

Heliquinomycin, a New Inhibitor of DNA Helicase, Produced by *Streptomyces* sp. MJ929-SF2

I. Taxonomy, Production, Isolation, Physico-chemical Properties and Biological Activities

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(Received for publication February 20, 1996)

Heliquinomycin was isolated as a part of a program designed to find inhibitors of DNA helicase from microbial sources. It was purified from the culture broth of *Streptomyces* sp. MJ929-SF2 by solvent extraction and serial chromatographies of centrifugal partition chromatography, Sephadex LH-20 and Capcell Pak C18 (HPLC). The isolated red powder was analyzed to have the molecular formula of $C_{33}H_{30}O_{17}$. It inhibited partially purified DNA helicase from HeLa cell in a non-competitive manner with the inhibition constant (K_i) of 6.8 mM.

Heliquinomycin exhibited biological activity against microorganisms including MRSA, and cultured cell lines.

DNA helicase is essential in the processes of DNA replication, repair, recombination and transcription by unwinding of double-stranded DNA to its reactive single strand form. This is an energy requiring process driven by the hydrolysis of deoxynucleoside 5'-triphosphate¹⁾. All known DNA helicases have been found to process intrinsic DNA-dependent ATPase activity^{2~4)}.

DNA helicase was discovered for the first time in 1976 in *E. coli*⁵⁾. Most of the DNA helicases need ssDNA adjacent to the duplex region to be unwound, with the notable exception of the SV40 T antigen⁶⁾, *E. coli* helicase⁷⁾, and *E. coli* Rec⁸⁾ which are also able to unwind fully duplex linear DNA.

In the course of screening for inhibitors of DNA helicase, we discovered a novel compound named heli-quinomycin from the culture broth of *Streptomyces* sp. MJ929-SF2. In this communication, we report the taxonomy of the producing culture as well as the fermentation, production, isolation, physico-chemical properties and biological activities of heli-quinomycin. Determination of heli-quinomycin structure will be described in the following paper⁹⁾.

Materials and Methods

Microorganism

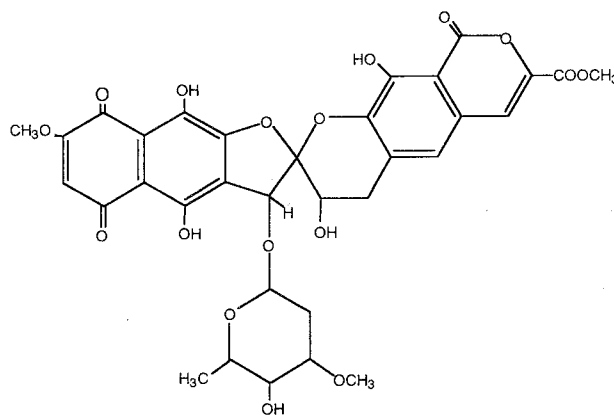
The heli-quinomycin producing organism, strain MJ929-SF2 was isolated from a soil sample collected at

Hachiohji, Tokyo, Japan and has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan under the accession number FERM P-14380.

Partial Purification of Human DNA Helicase

DNA helicase was prepared from HeLa cells. The logarithmically growing cells were collected and stocked at -80°C . The frozen cells were thawed on ice, and a nuclear extract fraction was prepared as described by DIGNAM *et al.*¹⁰⁾. Briefly, the nuclear extract protein was precipitated by slow addition of ammonium sulfate (0.35 g/ml) with constant stirring. The mixture was stirred for an additional hour and the precipitate was

Fig. 1. Structure of heli-quinomycin.



collected by centrifugation. The pellet was resuspended and dialyzed in buffer A (1 mM PMSF; 1 mM sodium bisulfite; 20 mM HEPES, pH 8.0; 0.1 M NaCl; 1 mM DTT; 1 mM EDTA and 20% glycerol). The fraction was passed through a Bio-Rex 70 column equilibrated with buffer A. The active fractions were precipitated with ammonium sulfate (0.35 g/ml), again, as described above. The protein pellet was resuspended and dialyzed in buffer B (1 mM PMSF; 1 mM sodium bisulfite; 50 mM Tris-HCl, pH 8.0; 50 mM KCl; 1 mM DTT; 1 mM EDTA and 10% glycerol) and stored at -4°C .

