REQUINOMYCIN, AN INHIBITOR OF R-FACTOR TRANSFER: ISOLATION, CHARACTERIZATION AND PROPERTIES

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A new member of the anthracycline group of antibiotics named requinomycin has been isolated from a strain of *Streptomyces filamentosus* which had been screened for anti-phage activity. The antibiotic was obtained as yellow crystals, whose molecular formula was assigned to $C_{40}H_{54}N_2O_{16}$. It inhibits the reproduction of phage f 2 in *E. coli* S-26, the transfer of R-factors between *E. coli* strains, the growth of Gram-positive bacteria and the growth *in vitro* of YOSHIDA rat sarcoma cells. Requinomycin resembles aklavin but they are apparently different in antibiotic activity against *E. coli* B and stability to acid hydrolysis.

The emergence and rapid spread of drug-resistant bacteria are serious problems in clinics. The drug-resistance of Gram-negative bacteria is transferred from resistant organisms to sensitive ones by recombination[1,2]. In this process an episome, called R-factor, which carries genetic information for several drug-inactivating enzymes, is passed on through a hair-like structure, an R-pilus. This process resembles an early step of phage f 2 infection in that genetic meterial is introduced into a recipient cell through a similar structure of either R or F pilus. Based on this notion, we proposed a working hypothesis that anti-phage compounds could inhibit R-factor transfer. From a culture broth which had been screened for anti-phage activity, an active material inhibiting R-factor transfer was isolated. It proved to be a new antibiotic and was named requinomycin. We describe in the present paper the isolation procedures and the properties of requinomycin.

Results and Discussion

Requinomycin-Producing Strain

The requinomycin-producing strain was isolated from a soil sample collected at Ojiya city, Niigata Prefecture, in 1969 and designated as MC 521-C 5 in our institute. Its morphological and physiological properties are listed in Table 1. The culture

Table 1 A. Morphological and physiological characteristics of *Streptomyces* No. MC 521-C 5

Medium	Characteristics
Morphology	Sporophores straight, long, without spirals. Spore : oval to elliptical. Spore surface : smooth
Sucrose-nitrate agar	AM* pale pink G colorless, spreading R pale pink SP none
Glucose-asparagine agar	AM white to pale pink G colorless R yellowish gray SP none
Glycerol-asparagine agar	AM white G colorless to light brownish gray R light brownish gray SP none
Starch agar	AM pale pink, aerial mycelium in form of white patches. G good, colorless R pale yellow SP none
Tyrosin agar	AM white to pale pink G colorless to pale yellowish brown R pale yellowish brown SP none
Nutrient agar	AM very poor, if any white G poor, colorless R colorless SP light brown
Yeast extract-malt extract agar	AM pale pink, aerial mycelium in form of white patches G good, clolorless to yellowish brown R yellowish brown SP light brown
Oatmeal agar	AM pale pink, aerial mycelium in form of white patches G colorless, spreading R brownish white SP none
Peptone-yeast extract iron agar	AM none G colorless to pale yellowish brown R yellowish brown SP none
Liquefaction of gelatin	No liquefaction
Hydrolysis of starch	Imcomplete clarification of hydrolytic zone by KI-I reaction
Milk coagulation	Positive
Milk peptonization	Positive
Melanin formation	Negative in tyrosin agar
Carbon utilization	L-arabinose, D-xylose, D-glucose, D-fructose, sucrose and rhamnose are utilized for growth. No growth or only trace of growth on *i*-Inositol, raffinose and D-mannitol.

* Abbreviations. AM : aerial mycelium. R : reverse. G : growth. SP : soluble pigment.

Table 1B. Comparison of *Streptomyces* No. MC521-C 5 and related microorganisms

			MC 521-C 5	*S. filamentosus* ISP 5022	*S. roseoporus* ISP 5122	*S. polychromogenes* ISP 5316
Cultural characteristics	Glucose asparagine	AM*	abundant	poor	poor	poor
	Sucrose-nitrate	AM	abundant	abundant	poor	abundant
	Glycerol-asparagine	AM	abundant	poor	abundant	poor
	Starch	AM	pale pink	pale pink	yellowish gray	light brownish gray
	Nutrient	SP	light brown	none	none	light brown
	Yeast extract-malt extract	AM	pale pink	pale pink	pale pink	light brownish gray
		SP	light brown	none	none	none
Utilization of carbon source	D-fructose utilization		+	±	±	+

* AM : Aerial mycelium. SP : Soluble pigment.

