FORTIMICINS C, D AND KE, NEW AMINOGLYCOSIDE ANTIBIOTICS

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(Received for publication October 13, 1978)

From the fermentation broth of *Micromonospora olivoasterospora* CS-26 that produced fortimicins A and B three new aminoglycoside antibiotics, fortimicins C, D and KE, were isolated. Fortimicins C and D exhibited potent, broad spectrum antibacterial activities against Gram-positive and negative bacteria, while fortimicin KE was only weakly active.

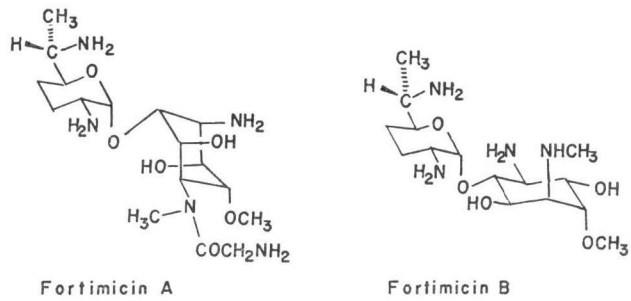
Micromonospora olivoasterospora was reported by NARA *et al.*[1,2] to produce fortimicins A and B in fermentation broth.

Fortimicins A and B are unique aminoglycoside antibiotics and their structures[3] are shown in Fig. 1. Fortimicin A exhibits broad antibacterial activities, and is not inactivated by many aminoglycoside inactivating enzymes[4].

Moreover, it was found by OKACHI *et al.*[2] that *M. olivoasterospora* elaborates other antibacterial compounds besides fortimicins A and B.

In the present paper we describe the isolation and some properties of these minor components.

Fig. 1. Structures of fortimicins A and B.



Materials and Methods

Microorganism

A mutant (CS-26) derived from *M. olivoasterospora* MK 70 and giving high yields of fortimicin was used throughout this investigation. The culture was maintained on an agar slant consisting of 0.4% yeast extract, 1.0% malt extract, 0.5% glucose and 2.0% agar.

Fermentation

The composition of the seed medium and the cultural conditions for the seed culture were the same as reported previously[2]. The production medium (pH 7.5 before sterilization) was composed of the following: 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% $CaCO_3$.

Fermentation was carried out in a 2,000-liter fermentor containing 1,000 liters of the production medium and cultivated for 7 days with agitation (120 r.p.m.) and aeration (400 liters/min.) at 30°C.

Quantitative Assay of Fortimicins

Each component of fortimicins was assayed by a chemial assay method reported previously[5].

Silica Gel Thin-Layer Chromatography

Thin-layer chromatography was performed at room temperature on thin-layer silica gel plate (E. Merck, 0.25 mm, 20×20 cm). The following solvent systems were used: (A) the lower layer of chloroform - methanol - 17% ammonium hydroxide (1: 1: 1); (B) isopropyl alcohol - chloroform - 17% ammonium hydroxide (2: 1: 1).

Preparation of Crude Fortimicin Mixture

A crude fortimicin mixture from the fermentation broth was prepared by a method reported previously[2].

Results and Discussion

Separation of Components in CS-26 Fermentation Broth

In the course of studies on the metabolites of the strain CS-26 it was found that some biologically active compounds accumulated in the culture broth. As can be seen in Fig. 2, these compounds were distinguished from fortimicins A and B. Among these compounds, designated fortimicins C, D and KE, the yield of fortimicin D was the largest.

Cultural Conditions for Fortimicin D Production

Although cultural conditions for fortimicin D production were similar to those for fortimicin A production, there were some differences between optimum conditions for fortimicin A production and those for fortimicin D production.

The addition of cobalt chloride at the concentration of 1×10^{-6}% gave better fortimicin A production than the control, while fortimicin D production was inhibited by the addition of cobalt ion (Table 1). This finding agrees with previous reports which demonstrated a regulatory effect of cobalt on gentamicin and fortimicin fermentations, as reported by Schering[6] and Kyowa Hakko workers[5,7,8], respectively.

On the other hand, the addition of mannitol had little effect on fortimicin A production, while fortimicin D production increased in a

Fig. 2. Silica gel thin-layer chromatogram of the fermentation broth of strain CS-26.
Silica gel plate, E. Merck, 0.25 mm, 20×20 cm. Solvent systems (A) and (B) as described in Materials and Methods. Detection, NBD chloride[5] and bioautography on *Bacillus subtilis*.

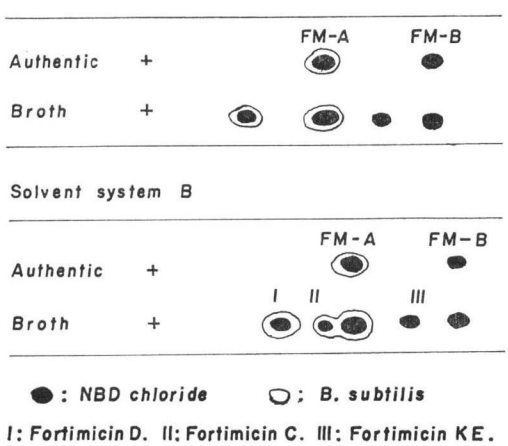


Table 1. Effects of cobalt ion and mannitol on the formation of fortimicins.