DNA Helicase Assay

DNA helicase activity was measured by the means of the release of $\alpha^{32}\text{P}$ -labelled DNA fragment (d(GTAAA-ACGACGGCCAGT)) from annealed circular M13mp18 DNA molecule. The reaction mixture (10 μl) containing 20 mM Tris-HCl (pH 7.5); 8 mM DTT; 2 mM MgCl_2 ; 2 mM ATP; 10 mM KCl; 4% (wt/vol) sucrose; 80 $\mu\text{g/ml}$ BSA; ^{32}P -labelled helicase substrate (1000 cpm) and the helicase fraction, was incubated at 37°C for 20 minutes. The reaction was terminated by the addition of 0.06% SDS, 2 mM EDTA, 1% glycerol and 0.02 mg/ml bromophenol blue at the final concentration. After further incubation at 37°C for 5 minutes the substrate and product were separated by electrophoresis on a 12% nondenaturing polyacrylamid gel. The gel was dried under vacuum and exposed to Hyperfilm with an intensifying screen for autoradiography. The IC_{50} value is the concentration of inhibitor at 50% inhibition of enzyme activity.

Fermentation

The strain, MJ929-SF2, was inoculated into 110 ml of seed culture medium consisting of galactose 2.0%; dextrin 2.0%; Bacto Soytone 1.0%; corn steep liquor 0.5%; $(\text{NH}_4)_2\text{SO}_4$ 0.2% and CaCO_3 0.2% (pH 7.4) in a 500-ml Erlenmeyer flask, and cultured by incubating at 27°C for 4 days on a rotary shaker (180 rpm). Two ml of this seed culture were inoculated into 110 ml of the same medium in a 500-ml Erlenmeyer flask and incubated at 27°C for 4 days on a rotary shaker (180 rpm).

Isolation

The culture broth was separated into mycelial cake and culture filtrate by filtration. The mycelial cake was extracted with methanol. The extract was filtered and concentrated *in vacuo* to an aqueous solution. The solution was combined with the culture filtrate and extracted with an equal volume of butyl acetate. The active extract was concentrated to dryness under reduced pressure. The dried material was chromatographed on a silica gel column ($\times 50$ w/w powder) with a linear gradient 0 to 100% aqueous MeOH. The active fractions were collected and evaporated to give a red powder. The powder was subjected to centrifugal partition chromatography using CPC-L.L.N model NMF (Sanki Engineering Limited) with a solvent system of CHCl_3 - MeOH -

H_2O (5:6:4). The upper phase of the solvent was introduced by ascending method. By changing of the flow direction (descending method), the active fractions were eluted with the lower phase of the solvent system to give a red powder. The powder was applied onto Sephadex LH-20 (1 liter) and eluted with solvent mixture of CHCl_3 - MeOH (1:1). The active fractions were concentrated under reduced pressure to give a red powder. The crude powder was purified by reverse phase HPLC using Capcell Pak C18 column (i.d. 2.0×25 cm) with a solvent mixture of CH_3CN - 50 mM KH_2PO_4 (pH 2.5) running at the flow rate of 8 ml/minute. The active fraction was extracted with an equal volume of butyl acetate and evaporated to dryness to obtain pure heliquinomycin.

Physico-chemical Properties

UV absorption spectra were measured with a Hitachi U-3210 spectrophotometer. IR absorption spectra were obtained using a Hitachi I-5020 FT-IR spectrometer. Mass spectra were obtained with a JEOL JMS-SX 102 spectrometer. NMR spectra were recorded on a JEOL JNM-GX500 spectrometer. MP was determined on a Yanagimoto micro melting point apparatus. Optical rotation was measured with a Perkin-Elmer 241 polarimeter.

Antimicrobial Activity

The minimum inhibitory concentrations (MIC) of heliquinomycin were examined by serial agar dilution method using Mueller Hinton agar (Difco) for an antibacterial test which was incubated at 37°C for 18 hours¹¹⁾.

Antitumor Activity

Tumor cells were incubated in 96-well plate for 24 hours prior to the addition of heliquinomycin into culture well at varied concentrations. After 2 to 3 days incubation at 37°C , MTT reagent was added and further incubated for 4 hours. Growth inhibition activity was determined according to the standard MTT assay method¹²⁾ and IC_{50} was calculated.

Fig. 2. Scanning electron micrograph of strain MJ929-SF2.

Bar represents 1.76 μm .

