which is Type NC[10] sugar pattern. The cell

Table 1. Cultural characteristics of strain RK-441.

| Medium | Growth | Reverse color | Aerial mycelium | Soluble pigment |
|---|---|---|---|---|
| Yeast extract-malt extract agar (ISP No.2) | Good | Chocolate (7ml) | Pale yellow brown (4ig) | Dark wine (7pi) |
| Oatmeal agar (ISP No. 3) | Poor | Yellowish brown (3cb) | None or scant | None |
| Inorganic salts-starch agar (ISP No. 4) | Poor | Yellowish brown (3cb) | None or scant | None |
| Glycerol-asparagine agar (ISP No. 5) | Poor | Light brown (4ng) | None or scant | Redwood (6ie) |
| Peptone-yeast extract-iron agar (ISP No. 6) | Poor | Light brown (4ie) | None or scant | None |
| Tyrosine agar (ISP No. 7) | Good | Brown (4lg) | None or scant | Light brown (3lg) |
| Starch-yeast extract agar | Good | Chocolate (7ml) | Rose wood (5ge) | Chocolate brown (5po) |
| Nutrient agar | Good | Beaver (4il) | None or scant | None |
| Glucose-asparagine agar | Poor | Camel (3ie) | None or scant | Grape (10ic) |
| Sucrose-nitrate agar | Poor | Yellowish brown (3cb) | None or scant | None |

Fig. 1. Scanning electron micrograph of spore chains of strain RK-441 on oatmeal agar medium diluted with V8 juice.

Incubated at 28°C for 14 days (bar indicates 5 $\mu$m in Fig. 1A and 2 $\mu$m in Fig. 1B).

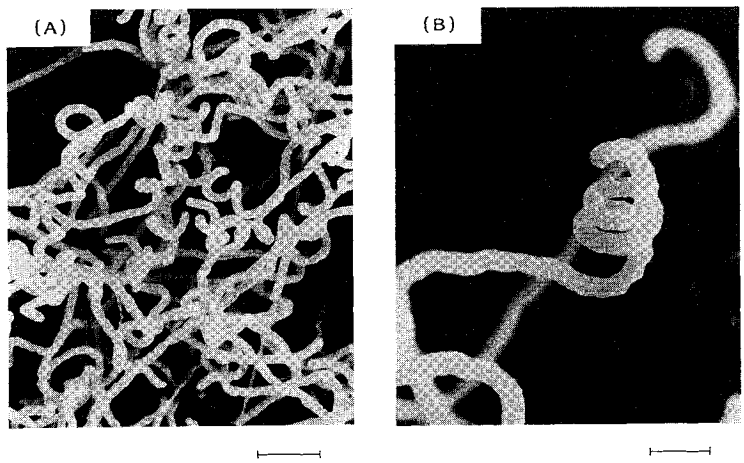


Table 2. Comparison of characteristics.

| | *Streptomyces bobili* | | *Streptomyces pulveraceus* | | RK-441 |
|---|---|---|---|---|---|
| | JCM 4012 | JCM 4627 | IFO 3855 | IFO 3857 | |
| Spore chain | Spiral | Spiral | Spiral | Spiral | Spiral |
| Spore surface | Smooth | Smooth | Smooth | Smooth | Smooth |
| Aerial mycelium[a] | None | None | Gray | White | Yellow brown |
| Reverse color[a] | Pink | Pink | Yellow | Yellow | Chocolate |
| Red pigment | − | − | − | − | + |
| Utilization of carbon source | | | | | |
| D-Glucose | + | + | + | + | + |
| D-Xylose | + | + | + | + | + |
| L-Arabinose | + | + | − | − | − |
| L-Rhamnose | + | + | + | + | + |
| D-Fructose | + | + | + | + | + |
| D-Galactose | + | + | + | + | + |
| Raffinose | + | + | + | + | + |
| D-Mannitol | − | − | − | − | − |
| *i*-Inositol | + | + | − | − | − |
| Salicin | − | − | + | + | + |
| Sucrose | + | + | − | − | − |

[a] These 5 strains were cultured on the 10 kinds of agar media listed in Table 1. Aerial mycelia and reverse color were described by the observation on the ISP No. 2 medium.

hydrolysate contained the L,L isomer of DAP which corresponds to cell-wall Type I[6].

