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Biological Activity of Acetoxycycloheximide and Its Producing Microoganism

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Acetoxycycloheximide의 생리활성 및 그 생산균주

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Strain No. 77-AG-567 showed antifungal activity against *Pyricularia oryzae* and *Sphaerotheca fuliginea*. In the process of purification of active component this strain was found to produce blasticidin S together with another antibiotic. This compound was identified as being acetoxycycloheximide, also referred to as E-73, by UV and ^{1}H NMR spectral data. Identification of this strain led us to conclude that strain No. 77-AG-567 is most probably be *Streptomyces atratus*. It showed different characteristics from *S. albulus*, so far known to produce both blasticidin S and acetoxycycloheximide. Particularly worthy of note was the difference in spore surface. In addition, acetoxycycloheximide has been known to have activity only against yeast and tumor cells but we found that it also has activity against *Pyricularia oryzae* and *Sphaerotheca fuliginea*.

Rice blast, which has been most extensively studied in Japan, is the major fungal disease in rice and it occurs in nearly all corners of rice cultivating countries in the world. A series of antibiotics active against various plant phathogens have been manufactured on a large scale in Japan [blasticidin S (1), cellocidin (2), kasugamycin (3), polyoxin (4) and validamycin (5)]. Outstanding results were achieved with blasticidin S, active against *Pyricularia oryzae* and with synthetically produced cellocidin. It is mostly due to these achievement that Japan is no longer compelled to import rice and even export a considerable amount.

On the other hand, *Sphaerotheca fuliginea* occurs on many plants and causes the worldwidely problematic powdery midew in cucurbits. Effective fungicides are hitherto known to be benzomyl, triarimol, and dimethirimol (6). Nontheless, efficient control of this disease in cucumber seems unlikely to be satisfactory. Finding an antibiotic effective against *Sphaerotheca fuliginea* is hindered by the fact that it is obligate parasite.

In the screening program for new antibiotics against problematic plant pathogens strain No. 77-AG-567 showed antifungal activity against *Pyricularia oryzae* and *Sphaerotheca fuliginea*. Thus made us to isolate the active compound and to identify the producing organism.

Materials and Methods

Microoganisms

Strain No. 77-AG-567 was isolated from the soil collected in Iwaki-shi Hukuoka-ken, Japan. *Pyricularia oryzae* IFO 5994 was employed for *in*

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vitro bioassay, and *Sphaerotheca fuliginea* and *Colletotrichum lagenarium* were used for the *in vivo* (pot test) investigation of preventive effect against powdery mildew and anthracnose in cucumber, respectively.

Fermentation conditions

Strain No. 77-AG-567 was cultured in antibiotic production medium (pH 7.3) which consisted of glucose; 2%, soluble starch; 1%, meat extract; 0.1%, dried yeast extract; 0.4%, soybean flour; 2.5%, NaCl; 0.2%, and KH_2PO_4; 0.005%. Inoculum flasks were rotated at a velocity of 250 rpm at 27°C for 24 hr and its content was used as a seed inocula. One m*l* of the seed culture was inoculated to each 20 flasks containing 70 m*l* of the same medium and incubated under the same conditions as in seed culture except 96 hr of cultivation.

Pot test

Twenty four hours after spraying sample soultion (30 m*l*/3 seedlings), spores of pathogenic fungi obtained by culturing on cucumber leaves were brushed off on cucumber seedlings at the growth stage of 1-2 leaves. They were then kept in green house for 10 days at 25°C. Preventive value was calculated as the following equation.

$$\text{Preventive value} = 100 - \frac{\text{lesion area (\%) in treated seedlings}}{\text{lesion area (\%) in untreated seedlings}}$$

Taxonomy

The methods described by Shirling and Gottlieb (7) were principally empolyed for the taxonomic studies and that of Pridam and Gottlieb (8) for carbon sources utilization test. Observation of the culture was made after incubation at 27°C for 2 weeks, except where otherwise mentioned. Cell wall analysis was performed on two-dimensional TLC in combination with HPLC described by Harper (9) and Tisdall (10), respectively. Color descriptions were assigned according to the "Color Haromony Manual" (11). The taxonomic keys of 8th ed. of Bergy's Manual (12), and ISP (13-17) were used to compare culture with recognized genera and species of *Streptomyces.*

