FORTIMICINS A AND B, NEW AMINOGLYCOSIDE ANTIBIOTICS

II. ISOLATION, PHYSICO-CHEMICAL AND CHROMATOGRAPHIC PROPERTIES

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(Received for publication April 13, 1977)

The aminoglycoside antibiotics fortimicins A and B produced by a naturally occurring strain *Micromonospora* sp. MK-70 were isolated from its fermentation beer. Fortimicins A and B were isolated as water-soluble, basic, white amorphous powders having molecular formula $C_{17}H_{35}N_5O_6$ and $C_{15}H_{32}N_4O_5$, respectively. Acid hydrolysis of fortimicin A indicated that it has one mole of glycine in its molecule while fortimicin B has not. Paper chromatography, silica-gel and carbon thin-layer chromatography revealed that fortimicins A and B are novel aminoglycoside antibiotics.

Our previous work showed that *Micromonospora* sp. MK-70 produced a mixture of water-soluble basic aminoglycoside antibiotics which are active against Gram-positive and Gram-negative bacteria[1]. *Micromonospora* species have been reported to produce such aminoglycoside antibiotics as gentamicin C complex[2], sisomicin[3~5], gentamicin A[2,6~8], A_1[9], A_2[10], A_3[9], A_4[9], B(Sch 14342)[8,11~13], B_1[8], X[8], verdamicin[14~17], Antibiotic G-418[18~21], Sch 17726 (Antibiotic G-52)[22~24], Antibiotic 66-40B, 66-40D[25~27], Antibiotic JI-20A, JI-20B[28], sagamicin[29~31] (gentamicin C_{2b}[32]), Antibiotic 460[33] and neomycin[34,35].

Fortimicins are produced as a mixture of several biologically active components as is usually the case with aminoglycoside antibiotic fermentations. Fortimicins A and B are two major components in the mixture. In this report, the large-scale fermentation, isolation, physicochemical properties and chromatographic behavior of fortimicins A and B will be described.

Materials and Methods

Fermentation of *Micromonospora* sp. MK-70

The seed medium contained 2% glucose, 0.5% peptone, 0.5% yeast extract and 0.1% $CaCO_3$. The pH was adjusted to 7.5 with diluted NaOH solution before autoclaving. The production medium was composed of 4% soluble starch, 2% soybean meal, 1% corn steep liquor, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.03% KCl and 0.1% $CaCO_3$. The pH was adjusted to 7.5 before sterilization. The fermentation was carried out at 30°C.

Quantitative Assay of Fortimicins

Total fortimicins were assayed by the paper disc diffusion method with paper discs of 8 mm, thick, Toyo Roshi Co., Ltd., Japan. The fermentation beer was acidified to pH 2.0 with 6 N H_2SO_4 and stirred for 15 minutes. The supernatant of the beer was diluted with 1/15 M phosphate buffer (pH 8.0). Paper discs were dipped in the buffered supernatant and assayed on agar plate seeded with *Escherichia coli* KY 4271. Total fortimicins were calculated from a standard curve obtained by use of appropriate dilutions of fortimicin A.

Paper Chromatography

Toyo filter paper No. 51 (Toyo Roshi Co., Ltd., Japan) was used for chromatographic separation of the components in *M.* sp. MK-70 fermentation broth (Fig. 2) and for salting-out chromatography (Fig. 7). Solvent systems (Fig. 2) were; (I) 20% aqueous ammonium chloride, (II) *n*-butanol saturated with water, (III) *n*-butanol - acetic acid - water (3: 1: 1, v/v), (IV) ethylacetate saturated with water, and (V) *n*-butanol saturated with water containing 2% *p*-toluene sulfonate and 2% piperidine. For salting-out chromatography (Fig. 7), water containing 0, 0.5, 1.5, 2.5, and 20% ammonium chloride was used.

To separate the fortimicin components, 1×40 cm strips of Whatman No. 1 chromatography paper (W. & Balston, Ltd., England) were used. Samples were spotted approximately 5 cm from the end of each strip. After drying under an air stream, the strips were placed in a glass jar containing the lower phase of a solvent mixture consisting of chloroform - methanol - 17% ammonium hydroxide in a ratio of 2: 1: 1 (v/v) and developed ascendingly for 17 hours at 28°C. The strips were dried in air and bioautographed on agar plates seeded with spores of *Bacillus subtilis* KY 4273.