Plate 1. MC 521-C 5 (×50) on yeast-malt agar

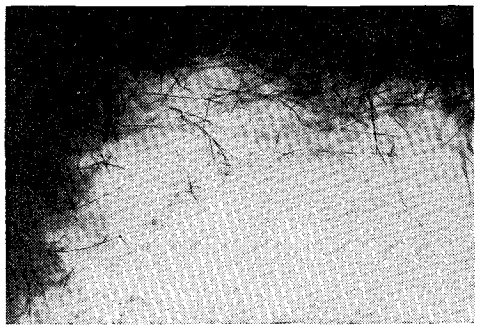


Plate 2. MC 521-C 5 (×2,250) on yeast-malt agar.

was incubated for 1~2 weeks at 27°C. Microscopic examination of the cultures grown on yeast extract - malt extract agar (ISP medium 2) revealed long straight sporophores, occasionally forming rings or bents (Plate 1). The electron micrograph showed oval spores with smooth surface (Plate 2). The morphological and physiological characteristics of MC 521-C 5 strain, as shown in Table 1 A, suggest this strain belongs to the genus *Streptomyces*. Among the known species of *Streptomyces*, *S. filamentosus* OKAMI & UMEZAWA, *S. roseosporus* FALCÃO DE MORAIS & DALIÁ MAIA and *S. polychromogenes* HAGEMANN, PÉNASSE & TEILLON were similar to strain MC 521-C 5 in cultural characteristics. Although some differences were noted as shown in Table 1 B, it is reasonably certain that strain MC 521-C 5 belongs to *S. filamentosus* OKAMI & UMEZAWA.

Production and Isolation

Requinomycin was produced by shaking cultures ranging from 25 to 30°C in media containing various carbon and nitrogen sources. A suitable medium (medium C) contained 2.0 % potato starch, 2.0 % glucose, 2.0 % soya meal, 0.5 % yeast extract, 0.25 % NaCl, 0.32 % $CaCO_3$, 0.0005 % $CuSO_4 \cdot 5H_2O$, 0.0005 % $MnCl_2 \cdot 4H_2O$, 0.0005 % $ZnSO_4 \cdot 7H_2O$ in water, and the pH was adjusted to 7.4. Small pieces of a mycelial mat were withdrawn from a slant culture, inoculated into a SAKAGUCHI flask containing 125 ml of medium C and incubated at 27°C with reciprocal shaking (seed culture). After 2 days of incubation, about 1 ml portions of the seed culture were transferred to 22 flasks each containing 125 ml of medium C and incubated in the same manner. After 7 days of incubation, as much as 100 mcg/ml of requinomycin was accumulated, when the pH was 8.0. Requinomycin could be determined by measuring the antibiotic titer against *Bacillus subtilis* by the paper disc method.

The following is an example of an isolation procedure. To 5 liters of 7-day-cultured broth, 50 g Hyflo Supercel was added and the suspension was filtered. The filter cake, after washing with a small volume of water, was mixed with 500 ml of methanol and was filtered. The methanol extract was dried *in vacuo* at a temperature below 40°C and the residue was dissolved in 300 ml of water, pH 8.0, and combined with the filtrate and the water wash. The combined solution was extracted with an equal volume of ethyl acetate at pH 8.0. The ethyl acetate layer was separated and concentrated to dryness (42 % yield). The residue was dissolved in 300 ml of methanol. After removal of insoluble materials, the solution was dried *in vacuo* and the residue

