NAPHTHYRIDINOMYCIN, A NEW BROAD-SPECTRUM ANTIBIOTIC

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A new antibiotic, naphthyridinomycin, was isolated in crystalline form from the culture filtrate of *Streptomyces lusitanus* AY B-1026. The antibiotic is active against a large number of both gram-positive and gram-negative bacteria, and inactive against *Candida albicans*, *Trichophyton granulosum* and *Microsporum gypseum*. The antibiotic is toxic in mice.

A new strain of streptomycete was isolated from a Easter Island (Rapa Nui) soil sample. Cultures of this microorganism were found to produce marked antibacterial activity and to bring about strong morphological changes in *Escherichia coli*. The active principle was isolated in crystalline form from the culture filtrates of the streptomycete grown in submerged, aerated liquid culture, and named naphthyridinomycin.

This paper deals with the characterization of the producing strain, the isolation and purification as well as the chemical, physical and biological properties of the antibiotic.

Characteristics of the Naphthyridinomycin-producing Streptomycete

Streptomycete strain AY B-1026 was isolated from a soil sample collected in Easter Island (Rapa Nui). The isolate was maintained on tomato paste-oatmeal agar[1] and preserved by lyophilization[2]. The methods used for characterization were those of the International Streptomyces Project (ISP) published by SHIRLING and GOTTLIEB[3]. Streptomycete strain AY B-1026 was found to be identical or very similar to *Streptomyces lusitanus* in NONOMURA's key of classification[4]; it was deposited in the ARS culture collection of USDA (Peoria, Ill.) and assigned the number NRRL 8034.

1. Morphological Characteristics

Streptomycete strain AY B-1026 sporulates abundantly on most media studied. Sporophores are rather loose, forming open loops (Retinaculi-Aperti) or open spirals (Spirae)[5] in 1 : 1 ratio. Spore chains consist of 10 conidia or more. Conidia are smooth with no sign of the presence of spines, warts or hair.

2. Cultural Characteristics

Optimum temperature for growth is about 25°C and the range is 20～35°C. On yeast extract-malt extract agar (ISP medium 2) and tomato paste-oatmeal agar[1] aerial growth is gray with tiny white spots developing at 14 days incubation at 25°C. On inorganic salts-starch agar (ISP medium 4) the grey color is lighter with small white spots. On oatmeal agar (ISP medium 3) and glycerol-asparagine agar (ISP medium 5), aerial growth is grayish white and less abundant than on other media.

Substrate growth is gray to whitish depending on the medium, grey on ISP medium 2, whitish on ISP media 3, 4 and 5. It is brown on tomato paste-oatmeal agar[1]. No soluble pigment is present on any medium.

3. Physiological Characteristics

Streptomycete AY B-1026 is hydrogen sulfide-negative on peptone-yeast extract-iron agar (ISP medium 6) and melanine-negative on tyrosine agar (ISP medium 7).

Carbohydrate utilization was studied in carbon utilization agar (ISP medium 9) supplemented with various carbohydrates. The following carbohydrates were well utilized: D-glucose, L-arabinose, sucrose, D-xylose, *i*-inositol, D-mannitol, D-fructose, rhamnose and starch. Carbohydrates not utilized were raffinose and cellulose.

Production of Naphthyridinomycin

Streptomyces lusitanus AY B-1026 was grown for 7 days at 28°C on tomato paste-oatmeal agar slants[1]. The inoculum was prepared by suspending the spores from each slant in 10 ml of sterile distilled water. The medium consisted of (g/liter): "cerelose", a pharmaceutical grade of glucose (Corn Products Corporation, New York, N.Y.), 20; Bacto-neopeptone (Difco Laboratories, Detroit, Mich.) 8; tomato paste, 10; corn meal, 8; "blackstrap" molasses, 20; sodium chloride, 3; in tap water. The medium was cooked at 121°C for 20 minutes, cooled to 60°C and adjusted to pH 7.8 with ammonium hydroxide. The medium was distributed into 500-ml Erlenmeyer flasks in quantities of 100 ml/flask; sterilization: 121°C, 20 minutes. Flasks were cooled to 28°C, inoculated with 2% of the spore inoculum and incubated for 18 to 24 hours at 28°C on a rotary shaker (2″-stroke) at 240 rev/min: this constitutes the first-stage inoculum. The second-stage inoculum was prepared in 24-liter round flasks containing 3.2 liters of the same medium and sterilized at 121°C for 1 hour. The flasks were cooled to 28°C, inoculated with 2% of first-stage inoculum and incubated at 28°C on a reciprocating shaker at 65 rev/min (4″-stroke) for 24 hours.