Silica-gel Thin-layer Chromatography

Glass plates (20×20 cm) were coated with 0.25 mm of silica-gel (silica-Rider 5B, Daiichi Pure Chemicals Co., Ltd., Japan). After drying in air, the plates were activated by heating at 115°C for 1 hour. Plates were developed ascendingly in a solvent consisting of 10% ammonium acetate - methanol (1: 1, v/v) at room temperature for 3 hours, and bioautographed on *B. subtilis* agar, or sprayed with ninhydrin.

Carbon Thin-layer Chromatography

Active carbon "Purified Shirasagi" (Takeda Chem. Ind. Co., Ltd., Japan) sifted through a 100-mesh stainless-steel sieve was used to prepare thin-layer plates. Ten grams of the carbon and 0.5 g $CaSO_4 \cdot \frac{1}{2}H_2O$ were suspended in a mixture of 30 ml $\frac{1}{2}$ N H_2SO_4 with 30 ml methanol. After spreading a 0.25-mm layer on glass plates, the plates were activated for 1.5 hours at 115°C. Ten to 20 mcg of each antibiotic were spotted on the plates, which were then developed ascendingly with $\frac{1}{2}$ N H_2SO_4 for one hour in a glass vessel. The developed plate was covered by a sheet of filter paper (20×20 cm, Toyo Filterpaper No. 51) wetted by 50% aqueous methanol. After pressing the sheet tightly for 15 minutes on the carbon surface, the filter paper was removed and exposed to ammonia gas for 5 minutes. The neutralized filter paper was bioautographed on an agar plate seeded with *B. subtilis*.

Acid Hydrolysis of Fortimicins A and B

The free bases of fortimicins A and B were sealed in small glass tubes with 6 N HCl and heated at 100 °C for 2 hours. The hydrolysates were concentrated to dryness *in vacuo* and dissolved in water. The concentration was repeated to remove excess hydrogen chloride. The hydrolysates were examined for amino acids using JEOL LCR-1 resin in a Nihondenshi 5AH amino acid analyzer. The resin was eluted with citrate buffer (pH 5.28 for basic amino acids, pH 3.1 and 4.25 for neutral and acidic amino acids). Basic amino acids were separated on an 8×150 mm column at a flow rate of 1.22 ml/min. Neutral and acidic amino acids were separated on an 8×700 mm column at a flow rate of 0.83 ml/min.

Reference Antibiotics

Gentamicin C_1 (sulfate, 645 mcg/mg), C_{1a}(sulfate, 764 mcg/mg), C_2(sulfate, 622 mcg/mg), sisomicin (Antibiotic 66-40, sulfate 610 mcg/mg), verdamicin (sulfate, 634 mcg/mg), Antibiotic G-418 (601 mcg/mg), Antibiotic JI-20A (710 mcg/mg), JI-20B (sulfate, 632 mcg/mg), gentamicin A_1, A_2, A_3, A_4, B_1, X, Antibiotic G-52, Antibiotic 66-40D, and gentamicin B (sulfate, 586 mcg/mg) were supplied by Drs. G. H. WAGMAN and W. DAVIS, Schering Corp., U.S.A. Nebramycin factor 2, 5′, 7, and tobramycin were gifts from Drs. R. C. HAMILL and K. F. KOCH, Eli Lilly & Co., U.S.A. Ribostamycin, NK-1001, -1003, -1012-1 and NK-1012-2 were supplied by Dr. T. NIIDA, Meiji Seika Co., Ltd. Japan. Lividomycins A, B and D were gifts from Dr. I. MORI, Kowa Co., Ltd. Japan. Butirosins A and B were given by Dr. H. E. MACHAMER, Parke-Davis & Co., U.S.A. Bu1709-E_1, E_2, Bu-1975-C_1, C_2 were supplied by Dr. H. KAWAGUCHI, Bristol-Banyu Res. Inst., Japan. Xylostasin was given by Dr. J. UEYANAGI, Takeda Chem. Ind., Japan. Nebramycin factor 4 (base) and bluensomycin (1,215 mcg/mg) were gifts from ICIA, Belgium.