Isolation and purification

As can be seen in figure 1, supernatant obtained

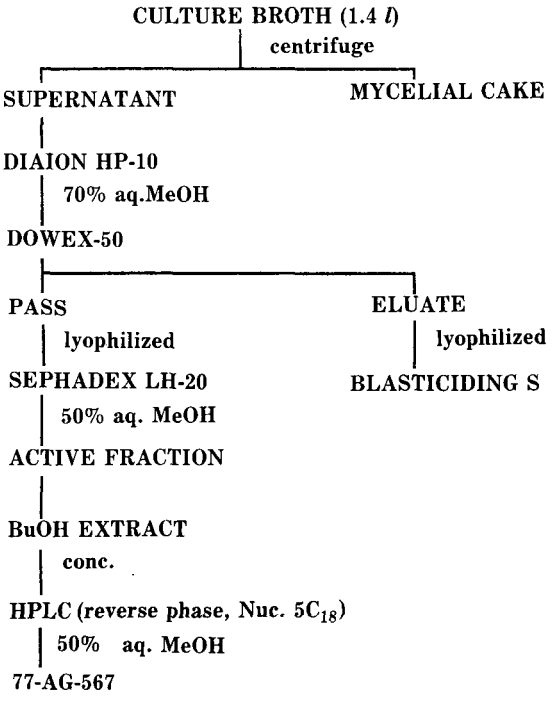

Fig. 1. Isolation procedure for the active component of strain No. 77-AG-567.

by centrifuging whole culture broth (1.4 *l*) was eluted on Diaion HP-10 (Nippon Rensui Co., Ltd.) by 70% aqueous methanol followed by percolation and H_2O-washing. The resultant was then subjected to Dowex 50 (X4; 100-200 mesh; H^+ form; Dow Chem. Co., Ltd.) column chromatography to eliminate basic water-soluble fraction which contained blasticidin S.

Pass was lyophilized to fractionate on Sephadex LH-20 (Seikagaku Co., Ltd.) by 50% aqueous methanol. Active fractions were collected to extract with butanol followed by HPLC (reverse phase, Nucleosil $5C_{18}$) fractionation with 50% aqueous methanol. Bioassay was carried out in every purification steps to track the active principle by agar diffusion method with *Pyricularia oryzae* as the test organism, i.e., suspension of homogenized spore and mycelium grown in submerged medium was inoculated uniformly on Petri dishes and paper disc for antibiotic examination (thick, size 8 mm dia.) dipped into sample solution was placed on the test plate.

Physico-chemical properties

UV spectrum was investigated only in absolute methanol because of insufficient sample volume and

Table 1. Preventive effect of broth filtrate of strain No. 77-AG-567 against 2 different cucumber diseases.

Disease	Dilution	Lesion area (%)	Preventive value (%)	Evaluation
Powdery	1	3.3	95.7	A
mildew	4	3.3	95.7	B
Untreated	–	76.7	–	–
Anthracnose	1	25.0	71.2	B
	4	31.7	63.4	C
Untreated	–	86.7	–	–

Ninety to 100% prevention was evaluated as A, 60-89.9% B. and 0-59.9% C.

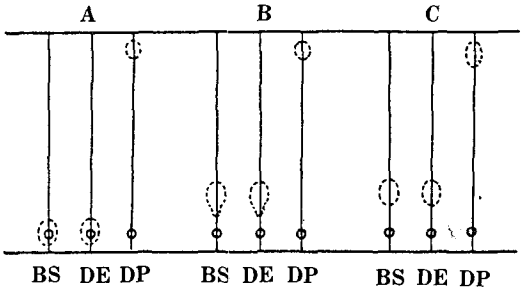

Fig. 2. Comparison of Dowex 50 pass (DP) and eluate (DE) with blasticidin S (BS) on cellulose TLC following bioautography.
Solvent system of A:butanol-methanol-H_2O (4:1:2), B: butanol-acetic acid-H_2O (4:1:2), C: propanol-1N NH_4OH (7:3). Test microorganism was *Pyricularia oryzae*. Dotted line indicates inhibition zone.

^1H NMR in $CDCl_3$. ^1H NMR spectrometer was JEOL 500 MHz and TMS was used as the internal reference.

Results

In the course of new antibiotic screening for agricultural use, we found that strain No. 77-AG-567 produced antibiotics active against *Pyricularia oryzae* and *Sphaerotheca fuliginea*. Broth filtrate of this strain represented 95.7% preventive effect, as shown in Table 1, against cucumber powdery mildew caused by *Sphaerotheca fuliginea*.

