

INHIBITION OF NUCLEIC ACID BIOSYNTHESIS IN PROCARYOTIC AND EUCARYOTIC CELLS BY CYANOCYCLINE A

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Cyanocycline A was cytotoxic against Meth A cells *in vitro*, and also showed marked activity against the same cell line grown as an experimental ascites tumor. This antibiotic inhibited nucleic acid synthesis in *Escherichia coli* and Meth A cells. The antimicrobial activity of the antibiotic was reversed by addition of exogenous herring sperm DNA. Cytofluorometric analysis of cyanocycline A-treated Meth A cells showed an unusual pattern of the relative content of DNA and RNA per cell. These results suggested that cyanocycline A binds to DNA, and that it inhibits nucleic acid synthesis.

Cyanocycline A is a broad spectrum antibiotic isolated from the culture filtrate of *Streptomyces flavogriseus* strain No. 49.^{1,2)} The structure of this compound elucidated by X-ray crystallography consists of a benzoquinone moiety and a cyano group, and belongs to the group of heterocyclic quinone antibiotics³⁾. Cyanocycline A exhibits potent cytotoxicity toward cultured cells and has antitumor activity against ascites carcinoma Meth A.

Because of the demonstration of daunomycin's clinical utility, there has been considerable interest in the search for further DNA binding antibiotics. Thus cyano containing antibiotics such as naphthyridinomycin and saframycin A have been reported as a new group of DNA binding antibiotics.^{4,5)} Cyanocycline A, described here, is structurally similar to these antibiotics.^{6,7)} This structural similarity suggested that cyanocycline A might also bind to DNA. In the present paper we report the antitumor activity of cyanocycline A against an ascites tumor in mice, and the cytopathic effect on tumor cells *in vitro*. The mode of action of cyanocycline A on *Escherichia coli* is also discussed.

Materials and Methods

Chemicals

Cyanocycline A was isolated from fermentations of *Streptomyces flavogriseus* strain No. 49,²⁾ and actinomycin D was prepared by our company. Mitomycin C and adriamycin were purchased from Kyowa Hakko Kogyo Co. Calf thymus DNA from Boehringer Mannheim GmbH. Herring sperm DNA and yeast RNA were purchased from Sigma Co. [*Methyl*-³H]thymidine (40 Ci/mmol), [5-³H]-uridine (25 Ci/mmol) and [³H]leucine (150 Ci/mmol) were purchased from the Radiochemical Centre (RCC), Amersham. Dithiothreitol was purchased from Nakarai Kagaku Co.

Cell Culture

The Meth A No. 2 cell line was cloned from a Meth A ascites cell of BALB/c mice. The cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, benzylpenicillin 100 μ g/ml and streptomycin 100 μ g/ml at 37°C in 5% carbon dioxide. The cells, in logarithmic phase of growth (4×10^4 cells/ml), were dispensed into 1 ml well (Falcon Multi-well, Falcon 3008), with various concentrations of cyanocycline A followed by incubation for 48 hours at 37°C. At the end of the incubation period, viable cells were counted by microscopic observation with trypan blue. Cells were collected on micro-

scope slides by cytocentrifugation, treated with Giemsa stain, and then morphological changes were looked for under the microscope.

Cytofluorograph

Meth A cells (1×10^5 cells/ml) were grown in Petri dishes (50 mm diameter), and exposed to a concentration of 10 $\mu\text{g/ml}$ cyanocycline A. After 1- and 6-hour incubation, the cells were treated with 1/10 volume of 1×10^{-5} M acridine orange solution for 10 minutes at room temperature and analyzed by a Cytofluorograf[®] (Bio/Physics System Inc.) for the relative content of DNA and RNA per cell.

Effects on Eucaryotic Macromolecular Synthesis

To determine the effect of cyanocycline A on the synthesis of DNA, RNA and protein in Meth A cells, cells in the logarithmic phase of growth (2.5×10^5 cells/ml) were treated with cyanocycline A and labeled precursors; [³H]thymidine, 1.0 $\mu\text{Ci/ml}$, [³H]uridine, 3.0 $\mu\text{Ci/ml}$ or [¹⁴C]leucine, 5.0 $\mu\text{Ci/ml}$. After incubation for 120 minutes, the cells were collected on glass fiber filters (Whatman GF/A 25 mm), washed four times with phosphate buffer saline (pH 7.0), twice with cold 5% trichloroacetic acid and once with ethanol. The acid insoluble radioactivity on filters was determined in vials containing toluene scintillation fluid (0.5% POP, 0.03% dimethyl-POPOP).