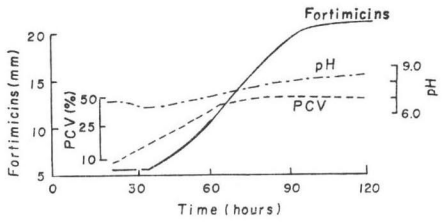
Results

Large-scale Fermentation of *Micromonospora* sp. MK-70

M. sp. MK-70 was inoculated into 10 ml of a seed medium in a 50-ml test tube and incubated for 5 days on a reciprocating shaker. The first seed broth was transferred to 30 ml of the second seed medium in a 250-ml Erlenmeyer flask with an inoculum size of 10% (v/v), and incubated for 2 days on a rotary shaker. The second seed culture was inoculated into 300 ml of the seed medium in a 2-liter Erlenmeyer flask and incubated for 2 days. The third seed culture broth was transferred to 15 liters of the final seed medium in a 30-liter stainless steel jar fermentor in a ratio of 5% (v/v) and cultivated for 2 days with agitation (350 r.p.m.) and aeration (15 liters/min.). The production medium was prepared by weighing each component, adding water to 150 liters, and sterilizing at 120°C for 30 minutes with steam. The final seed culture was transferred to the production medium in a 300-liter-tank fermentor and cultivated for 6 days with agitation (150 r.p.m.) and aeration (80 liter/min.). As shown in Fig. 1, the potency of fortimicins reached a maximum at 100~120 hours of fermentation age.

Fig. 1. Time course of fortimicin fermentation with *Micromonospora* sp. MK-70

Potency of fortimicins are shown in diameter of inhibition zone against *E. coli* by paper disc assay. Packed cell volume (PCV) are measured after centrifugation for 15 minutes at 3,000 rpm.



Isolation and Purification of Fortimicins A and B

Fermentation broth harvested from a 300-liter stainless-steel fermentor was adjusted to pH 2.0 with sulfuric acid, stirred for 30 minutes, and adjusted to pH 7.0 with sodium hydroxide. Filter aid (Radiolite # 600, Showa Kako Co., Ltd., Japan) was added and the mixture was filtered. The filtrate was passed through a cation-exchange resin IRC-50 in the ammonium cycle. After washing the resin column with deionized water, the resin was eluted with 0.5 N ammonium hydroxide. Fractions active against *B. subtilis* were collected and concentrated *in vacuo* to 1/3 of the original volume. The concentrate was passed through a column of anion-exchange resin Dowex 1×2 (OH^-) and washed with deionized water. The effluent and washings were collected, concentrated *in vacuo*, adjusted to pH 8.0 with sulfuric acid and charged on a carbon column (Chromatography grade, Wako Pure Chemicals Co., Ltd., Japan). After washing the column with deionized water, fortimicins were eluted with 0.2 N sulfuric acid. The active fractions were neutralized with Dowex 44 (OH^-) and freeze-dried to obtain the crude fortimicin sulfate mixture.

The crude powder was charged on a silicic acid column (Silicic acid AR, 100 mesh, Mallinckrodt Co., U.S.A.) packed with the lower phase of a solvent mixture of chloroform - isopropanol - 17% ammonium hydroxide in a ratio of 2 : 1 : 1 (v/v) and eluted with the same solvent system. Eluates were monitored by the paper disc diffusion assay and paper chromatography. Fortimicin B was eluted as the first major component. Fortimicin A was eluted as the second major component possessing broad spectrum antibacterial activity. The fractions containing fortimicins A and B were pooled separately and concentrated *in vacuo*. The concentration was repeated several times and the solids were finally dissolved in water followed by freeze-drying. Thus, the free bases of fortimicins A and B were obtained as white amorphous powders.

Since fortimicin A prepared as above still contained a small amount of ninhydrin-positive impurities, it was chromatographed again on a cellulose column (Avicel for column chromatography, Funakoshi Yakuhin Co., Ltd., Japan) and eluted with a solvent mixture of *n*-butanol - pyridine - acetic acid - water in a ratio of 6: 4: 2: 4 (v/v). The ninhydrin-positive impurities were removed by this chromatography.

