Notes

AZOXYBACILIN, A NOVEL ANTIFUNGAL AGENT PRODUCED BY *Bacillus cereus* NR2991

PRODUCTION, ISOLATION AND STRUCTURE ELUCIDATION

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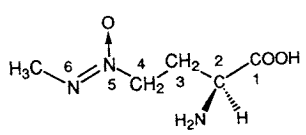
(Received for publication January 14, 1994)

In our screening program for novel antifungal agents of microbial origin, an azoxy-containing antifungal named azoxybacilin was found in the culture broth of *Bacillus cereus* Frankland and Frankland NR2991. Azoxybacilin shows potent antifungal activity *in vitro* in an amino acid free medium, especially against mycelial fungi such as *Aspergillus fumigatus* and *Trichophyton mentagrophytes*. In the present report, we describe its fermentation, isolation and structure elucidation.

The producing organism, NR2991, was isolated from a soil sample collected in Odawara City, Kanagawa Prefecture, Japan, and was identified as *B. cereus*. A seed broth was prepared by inoculating spores of the producing strain into a 500-ml of Erlenmeyer flask containing 100 ml of medium consisting of 1% dextrin, 1% glucose, 1% Pharmamedia (Traders Protein), 1% S-3 Meat (Ajinomoto), 0.06% K_2HPO_4 and 0.025% KH_2PO_4. The flask was shaken at 200 rpm for 1 day at 27°C. The resultant culture (0.6 ml) was transferred to a 3-liter flasks containing 600 ml of the same medium above. Fermentation was conducted on a rotary shaker at 100 rpm at 27°C. After four days of cultivation, the production of azoxybacilin reached its maximum.

Fig. 1. Structure of azoxybacilin.



The culture broth (6.8 liters) obtained as described above was acidified (pH 2.0) with 1 N HCl, and the mycelial cake was filtered over Celite. The filtrate was neutralized with 1 N NaOH (pH 7.5) and the active principle in the filtrate was adsorbed on SK104 (700 ml) (H^+-form, Mitsubishi Chemical). The resin was washed with water (2,000 ml) and the adsorbed azoxybacilin was eluted with 0.5 N NH_4OH (2,000 ml). The active fractions were collected and neutralized with 1 N HCl after removal of excess ammonia by evaporation under reduced pressure. The resultant solution was lyophilized and the residue was treated with MeOH (500 ml). The MeOH insoluble substance was removed by filtration and the filtrate was concentrated to give a crude powder (11.5 g). Subsequently, the crude powder was dissolved in 100 ml of methanol and this solution was mixed with 50 ml of silica gel (Kieselgel 60, 70~230 mesh, Merch Co. Ltd.). After evapolating the solvent, the resultant dry powder was poured on the column of the same silica gel (150 ml) which was prepacked with CH_2Cl_2. The active principle was eluted with CH_2Cl_2-MeOH-25% NH_4OH (70 : 30 : 1 to 70 : 30 : 2 in volume). The combined active fractions (2.24 g) was further purified by Sephadex LH-20 (700 ml) column chromatography developed with MeOH. The active fractions were collected and evaporated under reduced pressure to give a colorless powder (208 mg). The powder was crystallized from aqueous MeOH to give azoxybacilin (128 mg) as colorless needles.

The physico-chemical properties of azoxybacilin are summarized in Table 1. The molecular formula

Table 1. Physico-chemical properties of azoxybacilin.

Appearance	Colorless needles
MP (°C)	203~205
$[\alpha]_D^{24}$	+9.4° (*c* 1.0, H_2O)
Molecular formula	$C_5H_{11}N_3O_3$
HRFAB-MS (*m/z*)	
Found:	162.0877 $(M+H)^+$
Calcd:	162.0877
UV λ_{max}^{MeOH} nm (ε)	215 (8,400), 280 (sh, 160)
IR ν_{max} (KBr) cm^{-1}	3600~2400 (br), 1620, 1590
Solubility	Soluble in H_2O, DMSO, MeOH insoluble in *n*-hexane, acetone
Color reaction	Ninhydrin, Dragendorff reagent
positive	

Table 2. ^{1}H and ^{13}C NMR assignments of azoxybacilin (in D_2O).