The production stage was carried out in 250-liter fermentors (Model F-250, New Brunswick Scientific Co., New Brunswick, N.J.) equipped with automatic antifoam addition systems and pH recorder-controller. Each fermentor was filled with 160 liters of the liquid medium described above; 160 ml of lard oil (Larex No. 1, Swift Canadian Corporation, Toronto, Ont.) were added and served as an antifoam agent during sterilization. The fermentors were sterilized at 121°C for 30 minutes, cooled to 28°C and inoculated with 3.2 liters (2%) of second-stage inoculum. Aeration was maintained at 0.5 vol air/vol medium/min, and agitation at 250 rev/min. The antifoam agent, added on demand during fermentation, was Mazu DF-M3PX (Mazer Chemical Inc., Gurner, Ill.); pH was maintained at 6.0 by automatic addition of a 25% sodium hydroxide solution. Maximum production of naphthyridinomycin generally occurred after 96 hours.

Fermentation broth activity was assayed by a cylinder-plate diffusion method using *Pseudomonas aeruginosa* or *Bacillus subtilis* as test organism. When maximum titers were observed, fermentation was terminated by addition of concentrated HCl to pH 4.0, and diatomaceous earth (5% w/v) was added. The mixture was filtered and the mycelium discarded.

Isolation and Purification of Naphthyridinomycin

The-filtrate (*ca.* 150 liters) was adjusted to pH 7.0 with 1 N ammonium hydroxide and passed through a column containing 7 liters of Amberlite IRC 50 H^+ resin at the rate of 10 liters an hour. The antibiotic was completely adsorbed onto the resin and the effluent discarded. The resin was washed extensively with deionized water until the washings were colourless. This was followed by a wash with methanol to displace the water from the column. The antibiotic was eluted from the resin with 12~15 liters of 0.2 N HCl in methanol. The eluate was decolorized with 1% activated charcoal (Darco G-60) and concentrated under reduced pressure to about 200 ml. The concentrate was mixed with 2,000 ml of methylene dichloride and the resulting upper aqueous layer discarded. The methylene dichloride extract was dried with Na_2SO_4 and concentrated to 30~50 ml under reduced pressure. From this concentrate, the crude antibiotic was precipitated with *n*-hexane. The yellowish precipitate was dried thoroughly under vacuum.

Thin-layer chromatography of the crude antibiotic indicated a mixture of active and inactive materials. Two systems were employed: TLC on Silica G (Merck) using a solvent mixture of acetone, propanol and ethylene chloride (6 : 2 : 3); and aluminum oxide (Merck, Type T) with a solvent system consisting of benzene, acetone and methanol (7 : 1.5 : 1.5).

The crude material (yellowish precipitate) was subjected to column chromatography on neutral aluminum oxide (activity V; Woelm, Germany) using a solvent system consisting of benzene and chloroform (80 : 20). Fractions were collected and samples thereof analyzed by TLC as well as by bioautography using paper discs deposited on agar plates seeded with *Bacillus subtilis*. The antibiotic-containing fractions were pooled and evaporated to a small volume under vacuum. The antibiotic crystallized spontaneously on the walls of the vessel. The compound was recrystallized from ethyl ether and dried thoroughly under high vacuum for 24 hours. From 160 liters of fermentation broth 1.6 g of pure naphthyridinomycin crystals (ref. AY-23,649) was routinely obtained.

Physical and Chemical Properties of Naphthyridinomycin

Naphthyridinomycin is a ruby red crystalline substance, m.p. 108~110°C (decomposition). It is soluble in water, methanol, acetone, chloroform, methylene dichloride, ethyl acetate and ether; insoluble in hexane.