Separation of the Components in a MK-70 Fermentation Broth

M. sp. MK-70 produced some biologically active components besides fortimicins A and B in a fermentation broth. The agar dilution assay of the MK-70 broth showed antibacterial activity mainly against Gram-positive bacteria (Table 1-A). Paper chromatograms of the broth showed at least three inhibition zones on solvent III (Fig. 2-(a)). Spent broth of MK-70 passed through IRC-50 (H^+) resin (Table 1-B) contained an antibacterial substance shown in Fig. 2-(b) which is active against *Staphylococcus aureus* and only slightly active against *B. subtilis* (Table 1-B). This substance was thus removed from fortimicins which were adsorbed on IRC-50(H^+).

In an eluate from IRC-50 (H^+) resin with 0.5 N HCl, fortimicins and one more unknown substance were included. (Fig. 2-(c), Table 1-C). The eluate was adjusted to pH 4.0 with aqueous sodium hydroxide and passed through HP-10 (Mitsubishi Kasei Co., Ltd. Japan). Fortimicins are eluted with water from the resin (Table 1-E). An unknown substance which is active against *Streptococcus faecalis* and *B. subtilis* was ad-

Fig. 2. Paper chromatograms of antibiotic components in fermentation broth
Filter paper: Toyo No. 51 (2×40 cm, Toyo Roshi Co., Ltd.)
Solvent system: I～V are described in Materials and Methods. I and IV were developed for 3 hours, II, III and V were developed for 18 hours at 28°C.

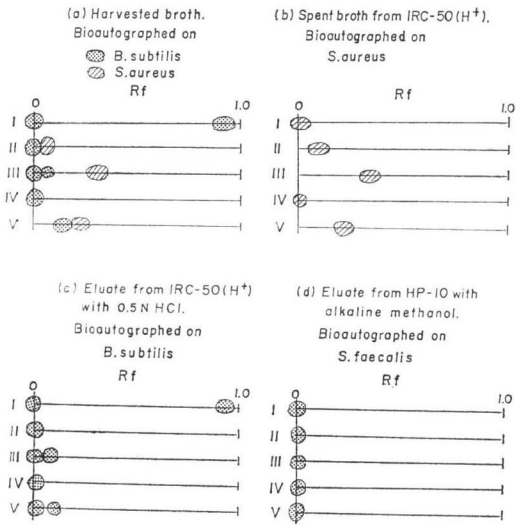


Table 1. Antibacterial activities of components in fermentation broth of *M.* sp. MK-70

Test organisms	Preparations						
	A	B	C	D	E	F	G
Bacillus subtilis	1,280	80	320	640	320	640	320
Streptococcus faecalis	640	10	320	1,280	0	320	0
Staphylococcus aureus	2,500	1,280	160	0	160	1,280	160
Escherichia coli	10	0	40	0	40	0	40
Proteus vulgaris	10	10	10	0	40	0	40
Salmonella typhosa	10	10	80	0	40	0	40
Klebsiella pneumoniae	40	0	80	0	80	0	80

Relative dilution units are given as the dilution factor of the preparations which show minimal inhibitory concentrations.

A: Harvested broth concentrated to ten times
B: Spent broth from IRC-50(H^+) resin
C: Eluate from IRC-50(H^+) resin with 0.5 N HCl
D: Eluate from HP-10 resin with alkaline methanol
E: Eluate from HP-10 resin with water
F: Spent broth from IRC-50(NH_4^+) resin
G: Eluate from IRC-50(NH_4^+) resin with 0.5 N NH_4OH

Fig. 3. Separation of minor components in crude fortimicin powder
[A] Rf values on silica-gel thin-layer chromatography developed with 10% CH_3COONH_4 - CH_3OH (1: 1, v/v)
[B] Rf values on paper chromatography developed with a lower layer of $CHCl_3$ - CH_3OH - 17% NH_4OH (2: 1: 1, v/v)
[C] Diameter of inhibition zones on *B. subtilis* (mm)
* GM; gentamicin C complex

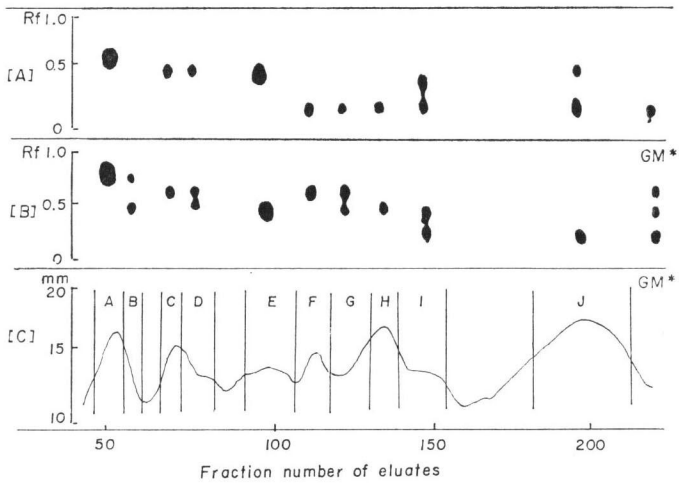


Table 2. Physico-chemical properties of fortimicins A and B (base).

