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Purification of Antifungal Antibiotic NH-B1 from Actinomycete NH 50 Antagonistic to Plant Pathogenic Fungi

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식물병원진균에 길항효과가 있는 방선균 균주 NH 50에서 항진균성 항생물질 NH-B1의 순수 분리

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ABSTRACT : About 300 actinomycetes were isolated from two forest and one sea-shore soil and tested for inhibitory effects on mycelial growth of six plant pathogenic fungi *Magnaporthe grisea, Alternaria mali, Colletotrichum gloeosporioides, Phytophthora capsici, Fusarium oxysporum* f. sp. *cucumerinum*, and *Rhizoctonia solani*. Among 300 actinomycetes tested, only 16 actinomycetes showed the antifungal activity against the test fungi. Isolate NH 50 was selected for production and purification of antifungal antibiotic substances. Actinomycete isolate NH 50 displayed the broad antifungal spectra against 11 plant pathogenic fungi. To identify actinomycete isolate NH 50, cultural charateristics on various agar media, diaminopimelic acid type, and morphological charateristics by scanning electron microscopy were examined. As a result, actinomycete isolate NH 50 was classified as a rare actinomycete that had LL-DAP type and did not produce spores. After incubation of isolate NH 50 in yeast extract-malt extract-dextrose broth, antifungal compound NH-B1 that inhibited mycelial growth of some plant pathogenic fungi was purified from the methanol eluates of XAD-16 resins by a series of purification procedures, i.e., silica gel flash chromatography, C_{18} flash chromatography, Sephadex LH-20 column chromatography, silica gel medium pressure liquid chromatography (MPLC), C_{18} MPLC, and high pressure liquid chromatography (HPLC). UV spectrum and ^{1}H-NMR spectrum of antifungal compound NH-B1 dissolved in methanol were examined. The antibiotic NH-B1 showed the major peaks at 230 and 271.2 nm. Based on the data of ^{1}H-NMR spectrum, NH-B1 was confirmed to be an extremely complex polymer of sugars called polysaccharides. The antibiotic NH-B1 showed strong antifungal activity against *Alternaria solani* and *Cercospora kikuchi*, but weak activity against *M. grisea*.

Key words : **actinomycete antagonist, forest and sea-shore soils, antifungal antibiotics, plant pathogenic fungi.**

The problems of fungicide resistance of pathogens and some side effects on the environment have been caused by the careless use of synthetic fungicides for control of plant diseases (3, 6). These problems prompted researchers to undertake screening for safe compounds from microorganisms. For example, blasticidin S was thus discovered and introduced into agriculture for the control of rice blast caused by *Magnaporthe grisea* (36). The success of blasticidin S encouraged further screening, which eventually brought about polyoxin (11), kasugamycin (37), validamycin (10) and more recently mildiomycin (7). These antibiotics of microbial origin have few side effects to the environment and show little toxicity to plants. They also have the selective activity against plant pathogens, can be decomposed quickly after use, and do not bring about residual toxicity in soils (18).

A large number of bioactive metabolites has been isolated from various microbial sources such as actinomycetes, bacteria, fungi, mushrooms, etc. Actinomycetes have been described as the greatest source of antibiotics, since Waksman introduced streptomycetes into his systematic screening program for new antibiotics in the early 1940s (41). The new antibiotics found in 1980 were derived from actinomycetes (80.4%), bacteria (11.4%) and fungi including mushrooms (8.3%); about 75% of new antibiotics discovered during 1971~1980 were also from actinomycetes (8). The 75% of antibiotics produced from ac-

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tinomycetes were also derived from the genus *Streptomyces* (19). Antibiotics of actinomycete origin show the varieties of chemical structures encompassing aminoglycosides, β-lactams, anthracyclines, glycopeptides, macrolides, polyethers, nucleosides, peptides, polyenes, and tetracyclines (23).

An efficient way of finding new antibiotics is the discovery of new microorganisms. From a microbiological point of view, one of the most important investigations in the history of antibiotic screening was Weinstein's discovery of gentamycin (43) from a strain of *Micromonospora*, a genus of the so-called rare actinomycetes that had been very little investigated for antibiotic production before that time. With this study as a turning point, various kinds of rare actinomycetes were subjected to screening, resulting in the discovery of a variety of new antibiotics (12). Mildiomycin (7) from *Streptoverticillium*, setamycin (26) and cystargin (39) from *Kitasatosporia*, Spartanamicin A and B (20) from *Micromonospora*, and pradimicin analogs (31) from *Actinomadura* were the antibiotics recently found from rare actinomycetes.

Compared with *Streptomyces* strains, rare actinomycetes show the following characteristics: (a) slower growth, (b) more complex nutritional requirements, (c) poor sporulation, (d) instability toward preservation. In addition to these disadvantages, the amount of the desired antibiotics produced by the rare actinomycete frequently has been very limited (12). Therefore, the researchers have investigated selective isolation methods for rare actinomycetes by the use of antibiotics and various soil pre-treatments. The selective isolations of *Nocardia* (27), *Micromonospora* (40), *Actinomadura* (1), *Actinoplanes* (28), *Streptosporangium* (22), and *Kitasatosporia* (35) were reported previously.

In the present study, we isolated various rare actinomycetes antagonistic to various plant pathogenic fungi. Antifungal compound NH-B1 was purified from the XAD-16 eluates of the actinomycete isolate NH 50 cultured, which were highly antagonistic to plant pathogenic fungi.

MATERIALS AND METHODS

Isolation of antagonistic actinomycete isolates. Soil samples from the mountain Il-Jang (Kyoungki-Do) were collected from 10 to 15 cm deep from the soil surface and stored at 4°C until used. The soil sample (5 g) was suspended in 50 ml of sterilized saline containing 0.01% $MgSO_4 \cdot 7H_2O$ and 0.002% Tween 20, and stirred vigorously by a mixer. The suspension was immersed in a water bath at 55°C for 7 min before further dilution. After cooling, soil suspension was filtered through a Whatman No. 1 paper and then diluted to 1 : 100 (v/v) with M3 agar medium (30) in a Petri-dish. The M3 agar medium contained 0.466 g KH_2PO_4, 0.732 g Na_2HPO_4, 0.10 g KNO_3, 0.29 g NaCl, 0.10 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCO_3$, 0.20 g sodium propionate, 200 μg $FeSO_4 \cdot 7H_2O$, 180 μg $ZnSO_4 \cdot 7H_2O$, 200 μg $MnSO_4 \cdot 7H_2O$, 18 g agar, 50 mg cycloheximide, and 4 mg thiamine · HCl, pH 7.0 in 1 L distilled water. A solution containing cycloheximide and thiamine · HCl was sterilized by membrane filtration and added to the autoclaved, cooled agar medium to give the final concentrations specified. The seed agar plates were incubated at 28°C for 2~4 weeks.

