A NEW ANTIBIOTIC, KINAMYCIN: FERMENTATION, ISOLATION, PURIFICATION AND PROPERTIES

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A new antibiotic, named kinamycin[1,2], has been isolated from the fermentation broth of *Streptomyces murayamaensis* sp. nov. HATA *et* OHTANI, which was obtained from a soil sample collected in Murayama, Saitama-ken, Japan. Kinamycins, produced as components A, B, C, and D as a mixture, are quinone antibiotics which show strong inhibitory activities against gram-positive bacteria. Details of the fermentation, isolation, and physico-chemical and biological characteristics are given.

In a previous communication[1], characteristics of the new antibiotic complex, kinamycins A, B, C and D, were reported briefly. The present paper reports the fermentation, isolation, purification, and properties of the four components.

Materials and Methods

In the initial investigation on kinamycin fermentation, reproducible data could not be obtained, since kinamycin was unstable in the fermented broth and potencies of the antibiotic decreased rapidly during the fermentation.

In order to obtain a good yield of kinamycin and stable fermentation, various conditions of the fermentation were examined and the cultural conditions for the antibiotic production were established. Further, rapid qualitative and quantitative analyses of kinamycin in the fermented broth were established.

Strains: The antibiotic-producing strain, *Streptomyces murayamaensis* sp. nov. HATA *et* OHTANI was maintained on KRAINSKY's agar slant and freeze-dried stock in the usual way.

Fermentation: The medium shown in Table 1 was always used for the culture including preincubation and fermentation, and sterilized at 121°C for 15 minutes. Adekanol LG-109 (Asahi Electro-Chemical Co., Ltd.) was used as an antifoam agent.

Table 1. Composition of medium (Preincubation and fermentation medium)

Glucose	2.0 %
Soybean meal	2.0 %
NaCl	0.3 %

The pH of medium was adjusted to 7.0 with NaOH before steriiization.

Standard fermentations in 30-liter jar fermentor were made under the following conditions: A 500-ml shake flask containing 125 ml of the liquid medium was inoculated from the agar slant, incubated on a reciprocating shaking machine at 27°C for 40~48 hours as a

Table 2. Effect of temperature and presterile pH on kinamycin fermentation in 5-liter fermentor

Temp. (°C)	pH of medium		Age (hrs.)	pH	Mycelium ml/10 ml	Residual sugar (%)	Potency	
	before sterilization	after sterilization					Kinamycin formed (mcg/ml)	O. D. at 390 mμ
24	6.0	5.8	30	5.8	2.0	1.37	1.7	0.200
	7.0	6.0	72	5.9	2.8	trace	34.4	0.788
	8.0	6.3	72	5.9	2.5	0.38	41.2	0.840
27	6.0	5.7	72	5.8	2.9	trace	4.3	0.290
	7.0	6.0	72	5.9	2.9	trace	22.0	0.410
	8.0	6.3	72	6.0	2.3	0.38	44.0	1.410

The 600 r.p.m. agitation and 5 liters/min aeration were used.

seed culture and transferred to 20 liters of fermentation medium in a 30-liter stainless steel jar-fermentor. Ten percent of vegetative inoculum, by volume, was used.

The fermentation conditions were as follows: Temperature, 26~27°C; aeration, 30 liters/min; agitation, 300 r.p.m.; pressure, 0.5 kg/cm^2. Samples were collected at 8-hour intervals to determine pH, mycelium, volume, and residual sugar in the usual way. In addition, examination of various conditions for kinamycin fermentation was made with 5-liter jar fermentors as shown in Table 2.

Kinamycin production in 400-liter tank was carried out in the same way as that in a 30-liter jar-fermentor except for aeration of 80 liters/min and agitation of 200 r.p.m.

Analysis of kinomycin: Kinamycin in the fermented broth was analyzed by the following three procedures:

(1) Agar diffusion method: The antibacterial activity was estimated by the paper disc or cup method in the usual way. A mixture of kinamycins C and D, the main components, was used as the standard for estimation of the antibiotic.

(2) Colorimetric method: The ultraviolet and visible spectra of kinamycin show characteristic absorption at 390~450 mμ, assigned to the naphthoquinone skeleton. After fermentation, the culture broth was withdrawn and its filtrate was extracted with the same volume of benzene. The optical density of the benzene extract was determined at 390 mμ.

