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Isolation, Physico-chemical Properties, and Biological Activity of New Thiopeptide Antibiotics, Kimorexins

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An isolate 90-GT-302, identified as *Kitasatosporia kimorexae*, was found to produce antibiotics that induce mycelial swelling in *Magnaporthe grisea* and *Fusarium solani*. The strain produced at least 5 antibiotics. Among them, the main active compound designated as kimorexin A was isolated and its physico-chemical properties and biological activities were examined, and as a result was found to be of the thiopeptide antibiotic. A comparison between the properties of kimorexin A and those of the known thiopeptide antibiotics led us to conclude that kimorexin A was a new thiopeptide polythiazolyl antibiotic. Kimorexin A showed a narrow antimicrobial spectrum against very limited genus of phytopathogenic fungi. It prevented host plants from infections of *Rhizoctonia solani* and absolute parasitic fungi, such as *Sphaerotheca fuliginea* and *Puccinia recondita*, almost completely at the treatment concentration of approximately 20 ppm.

The majority of fungal cells are surrounded by a rigid cell wall which mainly consists of a variety of polysaccharides (80 to 90%). Of the polysaccharides most important are chitin and cellulose, both polymers of glucose. Most fungi in their cell walls have chitin and no cellulose, whereas for few fungi the reverse is true. In the cell walls, microfibrils of chitin or cellulose are intertwined and embeded in an amorphous matrix which cements them together, providing a skeletal framework that gives wall their morphological forms. The matrix contains protein and polysaccharides such as glucans or mannans. A target-directed antibiotic screening against chitin, glucans or mannans is promising because they do not occur in mammals or plants.

Since the discovery of polyoxin (1) and elucidation of its mode of action on fungal cell wall biosynthesis (2), inhibitor for the fungal cell wall biosynthesis has received a great deal of attention due to its extremely low toxicity. Inhibitors for fungal cell wall induce mycelial swelling in fungi due to direct contacts of cellular membrane to the extemal environment. This phenomenon can easily be detected by observing the inhibited area under the microscope. Gunji *et al.* (3) screened antifungal agents inducing morphological abnormalities and Selitrennikoff (4) employed temperature sensitive, protoplast-forming *Neurospora crassa* for the screening of antifungal agents.

In the course of our selective screening program for antibiotics with an inhibitory activity against the fungal cell wall biosynthesis, an isolate of *Kitasatosporia* sp. isolated from the soil collected in Goesan, Choongbook Prefecture, Korea, was found to produce antibiotics that induce mycelial swelling in *Magnaporthe grisea* and *Fusarium solani*. Taxonomy of the producing microorganism is presented elsewhere in the accompanying publication (5).

MATERIALS AND METHODS

Fermentation Conditions

Fermentation was carried out at 32°C for 96 h in a 50 litre jar fermentor containing 30 litre medium consisted of: 2.0% glucose, 1.0% soluble starch, 0.1% meat extract, 0.4% yeast extract, 2.5% soybean flour, 0.2% NaCl, 0.005% K_2HPO_4 and 0.1% antifoaming agent. Agitation and aeration were controlled to 50~350 rpm and 5~30 litre/min, respectively depending on the growth phase. Antimicrobial activity of the culture broth was monitored with *Magnaporthe grisea* IFO 5994 as the test microorganism. Paper disc agar diffusion method was employed for the assay of antimicrobial activity.

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Key words: New thiopeptide antibiotics, physico-chemical properties, biological activity, *Kitasatosporia* sp.

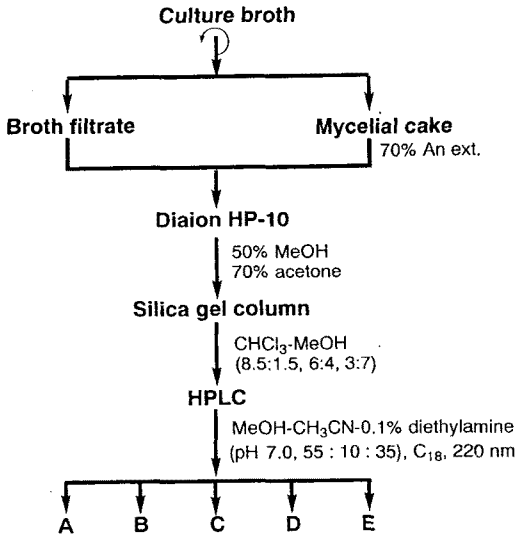


Fig. 1. Purification scheme of active compounds produced by *Kitasatosporia kimorexae*.