Among a number of colonies appearing, only actinomycete-like microorganisms were selected. Modified Bennet's agar medium (1% glucose, 0.2% casamino acids, 0.2% yeast extract, 0.1% beef extract, 1.5% agar, pH 7.0) was used for the growth and maintenance of the actinomycetes. To test antagonistic effects of actinomycetes inhibitory to six plant pathogenic fungi such as *Magnaporthe grisea, Alternaria mali, Colletotrichum gloeosporioides, Phytophthora capsici, Fusarium oxysporum* f. sp. *cucumerinum*, and *Rhizoctonia solani*, the actinomycetes isolated were streaked at the middle of plates on V8 juice agar and incubated at 28°C. After 4 days, a mycelial disc (7 mm in diameter) cut from the margin of actively growing cultures was placed on both sides of 30 mm apart from streak-inoculation. Inhibition of mycelial growth was rated 5 days after incubation at 28°C. The antagonistic actinomycetes were stored in modified Bennet's broth with 15% glycerol at -70°C. To examine the antifungal spectra of actinomycete isolate NH 50, eleven plant pathogenic fungi such as *A. mali, A. solani, Botrytis cinerea, Botryosphaeria dothidea, C. gloeosporioides, F. oxysporum* f. sp. *cucumerinum, M. grisea, Mycosphaerella melonis, P. capsici, R. solani* and *Sclerotinia sclerotiorum* were used.

Classification of actinomycete isolate NH 50. Analysis of DAP in the actinomycete isolate NH 50 was done using the method modified from Becker *et al.* (2) and Kutzner (14). The actinomycete isolate NH 50 was incubated on yeast extract-malt extract agar (4 g yeast extract, 10 g malt extract, 4 g dextrose and 20 g agar in 1 L water) at 30°C for 15 days. The mycelia

on the agar surface were scraped and marcelated with tap water. Marcelated cells were centrifuged and the precipitates were then washed twice with ethyl alcohol, and air-dried at room temperature.

Dried cells (12 mg) were hydrolyzed with 1 ml of 6 N HCl in a sealed Pyrex tube (10×130 mm) at 100°C for 18 hr. After cooling, the hydrolysate was filtered through a Whatman No. 1 paper and the residue was washed with 1 ml of distilled water. To remove the HCl, the filtrate was evaporated on a rotary evaporator at 40°C. This step was repeated twice after addition of distilled water. The residue of finally washed extract was taken up in 0.3 ml of distilled water and spotted onto cellulose-coated, thin-layer chromatography (TLC) plastic sheets (Merck 5577). Five μl of 0.01 M standard solution containing the mixture of meso- and LL-DAP isomers (Sigma Chemical Co.) were also spotted. Separation of the amino acids was achieved by ascending single-dimensional TLC in a solvent system containing methanol-pyridine-10 N HCl-water (80 : 10 : 2.5 : 17.5, v/v). Development was achieved for about 8 hr. After the chromatogram was air-dried, amino-acid spots were visualized by spraying the chromatogram with ninhydrin in acetone (0.1%, w/v), followed by air drying and heating at 100~110°C for 2 min. The isomers of DAP produced slowly moving olive-green spots that faded to yellow. Most of the other amino acids present in whole cell hydrolysates exhibited purple spots.

To observe cultural characteristics, the actinomycete isolate NH 50 was grown on various agar media at 28° C for 4 wks. The microorganism was inoculated on agar media using the cross-hatch streaking method by Bergey's Manual of Systematic Bacteriology (48). The test agar media were yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), tyrosine agar (ISP 7), nutrient agar, Bennett's agar, skim milk agar, and chitin agar. Cultural characteristics were observed under the naked eye and light microscopy at 400× magnification for 4 weeks.

To examine the spore morphology, isolate NH 50 was cultured on yeast extract-malt extract agar medium (ISP 2) for 4 weeks. Yeast extract-malt extract agar consisted of 4 g yeast extract, 10 g malt extract, 4 g dextrose, and 20 g agar in 1 L water. Spore morphology was examined under the light microscopy at × 400 magnification and SEM. Specimen for SEM was prepared by the method of King and Brown (13).

Selection of an optimal medium for a large-scale production of antibiotics. To select the medium favorable for a large-scale production of antibiotics from actinomycete isolate NH 50, five media consisting of various nutritional elements were tested. Five media used in this study were as follows : yeast extract-starch-casein medium (YSC; 5 g yeast extract, 20 g starch, 5 g casein hydrolysate, 10 g dextrose and 4 g $CaCO_3$ in 1 L H_2O) (45), yeast-malt extract-dextrose medium (YMD; 4 g yeast extract, 10 g malt extract and 4 g dextrose in 1 L H_2O) (20), yeast extract-starch-beef extract-tryptone medium (YSBT; 0.5% yeast extract, 2.4% soluble starch, 0.3% beef extract, 0.5% tryptone, 0.1% glucose and 0.2% $CaCO_3$ in 1 L H_2O) (5), starch-soybean meal medium (SS; 3% soluble starch, 3% soybean meal, 0.4 % $CaCO_3$, pH 7.2 in 1 L H_2O) (21), and soybean meal-dextrin medium (SD; 5 g soybean meal, 50 g dextrin, 5 g dextrose, 7 g $CaCO_3$ and cobalt chloride 10^{-4}~10^{-6} M in 1 L H_2O) (44).

To prepare seed inoculum for the production of antibiotics, a loopful of cells of actinomycete isolate NH 50 incubated on YMD agar at 32°C for 5 days was inoculated into a 250-ml Erlenmeyer flask containing 100 ml of YMD broth. After incubation for 5 days at 32°C on a rotary shaker at 180 rpm, seed inoculum (3 ml) was inoculated into a 500-ml Erlenmeyer flask containing 300 ml of each of the above five media in the presence of Amberlite XAD-16 adsorber resin (Hydrophobic polyaromatic resin, Sigma). The culture was carried out at 32°C for a week. XAD-16 resin was separated from the culture broth with a sieve and was eluted with methanol. XAD-16 eluates were concentrated *in vacuo* using a rotary evaporator. Antifungal activity of the XAD-16 eluates was tested against *M. grisea, P. capsici, A. mali*, and *R. solani* using a paper disk method.