(3) Thin-layer chromatography: The sample prepared from the benzene extract was applied on a thin-layer chromatographic plate of Kieselgel G (Merk) treated with 2% H_2SO_4 and a mixed solvent of benzene-acetone (5:1, v/v). Kinamycins A and B (Rf: 0.60), and C and D (0.25) were detected on the thin-layer chromatogram.

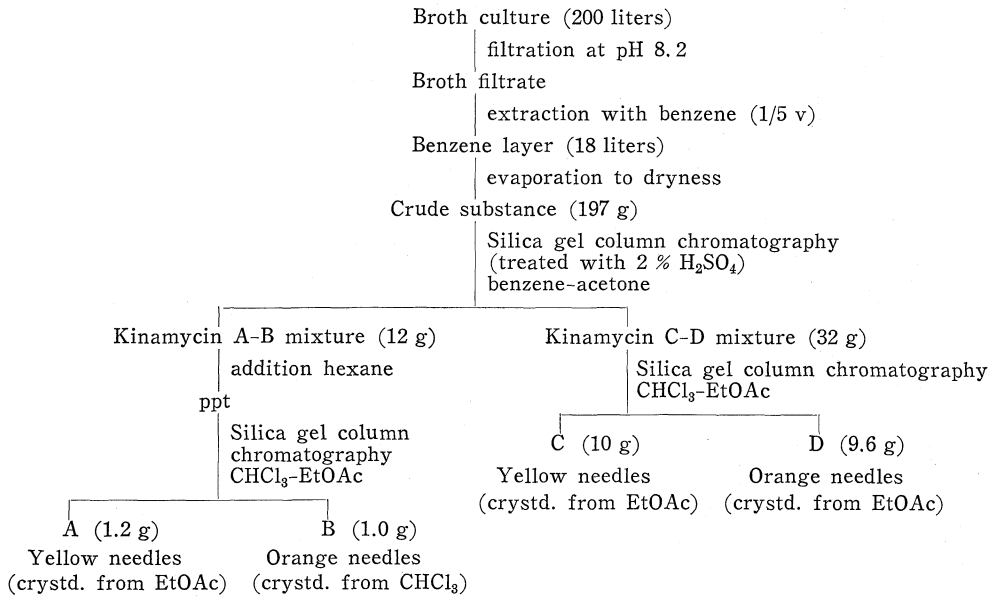
Results

Isolation of Main Components of Kinamycin

During the isolation and purification process, antibacterial activity was assayed by paper disc method with *Bacillus subtilis* PCI-219 as a test organism. Four active components of kinamycins were isolated from the broth culture in a 400-liter tank by extraction with an organic solvent as shown in Fig. 1.

Kinamycins from the filtered broth (200 liters) were extracted with benzene (1/5,

Fig. 1. Isolation and purification of kinamycins A, B, C and D



v/v) at pH 8. Benzene extract was then evaporated under reduced pressure and a syrup (197 g) was obtained, which solidified on treatment with 600 ml of hexane. The crude powder (126 g) thus obtained was column chromatographed over Kieselgel (0.05 ~0.2 mm) treated with 2 % H_2SO_4 using a mixture of benzene and acetone. First active fractions eluated with a mixture of benzene and acetone (20 : 1, v/v) were collected and evaporated *in vacuo* to give A and B mixture (12 g). A mixture of C and D (32 g) was eluted with a more polar mixed solvent (benzene - acetone, 5 : 1, v/v). A and B mixture was chromatographed over Kieselgel (0.05~0.2 mm) using a mixture of chloroform and ethyl acetate (15 : 1, v/v) as a developer and components A (1.2 g) and B (1.0 g) thereby obtained crystallized from chloroform as yellow and orange needles, respectively. By the same procedure, the components C (10 g) and D (9.6 g) were separated from their mixture by elution with chloroform - ethyl acetate (5 : 1, v/v) and crystallized from ethyl acetate to yellow and orange needles, respectively. Thin-layer chromatography of the complex on Kieselgel G (solvent system : $CHCl_3$ - EtOAc, 3 : 2) gave Rf values of 0.89, 0.82, 0.47 and 0.39 for these four components, A, B, C, and D, respectively.