Antitumor Tests

The Meth A No. 2 cell line, was maintained by intraperitoneal passage in BALB/c mice as an ascites tumor. For antitumor tests, 1×10^8 Meth A cells were transplanted intraperitoneally in BALB/c mice. Antibiotic solutions were injected intraperitoneally once on days 1, 2, 3, 6, 7 and 8. The mice were housed in plastic cages in a room maintained at $24 \pm 1^\circ\text{C}$, at relative humidity of $55 \pm 5\%$ and were fed on laboratory chow CE-2 (Clea Japan, Inc., Tokyo).

Microorganism and Medium

Escherichia coli K-12 YA21 (Leu⁻, Met⁻ and Thy⁻) was kindly furnished by Dr. A. TAKATSUKI, Department of Agricultural Chemistry, University of Tokyo. *Micrococcus luteus* B was a strain from the Chugai Type Culture Collection. Nutrient broth medium was purchased from Eiken Co.

Procaryotic Macromolecular Synthesis Inhibition

A 0.1 ml sample of cells of *E. coli* in the logarithmic growth was inoculated into 10 ml of diluted (20% v/v with water) fresh nutrient broth medium in L-tubes, the tubes were incubated at 37°C with shaking. When the cell density reached on OD 550 nm=0.1, 0.1 $\mu\text{Ci/ml}$ of each of radiolabeled precursors ([³H]thymidine, [³H]uridine or [³H]leucine) was added simultaneously with cyanocycline A to the culture. At designated times, 1 ml samples of each culture were taken and 4 ml of ice-cold 6.45% trichloroacetic acid added, and kept for 1 hour on ice. The insoluble materials were collected on glass fiber filters, washed twice with cold 5% trichloroacetic acid, and with once ethanol, and finally dried. The acid-insoluble radioactivity was determined in a scintillation spectrometer (see Materials and Methods).

Effect of Exogenous DNA on Antimicrobial Activity

Herring sperm DNA and yeast RNA were added at final concentrations of 250 $\mu\text{g/ml}$ in nutrient broth agar media, and 8 mm paper discs soaked with the antibiotic solution were placed on the agar plates which were inoculated with 1.0×10^7 cells/ml of *M. luteus* B. After 16-hour incubation at 37°C , the diameters of inhibition zones were measured.

Results

Anti-Meth A Activity *In Vitro*

Cyanocycline A showed significant growth inhibition of Meth A cells *in vitro* (Fig. 1). The concentration for 50% growth inhibition (IC_{50}) was 3.4 ng/ml calculated on the basis of the number of viable cells at 48 hours. Cyanocycline A shows different mode of anti-Meth A cell activity at low and high doses; at higher concentrations of the antibiotic (3~10 $\mu\text{g/ml}$) it exhibited a cytotoxic activity, and at low concentrations (0.03~1.0 $\mu\text{g/ml}$) a cytostatic activity. After 3-hour treatment with 3~10 $\mu\text{g/ml}$ of

cyanocycline A no growth of cells in culture was observed, and both condensation of chromatin and decreased staining of the nucleus and cytoplasm were visible under the microscope with denaturation of the cells. After treatment with 0.01~1.0 $\mu\text{g/ml}$ of cyanocycline A, the major pathologic change of the cells was the appearance of the nucleolus, being slightly denatured. After 24 hours, treatment with the lowest concentration of cyanocycline A (0.01 $\mu\text{g/ml}$) showed the appearance of small-multi-nuclei and the condensation of chromatin in irregular shape cells.

Cytofluorograph

The relative content of both DNA and RNA per Meth A cell treated with 10 $\mu\text{g/ml}$ of cyanocycline A was analyzed with a cytofluorograph (Fig. 2)⁹. In the control, the cytofluorograph of the relative content of both DNA and RNA showed the same pattern as the graph at zero time even after 6-hour incubation as indicated in Fig. 2-(a) and (d). The pattern of the relative content of DNA per cell treated with 10 $\mu\text{g/ml}$ of cyanocycline A for 1 hour changed to an enlarged broad peak at 2.5 (4n, corresponds to tetraploid) with a minor peak at 1.0, whereas the peaks of control cells appeared at 1.5 (2n, corresponds to diploid) (Fig. 2-(b)). After 6-hour treatment with cyanocycline A, the enlarged broad peak resolved into two sharp peaks (1.0 and 2.5 in Fig. 2-(c)). The relative content of RNA per cell treated with 10 $\mu\text{g/ml}$ of the antibiotic increased as indicated the peak at 2.0 after 1 hour and at 3.0 after 6 hours (Fig. 2-(e) and (f)), as the incubation progressed.