Position	δ_H [a,b]	δ_C [a,c]
1	—	176.0
2	3.78 (1H, t, J=6.5 Hz)	55.1
3	2.45 (2H, m)	31.1
4	4.39 (2H, t, J=6.5 Hz)	68.4
N-6-CH_3	3.21 (3H, s)	42.1

[a] 3-(Trimethylsilyl)-propionic acid-d_4 sodium salt as external reference.
[b] 400 MHz: δ in ppm.
[c] 100 MHz: δ in ppm.

of azoxybacilin was determined to be $C_5H_{11}N_3O_3$ from its positive HRFAB-MS data [m/z 162.0877 (M+H) calcd 162.0877 for $C_5H_{12}N_3O_3$; m-nitrobenzylalcohol matrix] and its ^{1}H and ^{13}C NMR spectral data (Table 2). The positive color reaction to Ninhydrin suggested the presence of a primary amino group. The IR spectral data of azoxybacilin also suggested the presence of $-NH_3^+$ (3400~2500 and 1620 cm^{-1}) and carboxylate (1590 cm^{-1}). The UV absorption maxima of azoxybacilin at 215 nm (ε 8,400) and 280 nm (sh, ε 160) suggested the presence of azoxy functionality[1] in the molecule.

The ^{1}H NMR spectrum (in D_2O) of azoxybacilin indicated the presence of one methyl (δ 3.21), two methylenes (δ 2.45, 4.39) and one methine (δ 3.78) (Table 2). The presence of these groups were confirmed by the DEPT experiment of azoxybacilin. The connectivity of two methylenes (C-3 and C-4) and one methine (C-2) was accomplished by the ^{1}H decoupling experiments. The hydrogenolysis (PtO_2 catalyst) of azoxybacilin in AcOH[2] gave 2,4-diamino butyric acid. This result suggested the connectivity of C-4 to the azoxy group in azoxybacilin because C-4 methylene protons were observed at δ 4.39 and in the ^{1}H NMR spectrum of azoxybacilin. The location of the oxygen atom in the azoxy moiety was assigned to be on the C-4 side on the basis of the ^{1}H and ^{13}C chemical shifts of 4-methylene (δH 4.39, δC 68.4) which were obviously downfield shifted by the adjacent =N(O)– group[3]. The geometry of the azoxy moiety was determined to be Z because the UV absorption maximum of azoxybacilin (215 nm) reveals a good accordance with those of the reported Z-azoxy compounds (ranging 220±3 nm, 230±3 nm for E-isomers)[4]. The absolute configuration at the C-2 position was determined to be (S) by the chiral HPLC analysis (Tosoh column TSK-GEL ENANTIO-L1, 4.6×250 mm, 1 mM $CuSO_4$, UV at 210 nm, 1 ml/minute, retention time 6.90 minutes) of 2,4-diaminobutyric acid, which was obtained from the hydrogenolysis of azoxybacilin, described above. Based on these findings, the structure of azoxybacilin was determined to be (S)-2-amino-4-(Z-methyl-N,N,O-azoxy) butanoic acid as shown in Fig. 1.

Azoxybacilin showed potent antifungal activity especially against mycelial fungi such as *Aspergillus fumigatus* (IC_{80}; 0.71~1.3 μg/ml), and *Trichophyton mentagrophytes* (IC_{80}; 0.03~0.24 μg/ml), and showed moderate activity against yeast-type fungi including *Candida albicans* (IC_{80}; 4.2~5.8 μg/ml) in an amino acid free media.

Several azoxy-containing antibiotics and antifungals such as elaiomycin[5], LL-BH872α[6], valanimycin[7], and maniwamycins[8,9] have so far been isolated from the cultured broth of *Streptomyces*. All the compounds are not derivatives of amino acids. Azoxybacilin is the first azoxy-containing antifungal isolated from bacterial origin. Further biological investigation of azoxybacilin including its mode of action will be reported elsewhere.

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