STRUCTURE OF ENZYMATICALLY ACETYLATED SISOMICIN BY *PSEUDOMONAS AERUGINOSA*

Sir:

As described in the previous paper[1], most *Pseudomonas aeruginosa* strains isolated from clinical specimens are sensitive to sisomicin (SS) but we found three SS-resistant (to 50 μg /ml or more) strains of *P. aeruginosa* GN269, GN315 and GN362 among our stock cultures. It was also found that these strains could inactivate SS by acetylation, although the position of acetylation was not clear[1]. *P. aeruginosa* GN269 showed the greatest resistance against SS and was used in this experiment.

The strain was cultured in 3 liters of nutrient broth with shaking at 37°C. The cells at the logarithmically growing phase (OD_{600nm}, 0.6) were harvested by centrifugation, washed three times with TMK solution (0.06 M KCl, 0.01 M magnesium acetate and 0.006 M 2-mercaptoethanol in 0.1 M tris-HCl buffer, pH 7.8) and suspended in 18 ml of the same solution. The cell suspension was disrupted by a supersonic apparatus (Ohtake Co., Tokyo) at 20Kc for 10 minutes and centrifuged at $105,000\times g$ for 2 hours to remove disrupted cell particles. The supernatant thus obtained was diluted with TMK solution to 20 mg protein/ml[2] and used as the S-105 fraction (crude extract).

The inactivation reaction was carried out by the method reported previously.[1] SS was treated with the crude extract of GN269 strain at 37°C for 48 hours in a reaction mixture consisting of 30 ml of the S-105 fraction, 200 mg of disodium ATP neutralized with $NaHCO_3$, 20 mg of CoA, 100 mg of magnesium acetate and 50 mg of SS. The reaction was stopped by heating at 100°C for 5 minutes and the residual antibiotic activity in the reaction mixture was bioassayed with *Bacillus subtilis* PCI-219 as the test organism. It was found that SS was completely inactivated.

The mixture containing the inactivated SS was centrifuged at $10,000\times g$ for 30 minutes; the supernatant was adjusted to pH 4.5 with sulfuric acid and the inactivated SS-sulfate was precipitated by addition of excessive ethanol. The precipitate was resuspended in redistilled water and excess ethanol was added to the solution. The same procedure was repeated 3 times. The precipitate thus obtained was dissolved in 5 ml of redistilled water and the solution was passed through a Sephadex G-25 column (1.8×32 cm^2), those fractions (10 ml) which showed both positive ninhydrin reaction (0.2% ninhydrin in *n*-butanol saturated with water) and reactivation of the inactivated SS[3] by treatment with 2 N NaOH (final concentration) at 100°C for 1 hour, was mixed and lyophilized. The yellow powder thus obtained was dissolved in 2 ml of redistilled water, and the solution was passed through a Dowex 1 $\times$2 column (1.7×34 cm^2, OH^- form). Paper chromatography and thin-layer chromatography were carried out on each 5-ml fraction. Fractions 6 and 7 showed a single spot by ninhydrin spray on paper chromatography and were mixed and lyophilized, yielding 4.1 mg of white powder. The calculated value from the reactivation test was 4.5 mg.

In paper chromatography (Toyo filter paper No. 50) using the following solvent systems; *n*-butanol - pyridine - water - acetic acid (6 : 4 : 3 : 1), **I**, and chloroform - methanol - 28 % NH_4OH (1 : 1 : 1), **II**, Rf values of SS and the inactivated SS were 0.05 and 0.12 with solvent **I**, and 0.40 and 0.55 with solvent **II**. In thin-layer chromatography (silica gel, Tokyo Kasei Co., Ltd.) using the following solvent system; chloroform - methanol - 28 % NH_4OH (2:1:1), Rf values of SS and the inactivated SS were 0.25 and 0.32, respectively. The chromatogram was developed by the ascending technique and visualized with either 0.2 % ninhydrin or 50 % sulfuric acid. The color of SS and the inactivated SS were yellow and violet during the early stages of heating (120°C, 3 minutes), respectively.

When the solution containing ^{14}C-acetylated SS was used, the counts per minute of labeled acetate were recorded by Aloka paper scanner TRM-1 type. The Rf value of the incorporated ^{14}C-acetate into SS (^{14}C-acetylated SS) in the reaction mixture was 0.11 with solvent I. Chromatographic behavior of the ^{14}C-acetylated SS, extracted with 1 M NaCl from phosphocellulose paper P-81, showed the same Rf value of 0.12 with solvent I.

The inactivated SS melts at 197~200°C with bubbling compared to a melting point of 198~203°C for SS, and 191°C for gentamicin mixture. The infrared spectrum (Hitachi grating

infrared spectrophotometer EPI-G3) of the inactivated SS showed amide bands I and II at 1640 cm^{-1} and 1570 cm^{-1}.

The mass spectra of inactivated SS and SS were compared with some key features of the mass spectral fragmentation patterns of aminocyclitol antibiotics presented by DANIELS *et al.*[4], COOPER *et al.*[5,6,7] and INOUYE.[8] The mass spectrum exhibited a molecular ion at *m/e* 489 and a somewhat more intense $(M+1)^+$ at *m/e* 490 consistent with a mono-N-acetylated SS. Intense peaks at *m/e* 191, 173, 163, 145, and 160 could be attributed to the 2-deoxystreptamine and garosamine ions (COOPER *et al.*[6,7]), indicating that the acetyl group was not attached to either of these parts of the molecule. A prominent peak observed in SS at *m/e* 127 associated with 4′, 5′-didehydropurpurosamine unit,[4] was absent from the spectrum of the acetylated compound. However, there was a peak in the inactivated SS at *m/e* 169, indicating that the 4′, 5′-didehydropurpurosamine moiety was acetylated. Mass spectra were determined on a Hitachi double focusing mass spectrometer RMU-7L using a direct inlet system.

The nuclear magnetic resonance (nmr) spectra of the inactivated SS (base and sulfate) were compared with that of SS (base and sulfate). The inactivated SS sulfate was prepared from the base by adjustment to pH 4.5 with sulfuric acid. The nmr spectrum of SS was studied comparing with those of gentamicin C_{1a}, gentamicin-C_1, kanamycin and 6′-N-acetylated kanamycin prepared by the crude enzyme of *P. aeruginosa* GN315.[9] The nmr spectra were determined on a Model JMN-4H-100 spectrometer (Japan Electron Optics Lab. Co., Ltd.) at 100 MHz in deuterium oxide (D_2O) solution using sodium trimethylsilane as the internal reference ($\sigma=0$) or tetramethylsilane as the external reference. In the nmr spectrum of the inactivated SS-base in D_2O, the signal of the C-6′ methylene protons at $\sigma=3.52$ in SS-base shifted to $\sigma=3.73$, and sulfate showed a methyl proton signal from the acetyl group at $\sigma=2.13$. Thus, the structure of the inactivated SS was concluded to be 6′-N-acetylsisomicin.

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