Notes

ISOLATION OF AZOMYCIN FROM *PSEUDOMONAS FLUORESCENS*

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In recent years, it has been recognized that bacteria can produce antibiotics of wide structural diversity. Many examples for the production of the antibiotics known as products of Actinomycetales by eubacteria have been reported[1]. Here, we will report the isolation of azomycin from the culture broth of a strain identified as *Pseudomonas fluorescens*. Azomycin was first isolated from a strain resembling *Nocardia mesenterica*[2,3] and also from *Streptomyces eurocidicus*[4,5].

The producing organism numbered PB-6,282 was isolated from river water in Matsusaka-city, Mie Prefecture, and identified as *P. fluorescens*[6] by the following characteristics. The organism is aerobic, Gram-negative, non-sporulating rods ($0.5 \times 1.0 \sim 2.0\ \mu m$) with rounded ends. Motility is observed with polar multi-trichous flagellation. Colonies on heart infusion agar are circular, slightly convex, smooth and shiny with yellowish orange color. Glucose is metabolized oxidatively. The following tests gave positive results: Catalase, oxidase, gelatin liquefaction, citrate utilization, arginine dihydrolase, and formation of fluorescent pigments. But, the followings were negative: Accumulation of poly-β-hydroxybutyrate, nitrate reduction, lysine and ornithine decarboxylases. Growth was observed at 28 and 5°C.

The cell suspension of strain PB-6282 was inoculated into 100 ml of a medium consisting of starch 2.0%, glycerol 0.5%, Bacto Soytone 1.5%, corn steep liquor 0.5%, NaCl 0.3%, $CaCO_3$ 0.3% (pH 7.0) in a 500-ml Erlenmeyer flask, which was cultured at 23°C for 2 days on a rotary shaker (180 rpm, stroke 70 mm).

The culture broth (5 liters) was adjusted to pH 2.0 with HCl and mixed with BuOH (1 liter). The mixture was stirred for half an hour and then filtered. The BuOH layer was separated and the residual aqueous solution was further extracted with BuOH (1 liter). The BuOH extracts were combined and concentrated to *ca.* 500 ml. The same volume of ethyl ether was added to the concentrate, and the antibiotic contained was transferred into 3% aqueous $NaHCO_3$. The antibiotic was re-extracted with BuOH at pH 2.0. The BuOH extract was washed with H_2O and concentrated to an oily residue, which was triturated with acetone to give a crude powder (1.4 g).

The crude powder (0.7 g) was applied to a Sephadex LH-20 column (2.6×90 cm) and developed with MeOH. The active eluate fraction was concentrated and applied to a silica gel column (Merck, Silica gel 60, 2.2×20 cm) which was developped with $CHCl_3$ - MeOH (20 : 1). The active eluate from the column was concentrated and dissolved in BuOH, which was washed with diluted HCl and water successively and concentrated to an oily residue. The residue was crystallized from MeOH to give a crystal (65 mg) of the antibiotic.

The antibiotic is an acidic substance. The free form is obtained as colorless prisms and decomposes

Fig. 1. IR spectrum of the antibiotic produced by PB-6,282 (KBr).

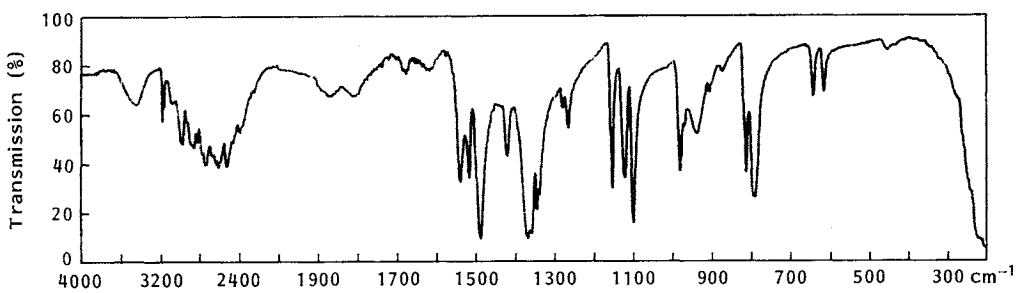

Fig. 2. Structure of azomycin.

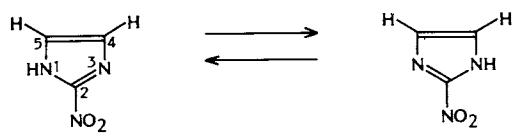

Table 1. Antibacterial spectrum of azomycin against anaerobic bacteria.

Test organism	MIC (μg/ml)
Peptococcus asaccharolyticus ATCC 14963	3.13
P. prevotii ATCC 9321	6.25
Peptostreptococcus micros VPI 5464-1	1.56
Streptococcus constellatus ATCC 27823	>100
Eubacterium limosum ATCC 8486	3.13
E. aerofaciens ATCC 25986	6.25
Propionibacterium acnes ATCC 11827	50
Bifidobacterium adolescentis JCM 1250	6.25
B. bifidum JCM 1122	6.25
B. longum ATCC 15707	12.5
Clostridium perfringens ATCC 13124	0.78
C. difficile ATCC 17857	0.78
Veillonella parvula ATCC 10790	1.56
Bacteroides fragilis GM 7000	3.13
B. fragilis ATCC 25285	3.13
B. thetaiotaomicron WAL 3304	6.25
B. vulgatus ATCC 29327	0.39
B. melaninogenicus GAI 0413	0.39
Fusobacterium varium ATCC 8501	1.56
F. necrophorum ATCC 25286	0.78
F. nucleatum ATCC 25586	1.56
F. mortiferum ATCC 9817	0.78

Inoculum size: One loopful of 10^6 cfu/ml.
Medium: GAM Agar (Nissui).

above 250°C. It is soluble in aqueous alcohols and dimethyl sulfoxide, slightly soluble in methanol and ethanol, and essentially insoluble in acetone, ethyl acetate, chloroform and water. Elemental analysis and MS, electron impact (EI)-MS: m/z 113 (M^+), indicated a molecular formula, $C_3H_3N_3O_2$.

Anal Calcd for $C_3H_3N_3O_2$: C 31.86, H 2.67, N 37.16.
Found: C 31.86, H 2.92, N 37.10.

The UV absorption, $\lambda_{max}^{95\%EtOH}$ nm (ε) 219 (3,900), 315 (8,600); $\lambda_{max}^{0.01\,N\,HCl\text{-}95\%EtOH}$ nm (ε) 219 (3,900), 315 (8,400); $\lambda_{max}^{0.01\,N\,NaOH\text{-}95\%EtOH}$ nm (ε) 223 (3,500), 365 (10,700), and the IR spectrum (Fig. 1) are quite similar to the reported data of azomycin[4]. No optical activity is shown by CD measurement.

^1H and ^{13}C NMR of the antibiotic in DMSO-d_6 using TMS as an internal reference were measured with a Varian XL-200 spectrometer. All the signals observed were assignable to azomycin (2-nitroimidazole) (Fig. 2) as follows: δ 7.41 (s, 4-H, 5-H) in the ^1H NMR δ 126.3 (d) $^1J_{CH}=195$ Hz, long range coupling (d), $^2J_{CH}=12$ Hz (C-4 and C-5), and δ 146.1 (s, C-2) in the ^{13}C NMR.

Azomycin has been reported to be active against aerobic Gram-positive and Gram-negative bacteria[1,4]. It was found in this experiment that the antibiotic was also active against a variety of anaerobic bacteria (Table 1).

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