According to BERGEY's Manual[11], these chemotaxonomic and general characteristics suggest that strain RK-441 resembles *S. pulveraceus* and *S. bobili*. Therefore, the culture characteristics of the strain RK-441 were compared with *S. pulveraceus* IFO 3855 and IFO 3857 and *S. bobili* JCM 4012 and JCM 4627, side by side. Morphology of spore-chain, ornamentation of spores and utilization of carbon source of strain RK-441 were consistent with those of *S. pulveraceus* IFO 3855 and 3857. In contrast, *S. bobili* JCM 4012 and JCM 4627 formed pink or pink-violet vegetative mycelium on the ISP No. 2, No. 3 and No. 4 agar media, which was different from those of RK-441. In this respect, strain RK-441 is more similar to *S. pulveraceus* than *S. bobili*. However, RK-441 produced a characteristic soluble reddish pigment. Production of the pigment was not observed both with *S. pulveraceus* and *S. bobili* (Table 2). Therefore, it was concluded that the strain RK-441 is a variety of *S. pulveraceus* and is designated as *Streptomyces pulveraceus* subsp. *epiderstagenes* considering the production of a new antibiotic, epiderstatin.

## Fermentation

The time course of fermentation in a 30-liter jar fermenter was monitored. Both inhibitory activity of incorporation of [$^3$H]thymidine into quiescent Balb/MK cells induced by EGF and antifungal activity against *P. oryzae* reached maximum after 72 hours-fermentation. At the time, pH of the fermentation broth reached around 7.7.

## Isolation

Epiderstatin was isolated from the culture filtrate (72 liters) by the procedure presented in Scheme 1. The activity of epiderstatin was monitored by an assay measuring inhibitory effect on the incorporation of [$^3$H]thymidine into quiescent Balb/MK cells after stimulation with EGF. The solvent layer of ethyl acetate extraction contained both anti-EGF and antifungal activities. After the second silica gel column

chromatography, epiderstatin was separated from the known glutarimide antibiotics (cycloheximide 1.2 g, acetoxycycloheximide 180 mg, streptovitacin A 16 mg and streptovitacin B 3.2 mg) which were identified after purification. Finally, 9.2 mg of pure epiderstatin was obtained by the reverse phase HPLC (Senshu Pak N5171 column).

Scheme 1. Purification procedure.

Culture filtrate (72 liters)
↓ EtOAc extraction
Solvent layer
↓ evaporation *in vacuo*
27.7 g of oily substance
↓ silica gel column chromatography
column: 7.4 i.d. × 40 cm
solvent: $CHCl_3$ - MeOH (96:4)
3.5 g of active material after evaporation
↓ silica gel column chromatography
column: 2.4 i.d. × 56 cm
solvent: $CHCl_3$ - MeOH (92:8)
108 mg of active substance after evaporation
↓ Sephadex LH-20 column chromatography
column: 2.6 i.d. × 96 cm
solvent: 80% MeOH
44 mg of active substance after lyophilization
↓ 1st HPLC
column: Senshu Pak N5171 (20 i.d. × 250 mm)
solvent: 50% MeOH
2nd HPLC
column: Senshu Pak N5171 (20 i.d. × 250 mm)
solvent: 45% MeOH
9.2 mg of white powder after lyophilization

Table 3. Physico-chemical properties of epiderstatin.

| | |
|---|---|
| Appearance | White amorphous powder |
| $[\alpha]_D^{21}$ ($c$ 0.22, MeOH) | +5.3° |
| MP | 185~187°C |
| UV $\lambda_{max}^{MeOH}$ nm ($\varepsilon$) | 295 (16,700) |
| IR (KBr) $cm^{-1}$ | 3430, 1725, 1700, 1676 |
| HREI-MS $m/z$ | Calcd for $C_{15}H_{20}N_2O_4$: 292.1422 |
| | Found: 292.1423 |
| Color reaction: | |
| Positive | Rydon-Smith, anthrone, anisaldehyde - $H_2SO_4$ |
| Negative | Periodate - benzidine, $FeCl_3$, Lemieux, ninhydrin, 2,4-dinitrophenylhydrazine |
| Rf[a] | 0.87 ($CHCl_3$ - MeOH, 4:1) |
| | 0.55 ($CHCl_3$ - MeOH, 9:1) |

[a] TLC (Kieselgel $F_{254}$, Merck).

Fig. 2. UV profile of epiderstatin in MeOH and acidic MeOH solution.