	Fortimicin A base	Fortimicin B base
Nature	White amorphous basic powder Soluble in water, lower alcohols Insoluble in organic solvents	White amorphous basic powder Soluble in water, lower alcohols Insoluble in organic solvents
Elemental analysis	Found: C 50.2, H 8.67, N 17.5 Calcd.: C 50.4, H 8.64, N 17.3	Found: C 51.72, H 9.19, N 16.16 Calcd.: C 51.71, H 9.26, N 16.08
Molecular weight	405 (mass spectrometry)	348 (mass spectrometry)
Empirical formula	$C_{17}H_{35}N_5O_6$	$C_{15}H_{32}N_4O_5$
Melting point	>200°C (dec.)	101~103°C
UV spectrum	End absorption	End absorption
Optical rotation	$[\alpha]_D^{25}+87.5°$(*c* 0.1, H_2O)	$[\alpha]_D^{25}+22.2°$(*c* 0.1, H_2O)
Color reaction	Positive ninhydrin, RYDON-SMITH, $KMnO_4$ Negative ELSON-MORGAN, biuret, SAKAGUCHI	Positive ninhydrin, RYDON-SMITH, $KMnO_4$ Negative ELSON-MORGAN, biuret, SAKAGUCHI

sorbed on the HP-10 resin and eluted by methanol containing 0.1 N ammonium hydroxide (Fig. 2-(d), Table 1-D). Thus, the two unknown substances that are active only against Gram-positive bacteria are separated from fortimicins by IRC-50 (H^+) and HP-10 resins.

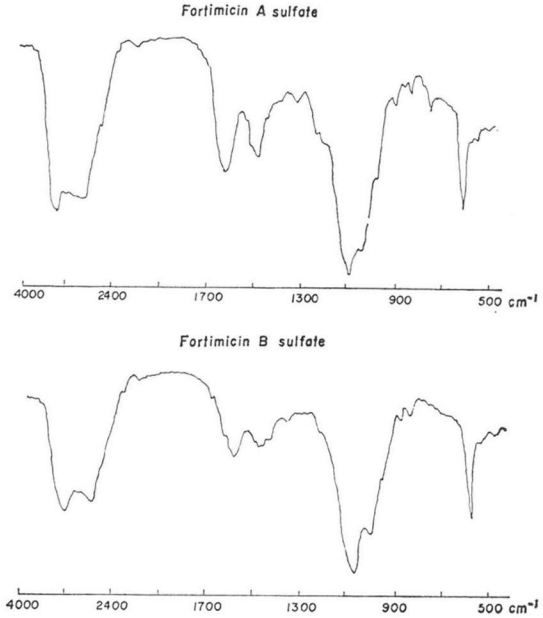
These unknown substances are removed simultaneously by using IRC-50 resin in ammonium cycle instead of H^+ cycle. Fortimicins are adsorbed on IRC-50 (NH_4^+) resin and eluted with 0.5 N NH_4OH, while the other com-

Table 3. pH and temperature stabilities of fortimicins A and B

Temperature	Fortimicin A		Fortimicin B	
	25°C	60°C	25°C	60°C
pH 2.0	95.8	63.4	97.8	96.4
pH 7.0	100	92.5	100	97.3
pH 10.0	69.8	38.1	95.1	93.2

One mg/ml solution of fortimicin A or B was kept for 4 hours in the conditions indicated. Residual activities assayed by the paper disc method on *B. subtilis* agar are shown as relative ratio to pH 7.0 at 25°C for 4 hours.

Fig. 4. Infrared spectra of fortimicins A and B sulfates in KBr tablets



ponents are not adsorbed on the resin and remain in the spent broth (Table 1-F and G).