Fig. 1. Infrared spectrum of naphthyridinomycin (in chloroform)

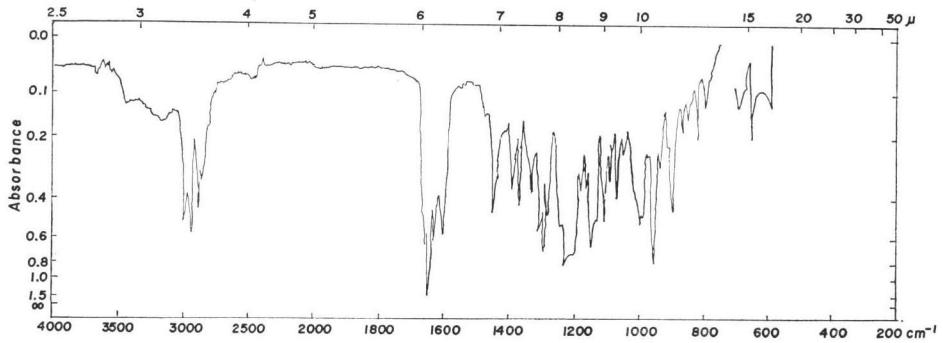
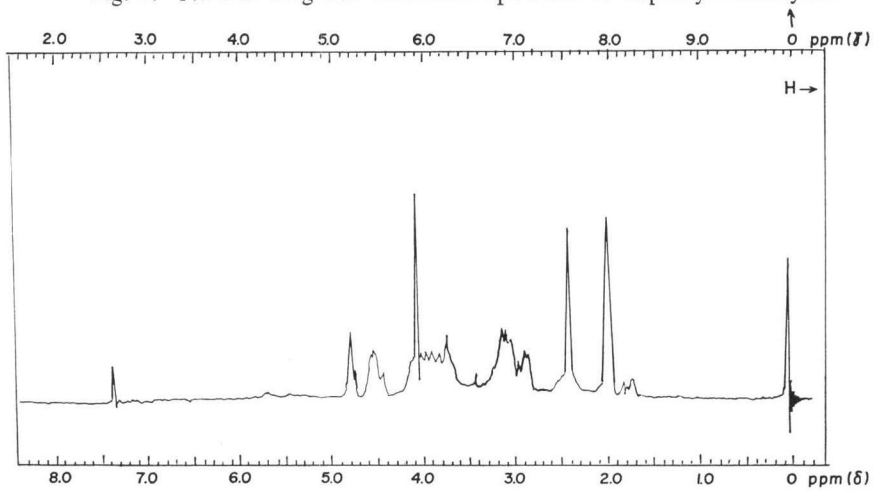


Fig. 2. Nuclear magnetic resonance spectrum of naphthyridinomycin

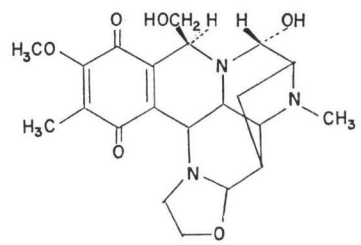


Naphthyridinomycin analysed for $C_{21}H_{27}N_3O_6$ (M.W. 417.47):

Calcd. for: C 60.42, H 6.52, N 10.07.

Found: C 60.29, H 6.52, N 9.93.

Fig. 3. Structure of naphthyridinomycin



Naphthyridinomycin

Table 1. Antibacterial activity of naphthyridinomycin

Bacteria	Minimum inhibitory concentration (μg/ml)
Staphylococcus aureus pen^S	<0.025
Staphylococcus aureus pen^R	<0.025
Streptococcus faecalis	<0.025
Escherichia coli	0.8
Enterobacter aerogenes	0.2
Salmonella pullorum	0.2
Pseudomonas aeruginosa	0.2
Pseudomonas fluorescens	1.6
Proteus mirabilis	0.4
Proteus vulgaris	0.4
Klebsiella pneumoniae	0.05
Serratia marcescens	0.05

Its specific rotation is $[\alpha]_D^{25}+69.4°$ (c 1, $CHCl_3$). The infrared spectrum (in chloroform) shows characteristic absorption bands at 3000, 2940, 2880, 2845, 1715, 1690, 1650, 1604 and 1495 cm^{-1} (Fig. 1). The nuclear magnetic resonance spectrum of naphthyridinomycin is shown in Fig. 2.

The ultraviolet spectrum shows a characteristic absorption maximum at 270 nm, $E_{1cm}^{1\%}$ 248.5 (methanol).

Structure elucidation of naphthyridinomycin has been carried out by X-ray crystallography and was published by SYGUSCH *et al.*[6] Structure is shown in Fig. 3.