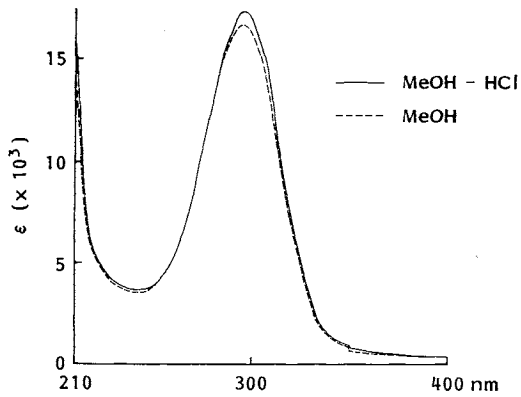



Fig. 3. IR spectrum of epiderstatin (KBr).

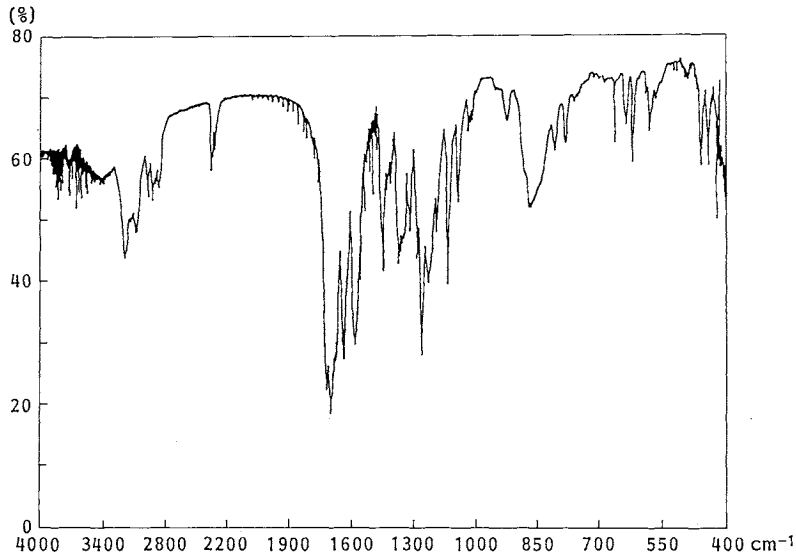


Fig. 4. $^{13}C$ NMR spectrum of epiderstatin (in $CDCl_3$).

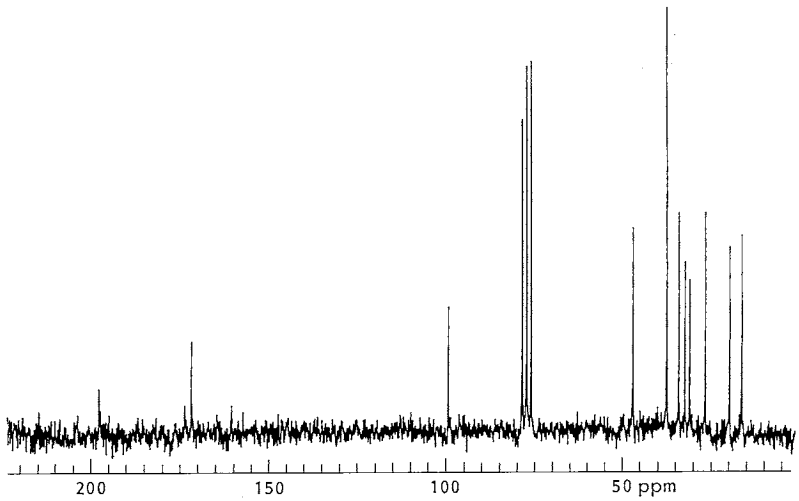
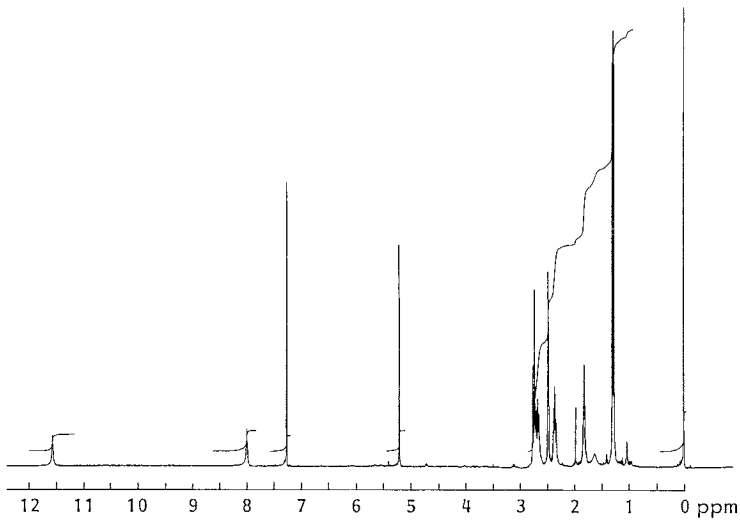



Fig. 5. $^{1}H$ NMR spectrum of epiderstatin (in $CDCl_3$).



