A NEW ANTIBIOTIC, NEGAMYCIN

Sir:

After more than 20 years of screening studies, one rarely encounters new antibacterial antibiotics with low toxicity. However, negamycin was found by an ordinary screening method and is unique type of antibiotic.

Negamycin was obtained from culture filtrates of three strains of *Streptomyces*; strain No. M890-C2 was isolated from a soil sample collected at Myogisan, Gunma Prefecture in 1964, the strain MA91-M1 from Nojiriko, Nagano Prefecture in 1965 and strain MA104-M1 from Sakomachi, Tokushima Prefecture in 1965. These strains are very closely related to *S. purpeofuscus*. In a medium containing 2.0 % glucose, 2.0 % starch, 2.0 % soybean meal, 0.5 % dry yeast, 0.25 % NaCl, 0.35 % $CaCO_3$, 0.0005 % $CuSO_4 \cdot 5H_2O$, 0.0005 % $MnCl_2 \cdot 4H_2O$, 0.005 % $ZnSO_4 \cdot 7H_2O$ after shaking culture of the strain No. M890-C2 for 3~8 days negamycin accumulated in the culture liquid. It was adsorbed on a cation-exchange resin containing carboxyl or sulfonic acid group and eluted with dilute aqueous ammonia. Negamycin in the active eluate was adsorbed on a column of Dowex 1 X2 (OH^- form) and after washing with water, was eluted with dilute hydrochloric acid. After neutralization of the active eluate, evaporation yielded crude negamycin hydrochloride. An aqueous solution of the hydrochloride was passed through a column of Amberlite CG 50 (NH_4^+ form) and the chromatograph was developed with 0.1 % ammonia water. Lyophilization of the active fraction yielded pure negamycin.

Negamycin is a colorless powder, m. p. 110~120°C (decomp.), $[\alpha]_D^{29}$ +2.5° (*c* 2, H_2O). The antibiotic is soluble in water, and practically insoluble in methanol, ethanol, butanol, ethyl acetate, butyl acetate, chloroform and benzene. It shows no ultraviolet absorption except end absorption. It gives positive ninhydrin, red tetrazolium and RYDON-SMITH reactions, and heating at 105°C for 6 hours in 6 N HCl gives more than three ninhydrin-positive degradation products. Under paper electrophoresis, 3,500 V for 15 minutes in formic acid - acetic acid - water (25 : 75 : 900), it moved 12 cm to cathode with Rm (L-alanine 1.0) of 1.4. Thus, negamycin has basic properties. Negamycin gives salts with picric acid and *p*-hydroxyazobenzene-*p'*-sulfonic acid. Negamycin *p*-hydroxyazobenzene-*p'*-sulfonate crystallizes as yellowish orange plates, m. p. 180~182°C (decomp.).

In the infrared spectrum of negamycin, carboxylate absorption is shown at 1590 cm^{-1} and in that of the *p*-hydroxyazobenzene-*p'*-sulfonate the carboxyl band is shown at 1730 cm^{-1}. Treatment of negamycin with hydrochloric acid-methanol gives negamycin methyl ester hydrochloride which shows an ester carbonyl band at 1740 cm^{-1}. pK values of 3.55, 8.10, 9.75 were found by titration with an equivalent weight of 287. Thus, negamycin is an amphoteric compound containing a carboxyl and two basic groups. Elemental analyses of negamycin and its *p*-hydroxyazobenzene-*p'*-sulfonate are as follows:

Negamycin; found:
C 40.63, H 7.90, N 21.11, O 29.70.
Calcd. for $C_9H_{20}N_4O_4 \cdot H_2O$:
C 40.59, H 8.33, N 21.04, O 30.04.
Negamycin *p*-hydroxyazobenzene-*p'*-sulfonate; found:
C 48.45, H 5.11, N 13.10, O 24.84, S 7.67.
Calcd. for $C_9H_{20}N_4O_4(C_{12}H_{10}N_2O_4S)_2 \cdot H_2O$:
C 48.16, H 5.14, N 13.62, O 25.28, S 7.79.

The molecular formula was derived from the high-resolution mass spectrum of di-N-acetylnegamycin methyl ester: calcd. mol. wt. for $C_{14}H_{26}N_4O_6$, 346.185; found, *m/e* 346.188 ± 0.005.