Effect of Cyanocycline A on DNA, RNA and Protein Biosynthesis of Meth A Cells

The effect of cyanocycline A on macromolecular synthesis of Meth A cells was studied in an attempt to elucidate the mechanism of action of this antibiotic against eucaryotes. The cells were exposed to both cyanocycline A and radio-labeled precursors of DNA, RNA or protein, and radioactivities incorporated into the corresponding fractions were measured at 120 minutes. Fig. 3 shows that cyanocycline A is an inhibitor of nucleic acid synthesis. The incorporation of [³H]uridine was inhibited by 84.6% and 96.9% at 0.1 and 1.0 $\mu\text{g/ml}$ of cyanocycline A, respectively, while the respective values for incorporation of [³H]thymidine were 48.6% and 70.8%. The synthesis of RNA was the most sensitive to this antibiotic, although the synthesis of DNA was also severely inhibited. The synthesis of protein was unaffected at a concentration of 10 $\mu\text{g/ml}$ of this antibiotic.

Fig. 1. Effect of cyanocycline A on the growth of Meth A cell *in vitro*.

Meth A cells (4×10^4 cell/ml) were inoculated into fresh medium in 1.0 ml wells, and various concentration of cyanocycline A were added simultaneously. The cells were sampled at 24 and 48 hours, and viable cells were counted as described in "Materials and Methods". Numbers in Figure indicate concentration of the antibiotic.

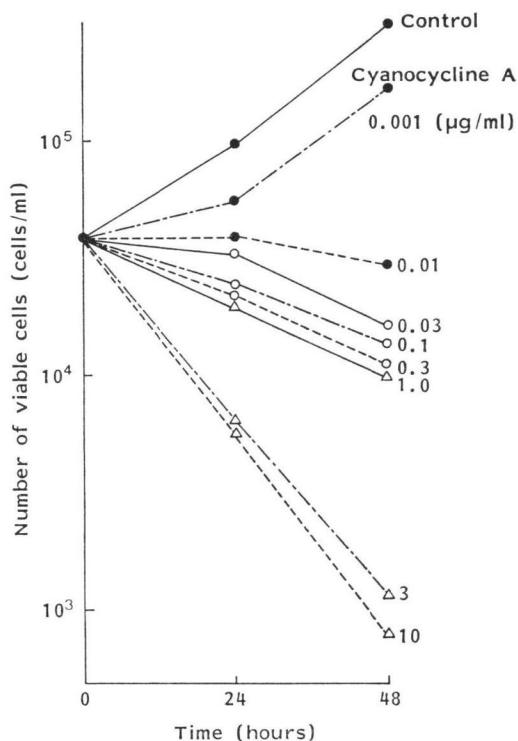
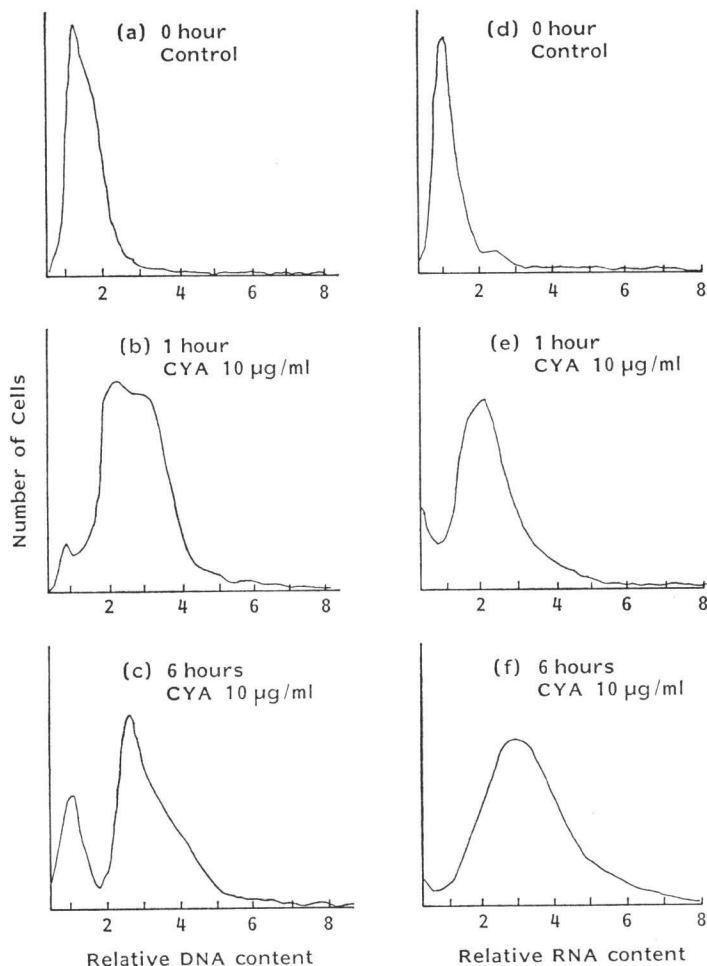