was dissolved in 500 ml of water, pH 8.0, and extracted with an equal volume of ethyl acetate. Requinomycin in the ethyl acetate extract was transferred into an equal volume of water at pH 3.0 and retransferred into equal volume of chloroform at pH 8.0 (with 1 N NaOH). The chloroform layer was dried resulting in 350 mg of reddish yellow powder. Requinomycin was slightly lost in the solvent extraction process. One hundred mg of the powder were applied on a column (3×10 cm) of powdered silicic acid (13 g, 100 mesh) which had been saturated with benzene. A stepwise elution with (1) 500 ml of benzene, (2) 500 ml of benzene-methanol (19 : 1, in volume), (3) 1,500 ml of benzene-methanol (9 : 1, in volume), and (4) 500 ml of benzene-methanol (1 : 1, in volume) was conducted. Most of the requinomycin was found in the eluate of (4), from which the solvents were removed by evaporation *in vacuo* and 66 mg of reddish yellow powder (89 % purity, 40 % overall yield) were obtained. The powder was dissolved in a minimum amount of ethanol at 60°C and left standing at room temperature to obtain requinomycin crystals which were collected by filtration. The filtrate was further concentrated and left standing to obtain additional crystals. After repeting these procedures, 37 mg of yellow crystals of pure requinomycin were obtained. The overall yield of requinomycin was 15 %.

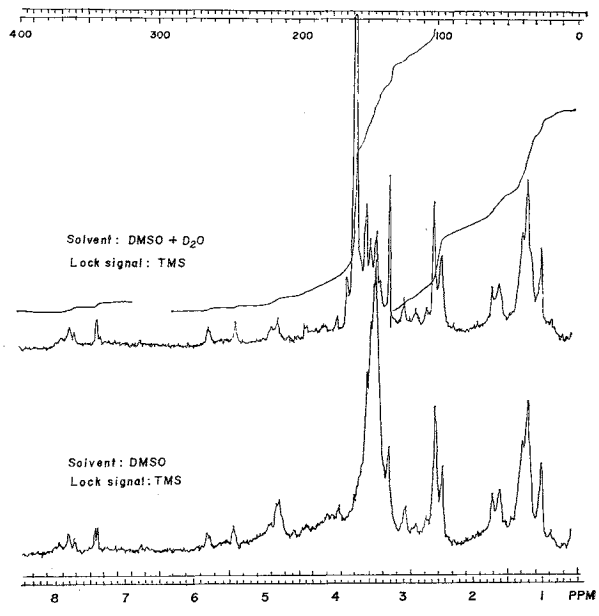
Physical and Chemical Properties

Requinomycin is obtained as yellow crystals melting at 215°C under decomposition, $[\alpha]_D^{28}+214°$ (*c* 1.09, $CHCl_3$).

Anal. Calcd. for $C_{40}H_{54}N_2O_{16}$: C 58.67, H 6.65, N 3.42, mol. wt. 818, 848.
Found: C 58.41, H 6.79, N 3.40, mol. wt. 600 (vapor pressure osmometer), 767 (RAST).

Requinomycin showed reasonable proton magnetic resonance spectral data for the empirical formula. The PMR spectrum in deuterodimethylsulfoxide, the ultraviolet and visible light absorption spectra and the infrared absorption spectrum in KBr tablet are shown in Figs. 1, 2 and 3.

Fig. 1. PMR spectrum of requinomycin.



The antibiotic is soluble in water, chloroform and ethyl acetate, moderately soluble in methanol, ethanol, acetone and pyridine while sparingly soluble or insoluble in ethyl ether, carbon tetrachloride and *n*-hexane. On silicic acid thin-layer chromatography developed by the solvent systems of *n*-butanol-pyridine-water (3:2:1.3), *n*-butanol-pyridine-benzene-water (5 : 3 : 1 : 3) and benzene-methanol (1 : 1), requinomycin gave Rf values of 0.77, 0.89 and 0.59, respectively. On paper-electrophoresis conducted with a solvent system of formic acid-acetic

Fig. 2. Absorption spectra of requinomycin.