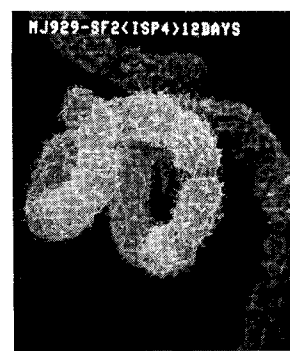


Table 1. Cultural characteristics of strain MJ929-SF2.

Medium	Aerial mycelium	Growth	Soluble pigment
Sucrose nitrate agar	White	Bright brown [4ge, Rose beige]	None
Glucose-asparagine agar	Thin, natural	Red brown [6pi, Brown mahogany]	Faint, brownish
Glycerol-nitrate agar	None	Grayish red purple [8ng, Old wing]	Faint, purple
Starch agar	None	Brownish purple [8pg, Claret wing]	Faint, purple
Calcium malate agar	None	Colorless	None
Nutrient agar	None	Yellowish brown [3ni, Clove brown]	Brownish
Yeast extract-malt extract agar (ISP-2)	Silver gray	Yellowish brown [3ni, Clove brown]	Brownish
Oatmeal agar (ISP-3)	Covert gray	Pale brown [5 l g, Cocoa brown]	Purple
Inorganic salts-starch agar (ISP-4)	Dk Covert gray	Dark brownish purple [8pl, Buegundy]	Purple
Glycerol asparagine agar (ISP-5)	Ashes	Brownish purple [7nl-7pl, Dk rose Brown to burgundy]	Brownish
Tyrosine agar (ISP-7)	Dk covert gray	Red purple [5po, Chocolate brown]	Brownish

Cultural characteristic were determined by the methods of SHIRLING and GOTTLIEB¹⁴⁾ and WAKSMAN¹⁵⁾.

Observation after incubation at 27°C for 21 days.

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

Table 2. Physiological properties of strain MJ929-SF2.

Temperature range for growth (°C)	10~37
Optimum temperature (°C)	27~37
Formation of melanoid	+
Liquefaction of gelatin (20°C)	+
Liquefaction of glucose peptone gelatin (27°C)	+
Coagulation of milk (37°C)	+
Peptonization of milk (37°C)	+
Hydrolysis of starch	-
Reduction of nitrate	+
Decomposition of cellulose	-
Solution of calcium malate	-

Physiological characteristics determined by the methods of WAKSMAN¹⁵⁾.

+: Utilized.

-: Not utilized.

Table 3. Utilization of carbon sources of strain MJ929-SF2.

L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
Rhamnose	+
Sucrose	+
Raffinose	+
Lactose	+
Inositol	-
D-Mannitol	-

Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB¹⁶⁾.

+: Utilized

-: Not utilized.

Results and Discussion

Taxonomy of the Producing Strain

Strain MJ929-SF2 produced branched substrate mycelia developing aerial hyphae which usually extended straightforward and had spiral spore chains with more than 50 spores measuring $0.93 \sim 0.99 \times 1.43 \mu\text{m}$. The surface of conidia was spiny. Its shown in Fig. 2. The cultural characteristics of the strain MJ929-SF2 are summarized in Table 1. The color of vegetative growth was chocolate brown to burgundy. Soluble pigment color was brownish to purple. Melanoid pigments were

produced. The physiological properties of the strain MJ929-SF2 are summarized in Table 2. Utilization of carbon source by this strain is presented in Table 3. Analysis of the whole-cell hydrolysate of the strain indicated the presence of LL-diaminopimelic acid.

On the basis of the characteristics described above, the strain MJ929-SF2 is assessed to belong to the genus *Streptomyces*.

Production and Isolation of Heliquinomycin

The strain of MJ929-SF2 was cultured and incubated in Erlenmeyer flasks at 27°C for 4 days on a rotary shaker. The time course of the production is shown in