Isolation and Purification of the Active Compounds

As shown in Fig. 1, culture broth (30 litre) was centrifuged to separate broth filtrate and mycelial cake. The mycelial cake was extracted overnight with 70% aqueous acetone at room temperature and concentrated under reduced pressure to eliminate acetone. Broth filtrate and mycelial cake acetone extract were pooled and adsorbed to Diaion HP-10 (8 litre) and the resin was washed with distilled water (20 litre) and 50% aqueous MeOH (25 litre). Active compounds were subsequently eluted with 70% acetone and concentrated *in vacuo* to obtain brown powder. It was then subjected to silica gel column chromatography and developed stepwise with $CHCl_3$-MeOH (8.5:1.5, 6:4, 3:7). Active fraction was further purified by HPLC with MeOH-CH_3CN-0.1% diethylamine (pH 6.5, 55:10:35, ODS-H80, YMC-PAK, 220 nm). Active fractions were concentrated *in vacuo* and subjected to Diaion HP-10 again to eliminate buffer ingredients. The adsorbate was washed with H_2O and active compound was eluted with 70% aqueous acetone, concentrated and lyophilized to obtain white powder. Paper disc agar diffusion method was employed for the tracking of active compounds.

Physico-chemical Propertie

UV spectrum was recorded on Hewlett Packard spectrophotometer with the sample dissolved in $CHCl_3$-MeOH (1:1). Melting point was determined by Fisher-Johns melting point apparatus. Solubility was investigated by dissolving the sample in a solvent which was then spinned at 3,000 rpm for 15 min. Solubility was determined by the antifungal activity of the supernatant against *Magnaporthe grisea* IFO 5994.

Biological Activity

Minimum inhibitory concentration (MIC) was determined by the conventional agar dilution method on potato sucrose or Mueller Hinton agar medium for fungi and bacteria, respectively. *In vivo* (pot test) biological assay was carried out with flask-cultured broth filtrate without purification, corresponding to a concentration approximately 20 ppm as the total active compounds. *Magnaporthe grisea* IFO 5994 and *Rhizoctonia solani* IFO 6258 were cultured on rice and wheat bran media, respectively to make them fully produce spore mass or sclerotia. *Sphaerotheca fuliginea* and *Puccinia recondita* were grown on their host plants, dried, and stored at -20°C until use. *Rhizoctonia solani* was homogenized to slurry, together with the wheat bran and sterilized distilled water. The phytopathogens were diluted to the final concentration of 10^5 spores/ml and sprayed onto the host plant. Antibiotic was applied to the host plants 24 h before the inoculation of pathogens. Preventive effect of the active compound was investigated by the following method:

$$\text{Preventive effect (\%)} = \left(1 - \frac{\text{No. of necrotic spots on treated plants}}{\text{No. of necrotic spots on control}}\right) \times 100$$

Test Microorganisms

Test microorganisms employed for *in vitro* bioassay were obtained from the Institute of Fermentation, Osaka (IFO), Institute of Applied Microbiology (IAM), Tokyo University, Japan Collection of Microorganism (JCM), RIKEN, Japan, and the American Type Culture Collection (ATCC).

RESULTS AND DISCUSSION

Isolation and Purification of the Active Compounds

As shown in Fig. 2, we could isolate 5 active compounds from the culture broth of *Kitasatosporia kimorexae* at the yield of 20 (A), 1 (B), 2 (C), 2 (D) and 3 (E) mg/litre culture broth, respectively and designated them as kimorexin A, B, C, D, and E. The main active compound, kimorexin A, was found to be degraded in DMSO when standing at room temperature for a week. But HPLC profiles of kimorexins B-E in DMSO were not changed for several months. All of the kimorexins in dried form were very stable at room temperature for up to 6 months as investigated by HPLC profiles. Therefore, Kimorexin B-E can not be regarded as artifacts. All of the active compounds were relatively heat stable at pH 7.0 but lost activity completely by heat treatment at 80°C for 30 min in acidic or alkaline solutions. Antimicrobial activities of the active compounds were not decreased in phosphate buffer solutions (pH 3.0-10.0) when sta-

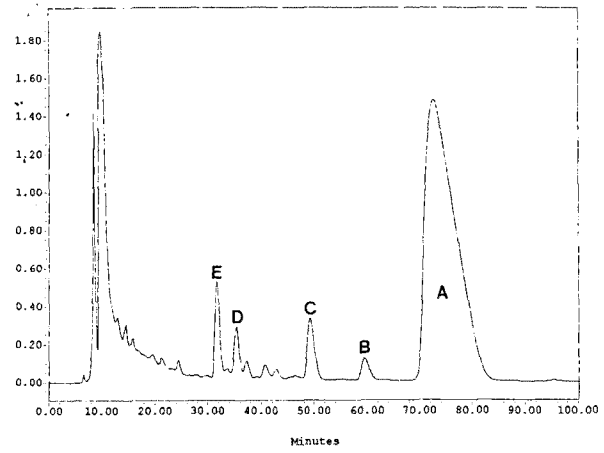


Fig. 2. HPLC profile of active compounds produced by *Kitasatosporia kimorexae* KCTC 0119BP.