Fig. 2. Cytofluorographic analysis of action of cyanocycline A on Meth A cells.

Meth A No. 2 cells were treated with a concentration of $10 \mu\text{g/ml}$ of cyanocycline A. After 1 and 6 hours, the cells were treated with acridine orange solution as described in the text, and analyzed by cytofluorograph. X-Axis indicates amounts of fluorescence per cell (relative content of DNA and RNA), Y-axis indicates number of cells. CYA: cyanocycline A



Antitumor Activity *In Vivo*

Fig. 4 shows the effect of cyanocycline A on the life span of mice implanted with Meth A ascites tumor. All control mice died within 12 days. Cyanocycline A showed a marked activity against Meth A ascites tumor with an optimum dose of $240 \mu\text{g/kg}$. The antitumor activity of cyanocycline A was similar to that of mitomycin C ($240 \mu\text{g/kg}$).

Effect of Cyanocycline A on DNA, RNA and Protein Biosynthesis of *E. coli*

The effect of cyanocycline A on DNA, RNA and protein was examined by incorporation of radio-labeled precursors into acid-insoluble products. After 60-minute treatment, as shown in Fig. 5, incorporation of [^3H]thymidine, [^3H]uridine and [^3H]leucine was inhibited by 76, 62 and 47%, respectively, in the presence of $2 \mu\text{g/ml}$ cyanocycline A. Incorporation pattern of [^3H]thymidine was slightly different from those of [^3H]uridine and [^3H]leucine. These results indicated that cyanocycline A primarily in-

hibits DNA synthesis and has also considerable inhibitory effect on RNA and protein synthesis.

Effect of Exogenous DNA and RNA on Antibacterial Activity

Cyanocycline A is fairly active against Gram-positive and Gram-negative bacteria. In a study on incorporation of labeled precursors into macromolecules of *E. coli*, DNA synthesis was slightly more inhibited than RNA synthesis and protein synthesis (Fig. 5). The structural similarity of cyanocycline A to saframycin A and naphthyridinomycin, which were known to bind to DNA, suggested that cyanocycline A may also bind to DNA, and that its antibacterial activity might be reversed by addition of exogenous DNA. To determine the effect of exogenous DNA on the antibacterial activity of cyanocycline A, the agar plate method with *M. luteus* B as described in "Materials and Methods" was used.

The inhibition zone of paper discs soaked with 20 $\mu\text{g/ml}$ of cyanocycline A was decreased to 25.5 mm (2.6 $\mu\text{g/ml}$) from 33.8 mm by addition of 250 $\mu\text{g/ml}$ herring sperm DNA to the medium (Table 1). On the other hand, addition of yeast RNA (250 $\mu\text{g/ml}$) did not change the activity. In a control experiment with actinomycin D and adriamycin, inhibition zones were decreased similarly by addition of herring sperm DNA, and no change observed by addition of yeast RNA. These results suggest that cyanocycline A binds to DNA in a similar fashion to actinomycin D and adriamycin. In the experiments with

Fig. 3. Effect of cyanocycline A on macromolecular synthesis in Meth A cell.

Meth A No. 2 cells were treated with cyanocycline A and each radiolabeled precursors; 0.1 $\mu\text{Ci/ml}$ [^3H]thymidine, [^3H]uridine and 0.2 $\mu\text{Ci/ml}$ [^{14}C]leucine. After 120 minutes, cells were treated as described in "Materials and Methods".