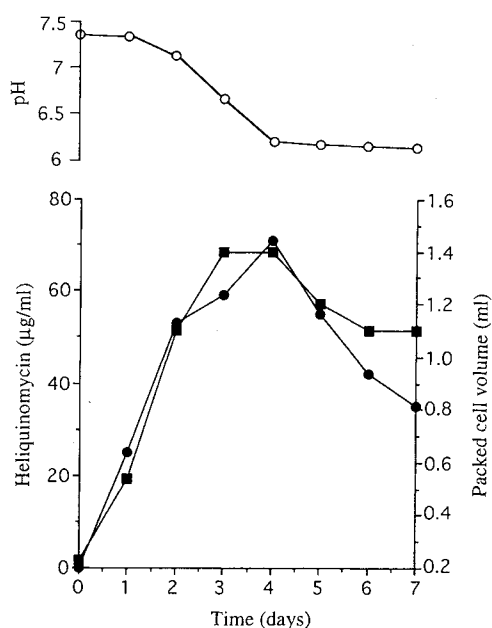
Fig. 3. The maximum production of heliquinomycin in the flask was obtained 4 days, thereafter the production slowly decreased with a pH change to acid. The flow diagram for the isolation is shown in Fig. 4. The yield of pure heliquinomycin was 129 mg from 5 liters of culture filtrate.

Physico-chemical Properties of Heliquinomycin

The physico-chemical properties of heliquinomycin are summarized in Table 4. The molecular formula of heliquinomycin was determined to be $C_{33}H_{30}O_{17}$ by HRFAB-MS. Heliquinomycin is soluble in DMSO, MeOH, $CHCl_3$ and EtOAc, but insoluble in H_2O ,

Fig. 3. Time course of heliquinomycin production.

● Heliquinomycin, ■ packed cell volume, ○ pH.



Mycelial growth was expressed as packed cell volume obtained after centrifugation of 10 ml of the culture broth at 2,000 rpm for 10 minutes.

1-propanol and *n*-hexane. The UV spectrum of heliquinomycin were similar to those of griseorhodins¹³).

Biological Activities of Heliquinomycin

In order to determine the inhibition kinetics to DNA helicase by heliquinomycin, the concentrations of enzyme substrate and heliquinomycin were varied in the reaction mixture. As shown in Fig. 5, inhibition of heliquinomycin against DNA helicase was in a noncompetitive manner. Its K_i value was determined to be $6.8 \mu M$.

The antimicrobial activities of heliquinomycin were also examined and the results are shown in Table 5. Heliquinomycin showed strong inhibitory activity against Gram-positive bacteria strains including methicillin-resistant strains, but not against Gram-negative organisms or fungi.

The antitumor activity of heliquinomycin against tumor cell lines listed in Table 6 was examined. Heliquinomycin exhibited the growth inhibitory effect on several tumor cell lines. The concentrations inhibiting

Fig. 4. Isolation of heliquinomycin.

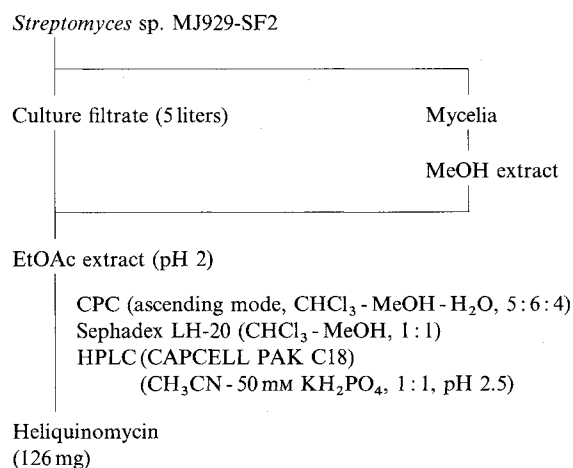


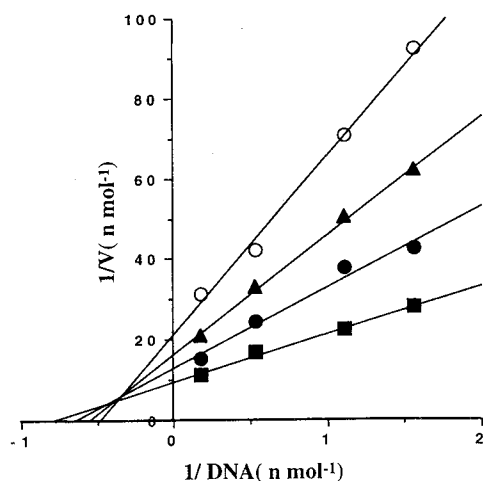
Table 4. Physico-chemical properties of heliquinomycin.