Culture time for the production of antibiotics. To determine the optimal culture time for the production of antibiotics, the actinomycete isolate NH 50 was cultured in YMD broth in the presence of XAD-16 adsorber resin at 32 °C. Actinomycete isolate NH 50 was pre-cultured for 5 days at 32°C in 100 ml of YMD broth flask. The 3 ml portions of seed culture were inoculated in 300 ml of YMD broth supplemented with 3% XAD-16 resin. XAD-16 resin was collected from culture broth at intervals of two days after incubation, and eluted with methanol. XAD-16 eluates were concentrated *in vacuo* using a rotary evaporator. Inhibitory effects of XAD-16 eluates were tested against *A. mali* using a paper disk assay method. Change in cell mass

during culture period was also examined. Cell mass was harvested by centrifuging culture broth without XAD-16 resin. Harvested cell mass was measured after drying at 50°C. In addition, change in pH of culture broth was measured.

Large-scale production and purification of the antibiotics. A loopful of cells of actinomycete isolate NH 50 from the stock culture was inoculated on YMD agar. A loopful of cells from YMD agar were transferred into 500 ml-Erlenmeyer flask containing 100 ml of YMD broth. The 6 ml portion of seed culture incubated for 5 days at 32°C were inoculated into 1 L Erlenmeyer flask containing 600 ml of YMD broth and 3% XAD-16 resin. Large-scale production of antibiotics was carried out at 32°C for 11 days on a rotatory shaker (150 rpm). The antibiotic substances produced by the isolate NH 50 were purified as described in Fig. 1.

XAD-16 resin from cultures (64 L) was put together with 2-mm mesh sieve. The XAD-16 resin was then eluted twice with methanol in 1 L-Erlenmeyer flask under a rotatory shaker and concentrated *in vacuo*. The same volume of ethyl acetate was added to methanol eluates from the XAD-16 resin. The prepared sample was applied to silica gel flash chromatography (Silica gel 60 F254, 70-230 mesh, Merck). The elution was carried out using stepwise gradients of ethyl acetate-methanol (50 : 50), ethyl acetate-methanol (30 : 70), ethyl acetate-methanol (10 : 90), 100% methanol, methanol-H_2O (80 : 20), and methanol-H_2O (60 : 40). The eluates fractionated from silica gel flash chromatography were taken to dryness under reduced pressure at 40°C in a rotary evaporator and dissolved with methanol. To detect the antifungal-active fractions, each fraction was bioassayed against *M. grisea*. The fragmented mycelial suspentions of *M. grisea* mixed with molten potato dextrose agar (PDA) were used for the paper disk bioassay. The antifungal-active fractions were chromatographed on a column (Pyrex® 65×40 mm, Iwaki) of C_{18} (LiChroprep® RP-18, particle size 40~63 μm, Merck). The C_{18} column was eluted by step-gradients of 100% H_2O, methanol-H_2O (20 : 80), methanol-H_2O (40 : 60), methanol-H_2O (60 : 40), methanol-H_2O (80 : 20), and methanol 100%. All fractions were tested for the inhibitory effects against *M. grisea*. The fractions that showed antifungal activity were pooled and concentrated *in vacuo* to small volumes, followed by gel filtration on Sephadex LH-20 column (20×970 mm). Methanol was used as an eluting solvent at the flow rate of 0.05 ml/min. Each of fractions (2 ml/frac.) was collected using a fraction collector (Parmacia Redi-Frac, Sweden) and bioassayed against *M. grisea* by the paper disk method. The active fractions were pooled and concentrated in the small volume of methanol. For further purification, MPLC was performed using Yamazen GR-200 and Yamazen pump 540. The column (38×320 mm, Yamazen) packed with C_{18} (LiChroprep® RP-18, particle size 40~63 μm, Merck) was eluted with linear gradients from methanol-H_2O (50 : 50) to methanol 100% within 50 minutes at flow rate of 20 ml/minute. Each fraction was collected to 100 ml. The active fractions were combined and evaporated to dryness. The residue dissolved in methanol was subjected to silica gel (Silicagel 60, 230~400 mesh, Merck) MPLC (38×320 mm, Yamazen). A mixture of ethyl acetate-methanol-H_2O (5 : 5 : 2) was isocratically used as an eluting solvent. The flow rate was 30 ml/minute and fractionation volume was 100 ml. The concentrated fractions were tested for antifungal activity against *M. grisea*. The frac-

Cultures (64L) with XAD-16 adsorber resin
| sieved XAD-16 resin
| eluted with MeOH
XAD-16 eluates (640 ml)
| concentrated *in vacuo*
Silica gel flash chromatography
| eluted with step gradient of EtOAc-MeOH (50 : 50), EtOAc-MeOH (30 : 70), EtOAc-MeOH (10 : 90) 100% MeOH, MeOH-H_2O (80 : 20), MeOH-H_2O (60 : 40)
C_{18} flash chromatography
| eluted with a step gradient of 0%, 20%, 60%, 80%, and 100% MeOH
Sephadex LH-20 column chromatography
| eluted with MeOH
MPLC (C_{18})
| eluted with linear gradient from MeOH-H_2O 50 : 50 to 100% MeOH
| evaporated *in vacuo*
MPLC (silica gel)
| eluted with ethyl acetate-methanol-H_2O 5 : 5 : 2
| active fractions concectrated *in vacuo*
HPLC
| analyzed by Prep Nova-pak HR C_{18} column (3.9×300 mm, Waters)
| active regions collected by Delta-pak C_{18} column (100 Å, 15 μm, 19×300 mm, Waters)
pure sample (6.12 mg)

Fig. 1. Purification procedures of antifungal substance NH-B 1 from the cultures of actinomycete isolate NH 50.

tion with antifungal activity against *M. grisea* was analyzed by Prep Nova-pak HR C_{18} column (3.9×300 mm, Waters). HPLC system was composed of Waters 600 controller, 600 TM Pump, and Waters 996 Photodiode Array Detector. The antibiotic NH-B1 was developed with linear gradients from methanol-H_2O (50 : 50) to methanol-H_2O (60 : 40) within 10 min, from methanol-H_2O (60 : 40) to methanol-H_2O (70 : 30) within 20 min, from methanol-H_2O (70 : 30) to methanol 100% within 10 min, and maintenance of 100% methanol for 10 min at flow rate of 0.7 ml/min. Preparative HPLC (column : C_{18} Delta-pak, 15 μm, 19×300 mm, Waters) was further performed to completely purify antifungal antibiotics.

