CYCLOTHIALIDINE, A NOVEL DNA GYRASE INHIBITOR

I. SCREENING, TAXONOMY, FERMENTATION AND BIOLOGICAL ACTIVITY

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(Received for publication June 30, 1993)

Streptomyces filipinensis NR 0484 produced a new DNA gyrase inhibitor, cyclothialidine[1~3].
It showed potent activity against DNA gyrases from *Escherichia coli* and *Micrococcus luteus*.

DNA gyrase belongs to the topoisomerase II group. Although both prokaryotic and eukaryotic cells have topoisomerase II, DNA gyrase is unique in showing characteristics different from those of other topoisomerases as it exists in bacteria only. It can catalyze both superhelix formation and supercoiled DNA relaxation, whereas mammalian topoisomerases can only relax supercoiled DNA. The enzyme has proved to be essential for bacterial growth. These characteristics provide the basis for the ability of DNA gyrase inhibitors to exert antibacterial activity with selective toxicity. It has been long known that DNA gyrase is the target of two classes of antibiotics: the synthetic quinolones, and the natural coumarin-type compounds such as novobiocin and coumermycin. Recently, three antibacterials, cinodine[4], microcin[5] and clerocidin[6], which fall outside the quinolone and coumarin classes, have been reported to have DNA gyrase as their target. Although synthetic quinolones are commercially successful antibacterials, work still needs to be done to reduce their toxicity. Natural novobiocin derivatives are also toxic. These problems led us to initiate microbial broth screening for DNA gyrase inhibitors, aiming at identification of new lead compounds. We have now identified a novel DNA gyrase inhibitor, cyclothialidine, produced by *Streptomyces filipinensis* NR 0484. In this report, we describe the screening procedures, taxonomy of the producing strain, fermentation, and biological activity. We also describe the isolation, characterization and structural elucidation of cyclothialidine in the accompanying paper[7].

Results and Discussion

Screening

We screened about 20,000 microbial culture broths, including actinomycetes, bacteria, and fungi, by performing the DNA supercoiling assay for determination of the DNA gyrase activity. DNA gyrase was partially purified from *Escherichia coli* D110 (thy^-, $endI^-$) by novobiocin-sepharose column chromatography. All microbial broths were filtered through Molcut TGC (molecular mass cut-off at 10,000 daltons, Millipore Ltd.). Then, 5 μl of each filtrate was added to 15 μl of the reaction mixture. That contained 50 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, 5 mM spermidine·HCl, 50 μg of bovine serum albumin per ml, 125 μg of *E. coli* *t*RNA per ml, 2 mM DTT, 5 mM ATP, 0.5 μg of relaxed ColE1 DNA, and DNA gyrase. The mixture was incubated for 30 minutes at 30°C. The production of supercoiled ColE1 DNA was monitored by agarose gel electrophoresis. The IC_{50} was defined as the inhibitory concentration at

which the thickness of the DNA supercoiling band was half that of the control.

Taxonomy

Strain NR 0484 was isolated from a sandy soil sample (collected at Ohse-machi, Hamamatsu, Shizuoka Prefecture, Japan, in 1985) by spreading it directly onto thin potato-carrot agar medium supplemented with 10% grated radish. Taxonomic studies were carried out according to the procedures of the International Streptomyces Project[8].

The aerial mycelium formed spiral chains of spores with more than 50 spores per chain. The spores were oval to cylindrical ($0.6 \sim 0.9 \times 0.9 \sim 1.2\,\mu m$), and their surface was spiny (Fig. 1). Sclerotic granules, sporangia, and flagellated spores were not observed. Fragmentation of the vegetative mycelium was not observed on any of the agar media tested. The cultural characteristics of strain NR 0484 grown on various agar media are summarized in Table 1. All cultures were incubated at 27°C for 15 days. Media ISP 2, 3, 4, 5, and 7 supported the best growth and abundant sporulation. The aerial mass color was gray. Pigmentation of the substrate mycelia was not distinctive. No soluble pigment was observed.

The physiological characteristics and the utilization of carbohydrates are shown in Tables 2 and 3, respectively. Starch hydrolysis, milk peptonization, and melanin production were positive. Gelatin liquefaction, milk coagulation, and nitrate reduction were negative. Growth was observed on 4% NaCl but not on 10%. The strain was sensitive to streptomycin at $6.3\,\mu g/ml$. The temperature range for growth was $20 \sim 45$°C.

Whole cell hydrolysates of strain NR 0484 contained L,L-diaminopimelic acid. Glycine was detected in the cell wall.