Physico-chemical Properties

Physico-chemical properties of each of these components, A, B, C, and D, of kinamycin are summarized in Table 3.

The kinamycins are soluble in methanol, chloroform, ethyl acetate, and benzene, hardly soluble in ether, and insoluble in petroleum ether, hexane, and water. Kinamycins are stable in neutral or acidic solution, but not in alkaline solution and acetone.

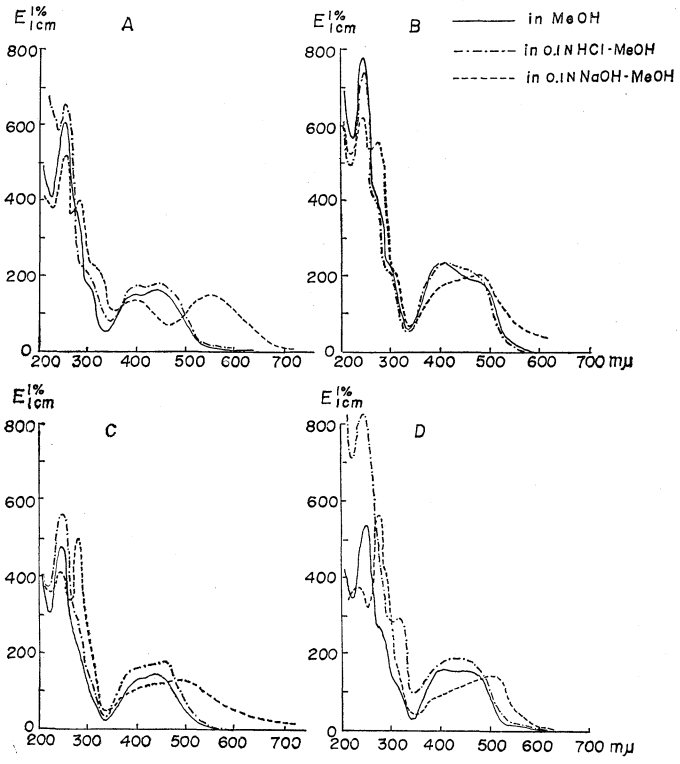
The ultraviolet and visible absorption spectra of kinamycins in neutral, acidic, and alkaline methanol indicate an absorption characteristic of quinone[3] as shown in Fig. 2.

Table 3. Physicochemical properties of kinamycins A, B, C and D

		A	B	C	D
m. p. (°C)		139~142 (dec.)	190~193 (dec.)	150~153 (dec.)	170~175 (dec.)
$[\alpha]_D^{25}$ (c 1, $CHCl_3$)		−60°	−48°	−24°	−37°
Elementary analysis found	C	58.30%	58.52%	58.40%	58.13%
	H	4.03	3.91	4.15	3.94
	N	5.42	6.74	5.33	6.17
	O	32.25	30.83	32.10	30.76
Molecular weight	Mass M^+ (m/e)	——	——	496	——
	Osmotic pressure method	515	415	515	513
Molecular formula		$C_{24}H_{20}N_2O_{10}$	$C_{20}H_{16}N_2O_8$	$C_{24}H_{20}N_2O_{10}$	$C_{22}H_{18}N_2O_9$

As shown in Fig. 3, the infrared spectra of kinamycins show absorptions assigned to quinone carbonyl (1660~1620 cm^{-1}), ester carbonyl (1725 cm^{-1}), and isonitrile or nitrile (2155 cm^{-1}) as functional groups common to these components. The NMR spectra of kinamycins A, B, C, and D are given in Fig. 4.

Fig. 2. UV and visible spectra of kinamycins A, B, C and D



Based on analytical data, measurement of the number of protons in NMR spectrum and mass spectrum, molecular formulae of $C_{24}H_{20}N_2O_{10}$, $C_{20}H_{16}N_2O_8$, $C_{24}H_{20}N_2O_{10}$, and $C_{22}H_{18}N_2O_9$ are proposed for kinamycins A, B, C, and D, respectively. These molecular formulae were derived from the elemental analyses and mass spectra of derivatives obtained during structural studies on these components, which will be reported in the following paper. The molecular formulae given in our short communication are corrected to these. Since these components were positive to color reaction with ferric chloride and alkaline solution, presence of a phenolic hydroxyl group in the molecules can be established, and the presence of a quinone as the chromophore was confirmed from the reaction with rhodamine and ammonia, and with 1-phenyl-3-methylpyrazol-5-one[4].