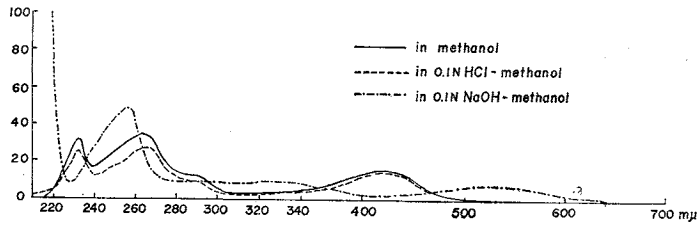
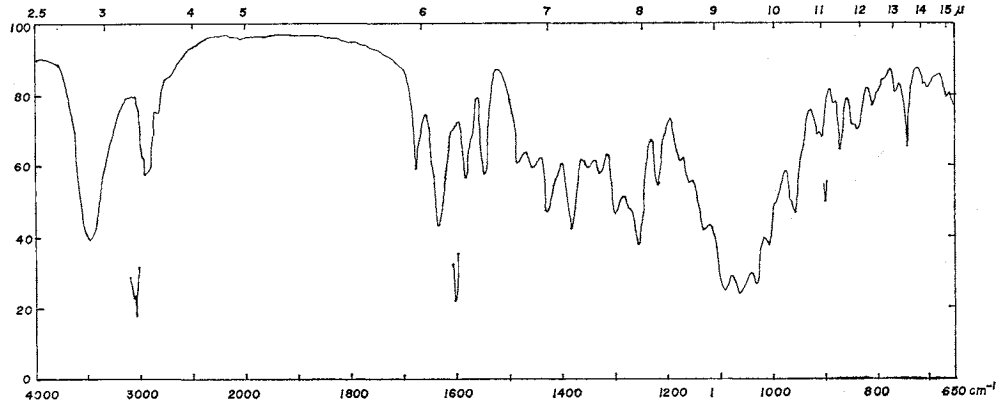


Fig. 3. Infrared absorption of requinomycin in KBr.



acid-water (25 : 75 : 900, pH 1.8) at 3,300 volts for 20 minutes, requinomycin moved to the cathode with an Rm of 0.5 (L-alanine ; 1.0).

A quinoid structure in the requinomycin molecule was suggested because of its positive magnesium acetate reaction[3] and its infrared absorption peaks at 1580, 1633 and 1670 cm^{-1}. The ultraviolet absorption spectrum at a neutral pH resembles that of aklavin[4,5] suggesting a similarity in the structure of their chromophore moieties, however, these antibiotics differ in stability to acid hydrolysis and in antibiotic activity against *E. coli* B. Based on physical and chemical properties, requinomycin is considered to be a new member of the family of anthracycline antibiotics.

Biological Properties

Requinomycin is active against Gram-positive bacteria, as shown in Table 2. Requinomycin inhibited the growth *in vitro* of YOSHIDA rat sarcoma

Table 2. Antibacterial spectra of requinomycin

Test microorganisms	Minimum inhibitory concentration (μg/ml)
Staphylococcus aureus FDA 209 P	3.12
〃 〃 Smith	3.12
Sarcina lutea PCI 1001	3.12
Micrococcus flavus FDA 16	12.5
Corynebacterium bovis 1810	25
Bacillus anthracis	1.56
Bacillus cereus IAM 1729	6.25
Bacillus subtilis NRRL B-558	6.25
Bacillus brevis IAM 1031	25
Mycobacterium phlei	25
Mycobacterium smegmatis ATCC 607	25
Candida albicans 3147	>100
Escherichia coli NIHJ	>100
〃 〃 K-12	>100
Shigella sonnei 191-66	>100
Salmonella typhosa T-63	>100
Klebsiella pneumoniae PCI 602	100
Pseudomonas aeruginosa A 3	>100
〃 〃 No. 12	>100
Proteus vulgaris OX 19	>100

Determined by the agar dilution method.

Fig. 4. Effect of requinomycin on the yield of phage-f_2.