Based on the taxonomic characteristics described above, we assigned strain NR 0484 to the

Fig. 1. Electron micrograph of strain NR 0484. (Oatmeal agar).

The bar represents $1.0\,\mu m$.

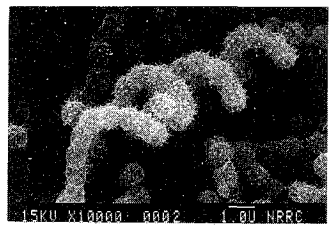


Table 1. Cultural characteristics of strain NR 0484.

Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Yeast extract - malt extract agar (ISP 2)	Good, colorless	Good, ashes (5fe)	Clove brown (3ni)	None
Oatmeal agar (ISP 3)	Good, yellow maple (3ng)	Good, ashes (5fe)	Beaver (3li)	None
Inorganic salts - starch agar (ISP 4)	Good, yellow maple (3ng)	Good, ashes (5fe)	Adobe brown (3lg)	None
Glycerol - asparagine agar (ISP 5)	Good, beaver (3li)	Good, ashes (5fe)	Beaver (3li)	None
Tyrosine agar (ISP 7)	Good, clove brown (3ni)	Good, ashes (5fe)	Clove brown (3ni)	None
Glucose - asparagine agar	Moderate, amber (3lc)	Thin, pussywillow gray (5dc)	Amber (3lc)	None
Sucrose - nitrate agar	Moderate, colorless	Thin, beige (3ge)	Beige (3ge)	None
Nutrient agar	Moderate, colorless	Thin, alabaster tint (13ba)	Clove brown (3ni)	None

The color names used in this table were based on the Color Harmony Manual (Container Corporation of America, Chicago).

Table 2. Physiological characteristics of strain NR 0484 and *Streptomyces filipinensis* IFO 12860.

	NR 0484	*S. filipinensis* IFO 12860
Gelatin liquefaction	−	−
Starch hydrolysis	+	+
Milk coagulation	−	−
Milk peptonization	+	+
Nitrate reduction	−	−
Melanin production		
ISP medium No. 1	+	+
ISP medium No. 6	+	+
ISP medium No. 7	−	−
NaCl tolerance	≥4%, but <10%	≥4%, but <10%
Streptomycin sensitivity (μg/ml)	6.3	6.3
Temperature range for growth	20~45°C	20~45°C
Optimum temperature for growth	30~37°C	30~37°C
Production of DNA gyrase inhibitor	+	−

+, Positive; −, negative.

genus *Streptomyces*. Among the known species of this genus, strain NR 0484 showed the closest resemblance to *Streptomyces filipinensis* Ammann, Gottlieb, Brock, Carter and Whitfield[9]. Therefore, the microbiological characteristics of strain NR 0484 were directly compared with those of *S. filipinensis* IFO 12860. Both strains had similar physiological characteristics and similarly utilized carbohydrates as shown in Tables 2 and 3, but *S. filipinensis* IFO 12860 did not produce the DNA gyrase inhibitor. In spite of this minor difference, strain NR 0484 and strain IFO 12860 showed numerous similarities in other morphological and cultural characteristics. Therefore, we concluded that strain NR 0484 could be assigned as a strain of *S. filipinensis* and designated it as *S. filipinensis* NR 0484. This strain was deposited on July 27, 1988, at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ibaraki Prefecture, Japan, under the Budapest Treaty, and was added to its collection of microorganisms as FERM BR-1982.

Table 3. Carbohydrate utilization by strain NR 0484 and *Streptomyces filipinensis* IFO 12860.

	NR 0484	*S. filipinensis* IFO 12860
L-Arabinose	+	+
D-Fructose	+	+
D-Glucose	+	+
Inositol	±	+
D-Mannitol	+	+
Raffinose	±	+
L-Rhamnose	±	±
Sucrose	±	+
D-Xylose	+	+