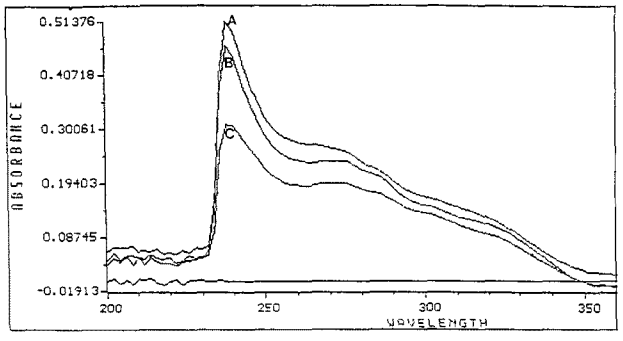


Fig. 3. UV spectrum of kimorexin A in $CHCl_3$-MeOH (1 : 1). A : pH 10.0, B : pH 7.0, C : pH 2.0

nding at room temperature for 3 weeks. They could be extracted with BuOH regardless of pH.

Physico-chemical Properties of Kimorexin A

Among the 5 active compounds, physico-chemical properties of the major active compound, kimorexin A was determined. It showed UV absorption maxima at: $\lambda_{max}^{CHCl_3-MeOH\ (1:1)}$ ($E_{1cm}^{1\%}$): 238 (1,254), 274 (644), 286 (555), 300 (408), 318 (314). UV absorption maxima were not shifted in acidic or alkaline condition as shown in Fig. 3. Kimorexin A (m.w. 1,472, FAB-MS) was readily soluble in DMSO and H_2O, slightly soluble in MeOH and EtOH, and insoluble in acetone, EtOAc, diethylether, chloroform and benzene. It was also readily soluble in $CHCl_3$-MeOH (9:1) mixture. Kimorexin A showed positive color reaction to Dragendorff and I_2 vapour, but negative to ninhydrin, anisaldehyde-sulfuric acid, bromcresol green, Rydon-Smith and anthron reagents. Melting point was determined to be 260-280°C (dec).

Although thiopeptide antibiotics such as saramycetin (6), cyclothiazomycin (7), jingsimycin (8), 5102-2 (9), cystargin (10) and globopeptin (11) have similar physico-chemical properties to kimorexin A their biological

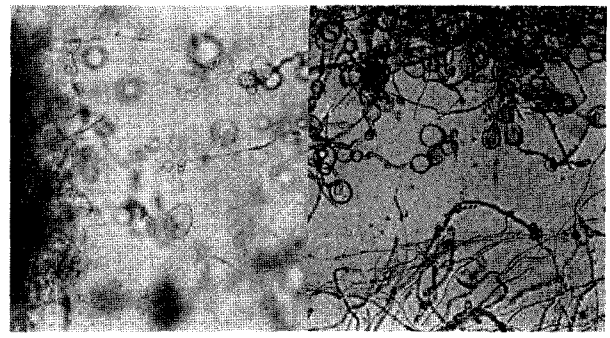


Fig. 4. Kimorexins-induced mycelial swelling in *Magnaporthe grisea*(A) and *Fusarium solani* (B).

activities were found to be quite different from kimorexin A. Cyclothiazomycin (m.w. 1472) has rennin inhibitory activity. Its antifungal activity is known to be very weak. In addition, ^{1}H-NMR and ^{13}C-NMR spectra of cyclothiazomycin revealed a clear difference from those of kimorexin A(data not shown here). Saramycetin, on the other hand, showed the closest similarity to kimorexin A, except for the difference in heat stability in acidic, neutral and alkaline conditions. A detailed information on biological activity of saramycetin is unavailable due to its very unstable pH ranges of 2.0~5.0 and 7.0~9.0 (6). On the other hand, kimorexins are very stable at neutral condition for several months and acidic (pH 3.0) or alkaline (pH 10.0) condition for 3 weeks. Isolation and purification of the kimorexins B-E, minor active compounds produced by *Kitasatosporia kimorexae*, are in progress. A structural determination of kimorexin A will be described elsewhere.

Biological Activity of Kimorexin A

As shown in Fig. 4, kimorexin A induced typical mycelial swelling in *Magnaporthe grisea* IFO 5994 and *Fusarium solani*. The minimum inhibitory concentration of kimorexin A is shown in Table 1. Kimorexin A showed no *in vitro* antimicrobial activity against bacteria, yeast and algae. It was active against a very limited genus of fungi. The most potent *in vitro* inhibitory activity was observed in *Magnaporthe grisea*. Minimum inhibitory concentrations against the test fungi ranged from 0.5 to 16 μg/ml.