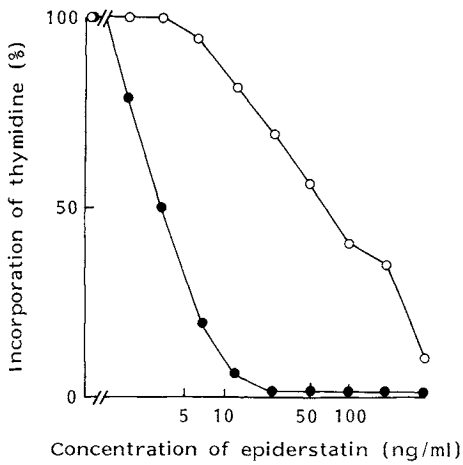

## Physico-chemical Properties

The physico-chemical properties of epiderstatin are summarized in Table 3. It is easily soluble in methanol, ethanol, chloroform and DMSO, and insoluble in water and hexane. The methanol solution exhibited an absorption maximum at 295 nm ($\varepsilon$ 16,700) as shown in Fig. 2. The antibiotic is more stable in acidic methanol than in alkali methanol solution. The solution in methanolic 0.05 N NaOH becomes turbid. The IR spectrum is shown in Fig. 3.

The molecular weight of the antibiotic was determined as 292 from its field desorption (FD)-MS. The high-resolution electron impact (HREI)-MS gave the molecular ion at $m/z$ 292.1423, which established the molecular formula of epiderstatin as $C_{15}H_{20}N_2O_4$. The number of carbon atoms is consistent with $^{13}C$ NMR spectrum (Fig. 4).

To our knowledge, there is only fusarochromanone[12] (a mycotoxin produced by *Fusarium roseum*)

Fig. 6. Incorporation of [$^3$H]thymidine into Balb/MK cells under the presence of various concentration of epiderstatin.




Incorporation was measured during 6 hour-period beginning at 17 hours following addition of epiderstatin and EGF to quiescent cells (●). Incorporation of [$^3$H]thymidine into growing cells being cultured in the serum/EGF-containing low calcium medium (○) was monitored.

Table 4. Antimicrobial spectrum of epiderstatin.

| Organisms tested | MIC ($\mu$g/ml) |
|---|---|
| *Pyricularia oryzae* IFO 5994 | 25 |
| *Botryotinia fuckeliana* IFO 5365 | 100 |
| *Alternaria mali* IFO 8984 | 100 |
| *Xanthomonas campestris* pv. *citri* | >200 |
| *Escherichia coli* AB1157 | >200 |
| *E. coli* BE1186 | >200 |
| *Salmonella typhimurium* TV119 | >200 |
| *Pseudomonas aeruginosa* IFO 13130 | >200 |
| *Staphylococcus aureus* IFO 12732 | >200 |

which has the same molecular formula as epiderstatin among the microbial products. However, the UV, $^1$H NMR (Fig. 5) and other spectra of epiderstatin is distinctly different from that of fusarochromanone. Thus, we concluded that it is a novel antibiotic. The chemical structure of epiderstatin is reported in the accompanying paper[13].

## Boiological Properties of Epiderstatin

The inhibitory activity of epiderstatin against the signal trandsuction of EGF in a mouse epidermal keratinocyte is worthnoting. There are many antibiotics which inhibit DNA synthesis of animal cells[14], but epiderstatin (50 ng/ml) showed relatively weak inhibition of the incorporation of [$^3$H]thymidine into growing cells compared with quiescent cells. Namely, the incorporation of [$^3$H]thymidine into quiescent cells stimulated by EGF was inhibited at a lower concentration of epiderstatin (10 ng/ml) as shown in Fig. 6. In contrast, actinomycin D and mitomycin C which are known to inhibit synthesis of nucleic acids showed nonspecific inhibition of [$^3$H]thymidine into growing cells and into quiescent cells stimulated with EGF in our assay system.

Epiderstatin did not inhibit the *in vitro* kinase activity of EGF receptor (details will be published elsewhere), therefore, the mechanism of action of epiderstatin is different from that of erbstatin[15] which is known to be an inhibitor of EGF-receptor kinase.

The *in vitro* antimicrobial activity of epiderstatin is shown in Table 4. The antibiotic showed weak antifungal activity but no antibacterial activity.

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