^{1}H-NMR spectrum analysis of the antibiotic NH-B 1. Nuclear magnetic resonance (NMR) spectra of the purified NH-B1 were performed on a Bruker AMX 500 NMR spectrometer. ^{1}H NMR spectra were obtained in CD_3OD.

Antifungal activity of the antibiotic NH-B1. Bioassay of the antibiotic NH-B1 for antifungal activity was done using a method modified from Nair *et al.* (20). Eight plant pathogenic fungi such as *A. mali, A. solani, C. gloeosporioides, C. cucumerinum, C. kikuchi, M. grisea, M. melonis*, and *P. capsici* were used as the test organisms. The test organisms were incubated on potato dextrose agar at 28°C for 14 days. One ml amounts of spore suspensions (10^6 spores/ml) was prepared in each micro-multiwell plate (Corning) including 0.5 ml potato dextrose broth. Solutions of the antibiotic NH-B1 at serial concentrations of 0.1, 1, 10, 50, and 100 μg/ml, respectively, were added into each of micro-multiwell plates. The plates including the antibiotic NH-B1 and spore suspensions were incubated at 28°C in a shaking incubator. Growth of the test orgarnisms was evaluated after incubation for 4 days.

RESULTS

Isolation of antibiotic-producing actinomycetes. To screen rare antagonistic actinomycetes, approximately 300 actinomycete isolates were isolated from two forest soils and one sea-shore soil. Their inhibitory effects on the mycelial growth were evaluated using *M. grisea, A. mali, P. capsici, C. gloeosporioides, F. oxysporum* f. sp. *cucumerinum*, and *R. solani*. Most of the actinomycetes isolated did not show the inhibitory effects against six plant pathogenic fungi tested. Only 16 actinomycetes were effective in inhibiting mycelial growth of the test fungi (Table 1). In par-

Table 1. Antifungal activity[b] of some actinomycetes from forest and sea-shore soils against six plant pathogenic fungi on M3 medium

Isolate[a]	Inhibition zone length (mm) of					
	Magnaporthe grisea	*Alternaria mali*	*Phytophthora capsici*	*Colletotrichum gloeosporioides*	*Fusarium oxysporum* f. sp. *cucumerinum*	*Rhizoctonia solani*
NH2	13	9	9	13	10	20
NH34	6	6	0	5	3	4
NH36	12	6	10	5	2	10
NH39	10	7	7	9	3	0
NH50	15	13	9	8	3	10
NH58	13	9	9	13	10	25
NH76	2	5	10	5	1	10
CM1	16	17	13	12	0	4
CM2	17	15	12	14	9	17
CM6	14	17	13	7	3	2
CM9	16	15	6	10	6	8
CM14	13	6	11	12	15	7
CM75	21	6	10	17	15	6
KSC26	20	20	12	15	19	10
KSC101	15	13	15	13	10	15
KSC108	15	14	8	16	13	9

[a] NH, CM, and KCS isolates were screened from forest soils of Mt. Il-Jang, Mt. Chun-ma, Kyoungki-Do and mud soil of Kangwha-do Changwhuri ashore, respectively.

[b] The antifungal activity was measured by placing a mycelial disk of each fungus on both sides of 30 mm far from steak-inoculation of actinomycete antagonistics in the middle of V8-juice agar plates. Inhibition zone of mycelial growth of each plant pathogenic fungus was rated 7 days after inoculation of the test fungi.

ticular, isolates NH 2 and NH 58 strikingly suppressed mycelial growth of *R. solani*, whereas NH 39 could not affect *R. solani*. The majority of the isolates effective in inhibiting of mycelial growth of the test fungi seemed to be *Streptomyces* spp.. Among the antagonistic actinomycetes isolated, isolate NH 50 which showed antifungal activity and regarded as rare actinomycetes was selected for large-scale production for antibiotic substance NH-B1.

Antifungal spectra of actinomycete isolate NH 50.

To examine the antifungal spectra of actinomycete isolate NH 50, inhibition of mycelial growth was evaluated against 11 plant-pathogenic fungi: *A. solani, A. mali, Botrytis cinerea, Botryosphaeria dothidea, C. gloeosporioides, F. oxysporum* f. sp. *cucumerinum, M. grisea, Mycosphaerella melonis, P. capsici, R. solani,* and *S. sclerotiorum* (Table 2). Isolate NH 50 displayed the broad antifungal spectra against all fungi tested. Inhibition zone length of fungal mycelial growth ranged from 4.5 to 18 mm. Especially, NH 50 strongly suppressed mycelial growth of *M. grisea*, but showed the relatively strong inhibition effects against *A. mali, B. cinerea, R. solani* and *S. sclerotiorum. F. oxysporum* f. sp. *cucumerinum* was poorly inhibited by the isolate NH 50.

Table 2. Antifungal activity[a] of actinomycete isolate NH 50 against various plant-pathogenic fungi

Test fungus	Inhibition zone length (mm) of mycelial growth
Alternaria mali	11.3±1.0[b]
Alternaria solani	6.3±0.5
Botrytis cinerea	10.0±0.8
Botryosphaeria dothidea	7.8±1.0
Colletotrichum gloeosporioides	6.3±1.0
Fusarium oxysporum f.sp. *cucumerinum*	4.5±1.0
Magnaporthe grisea	18.0±1.0
Mycosphaerella melonis	7.0±0.0
Phytophthora capsici	6.3±1.0
Rhizoctonia solani	9.3±0.5
Sclerotinia sclerotiorum	10.3±0.5

[a] The antifungal activity was measured by placing a mycelial disk of each fungus on both sides of V8-juice agar 30 mm far from streak-inoculation of actinomycete NH 50 in the middle of plates. Inhibition of mycelial growth of each fungus was rated 7 days after inoculation of the test fungus.