In vivo (pot test) biological activity of kimorexins is shown in Table 2. Kimorexins guarded the host plants almost completely against infection by the treatment of broth filtrate containing kimorexins at the concentration of approximately 20 ppm. Tobacco powdery mildew was shown to be prevented 80% at the concentration of 5 ppm as in Fig. 5. It was interesting to note that *in vitro* and *in vivo* effects of kimorexins were not consistent in some case, for example, occurrence of the rice blast

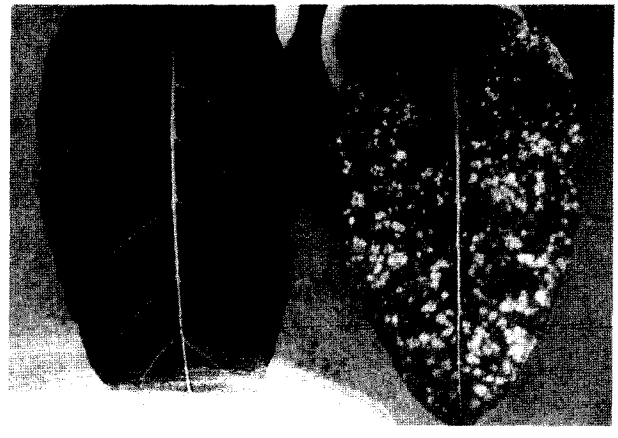
Table 1. Minimum inhibitory concentration (MIC) of kimorexin A.

Test microorganisms	MIC (μg/ml)
Gram positive bacteria	
Staphylococcus aureus FDA 209	>1,000
Mycobacterium phlei IFO 3158	>1,000
Bacillus subtilis IAM 1069	>1,000
Sarcina lutea	>1,000
Gram negative bacteria	
Escherichia coli AB 1157	>1,000
Salmonella typhimurium SL 1102	>1,000
Pseudomonas aeruginosa IFO 13130	>1,000
Pseudomonas fluorescens IAM 1201	>1,000
Xanthomonas campestris pv. *citri* IFO 3781	>1,000
Xanthomonas campestris pv. *oryzae* IFO 3312	>1,000
Erwinia carotovora IFO 12380	>1,000
Yeasts	
Candida albicans IAM 4905	>1,000
Saccharomyces cerevisiae IFO 1008	>1,000
Fungi	
Magnaporthe grisea IFO 5994	<0.5
Colletotrichum lagenarium IFO 751	1.0
Valsa ceratosperma	4.0
Botrytis cinerea IFO 5365	16.0
Alternaria kikuchiana IFO 8594	16.0
Bipolaris oryzae IFO 5277	8.0
Fusarium oxysporum IFO 9761	64.0
Trichoderma viride	250
Rhizoctonia solani IFO 6258	>1,000
Aspergillus niger ATCC 9642	>1,000
Mucor racemosus IAM 6258	>1,000
Algae	
Chlorella vulgaris	>1,000

Table 2. Preventive effect of kimorexins against fungal phytopathogens.

Disease	Preventive value (%)
Rice blast (*Magnaporthe grisea*)	0
Rice sheath blight (*Rhizoctonia solani*)	95
Cucumber gray mold (*Botrytis cinerea*)	0
Tomato late blight (*Phytophthora infestans*)	11
Wheat leaf rust (*Puccinia recondita*)	100
Bean powdery mildew (*Sphaerotheca fuliginea*)	99
Tobacco powdery mildew (*Erysiphe tabacina*)	80*

Treatment concentration was approximately 20 ppm as the content of total kimorexins, *Five ppm of kimorexins were applied.



A B

Fig. 3. Preventive effect of kimorexins against tobacco powdery mildew induced by *Erysiphe tabacina* kimorexins. (ca 5 μg/ml) were applied 24 h before inoculation of the pathogen. A indicates treated and B untreated groups.

induced by *Magnaporthe grisea* was not prevented but rice sheath blight induced by *Rhizoctonia solani* [illegible] was prevented very efficienctly. It is known that *Rhizoctonia solani* produces sclerotia but not spores. Therefore, results of *in vivo* biological activity of kimorexins may be because kimorexins inhibited fungal cell wall biosynthesis but not germination of fungal spores. It is also noteworthy that kimorexin is very effective for preventing absolute parasitic fungi such as *Sphaerotheca fuliginea, Puccinia recondita* and *Erysiphe tabacina* at a reasonably low concentration, 5 to 20 ppm. Most recently, polythiazolyl antibiotics were found to have an inhibitory activity against rennin or angiotensin converting enzyme (7). Therefore, antihypertensive effect of kimorexins is anticipated.

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