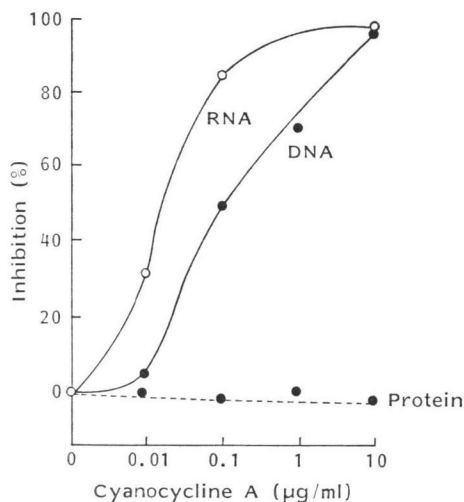


Fig. 4. Effect of cyanocycline A on the life span of mice with Meth A cell ascites tumor.

Meth A No. 2 cells (1×10^6 cells) were inoculated intraperitoneally in BALB/c mice. Arrows indicate the times of administration of cyanocycline A.

CYA: cyanocycline A, MMC: mitomycin C.

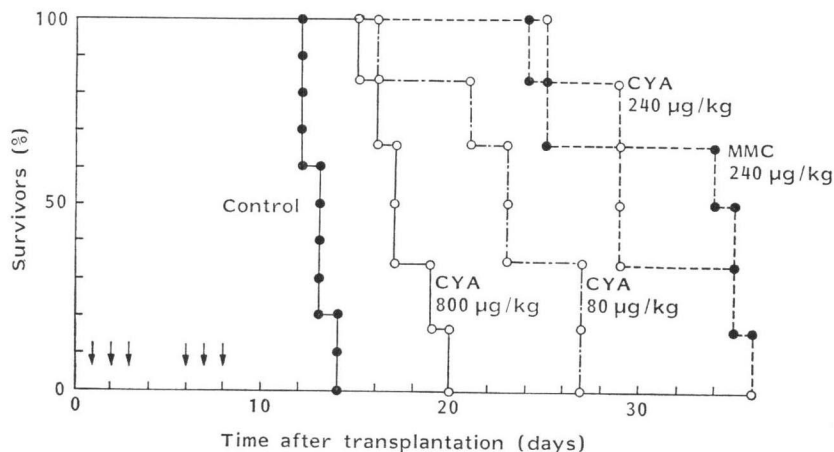


Fig. 5. Effect of cyanocycline A on macromolecular synthesis in *E. coli*.

When the cell density of *E. coli* in the nutrient broth medium (1/5 fold diluted medium) reached OD 550 nm=0.1, radiolabeled precursors (0.1 μ Ci/ml [3 H]thymidine, [3 H]uridine or [3 H]leucine) were added simultaneously with cyanocycline A, the incubation was carried out at 37°C with shaking. At the designated times, 1.0 ml samples were removed and treated as described in "Materials and Methods".

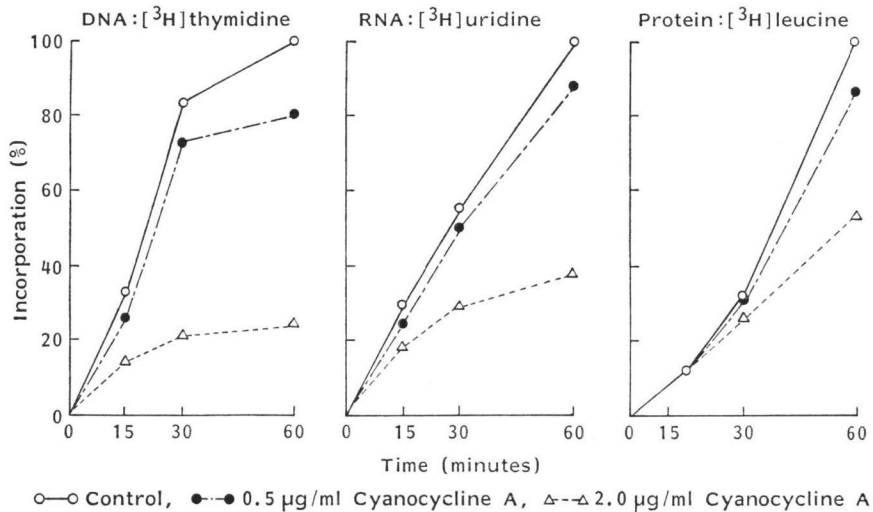


Table 1. Effect of exogenous DNA and RNA, and dithiothreitol on antimicrobial activity of cyanocycline A and related antibiotic.