E. coli S-26, the host strain, was grown in polypeptone broth at 37℃ under shaking until 0.18 O.D. 600 mμ was reached. From the cell suspension 1.7 ml portions were transferred into small test tubes, which had contained 0.2 ml of various concentration of requinomycin. After 10 minutes of incubation at 37℃ under shaking, each mixture received 0.1 ml of phage suspension (10^8 pfu) and incubation was continued another 90 minutes. After termination by chilling, each mixture was appropriately diluted with 0.85% NaCl - 2 mM $CaCl_2$ and 0.1 ml of the diluted suspension was submitted to plaque counting using *E. coli* S-26 as an indicator strain.

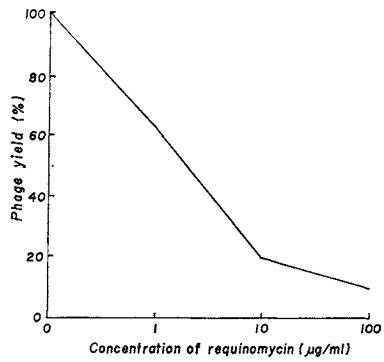
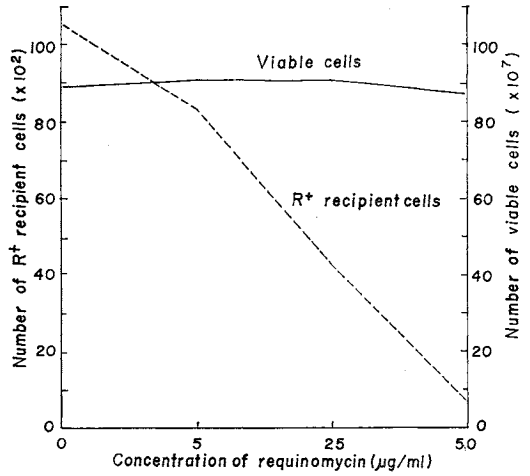


Fig. 5. Effect of requinomycin on R-transfer and viability of cells.

E. coli K-12 (T^-) R 1629[7] as a donor strain and *E. coli* K-12 as a recipient strain were grown separately in nutrient broth at 37℃ under shaking. When the cultures reached 0.4 O.D. 600 mμ, 0.9 ml aliquots were transferred from each culture to an Erlenmyer flask (30 ml vol.) which had contained 0.2 ml of requinomycin solution (0.85% NaCl solution for a control run). After gentle mixing for 60 minutes at 37℃, the mixture was chilled, appropriately diluted with 0.85% NaCl solution and 0.1 ml portions of the diluted cell suspension were submitted to colony counting for determination of the numbers of total viable cells and R^+ recipient cells (recipient cells which acquired the drug-resistance). By the pour plate method, the former was counted on nutrient agar plates while the latter on minimal agar plates supplemented with 70 mcg/ml of kanamycin sulfate.



cells[6] by 91%, 78% and 11% at 2 mcg/ml, 0.2 mcg/ml and 0.02 mcg/ml, respectively. Upon intravenous injection of requinomycin, mice died after 2 days and 9 days of injection at the doses of 2 mg and 1 mg per mouse, respectively, while the mouse which had received 0.5 mg of requinomycin survived.

As shown in Fig. 4, requinomycin inhibited the reproduction of phage f 2 in *E. coli* S-26. A separate experiment demonstrated that the growth of the host strain was not inhibited by requinomycin at 100 mcg/ml.

Transfer of R-factors between *E. coli* strains was specifically inhibited by requinomycin under conditions where neither the growth of the donor nor the recipient strain was significantly inhibited, as shown in Fig. 5.

As described above, requinomycin which had originally been selected for its anti-phage activity inhibits R-factor transfer, in support of our working hypothesis. It is unlikely that all anthracycline antibiotics are specific inhibitors of R-factor transfer. We have tested several which were inactive. The detailed mode of action of requinomycin concerning anti-phage activity and inhibition of R-factor transfer will be dealt with in a forthcoming paper.

AH 272α_2 and AH 272β_2/U.S.P. 3,592,925, July 13, 1971, American Cyanaid Co., exhibit some similarity to requinomycin. However, neither was identical with requinomycin based on comparison of analytical data and IR spectra.

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