[b] Each value represents a mean standard deviation of four replicates.

Classification of actinomycete isolate NH 50.

Analysis of DAP type in actinomycete isolate NH 50 was achieved using the method modified from Becker *et al.* (2) and Kutzner (14). Cell wall hydrolysates of the actinomycete isolate NH 50 were developed on a cellulose TLC plate. As a result, DAP type of actinomycete isolate NH 50 turned out to be LL-DAP. To observe cultural characteristics of the isolate NH 50, it was grown on various agar media at 28°C. Isolate NH 50 grew relatively well on various media described, but did not grow on inorganic salts-starch agar (ISP 4) and chitin agar (Table 3). On glycerol-asparagine agar (ISP 5), isolate NH 50 grew poorly. The aerial mycelia were not produced on all the media tested. The colors of substrate mycelia were yellowish to brown. In the colonies, isolate NH 50 had wrinkled, hard surfaces on yeast extract-malt extract agar (ISP 2), but had brown and smooth surfaces on oatmeal agar (ISP 3). Isolate NH 50 also had whitish yellow on glycerol-asparagine agar and nutrient agar. On Bennett's agar, isolate NH 50 had wrinkled, glistening and hard surfaces with amber-orange to brown colors in colonies. Isolate NH 50 did not produce soluble pigments on all the media studied.

For an examination of morphological charateristics by SEM, actinomycete isolate NH 50 was incubated for four weeks on YMD agar. Under the SEM, actinomycete isolate NH 50 had no spores on the smooth mycelial surface (Fig. 2).

Table 3. Cultural characteristics of the actinomycete isolate NH 50

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP 2)	Good	None	Yellow to brown, wrinkled and hard surface	None
Oatmeal agar (ISP 3)	Good	None	Brown, smooth surface	None
Inorganic salts-starch agar (ISP 4)	No growth			
Glycerol-asparagine agar (ISP 5)	Poor	None	Whitish yellow, mucoid	None
Tyrosine agar (ISP 7)	Good	None	Yellow to brown	None
Nutrient agar	Moderate	None	Whitish yellow, small colony	None
Bennett's agar	Good	None	Amber-orange to brown, wrinkled and glistening surface	None
Skim milk agar	Good	None	Creamlike color to brown, glistening surface	None
Chitin agar	No growth			

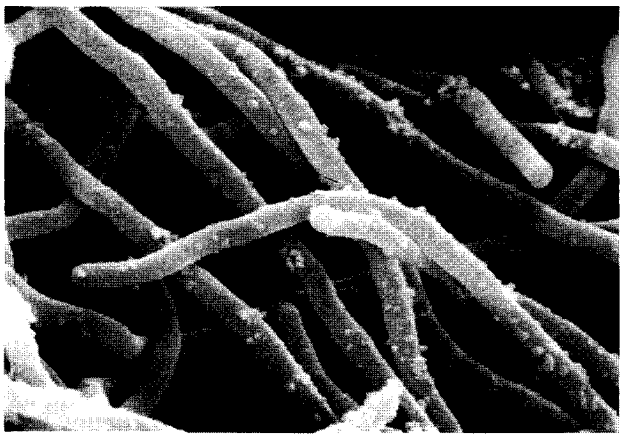

Fig. 2. Scanning electron micrograph of actinomycete isolate NH 50 grown on yeast extract- malt extract-dextrose agar for 4 weeks. Bar represents 1 μm.

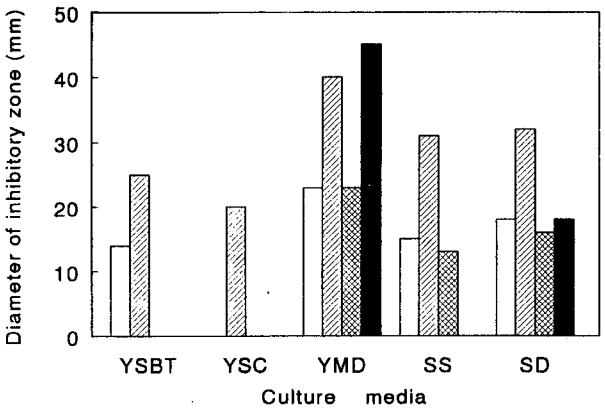

Fig. 3. Inhibitory effects of XAD-16 eluates from the culture of the actinomycete isolate NH 50 in different culture brothes against *Alternaria mali, Phytophthora capsici, Magnaporthe grisea,* and *Rhizoctonia solani.* YSBT : 2.4% soluble starch, 0.1% dextrose, 0.3% beef extract, 0.5% yeast extract, 0.5% tryptone, 0.2% $CaCO_3$, 1 L water, YSC : 5 g yeast extract, 10 g dextrose, 20 g starch, 5 g casein hydrolysate, 4 g $CaCO_3$, 1 L H_2O, YMD : 4 g yeast extract, 10 g malt extract, 4 g dextrose, 1 L H_2O, SS : 3% soluble starch, 3% soybean meal, 0.4% $CaCO_3$, 1 L H_2O, pH 7.2, SD : 30 g soybean meal, 50 g dextrin, 5 g dextrose, 7 g $CaCO_3$, 10^{-4}~10^{-6} M cobalt chloride, 1 L H_2O, □ : *Alternarla mali*, ▩ : *Phytophthora capsici*, ▨ : *Magnaporthe grisea*, ■ : *Rhizoctonia solani.*

Production and purification of antibiotics in actinomycete isolate NH 50. To select the medium favorable for a large-scale production of antibiotics, actinomycete isolate NH 50 was grown in five different culture brothes consisting of various nutritional elements and 3% XAD-16 adsorber resin. XAD-16 resin was collected from each culture and eluted with methanol. Each methanol eluate of XAD-16 resin from five media tested was examined for antifungal activity against *M. grisea, A. mali, P. capsici,* and *R. solani* using a paper disk method (Fig. 3). Antifungal activity against *M. grisea* was present in all the XAD-16 eluates, but was the most effective in the XAD-16 eluates from YMD broth. All eluates from various media, except YSC medium, greatly inhibited mycelial growth of *A. mali.* Moreover, inhibitory effect against *R. solani* existed in XAD-16 eluates only from YMD and SD media. XAD-16 eluates from YMD medium were more effective in inhibiting mycelial growth of *R. solani* than those from SD medium. In particular, *R. solani* was strongly inhibited by YMD culture eluates, as compared to other tested media. In contrast, the weak antifungal activity against *P. capsici* was found in all the culture brothes tested.

