NP-101A, Antifungal Antibiotic from *Streptomyces aurantiogriseus* NPO-101

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In the course of our continuing search for antifungal antibiotics from microbial sources, we found an antifungal antibiotic against phytopathogenic fungi, especially *Alternaria* sp. from Actinomycete strain NPO-101. In the present report, we describe fermentation, isolation, physico-chemical properties, structure elucidation, and biological activities of NP-101A.

The producing organism, strain NPO-101, was isolated from a soil sample collected in Sapporo, Hokkaido, Japan. The taxonomic characterization was carried out according to the methods used in the International Streptomyces Project (ISP)[1], the method of PRIDHAM and GOTTLIEB[2] and the method of LECHEVALIER[3]. The aerial mycelium mass color was gray color series. Flexious spore chains were oval-shaped with a smooth surface. Melanoid pigments were produced and hydrolysis of whole-cell of this strain gave LL-DAP. The results of morphological and physiological studies indicated that the strain NPO-101 was considered to belong to the genus *Streptomyces* and to be a strain of the Gray series of the PRIDHAM and TRESNER grouping[6]. According to the method of KÜSTER's classification[4] and in the literature description[5~8], the strain NPO-101 was found to most resemble *Streptomyces aurantiogriseus*. Therefore, this strain was named *Streptomyces aurantiogriseus* NPO-101.

A slant culture of *Streptomyces aurantiogriseus* NPO-101 grown at 27°C for 5 days on Hickey-Tresner medium[9] was inoculated into Sakaguchi flasks containing 100 ml of the seed medium composed of glucose 10 g, soluble starch 4 g, meat extract 3 g, yeast extract 5 g, tryptone(Bacto) 5 g and $CaCO_3$ 5 g per liter. The pH was adjusted to 7.0 before autoclaving. The flasks were incubated at 27°C for 3 days on a reciprocal shaker at 130 strokes per minute. The fermentation was carried out in a 10-liter jar-fermentor. The seed culture (210 ml) was transferred into a 10-liter jar-fermentor containing 7 liters of the fermentation medium consisting of glycerol 40 g, soybean meal 25 g, yeast extract 5 g, corn steep liquor powder 1 g, NaCl 0.5 g and $CaCO_3$ 2 g per liter. The pH was adjusted to 7.0 prior to autoclaving. The aeration and agitation of the fermentation were maintained at 7 liters per minute and 400 rpm, respectively, and the temperature at 27°C for 96 hours. The antibiotic titre reached the maximum at 60 hours culture and was 4.8 mg per liter equivalent to NP-101A using purified NP-101A as a standard. The production of the antibiotic as well as its purification were monitored by antifungal activity against *Alternaria* sp. S-1.

The fermentation broth (7 liters) was centrifuged at 3,000 rpm for 20 minutes. The mycelium cake was extracted with 2 liters of methanol. The methanol extract was diluted with 8 liters of deionized water and applied on a Diaion HP-20 resin (Mitsubishi Chemical Corporation) column. The supernatant (4.5 liters) of the fermented broth was also applied on another Diaion HP-20 resin column of the same size. The columns were washed with water followed by 40% MeOH and then antibiotics were eluted with 7 liters of 50% MeOH. The eluate was combined and concentrated *in vacuo*. This material was dissolved in 1 liter of water and the pH was adjusted to 7.0 with 1 N NaOH. The product was extracted three times with the same volume of *n*-butanol. The extracts (butanol layers) were concentrated and the residue was subjected to silica gel (Wakogel C-300) column chromatography using a mixture of chloroform : MeOH (9 : 1) as an eluting solvent. The active fractions were combined and evaporated to dryness. The residue was chromatographed on silica gel using chloroform. The active fractions were combined and concentrated *in vacuo* and rechromatographed using hexane-acetone eluting solvent (7 : 3). The concentrated active fractions were purified by silica gel PTLC with benzene : acetone (7 : 3) as a developing solvent (Rf = 0.3, twice development). NP-101A (35 mg) was given as white amorphous powder by recrystallization from diethylether-hexane solvent system.

The physico-chemical properties of NP-101A are summarized in Table 1. NP-101A was found to be soluble in methanol, ethanol, ethylacetate, benzene, pyridine and dimethylsulfoxide, but was insoluble in water. NP-101A reacted with ninhydrin reagent.