| Medium* | Inhibition zone (8 mm paper disc) | | |
|---------|-----------------------------------|----------------------------------|--------------------------------|
| | Cyanocycline A (20 μ g/ml) | Actinomycin D (50 μ g/ml) | Adriamycin (500 μ g/ml) |
| Control | 33.8 mm | 22.8 mm | 21.0 mm |
| DNA | 25.5 | 13.0 | 13.0 |
| RNA | 34.0 | 23.0 | 21.5 |
| DTT | 34.5 | 24.0 | 23.0 |
| DNA+DTT | 24.0 | 14.0 | 13.3 |

* Medium, nutrient broth agar medium. DNA, 250 μ g/ml of herring sperm DNA. RNA, 250 μ g/ml of yeast RNA. DTT, 2 mM dithiothreitol.

Paper disc impregnated with the antibiotic solution was placed on agar medium plate seeded with 1×10^7 cells/ml of *M. luteus* B.

the medium which contained herring sperm DNA, 2 mM dithiothreitol had no effect on the antimicrobial activity of cyanocycline A.

Discussion

Cyanocycline A has marked activity against Meth A cells both *in vivo* and *in vitro*. *In vivo* studies, showed that the activity of cyanocycline A against Meth A ascites tumor was similar to that of mitomycin C at the same doses. On the other hand, *in vitro* studies, established the IC₅₀ value of cyanocycline A against Meth A cells as 3.4 μ g/ml (Fig. 1). From the results (Fig. 2) of cytofluorographic analysis of cyanocycline A against Meth A cells at the concentration of 10 μ g/ml, it is suggested that the cells exposed for 1 hour were almost stopped in phase G₂ of the cell cycle, and therefore, the relative content of

DNA and RNA per cell increased. After 6-hour exposure to this antibiotic, a new small peak of DNA appeared at 1.0 (Fig. 2-(c)). The peak is considered to derive from phase G_1 in the cell cycle. This suggests that a fraction of the cells which was stopped at phase G_2 may begin to escape the inhibitory activity of cyanocycline A.

As indicated in Figs. 3 and 5, cyanocycline A is a potent inhibitor of nucleic acid synthesis in both eucaryotic and procaryotic cells. The synthesis of RNA is more sensitive to this antibiotic than that of DNA in Meth A cells, and *vice versa* in *E. coli*. At the present time, there is no precise explanation for this difference. Drugs such as saframycin A and adriamycin sometimes affect DNA and RNA synthesis in a different degree depending upon the species and test system¹¹⁻¹³). Thus cyanocycline A acts as an inhibitor of both DNA and RNA biosynthesis. Taking into account the above results, a study on the binding of cyanocycline A to DNA was carried out. As a result, we found that the antimicrobial activity of cyanocycline A against *M. luteus* was diminished by DNA (herring sperm). This indicates that cyanocycline A binds DNA. The addition of cyanocycline A alone to a buffered solution of herring sperm DNA did not result in any dramatic changes in the UV and visible regions of the absorption spectrum of the antibiotic (data not shown). Furthermore, calf thymus DNA was not modified by cyanocycline A, giving a T_m of 72°C in the presence of 20 mM phosphate buffer (pH 6.9). On the basis of these results, cyanocycline A might not function as an intercalating antibiotic. It is known that the hydroxyquinones formed from saframycin A and naphthyridinomycin by addition of reducing agent, bind to DNA.^{4, 5, 10}) Leaving the cyano group from the structure of saframycin A gives the same type of α -carbinolamine structure as naphthyridinomycin, and this α -carbinolamine structure is known to be necessary for binding to DNA. The cyano group of saframycin A was easily released with 0.1 N H_2SO_4 . On the other hand, neither treatment with conc. HCl or $TiCl_3$ have any effect on the cyano group of cyanocycline A.⁹) In the study with *M. luteus*, cyanocycline A was reduced with 2 mM dithiothreitol to hydroxycyanocycline A which gave almost the same level of antimicrobial activity as cyanocycline A. In the experiments with medium contained herring sperm DNA, 2 mM dithiothreitol had no effect on the antimicrobial activity of cyanocycline A and binding of the antibiotic to DNA. Considering these facts, the binding mechanism of cyanocycline A to DNA seems to differ from that of saframycin A^{4, 9}) and naphthyridinomycin⁹).

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