Isolation and purification

In the process of purification, Dowex 50 eluate revealed very peculiar inhibition zone (black large halo), which made us to doubt the presence of blasticidin S. It was later confirmed by cellulose TLC in 3 different solvent systems: butanol-methanol-H_2O (4:1:2), butanol-acetic acid-H_2O (4:1:2), and propanol-N NH_4OH (7:3), follwed by bioautography using *Pyricularia oryzae* as test organism. Furthermore, it is well known that blasticidin S and detoxin have antagonistic action (18) against each other, depending on the kind of test organism. Therefore, antagonistic effect of detoxin (Kaken Chem. Co., Ltd.) against Dowex 50 eluate was investigated by cross-placing filter paper scraps (0.5 × 5 cm) dipped into detoxin solution (500 ug/ml) with *Bacillus cereus* IAM 1279 as test organism. As shown in figure 2, Dowex 50 eluate revealed almost same Rf value as blasticidin S standard. In addition, antagonistic effect of Dowex 50 eluate against detoxin convinced the presence of blasticidin S in this fraction. However, passed fraction still had activity against *Pyricularia oryzae* and showed different properties in these tests, thus made us to keep further track of isolation and structure determination of the active compound, and identification of the producing strain.

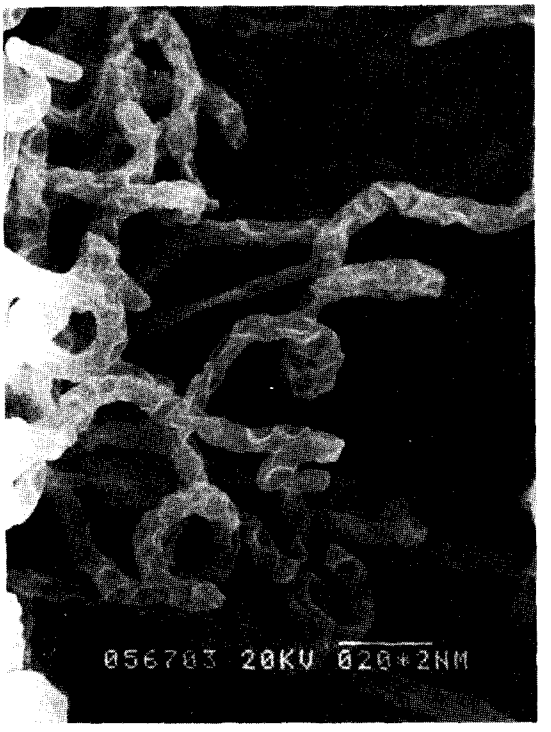

Fig. 3. Electron micrograph of strain No. 77-AG-567.

Table 2. Cultural characteristics of strain No. 77-AG-567.

Agar medium	Growth	Reverse color	Aerial mycelium	Souble pigment
Yeast-starch	excellent	dark brown (6nl)	fawn (4ig)	brownish
Yeast-malt	excellent	sepia brown (3pn)	beige brown (3ig)	dark brown
Oatmeal	moderate	covert gray (2fe)	beaver (3il)	none
Inorganic salt-starch	moderate	light brown (4ge)	fawn (4ig)	none
Tyrosine	good	deep brown (4pl)	pussywillow gray (5de)	none
Sucrose-nitrate	good	clove brown (3ni)	light brown (3ig)	none
Glucose-asparagine	poor	oyster white	white	none
Nutrient	moderate	ivory (2db)	none	none
Glycerol-asparagine	moderate	white	none	none
Peptone-yeast ext-iron	moderate	cinnamon (31e)	none	none

Color code was assigned according to "Color Harmony Manual".

Table 3. Morphological and physiological characteristics of strain No. 77-AG-567.

Cell wall composition	L,,L-DAP	Coagulation of skim milk	positive
Mycelial form	spirals	Peptonization of skim milk	positive
Spore surface	smooth	Hydrolysis of starch	positive
Sporangium & motile spore	none	Formation of melanoid pigment	negative
Gelatin liquefaction	positive	Formation of melanoid pigment	negative
Cellulose utilization	negative	Aerial mycelium color series	reddish brown
Nitrate reduction	positive		

Cultural characteristics

As shown in Table 2, growth of the strain No. 77-AG-567 was, in general, good on those media employed in this study but poor on glycerol-asparagine agar medium. Reverse side colors were gray to brown series and neither distinctive soluble pigment nor melanoid pigment was observed.