Fig. 3. IR spectra of kinamycins A, B, C and D (KBr)

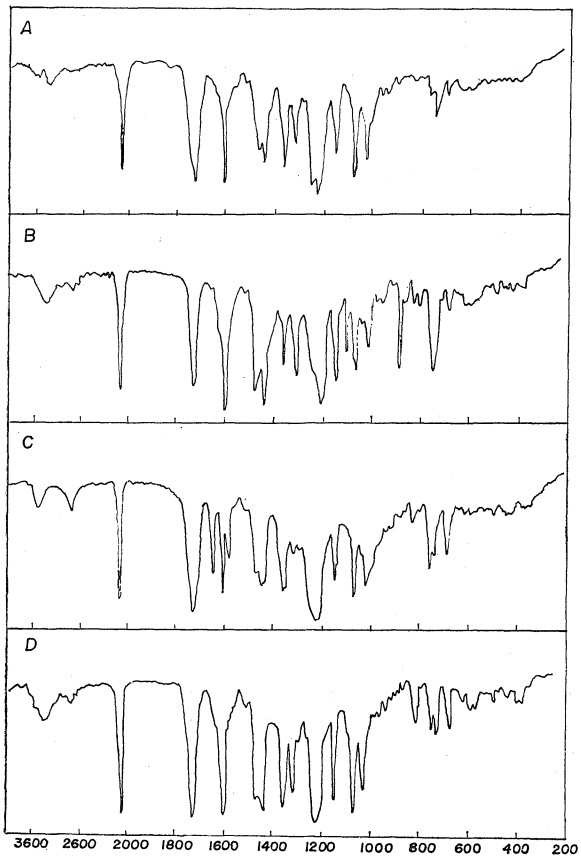
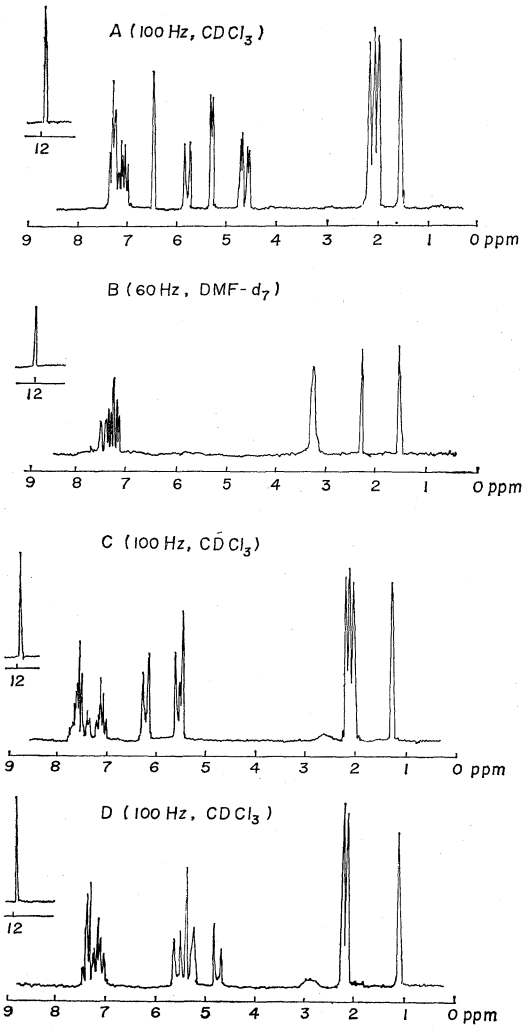


Fig. 4. NMR spectra of kinamycins A, B, C and D



Biological Properties

Antimicrobial activities of kinamycins by the agar dilution method are listed in Table 4. As can be seen from Table 4, each component is strongly active against gram-positive, but less active against gram-negative bacteria. Among these four components, B and D are more active than A and C. The minimum inhibitory concentration of component B is 0.012 mcg/ml against *Vibrio comma*. The antitumor tests of kinamycins C and D for EHRLICH ascites carcinoma or sarcoma-180 were carried out. C showed some survival effect by intraperitoneal injection in 0.1 and 1 mg/kg, but D showed no tumor inhibition effect[5]. The acute toxicity (LD_{50}) of the four components in mice was approximately 30~40 mg/kg each, intravenously.