Separation of Minor Components in Crude Fortimicins

In some conditions, *M.* sp. MK-70 produced many other minor components besides fortimicins A and B. These components were separated by silicic acid chromatography described in "Materials and Methods." The crude fortimicin powder was charged on the column and developed with the lower layer of the solvent mixture of chloroform - isopropanol - 17% ammonium hydroxide in a ratio of 2: 1: 1 (v/v). The eluates were separated into 10 fractions according to the antibacterial activities against *B. subtilis*. Each fraction was monitored by paper chromatography developed with the same solvent system. The elution pattern of this column chromatography is shown in Fig. 3. Thus, the crude powder of fortimicins contained at least ten components including fortimicins A and B. Fractions A and E (Fig. 3-(c)) correspond to

Fig. 5. Amino acids in the acid hydrolysate of fortimicins A and B

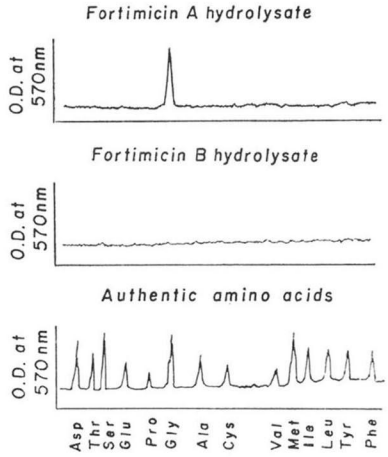


Table 4. Comparison between fortimicins A, B and other aminoglycoside antibiotics on paper chromatography

Antibiotics	Rf
Fortimicin A	0.43
Fortimicin B	0.70
Sagamicin, Antibiotic G-52	0.50
Gentamicin C_2, Verdamicin	0.42
Gentamicin C_1	0.62
Gentamicin C_{1a}, Sisomicin	0.19
Spectinomycin	0.41
Neomycins B, C, Neamine, Paromomycin, Paromamine, Kasugamycin, Hygromycin B, Kanamycins A, B, C, NK1001, NK1003, NK1012-1, NK1012-2, Lividomycins, A, B, D, Nebramycins factor 4,5′, 7, Apramycin, Tobramycin, Streptomycins, A, B, Hydroxy-, Bluensomycin, Antibiotic 460, Ribostamycin, Xylostasin, Antibiotic G-418, JI-20A, JI-20B, Gentamicins A_1, A_2, A_3, A_4, B, B_1, X, Seldomycins 1, 2, 3, 5, Butirosins A, B, Bu1709E_1, E_2, Bu1975C_1, C_2, Antibiotic 66-40D	0.00 ~0.02

Filter paper: Whatman No. 1 (1 cm × 40 cm)
Solvent system: Lower layer of $CHCl_3$ - CH_3OH - 17% NH_4OH (2: 1: 1, v/v)
Developed for 18 hours at 28°C
Bioautographed on *B. subtilis*

Fig. 6. Bioautograms of fortimicins A, B and related aminoglycoside antibiotics against sensitive [A] and resistant [B] *E. coli*

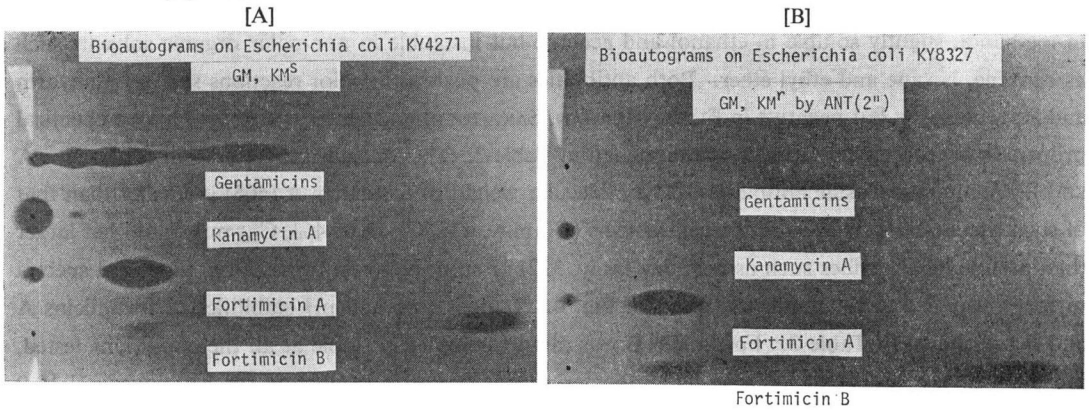


Fig. 7. Salting-out paper chromatography of fortimicins A, B and related aminoglycoside antibiotics.
Filter paper: Toyo No. 51 (0.5 cm×20 cm)
Developed in each solvent system for 2 hours at 28°C.
Bioautographed on *B. subtilis*