NP-101A gave molecular ion peak at 178 (100% rel. int.) by FD-MS, and the molecular formula of NP-101A was established as $C_9H_{10}N_2O_2$ by HREI-MS *m/z*

Table 1. Physico-chemical properties.

Appearance	White amorphous powder
MP	160~163°
MW	178
Molecular formula	$C_9H_{10}N_2O_2$
FD-MS	178 (M^+, 100% rel. int.)
HREI-MS (*m/z*, M^+)	
Calcd:	178.0743 for $C_9H_{10}N_2O_2$
Found:	178.0745
UV λ_{max} (MeOH) nm (log ε)	219 (3.56), 252 (3.31), 299 (2.72)
IR ν_{max} (KBr) cm^{-1}	3378, 3164, 1678, 1626, 1510, 1393, 1306, 769, 641

Table 2. ^1H and ^{13}C NMR data (270 and 67.8 MHz) of NP-101A in CD_3OD.

Position	δ_H[a] multiplicity (J=Hz)	δ_C[a] multiplicity
1		122.2 s
2		140.2 s
3	8.36 br d (8~9)	122.5 d
4	7.47 ddd (1.3, 7.9, 7.9)	133.4 d
5	7.14 ddd (0.7, 7.9, 7.9)	124.4 d
6	7.73 dd (1.3, 7.9)	129.4 d
1-$CONH_2$		173.5 s
2-$NHCOCH_3$		171.3 s
2-$NHCOCH_3$	2.16 s	24.8 q

[a] TMS (0 ppm) was used as internal standard. [b] CD_3OD (49 ppm) was used as internal standard. Assignments were based on DEFT, ^1H-^1H COSY, ^{13}C-COSY, and selective INEPT experiments[13].

Table 3. Antifungal spectrum of NP-101A.

Test organisms	MIC (μg/ml)
Alternaria sp. S-1	7.5
Penicillium roqueforti AHU8057	3.75
Pyricularia oryzae Ina168	3.75
Phytophthora infestans	7.5
Cladosporium herbarum AHU9032	7.5
Aspergillus oryzae AHU7134	7.5
Rhizopus oryzae AHU6536	15.0
Mucor javanicus AHU6052	30.0
Fusarium roseum AHU9056	30.0
Rhizoctonia zeae	30.0

MIC values were determined by serial dilution assay in MPG liquid medium (malt extract 1%, Polypepton 1%, glucose 1%, pH 5.5) inoculated with approximately 10^5 cells per ml of the respective test organisms at 27°C.

178.0745 (178.0743 calcd for $C_9H_{10}N_2O_2$; Table 1). The ^1H and ^{13}C NMR data of NP-101A are shown in Table 2. NP-101A indicated a simple ^1H NMR spectrum, and there were only four neighbouring aromatic proton signals and acetyl methyl singlet. The ^{13}C NMR and DEFT experiments realized that NP-101A has one acetyl methyl carbon, four aromatic methine carbons, two aromatic quaternary carbons, and two carbonyl carbons. The chemical shifts of the carbonyl carbon (δ_C 173.5 and 171.3) and molecular formula of NP-101A suggest that NP-101A has two amide groups. The IR spectrum of NP-101A showed major absorptions at 1678, 1626 cm^{-1} indicating the presence of amide groups.

Based on these results, it was concluded that NP-101A is 2-acetamidobenzamide. ^1H and ^{13}C NMR, and Rf value (0.3, Merck, Kiesegel $60F_{254}$: hexane : acetone = 7 : 3 solvent system) of 2-acetamidobenzamide, which was prepared from *O*-aminobenzamide by acetylation with Ac_2O/pyridine, were complete agreement with those of NP-101A. Thus, the structure of NP-101A was elucidated as 2-acetamidobenzamide.

The antibiotic NP-101A exhibited antifungal activities against phytopathogenic filamentous fungi (Table 3), while no activity was observed against *Saccharomyces cerevisiae* and bacteria, such as *Salmonella typhimurium* SL3770, *Escherichia coli* K-12 and *Bacillus subtilis*.

Benzamide and its derivatives with a substitution at the 3-position have been known as potent inhibitors of poly(ADP-ribose) synthetase[10,11]. 2-, 3-, and 4-acetamidobenzamides are also inhibitors of poly(ADP-ribose) synthetase[12]. However, their antifungal activity has not been reported to date. This is the first report on antifungal activity of 2-acetamidobenzamide, NP-101A, as a microbial product.

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