To determine the optimal culture time for the production of antibiotics, actinomycete isolate NH 50 was cultured in YMD broth in the prescence of XAD-16 resin at 32°C. XAD-16 resin was collected from culture broth at intervals of two days after incubation and eluted with methanol. Inhibitory effects of XAD-16 eluates were tested against *A. mali* in different time intervals (Fig. 4). Antibiotic production from actinomycete isolate NH 50 began to form 5 days after incubation and rapidly increased to 7 days after incubation. After 7 days of incubation, antibiotic production was slowly increased.

Change in cell mass was also measured during culturing of actinomycete isolate NH 50. Dry matter of cell mass distinctly increased to 9 days after incubation and thereafter declined to reach a stationary phase. In addition, change in pH of culture broth was observed during culturing of the isolate NH 50. The pH of culture broth increased slightly from 6.7 to 7.1 at 3 days, and 7.8 at 7 days after incubation. Based on all the culture conditions, the optimal culture time for the production of antibiotics was 11 days.

For the large-scale production of antibiotics, actinomycete isolate NH 50 was cultured for 11 days at 32° C in YMD broth with 3% XAD-16 hydrophobic polyaromatic resin. After 11 days of incubation, XAD-16 resin from the culture (63 L) was put together with a 2-mm mesh sieve and eluted with methanol. Methanol eluates (22.5 g) of XAD-16 resin were subjected to a silica gel flash chromatography using stepwise gradients of ethyl acetate-methanol (50 : 50, 30 : 70, 10 : 90), 100% MeOH, and MeOH-H_2O (80 : 20, 60 : 40). The eluates of EtOAc-MeOH (50 : 50, 30 : 70, 10 : 90) were highly active against *M. grisea.* The eluates of EtOAc-MeOH (50 : 50, 30 : 70, 10 : 90) showing the an-

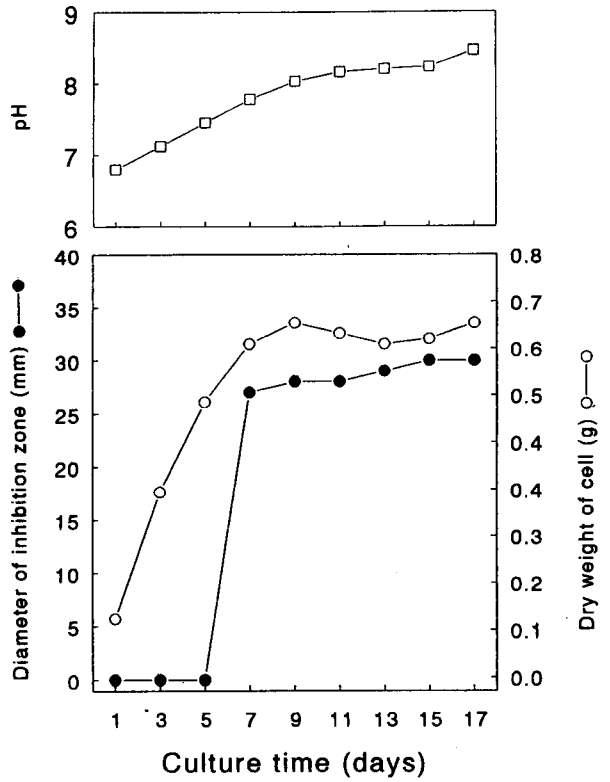

Fig. 4. Changes in pH of culture media, mycelial growth of the actinomycete isolate NH 50, and antifungal activity of XAD-16 eluates against *Alternaria mali* during the culturing of the actinomycete isolate NH 50 in YMD broth. A paper disk assay was used for measuring antifungal activity.

tifungal activity against *M. grisea* were pooled, concentrated and chromatographed on a C_{18} column. The C_{18} column was eluted with step gradients of MeOH-H$_2$O (0:100, 20:80, 40:60, 60:40, 80:20, 100:0). The fraction eluted with MeOH-H_2O (80:20) was most effective in inhibiting mycelial growth of *M. grisea*. Fraction of MeOH-H_2O (80:20) with strong activity was then purified by gel filtration on Sephadex LH-20 column with methanol. Among fractions obtained from Sephadex LH-20 column chromatography, fractions no. 53-98 showed high activity against *M. grisea* (Fig. 5). Antifungal-active fractions were combined and applied to further purification. MPLC was performed using C_{18} column. The column was eluted with a linear gradient from MeOH-H_2O 50:50 to 100% MeOH within 50 minutes at flow rate of 20 ml/min. Fraction no. 5~12 showed large inhibition effects against *M. grisea*. The active fractions were further pooled and given to MPLC with silica gel column using the eluting solvent system of ethyl acetate-methanol-H_2O (5:5:2). Among 20 fractions collected from MPLC with silica gel, fraction no.

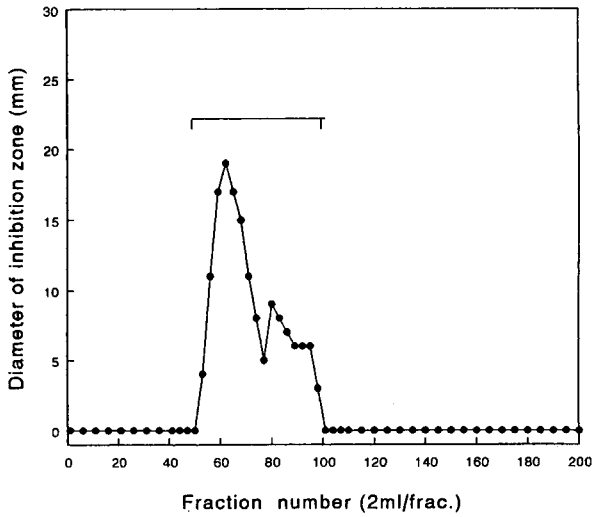

Fig. 5. Sephadex LH-20 column chromatogram of antifungal-active 80% MeOH eluate obtained from C_{18} flash chromatography. The column was eluted with methanol at 0.05 ml/min flow rate. *Magnaporthe grisea* was used as a test fungus for bioassay. Bar represents the active fractions to be purified in the next steps.