Morphological and physiological characteristics

Two-dimensional thin-layer chromatogram and high performamce liquid chromatogram made from whole cell hydrolysate clearly demonstrated L,L-diaminopimelic acid as a cell wall constituent of strain No. 77-AG-567 (Table 3). Spore chain was long open spiral with smooth surface as can be seen in Fig. 3, and sporangium and motile spore were not observed. Melanoid pigment was peptonized, and gelatin and starch were hydrolzed.

Carbon sources utilization

Among those 16 different carbon sources employed, only L-arabionose was not utilized, but D-xylose, lactose, galactose, and maltose were strongly utilized. And other sugars were weakly or doubtly utilized except D-glucose, raffinose, and melibose (Table 4).

Table 4. Carbon sources utilization of strain No. 77-AG-567.

L-Arbinose	−	Raffinose	+	Sorbose	∓
D-Xylose	++	Inositol	∓	Maltose	++
D-Glucose	+	Lactose	++	Sorbitol	∓
D-Fructose	±	Melibose	+	Salicine	∓
Sucrose	±	D-Mannose	∓	Control	∓
L-Rhamnose	±	Galactose	++		

Symbols: ++ ; strong utilization, + ; moderate utilization, ± ; weak utilization, ∓ ; doubtful utilization, − ; no utilization.

From these taxonomic results, we concluded that strain No. 77-AG-567 is most probably be *S. atratus*.

UV and ^{1}H NMR spectra

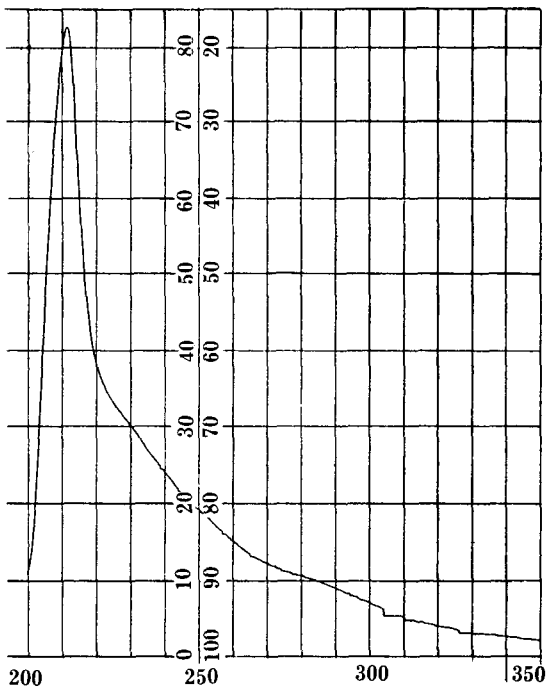

Fig. 4. UV spectrum of the active compound of strain No. 77-AG-567.

UV spectrum of the isolated active compound showed end absorption with λ_{max}^{MeOH} at 212 nm and shoulder at 230 nm (Fig. 4), which is typical UV spectrum of glutarimide group antibiotics. In addition, ^1H spectrum as shown in Fig. 5 revealed very similar pattern to cycloheximide and streptovitacin characterized by the signals at δ 7.75 and δ 4.25, thus led us us to compare those 2 compounds with the active compound of this strain on HPLC, but revealed different retention times.

From these resuts, we could understood that the active compound belonged to glutarimide group antibiotics and had 3 methyls characterized by the signals at δ 1.05 (d), δ 1.83 (s), and δ 2.00 (s). Furthermore, signal at δ 2.00 represented acetyl methyl.

Discussion

Amongst glutarimide group antibiotics, cycloheximide, streptovitacin, dehydrocycloheximide, streptomidone, dihydrocycloheximide, inactone, actiphenol, C-73, and C-73X contain 2 methyls, while promycin contains 4 methyls. Therefore, these compounds could be excluded from consideration. But acetoxycycloheximide, also referred to as E-73, contains 3 methyls. In addition, decoupling experiment and chemical shift analysis further supported our hypothesis that active compound other than blasticidin S of strain No. 77-AG-567 was acetoxycycloheximide. From these results, we could understand that *S. albulus* pruduces both blasticidin S and acetoxycycloheximide, and acetoxycycloheximide has been known to have activity only against yeast and