Biological Activity of Naphthyridinomycin

The minimum inhibitory concentration (M.I.C.) of naphthyridinomycin was determined by the broth-dilution method against selected strains of gram-positive and gram-negative bacteria. The results are given in Table 1. Naphthyridinomycin did not exhibit any significant activity against the pathogenic yeast *Candida albicans*, and the dermatophytes *Trichophyton granulosum* and *Microsporum*

gypseum.

The intraperitoneal injection of an aqueous solution of naphthyridinomycin (3.125 mg/kg) killed mice in 24 to 48 hours.

Mechanism of Action of Naphthyridinomycin

The mechanism of action of naphthyridinomycin was studied in *Escherichia coli* 15T$^-$ and *E. coli* ATCC 10536. Minimum inhibitory concentration of naphthyridinomycin for these cultures, as determined by the broth-dilution method, was 0.32 μg/ml; growth (determined by turbidity measurement) of strains 15T$^-$ and ATCC 10536 was significantly inhibited by 0.1$\sim$0.5 μg/ml. Exposure of exponentially growing *E. coli* cells to low levels of the antibiotic induced elongation of cells; the elongated cells were osmotically stable.

Respiration of *E. coli* with glucose as the substrate was stimulated by naphthyridinomycin (0.25 and 0.5 μg/ml) in the initial 2 hours of incubation; on longer incubation some inhibition was observed.

At low levels that effected considerable inhibition of growth and macromolecular synthesis, naphthyridinomycin did not cause any leakage of intracellular materials. However, at higher concentrations (1$\sim$2 μg/ml) some leakage of UV-absorbing materials was obtained, indicating cell membrane damage.

Naphthyridinomycin inhibited incorporation by *E. coli* 15T$^-$ of ^{14}C-thymidine and ^{3}H-leucine into DNA and protein respectively, but had no effect on incorporation of ^{14}C-lysine into cell wall fraction. Incorporation of ^{14}C-uracil into RNA was inhibited only at relatively higher concentrations. The primary effect of naphthyridinomycin on *E. coli* appears to be on the synthesis of DNA which is strongly inhibited within a few minutes of exposure to low concentrations (0.1$\sim$0.5 μg/ml) of the antibiotic. In its effect on DNA synthesis, the activity of naphthyridinomycin is similar to that of nalidixic acid[7,8] and mitomycin C[9]. However, unlike mitomycin and like nalidixic acid[10], naphthyridinomycin does not cause degradation of intracellular DNA.

A detailed account of the mechanism of action of naphthyridinomycin will be presented in a subsequent communication.

References

1) Pridham, T. G.; P. Anderson, C. Foley, L. A. Lindenfelser, C. W. Hesseltine & R. G. Benedict: A selection of media for maintenance and taxonomic study of *Streptomyces*. Antibiotics Annual 1956/1957: 947$\sim$953, 1957
2) Raper, K. B. & D. F. Alexander: Preservation of molds by the lyophil process. Mycologia 37: 499$\sim$525, 1945
3) Shirling, E. B. & D. Gottlieb: Methods for characterization of *Streptomyces* species. Internat. J. Syst. Bact. 16: 313$\sim$340, 1966
4) Nonomura, H.: Key for classification and identification of 458 species of the streptomycetes included in ISP. Ferm.Technol. 52: 78$\sim$92, 1974
5) Pridham, T. G.; C. W. Hesseltine & R. G. Benedict: A guide for the classification of streptomycetes according to selected groups. Appl. Microbiol. 6: 52$\sim$79, 1958
6) Sygusch, J.; F. Brisse, S. Hanessian & D. Kluepfel: The molecular structure of naphthyridinomycin, a broad-spectrum antibiotic. Tetrahedron Letters 1974-46: 4021$\sim$4023, 1974
7) Goss, W. A.; W. H. Deitz & T. M. Cook: Action of nalidixic acid on *Escherichia coli*. J. Bact. 88: 1112$\sim$1118, 1964

8) Goss, W. A.; W. H. Deitz & T. M. Cook: Mechanism of action of nalidixic acid on *Escherichia coli*. J. Bact. 89: 1068~1074, 1965
9) Szybalski, W. & V. N. Iyer: Cross linking of DNA by enzymatically or chemically activated mitomycins and porfiromycins, bifunctionally "alkylating" antibiotics. Federation Proc. 23: 946~957, 1964
10) Winshell, E. B. & H. S. Rosenkranz: Nalidixic acid and metabolism of *Escherichia coli*. J Bact. 104: 1168~1175, 1970