5~6 (NH-A), and 7~12 (NH-B) had high activity against *M. grisea*, whereas fraction no. 13~15 (NH-C) had relatively weak activity. The antifungal-active NH-B fraction was further purified using preparative HPLC (C_{18} Delta-pak column, 15 μm, 19×300 mm, Waters). The compound collected at the retention time 39'62 min produced a single peak by analytical HPLC at UV 254 nm (Fig. 6). The 6.12 mg of dark-brown oily compound

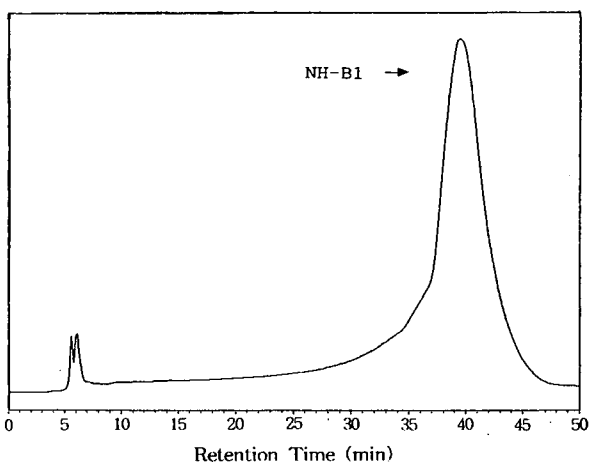

Fig. 6. High performance liquid chromatogram of the antibiotic NH-B1. Column: Prep Nova-pak C_{18}, 3.9 X 300 mm, Waters; solvent system: linear gradients from MeOH-H_2O 50:50 to 60:40 within 10 min, from 70:30 to 100% MeOH within 10 min, following 100% MeOH for 10 min; flow rate: 0.7 ml/min; detection at UV 254 nm.

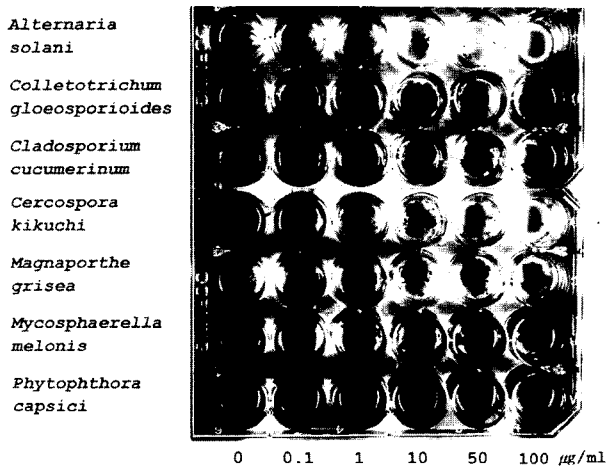

Fig. 7. Antifungal activity of the antibiotic NH-B1 against plant pathogenic fungi. The inhibition of mycelial growth in the multi-wells was examined 4 days after treatments with NH-B1.

NH-B1 was yielded.

^{1}H-NMR spectrum analysis of antibiotics NH-B1. The ^{1}H-NMR spectrum analysis of HPLC-purified antifungal compound NH-B1 dissolved in CD_3OD was performed on a Bruker AMX 500 NMR spectrometer. The ^{1}H-NMR spectrum did not show sharp peaks but broad ones. In general, carbohydrate monomer appears to be sharp, whereas polymer shows broad peaks. Therefore, antibiotic compound NH-B1 is thought to be extremely complex polymer of sugars called polysaccharides.

Antifungal activity of the antibiotic NH-B1. Bioassay of the antibiotic NH-B1 for antifungal activity was evaluated using a method modified from Nair *et al.* (20). Among eight plant pathogenic fungi tested, mycelial growths of *A. solani* and *C. kikuchi* were strongly inhibited (Fig. 7). *A. solani* and *C. kikuchi* grew poorly at 50 μg/ml of the antibiotic NH-B1, but did not at 100 μg/ml. The NH-B1 showed a weak activity against *M. grisea*. However, mycelial growths of other fungi were not inhibited even at a concentration of 100 μg/ml.

DISCUSSION

Generally, actinomycetes are well known as a rich source of antibiotics. About 75% of antibiotics discovered during 1971~1980 was originated from the actinomycetes (8). However, the 75% of antibiotics produced from actinomycetes was derived especially from one genus, the *Streptomyces* (19). This suggests that finding new antibiotics may be possible by the discovery of rare actinomycetes except *Streptomyces*. However, the amount of the desired antibiotics produced by rare actinomycete has been limited (12).

In this study, M3 medium (30) with pre-treatment of soil samples was used to isolate rare actinomycetes. Approximately 300 actinomycetes were isolated from forest soils of two mountains and mud soil of one seashore. Among them, only 16 actinomycetes were effective in inhibiting mycelial growths of six plant pathogenic fungi (Table 1). However, the majority of actinomycetes selected were not rare actinomycetes but genus *Streptomyces*.

Isolation procedures for rare and novel actinomycete isolates which produce useful antifungal antibiotics should be improved by various combinations of the concerning factors such as isolation sources, pre-treatment of soil samples, and isolation media (40, 9, 33, 34). More investigations of antibiotics derived from rare actinomycetes have been needed, although the genus *Streptomyces* is still considered an important source of new antibiotics.

To isolate new antibiotics of microbial origin showing antifungal activity, we selected the isolate NH 50 which could produce high amounts of antibiotics in culture. To identify the actinomycete isolate NH 50, we examined the DAP type analysis and morphological characteristics by SEM. The isolate NH 50 had LL-DAP in cell wall components and did not produce any spore on the mycelium (Fig. 2). Based on these data, the isolate NH 50 did not belong to genus *Streptomyces*, but was classified as a rare actinomycetes.

It was difficult to exactly identify a rare actinomycetes, because characters of rare actinomycetes for identification were rarely established. However, identification of genus *Streptomyces* had been successfully achieved in many studies. Numerical identifications of genus *Streptomyces* have been undertaken using TAXON program based on the data of chararters of *Streptomyces* species and probabilistic identification matrix proposed by Williams *et al.* (46, 47). TAXON is the computer program for identifying mostly unknown *Streptomyces* which was developed by Ward and Goodfellow, University of Newcastle upon Tyne, UK (42). To exactly identify the isolate NH 50, physiological and biochemical charateristics and DNA base composition need to be examined.