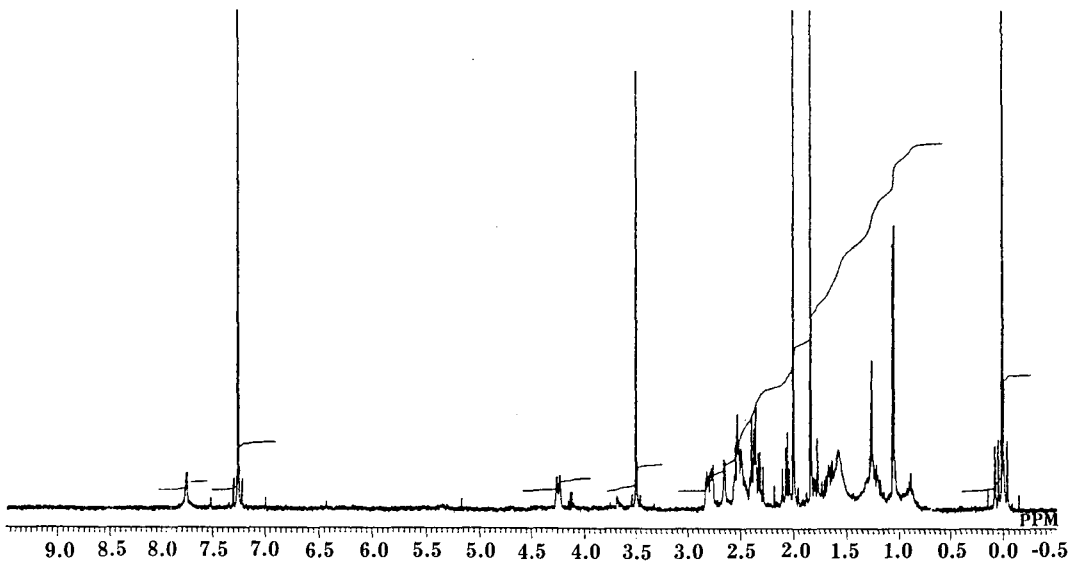

Fig. 5. ^1H NMR spectrum of the active compound of strain No. 77-AG-567.

tumor cells (19). In addition, blasticidin S has been known not to show biological activity against *Sphaerotheca fuliginea* (1).

Therefore, comparison of strain No. 77-AG-567 with *S. albulus* was made by the aid of references (12-17). But there were somewhat great differences between these 2 strains. Aerial mycelium of *S. albulus* was known to show gray or red color series on salts-starch agar and glycerol-asparagin agar. In addition, carbon utilization for growth of *S. albulus* also showed different properties from strain No. 77-AG-567. Distinctive difference was in D-xylose and raffinose, in that those 2 sugars are known not to be utilized by *S. albulus*. Among taxonomic characteristics, particularly worthy of note was the difference in spore surface: spore surface of *S. albulus* are spiny and that of strain No. 77-AG-576 was smooth. These results led us to conclude that strain No. 77-AG-576 was clearly different from *S. albulus* and most probably be *S. atratus*.

In this study, even though we could not discover a new antibiotic we found that a *Streptomyces* sp. which is clearly different from *S. albulus* and most probably thought to be *S. atratus* also produced acetoxycyloheximide together with blasticidin S. This finding adds another biological activity of acetoxycycloheximide against *Pyricularia oryzae* and *Sphaerotheca fuliginea*, and other *Steptomyces* sp. than *S. albulus*, so far known to produced both blasticidin S and acetoxycycloheximide (19) also produces those 2 antibiotics together.

요 약

77-AG-567 균주는 *Pyricularia oryzae* 와 *Sphaerotheca fuliginea* 에 대한 항균활성을 나타냈다. 활성물질을 정제해 가는 과정에서 이 균주는 blasticidin S와 또 다른 항생물질을 동시에 생산한다는 사실을 알았다. 이 화합물은 UV 및 ^{1}H NMR 스펙트럼에 의해 E-73이라고도 일컬어지는 acetoxycycloheximide로 밝혀졌다. 균주를 동정해본 결과 77-AG-567 균주는 *Streptomyces atratus* 로 판단되며 지금까지 blasticidin S와 acetoxycycloheximide를 동시에 생산하는 것으로 알려져 온 *S. albulus* 와는 다른 특성을 나타냈다. 특히 주목할만한 차이점은 포자의 표면이였다. 또한 acetoxycycloheximide는 지금까지 효모와 종양세포에만 생리활성을 나타내는 것으로 알려져 왔으나 본 실험결과 *Pyricularia oryzae* 와 *Sphaerotheca fuliginea* 에도 항균활성을 가진다는 사실을 알았다.

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