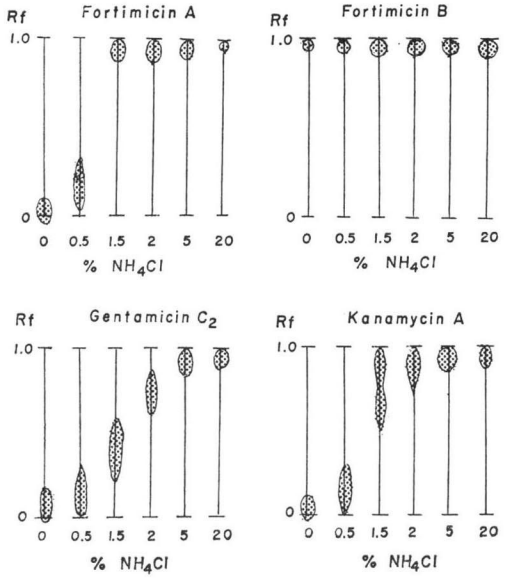


Fig. 8. Silica-gel thin-layer chromatogram of fortimicins A, B and related antibiotics.
Developed with 10% CH_3COONH_4 - CH_3OH(1 : 1, v/v) for 4 hours at 28°C
Bioautographed on *B. subtilis*

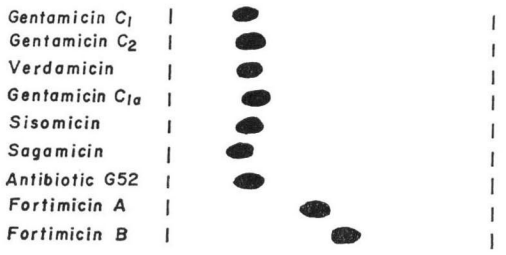
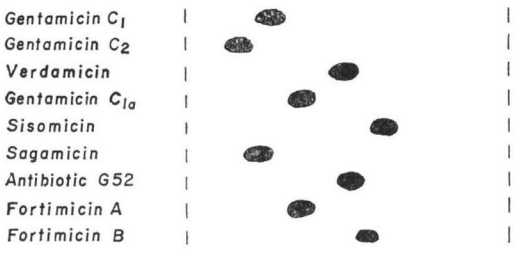


Fig. 9. Carbon thin-layer chromatogram of fortimicins A, B and related antibiotics.
Developed with 1/2 N H_2SO_4
Bioautographed on *B. subtilis*



fortimicin B and fortimicin A, respectively. Fractions B, C and D showed different Rf values from those of fortimicins A, B and gentamicins on paper or thin-layer chromatography. Fractions A, B, C, D and E exhibited antibacterial activities against *E. coli* KY 8327 which is resistant to gentamicins because of its aminoglycoside nucleotidyl transferase activity. Fractions F, G, H, I and J showed Rf values similar to certain components of the gentamicin C complex on paper and thin-layer chromatography. Among these minor components, some analogue derivatives of fortimicins A and B might be included.

Physico-chemical Properties of Fortimicins A and B

The free bases of fortimicins A and B are white amorphous powders freely soluble in water, soluble in methanol, slightly soluble in ethanol and acetone but insoluble in non-polar organic solvents such as benzene, hexane and ethyl ether. Both antibiotics are positive in color reactions such as ninhydrin and RYDON-SMITH but negative in ELSON-MORGAN, SAKAGUCHI and biuret reactions. Physico-chemical properties of fortimicins A and B are tabulated in Table 2. These results suggested that fortimicins A and B are aminoglycoside antibiotics. The molecular weight of fortimicin A (405) is smaller than that of such pseudotrisaccharides as the gentamicins (C_1, m.w. 477, C_2, m.w. 463, C_{1a}, m.w. 449) but larger than pseudodisaccharides such as neamine (m.w. 322) or spectinomycin (m.w. 332). Infrared spectra of fortimicin A and B sulfates are given in Fig. 4. Temperature and pH stabilities of fortimicins A and B are shown in Table 3. Fortimicin B was almost completely stable at all the conditions tested, but fortimicin A lost about 60% of its antibacterial activities after incubation for 4 hours at pH 10.0 and 60°C. TLC revealed the formation of fortimicin B from fortimicin A treated under such alkaline conditions.