Appearance	Red powder
Molecular formula	$C_{33}H_{30}O_{17}$
FAB-MS (m/z)	721 ($M + Na$) ⁺
HRFAB-MS (m/z)	
Calcd:	698.1483 (as $C_{33}H_{30}O_{17}$)
Found:	698.1486 (M^-)
UV λ_{max}^{MeOH} nm (log ϵ)	231 (4.71), 312 (4.30), 336 (4.06), 507 (3.86), 523 (3.84), 540 (3.87)
$\lambda_{max}^{MeOH-HCl}$ nm (log ϵ)	230 (4.15), 312 (4.09), 336 (1.03), 492 (3.83)
$\lambda_{max}^{MeOH-NaOH}$ nm (log ϵ)	224 (4.78), 322 (4.10), 379 (4.06), 573 (4.13)
IR ν_{max}^{KBr} cm^{-1}	3440 (br), 1720, 1618, 1458, 1250
MP (dec)	213~214°C
Rf value on TLC*	0.71 ($CHCl_3$ - MeOH, 10:1, silica gel)
Solubility	Soluble: DMSO, MeOH, $CHCl_3$, EtOAc Insoluble: H_2O , 1-propanol, <i>n</i> -hexane

* Silica gel TLC (Merck Art. 5715): $CHCl_3$ - MeOH (10:1).

Fig. 5. Lineweaver-burk plot inhibition of DNA helicase by heliquinomycin.

○ 13 µg/ml, ▲ 6.5 µg/ml, ● 3.25 µg/ml, ■ 0 µg/ml.



Inhibition of DNA helicase activity by heliquinomycin at various concentration was measured where the amount of enzyme substrate of ³²P-labeled annealing circular DNA was varied.

Thus obtained inhibition kinetics of heliquinomycin were analyzed by the Lineweaver-burk plot and showed to be the mixed type of inhibition.

50% of growth (IC₅₀) are shown in Table 6.

Heliquinomycin was discovered to be an inhibitor of DNA helicase, which is thought to be essential for DNA replication, repair, transcription and recombination. It showed antimicrobial and antitumor activity *in vitro*. We are now preparing to demonstrate whether heliquinomycin might show its antitumor and antimicrobial activity in animal models. Heliquinomycin did not show any toxicity (LD₅₀), in mice at 100 mg/kg with ip administration.

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Table 5. Antimicrobial activities of heliquinomycin.

Test organisms	MIC (µg/ml)
<i>Staphylococcus aureus</i> Smith	0.1
<i>S. aureus</i> FDA209P	0.1
<i>S. aureus</i> MS9610	0.39
<i>S. aureus</i> TY-00923(MRSA) ^a	0.1
<i>S. aureus</i> Y-00936(MRSA) ^a	0.1
<i>S. aureus</i> Y-01022(MRSA) ^a	<0.05
<i>S. aureus</i> TY-01806(MRSA) ^a	<0.05
<i>Micrococcus luteus</i> FDA 16	0.2
<i>M. luteus</i> IFO 3333	0.1
<i>M. luteus</i> PIC 1001	0.39
<i>Bacillus anthracis</i>	0.1
<i>B. subtilis</i> NRRL B-558	0.1
<i>B. subtilis</i> PCI 219	0.1
<i>B. cereus</i> ATCC 10702	0.1
<i>Corynebacterium bovis</i> 1810	0.39
<i>Escherichia coli</i> NIHJ	100
<i>E. coli</i> K-12	>50
<i>Shigella dysenteriae</i> JS1910	>50
<i>Salmonella typhi</i> T-63	>50
<i>Proteus vulgaris</i> OX19	>50
<i>Serratia marcescens</i>	>50
<i>Pseudomonas aeruginosa</i> A3	>50
<i>P. aeruginosa</i> GN315	>50
<i>Klebsiella pneumoniae</i> PCI602	>50
<i>Mycobacterium smegmatis</i> ATCC607 ^b	>50
<i>Candida albicans</i> 3147	>100

Mueller Hinton agar (Difco) 37°C 18 hours.

^a MRSA: Methicillin-resistant *Staphylococcus aureus*.

^b 37°C 42 hours.

Table 6. Growth inhibition of cultured cell lines by heliquinomycin.

Cells	IC ₅₀ (µg/ml)
HeLa S3	1.6
L1210 leukemia	0.97
IMC carcinoma	1.56
B16 melanoma	0.89
FS-3 fibrosarcoma	0.83

Growth inhibition activity was determined according to the MTT assay method.

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