On nutrient agar medium negamycin showed complete inhibition of test organisms at the following concentrations (the values in the parentheses are for partial inhibitions): *Staphylococcus aureus* FDA 209P, 50 μg/ml; *S. aureus* TERAJIMA strain, 12.5 μg/ml; *Sarcina lutea* PCI 1001, 12.5 μg/ml (1.56 μg/ml); *Bacillus subtilis* NRRL B-558, 25 μg/ml (12.5 μg/ml); *Escherichia coli* K-12, 3.12 μg/ml (1.56 μg/ml); *E. coli* K-12 ML

1629 (carrying multiple resistant R factor), 6.25 μg/ml (3.12 μg/ml); *E. coli* NIHJ, 12.5 μg/ml (3.12 μg/ml); *Shigella flexneri* 1a Ew8, 12.5 μg/ml; *Salmonella typhosa*, 3.12 μg/ml; *Proteus vulgaris* OX 19, 6.25 μg/ml; *P. rettgeri* GN 311, 12.5 μg/ml (6.25 μg/ml); *Serratia marcescens*, 12.5 μg/ml (6.25 μg/ml); *Pseudomonas aeruginosa* A3, 6.25 μg/ml; *Klebsiella pneumoniae* PCI 602, 12.5 μg/ml (6.25 μg/ml); *Mycobacterium smegmatis* ATCC 607, 100 μg/ml. Fifteen strains of *Pseudomonas aeruginosa* isolated from patients were inhibited completely at 12.5~50 μg/ml and partially at 6.25~25 μg/ml when one loopful of their broth culture was streaked on a nutrient agar medium containing negamycin. However, when one loopful of broth culture diluted 1,000 times was streaked, the complete and the partial inhibition were observed at half the above concentrations. When 0.5 % peptone agar was used instead of nutrient agar, the complete and the partial inhibitions were observed at lower concentrations as follows: *S. aureus* FDA 209P, 1.56 μg/ml; *E. coli* K-12, 1.56 μg/ml; *E. coli* K-12 ML 1629, 1.56 μg/ml (0.78 μg/ml); *S. flexneri* 1a Ew8, 3.12 μg/ml; *S. typhosa*, <0.78 μg/ml; *P. rettgeri* GN 311, 1.56 μg/ml; *Pseudomonas aeruginosa*, 3.12 ~ 12.5 μg/ml; *K. pneumoniae*, 6.25 μg/ml (3.12 μg/ml). The growth inhibitory effect was not reduced by serum. *E. coli* K-12 was inhibited at 1.56~3.12 μg/ml when serum was added to 0.5 % peptone water at 10~40 %.

The intramuscular injection of 50 mg/kg to a rabbit gave a serum concentration of about 100 μg/ml at 1 hour after administration with 80 % excreted in the urine in 24 hours resulting in a high concentration in urine, for instance 4480 μg/ml in urine taken 1~2 hours after the injection.

Negamycin was effective *in vivo* against infections of *Pseudomonas aeruginosa* No. 12, *Klebsiella pneumoniae* S-1802, *Salmonella typhosa* 63 and *Staphylococus aureus* SMITH S-424 in mice. The CD_{50} against these infections was 4.4, 5.0, 2.5 and 12.5 mg/kg, respectively, when 10 MLD was infected intraperitoneally and negamycin was subcutaneously injected immediately and 6 hours after the infection. The LD_{50} of negamycin to mice by intravenous injection was 400~500 mg/kg and daily intraperitoneal injection of 200 mg/kg for 10 days caused no toxicity.

As described above, negamycin is an antibiotic with low toxicity and effective *in vivo* against infections of Gram-negative and Gram-positive bacteria. Especially, an effect against resistant Gram-negative organisms including *Pseudomonas* was found. The structure and the pharmacology will be reported in other papers.

MASA HAMADA
TOMIO TAKEUCHI
SHINICHI KONDO
YOKO IKEDA
HIROSHI NAGANAWA
KENJI MAEDA
YOSHIRO OKAMI
HAMAO UMEZAWA

Institute of Microbial Chemistry
Shinagawa-ku, Tokyo, Japan

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