Production

The optimum conditions for kinamycin production in 5-liter fermentors were worked out by keeping all the factors constant except one which was varied within a reasonable limit and the following conditions were found necessary for keeping the good fermentation: presterile pH, 8.0; high agitation, 600 r.p.m.; adequate aeration,

Table 4. Antimicrobial spectra of kinamycins A, B, C and D

Test organism	Minimum inhibitory concentration (mcg/ml)			
	A	B	C	D
Bacillus subtilis PCI-219	0.024	0.012	0.19	0.012*
Bacillus anthracis	0.19	0.012	0.19	0.024*
Staphylococcus aureus FDA 209P	0.78	0.012	0.78	0.024*
Staphylococcus aureus SM-(R)	1.56	0.006	0.39	0.024*
Staphylococcus albus	0.024	0.012	0.39	0.024*
Streptococcus haemolyticus (Cook)	100	12.5	12.5	50 **
Streptococcus haemolyticus (NY-5)	1.56	3.15	12.5	12.5 **
Streptococcus haemolyticus (S-8)	6.25	6.25	12.5	12.5 **
Mycobacterium 607	25	6.25	6.25	6.25 *
Escherichia coli NIHJ	>100	3.12	100	12.5 *
Vibrio comma	>100	0.19	25	12.5 *
Vibrio comma Inaba 904	50	0.09		
Klebsiella pneumoniae	>100	12.5	100	25 *
Pseudomonas aeruginosa P-1	>100	>100	>100	>100 *
Pseudomonas aeruginosa P-2	>100	>100	>100	>100 *
Salmonella typhosa 901 W	>100	6.25	>100	12.5 *
Salmonella paratyphi A	>100	>100	>100	25 *
Salmonella enteritidis gaertnerii	>100	>100	>100	>100 *
Shigella dysenteriae	>100	25	>100	25 *
Proteus vulgaris OX-19	>100	12.5	>100	6.25 *
Neisseria gonorrhoeae	50	12.5	>100	50 **
Saccharomyces sake	>100	>100	>100	>100 ***
Candida albicans	>100	>100	>100	>100 ***
Aspergillus niger	>100	>100	>100	>100 ***
Penicillium chrysogenum Q-176	50	>100	>100	12.5 ***

Inoculum size: One loopful of test organism
Medium: * Nutrient agar ** Blood agar *** Potato agar

5 liters/min; inoculum size, 10%; temperature, 27°C.

In particular, the essential condition was to adjust presterile pH of the medium to 8.0 with NaOH.

Fig. 5. A typical time course of kinamycin fermentation

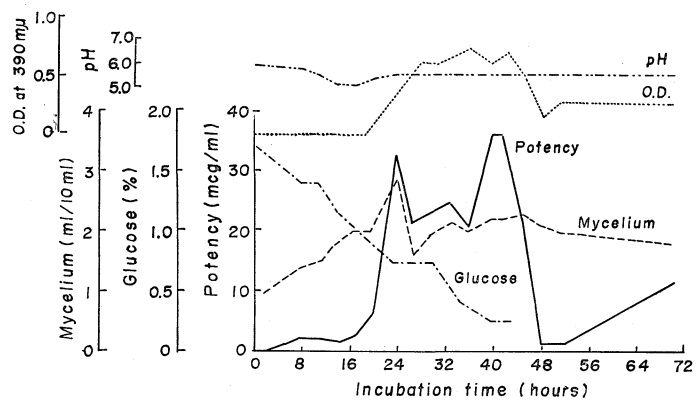
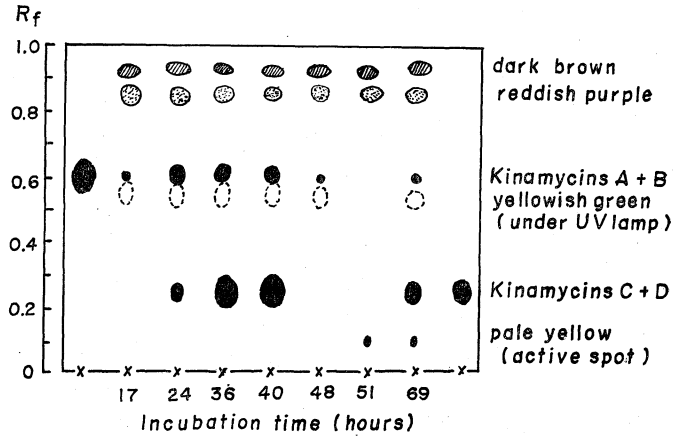