Acid Hydrolysis of Fortimicins

The acid hydrolysate of fortimicin A was found to be devoid of antimicrobial activity. As shown in Fig. 5, glycine was detected in the acid hydrolysate of fortimicin A, while no amino acids were detected in the acid hydrolysate of fortimicin B. Quantitatively, one mole of glycine was released from fortimicin A by acid hydrolysis.

Chromatographic Behavior of Fortimicins A and B

The antimicrobial activities and physico-chemical properties suggest that fortimicins are aminoglycoside antibiotics. Therefore, fortimicins A and B were compared with known aminoglycoside antibiotics by paper and thin-layer chromatography. Table 4 shows Rf values of fortimicins A and B and other aminoglycoside antibiotics on paper chromatography developed with the lower layer of chloroform - methanol - 17% ammonium hydroxide (2: 1: 1, v/v). Most of the aminoglycoside antibiotics except gentamicin-related antibiotics and spectinomycin remained at Rf 0.0 in this chromatographic system. Fortimicin A showed the same Rf value as gentamicin C_2 and verdamicin, but fortimicin B moved farther than any of the other antibiotics. A pair of chromatographic paper strips developed under the same conditions were assayed by different microorganisms. Fig. 6-A shows the bioautograms obtained against *E. coli* KY 4271 which is sensitive to gentamicins, kanamycin and fortimicins, while Fig. 6-B shows the bioautograms against *E. coli* KY 8327, which is resistant to gentamicins and kanamycin due to its possession of aminoglycoside 2″-nucleotidyl transferase. Fortimicins A and B exhibited the same inhibition zones against both microorganisms, but gentamicins and kanamycin were remarkably inactivated by the latter test organism. Rf values of fortimicins A and B on paper chromatography developed with different concentrations of aqueous ammonium chloride between zero and 20% are shown in Fig. 7. Fortimicin A and gentamicin C_2 showed different Rf values at 1.5% and 2% ammonium chloride. Fortimicin B moved to the front at 0.5% ammonium chloride solution while the other antibiotics tested remained at lower Rf values at this concentration.

On silica-gel plates developed with 10% aqueous ammonium acetate - methanol (1: 1, v/v) both fortimicins A and B could be distinguished from all the gentamicin and sisomicin analogues (Fig. 8).

BRODASKY[36] reported on the usefulness of carbon thin-layer chromatography for separation of the components of the neomycin complex. KONDO *et al.*[37] also used carbon chromatography for separa-

tion of water-soluble basic antibiotics. We modified these methods as described in "Materials and methods". The results are shown in Fig. 9. Sisomicin, verdamicin and Antibiotic G-52 were not separated from gentamicin C_{1a}, C_2 and sagamicin, respectively, by paper chromatography (Table 4). Sisomicin, however, could be distinguished by its dark gray color reaction with ninhydrin, while gentamicin C_{1a} showed a purple spot[4]. However, carbon TLC (Fig. 9) clearly separates sisomicin from gentamicin C_{1a}. Good separations of verdamicin from gentamicin C_2 and Antibiotic G-52 from sagamicin can also be seen in this system. These data confirm the usefulness of this carbon thin-layer chromatography as an effective analytical method to differentiate gentamicin components from their 4′,5′-dehydro derivatives. Fortimicin A was also separated from gentamicin C_2 and verdamicin on this chromatography. Fortimicin B showed a different Rf value from that of gentamicin-sisomicin analogues.

These results showed that fortimicins A and B are not identical with any known antibiotics, suggesting they are new aminoglycoside antibiotics so far not described.

Structural and *in vitro* studies of these antibiotics will be reported in succeeding papers[38,39].

Acknowledgements

The authors are very grateful to Dr. A. C. SINCLAIR and his associates, in Abbott Labs., U.S.A., for their kind advices and encouragements. We are also very thankful to those who supplied us with valuable authentic antibiotics as reference samples.

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