For large-scale production of antibiotics from actinomycete isolate NH 50, optimal medium and culture time

for producing antibiotics were examined. The production of antibiotic metabolites was most favorable when isolate NH 50 was cultured in YMD broth among five culture media tested. XAD-16 eluates from some cultures of isolate NH50 showed the antifungal activity against four plant-pathogenic fungi, whereas those from other cultures of isolate NH 50 did not (Fig. 3). It has been known that the kinds or quantities of targeted antibiotics may vary according to the components of media or culture conditions (16). The production of antibiotics is not rigorously species-specific. The same antibiotic can be produced by organisms of species or genera or even orders that are different. The reverse is also true, that is, strains classified taxonomically as members of the same species can produce different antibiotics. However, as a general rule, the more distant the organisms are on the taxonomic scale, the less probable it is that they will produce the same antibiotics (15).

The culturing time favorable for production of antibiotics from isolate NH 50 was examined during the culturing for 11 days (Fig. 4). In general, as shown above, the growth of rare actinomycetes was slower than that of genus *Streptomyces*. Most of the antibiotics from microorganisms are secondary metabolites produced at the beginning of idiophase when cell multiplication ceases (49), whose production is linked to nutrient depletion (38).

In our study, a non-ionic adsorber resin, 3% Amberlite XAD-16, was added to the culture from the beginning of purification of antibiotic substance. The resin is well tolerated by almost all microorganisms and has several advantages. It adsorbs all excreted substances if they are not extremely hydrophilic, and thereby concentrates and stabilizes the produced metabolites, so that one sampling at the end of the growth of the culture is sufficient and taking a series of samples along the growth curve is no more necessary (29). When inexactly autoclaved, XAD-16 resin has a risk to lose its affinity activity. Nevertheless, addition of XAD-16 resin to the culture of microorganisms at the beginning of incubation provides many advantages for purification of antibitic metabolites.

HPLC-purified antifungal compound NH-B1 on the silica gel TLC plate charred in black on heating after dipping in the solution of anisaldehyde-sulfuric acid. No reaction was detected by spraying with the ninhydrin solution in ethanol and heating the TLC chromatogram of NH-B1 (data not presented). This indicates that NH-B1 may not be a kind of peptides with free amino group terminals but a kind of carbohydrates. As supporting evidence for the chemical class of antibiotic substance NH-B1, it was eluted at the very beginning of the elution on the Sephadex LH-20 column chromatogram (Fig. 5). Moreover, ^{1}H-NMR spectrum in CD_3OD did not show sharp peaks but broad ones. In general, carbohydrate monomer appears to be sharp, whereas polymer shows broad peaks. As a series of our results described, antibiotic compound NH-B1 is thought to be extremely complex polymer of sugars called polysaccarides.

The antibiotic substances NH-B1 produced by the actinomycete isolate NH 50 showed a high level of antifungal activity against *A. solani* and *C. kikuchi* (Fig. 7). Inhibitory effects on mycelial growth of plant pathogenic fungi in laboratory may be different from those in the greenhouse. Although a large number of microbial metabolites are isolated every year in academic and industrial laboratories, effective screening for antibiotics that can be applied in plant disease control has led to a problem of difficulty. Most antifungal metabolites which were first detected *in vitro* did not show such an antimicrobial level in the greenhouse or field trials (4). In the past, the screening tests used have consisted of determining the minimal inhibitory concentration (MIC) of the antibiotic materials *in vitro* by a disk-plate method against fungal pathogens. The use of usual tests has, in many cases, resulted in the detection of a number of known antifungal antibiotics such as polyene, macrolides, antimycins and oligomycins which have no practical value in the use for plant disease control, because of their phytotoxity or instability (23).

In conclusion, we purified the antibiotic substance NH-B1 from the XAD-16 eluates of culture of actinomycete isolate NH 50 using silica flash chromatography, C_{18} flash chromatography, Sephadex LH-20 column chromatography, MPLC, and finally HPLC. The structure of purified antibiotic NH-B1 needs to be further elucidated. The practical disease control potential of NH-B1 should also be evaluated in the greenhouse and fields to determine the possibility of its use as a lead compound for the development of fungicides.

요 약

2개 산 토양과 1개 바닷가의 갯벌 토양에서 300개의 방선균 균주를 분리하여 6개 식물병원진균 *Magnaporthe grisea, Alternaria mali, Colletotrichum gloeospori-*

oides, Phytophthora capsici, Fusarium oxysporum f. sp. *cucumerinum*과 *Rhizoctonia solani*에 대한 균사 생장 억제 효과를 조사하였다. 이들 중 16개의 방선균 균주들이 항균 효과를 보이는 것으로 나타났고, 그중 NH 50 균주가 항균성 항생물질 분리를 위한 균주로 선발되었다. 길항방선균 NH 50은 11개 식물병원진균에 대해 비교적 넓은 항진균 스펙트럼을 보여 주었다. 균주 NH 50을 동정하기 위해 diaminopimelic acid(DAP) type과 주사전자현미경을 통해 형태적 특성을 알아보고 여러 배지에서의 배양적 특성을 관찰한 결과, 균주 NH 50은 LL-DAP type이며 포자를 형성하지 않는 희소 방선균으로 밝혀졌다. Yeast extract-malt extract-dextrose broth 에서 균주 NH 50을 배양한 후에 몇가지 식물병원진균의 균사생장을 억제하는 항균성 물질 NH-B1을 silica gel flash chromatography, C_{18} flash chromatography, Sephadex LH-20 column chromatography, silica gel MPLC, C_{18} MPLC, 그리고 HPLC의 순화 과정을 거쳐 XAD-16 resin의 methanol 용출액에서 순수 분리하였다. Methanol에 녹인 NH-B1의 UV 스펙트럼을 조사한 결과, 230 nm와 271.2 nm에서 최대 흡수 peak를 나타내었다. ^{1}H-NMR spectrum 의 data를 분석한 결과, 항균성 화합물 NH-B1은 다당류 계통의 여러 가지 당으로 구성되어 있는 탄수화물 복합체로 추정되었다. 순화된 항생물질 NH-B1은 *Alternaria solani*와 *Cercospora kikuchi*에 대해 강한 항균 활성을 보여 주었으나 *M. grisea*에 대해서는 약한 항균 활성을 나타내었다.

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