Fig. 5 shows the fermentation patterns from a 30-liter jar-fermentor. The qualitative analysis by thin-layer chromatography is summarized in Fig. 6. According to this result, there are two peaks of potency during the fermentation. The first peak was present at 24 hours and agreed with a maximal growth. The second peak appears at 48 hours and there is a marked accumulation of kinamycin in this phase. These two peaks seem to be closely related to the carbohydrate metabolisms because there was a marked tendency for the exponential decrease of the residual sugar in the broth, resulting in the acceleration of kinamycin production. It was

observed that a total amount of A and B at the first peak was higher than the second peak but, during the fermentation, C and D were the main components, and A and B were the minor. In addition, a slight amount of another active spot (Rf 0.1) was detected at a later stage of the fermentation.

Fig. 6. Thin-layer chromatogram of kinamycin fermentation
Absorbent : silica gel (treated with H_2SO_4)
Developer : benzene - acetone (5 : 1)
Detection : UV lamp and visible color



Discussion

On the basis of physicochemical properties, kinamycins A, B, C, and D seem to have a closely related structure. While several quinone antibiotics, such as frenolicin[6], aquayamycin[7], tetrangomycin[8], bostricin[9] and julimycin[10] were reported, kinamycins having an infrared absorption maximum at 2155 cm^{-1} assignable to a nitrile or isonitrile group can be differentiated from other antibiotics lacking this absorption.

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References

1) Itō, S.; T. Matsuya, S. Ōmura, M. Otani, A. Nakagawa, H. Takeshima, Y. Iwai, M. Ohtani & T. Hata : A new antibiotic, kinamycin. J. Antibiotics 23 : 315～317, 1970
2) Presented at the 169th Scientific Meeting of the Japan Antibiotics Research Association, Tokyo. Sept. 19, 1969
3) Singh, I.; R. T. Ogata, R. E. Moore, C. W. J. Chang & P. J. Scheuer : Electronic spectra of substituted naphthoquinones. Tetrahedron 24 : 6053～6073, 1968
4) Feigl, F. : Spot Tests in Organic Analysis. pp. 332～334, Elsevier Publishing Co., New York, 1966
5) Presented at the 90th Ann. Meeting of the Pharmaceutical Society of Japan, Sapporo, 1969
6) Van Meter, J. C.; M. Dann & N. Bohonos : Isolation and characteristics of frenolicin. Antimicr. Agents Ann. 1960 : 77～80, 1961
7) Sezaki, M.; T. Hara, S. Ayukawa, T. Takeuchi, Y. Okami, M. Hamada, T. Nagatsu & H. Umezawa : Studies on a new antibiotic pigment, aquayamycin. J. Antibiotics 21 : 91～97, 1968
8) Dann, M.; D. V. Lefemine, F. Barbstschi, P. Shu, M. P. Kunstamann, L. A. Mitscher & N. Bohonos : Tetrangomycin, a new quinone antibiotic. Antimicr. Agents & Chemoth.-1965 : 832～835, 1966
9) Noda, T.; T. Take, M. Otani, K. Miyauchi, T. Watanabe & J. Abe : Structure of bostricin. Tetrahedron Letters 58 : 6087～6097, 1968
10) Tsugi, N. & K. Nagashima : Studies on julimycins. II. Tetrahedron 24 : 4233～4247, 1968