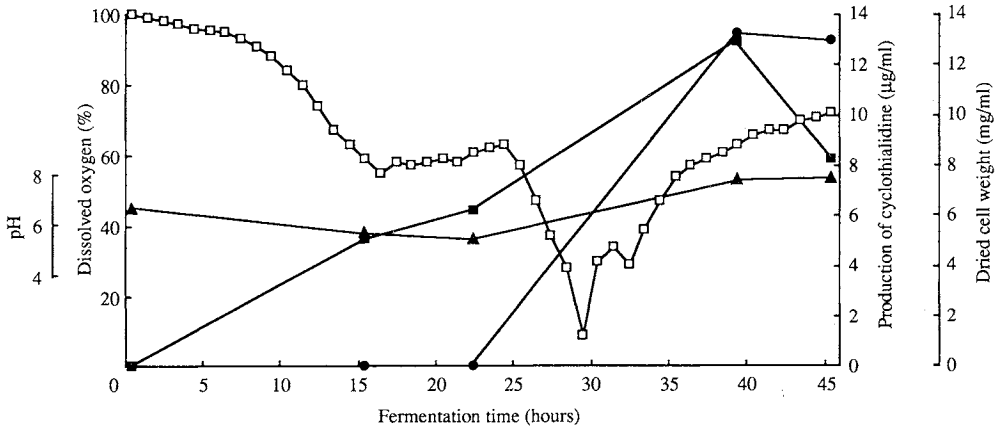
+, Utilization; ±, probable utilization.

Yield Improvement

It is well known that strain and medium improvement trials can increase the production of active substance(s) or decrease the production of undesired co-product(s). Since the original strain productivity was not enough for isolation and its co-products caused DNA breakage in the assay system, we conducted a series of trials for single colony isolation and medium improvement. Glucose was the best carbon source, and yeast extract supplemented with Toast soya was the best nitrogen source. NaCl was necessary for the production of cyclothialidine, whereas $CaCO_3$ caused co-products to be made. We finally obtained a strain which showed a 2-fold greater cyclothialidine productivity than that of the wild-type strain and it did not produce DNA breakage compounds in the new medium, namely A24.

Fig. 2. Time course of cyclothialidine production in a 200-liter fermentor.

□ Dissolved oxygen, ▲ pH, ■ dried cell weight, ● production of cyclothialidine.



Fermentation

The seed and fermentation medium, A24, used in the production of cyclothialidine contained the following: 2% glucose, 0.5% yeast extract, 2% Toast soya, 0.25% NaCl, 0.005% $ZnSO_4 \cdot 7H_2O$, 0.0005% $CuSO_4 \cdot 5H_2O$ and 0.0005% $MnCl_2 \cdot 4H_2O$ (adjusted to pH 7.0 before sterilization). An antifoaming agent (Nissan Disfoam CA-115) was added when necessary.

Fermentation was carried out in three stages. In the first stage, vegetative mycelia were prepared in a shake flask; a 500-ml baffled Erlenmeyer flask containing 100 ml of the seed medium was inoculated with a chunk of the agar containing spores and mycelia from the sporulated culture slant and incubated on a rotary shaker at 27°C for 3 days at 190 rpm. The second stage inoculum was also prepared in shake flasks; 500-ml baffled Erlenmeyer flasks containing 100 ml of the same medium were inoculated with 2% of the first stage culture and incubated on a rotary shaker under the same conditions as described above. For the third stage, production was carried out in a 200-liter fermentor containing 140 liters of the same medium which was inoculated with 2% of the second stage culture.

Jar fermentation was carried out at 27°C with an agitation rate of 250 rpm, air flow rate of 140 liters/minute and internal pressure of 0.5 kg/cm^2. The production of cyclothialidine reached maximum (13 μg/ml) at around 40 hours. A typical time course for cyclothialidine production in a 200-liter jar fermentor is shown in Fig. 2.

Biological Activity

In the DNA supercoiling assay by *E. coli* DNA gyrase, cyclothialidine was found to be the most potent inhibitor of all DNA gyrase inhibitors tested, with IC_{50}s (μg/ml) of 0.03 (cyclothialidine), 0.06 (novobiocin), and 0.66 (norfloxacin). Another DNA gyrase from *Micrococcus luteus* was also inhibited as strongly as DNA gyrase from *E. coli* with IC_{50} of 0.005 μg/ml.

Cyclothialidine exhibited antibacterial activity against *Eubacterium* among bacterial species tested. In the paper disk (8 mm) assay, 40 μg/disk of cyclothialidine displayed an inhibitory zone of a diameter of 17.5 mm against *Eubacterium moniliforme*. *E. coli*, *Bacillus subtilis*, and *Staphylococcus aureus* were not susceptible to this compound.

Cyclothialidine neither showed the inhibitory activity against calf thymus topoisomerase II up to 1,000 μg/ml, nor did it show the cytotoxicity against HeLa cells up to 800 μg/ml. Otherwise, cyclothialidine did not interact with DNA in the absence of DNA gyrase.

Therefore, it is concluded that cyclothialidine is a potent and selective inhibitor of DNA gyrase having a novel structure, but, its permeability to most bacterial cells appears to be poor. Detailed biological activity will be reported elsewhere[10].

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