Additives	%	Fortimicins (mcg/ml)	
		A	D
$CoCl_2 \cdot 6H_2O$*	0	274	92
	10^{-6}	305	64
	10^{-5}	265	55
Mannitol**	0	289	52
	0.05	274	92
	0.1	285	62

* Production medium + 0.05% mannitol.
** Production medium.

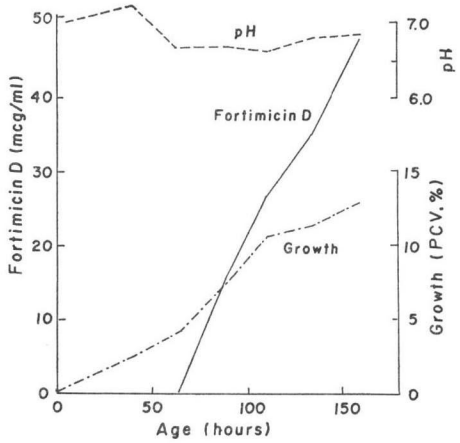
fermentation medium with 0.05% of mannitol.

Time Course of Fortimicin D Accumulation by the Strain CS-26

The time course of fortimicin D accumulation by the strain CS-26 in a 2,000-liter fermentor is shown in Fig. 3.

Fortimicin D began to be accumulated at 60 hours and 48 μg/ml of fortimicin D was accumulated at 160 hours. In this case, 150 μg/ml of fortimicin A and small amounts of fortimicins B, C and KE were produced together with fortimicin D.

Fig. 3. Time course of fortimicin D production by strain CS-26.
Fortimicin D was assayed by a chemical assay described in Materials and Methods. Growth (packed cell volume) was measured after centrifugation for 10 minutes at 3,000 rpm.



Isolation of Fortimicins C and D

A crude fortimicin mixture obtained from the fermentation broth was charged on an Amberlite CG-50 (NH_4^+ form) column. After washing with deionized water, elution was carried out with 0.11 N ammonium hydroxide containing 0.1 M ammonium chloride. Eluates were monitored by the thin-layer chromatography. The fractions containing fortimicins C and D were pooled separately.

After freeze-drying, the fortimicin C fraction was charged on a silica-gel column packed with the lower phase of a solvent mixture of chloroform - isopropyl alcohol - 17% ammonium hydroxide in a ratio of 2: 1: 1 (v/v) and eluted with the same solvent system. The fractions containing fortimicin C were collected and concentrated *in vacuo*. The concentrate was freeze-dried to give the free base of fortimicin C as a white amorphous powder.

The fortimicin D fraction obtained by Amberlite CG-50 column chromatography was adjusted to pH 6.5 with sulfuric acid and charged on an IRC 50 column (NH_4^+ form). After washing the column with deionized water, fortimicin D was eluted with 1 N ammonium hydroxide. The fraction containing fortimicin D was concentrated *in vacuo* and freeze-dried to give fortimicin D free base as a white amorphous powder.

Isolation of Fortimicin KE

The crude fortimicin mixture was charged on an Amberlite CG-50 (NH_4^+ form) column and eluted with 0.2 N ammonium hydroxide. As it was difficult on this column to separate fortimicin KE from fortimicin B, a silica-gel column chromatography followed. The silica-gel column charged fortimicin KE was eluted with a solvent mixture of chloroform - isopropyl alcohol - 17% ammonium hydroxide in a ratio of 2: 4: 1.

The fraction containing fortimicin KE was concentrated *in vacuo* and freeze-dried to give the free base of fortimicin KE as a white amorphous powder.

Chromatographic Behavior of Fortimicins C, D and KE

The Rf values of fortimicins C, D and KE in paper chromatography and silica gel thin-layer chromatography using various solvent systems are shown in Tables 2 and 3. Although fortimicins C, D and

Table 2. Rf values of fortimicins C, D and KE on paper chromatography.

Solvent systems	Rf values of			Period of development (hours)
	C	D	KE	
I	0.96	0.96	0.96	3
II	0.00	0.00	0.00	15
III	0.06	0.06	0.06	15
IV	0.00	0.00	0.00	4
V	0.04	0.04	0.04	15

I; 20% Ammonium chloride, II; Water-saturated 1-butanol, III; 1-Butanol - acetic acid - water (3: 1: 1), IV; Water-saturated ethyl acetate, V; Water-saturated 1-butanol containing 2% (w/v) *p*-toluenesulfonic acid and 2% (w/v) piperidine.

Table 3. Rf values of fortimicins A, B, C, D and KE on silica gel thin-layer chromatography.

Solvent systems	Rf values of				
	A	B	C	D	KE
I	0.74	0.80	0.75	0.74	0.78
II	0.37	0.62	0.40	0.37	0.58
III	0.47	0.62	0.44	0.40	0.54

I; The upper layer of chloroform - methanol - 17% ammonium hydroxide (2: 1: 1), II; 10% Ammonium acetate and methanol (1: 1), III; Isopropyl alcohol - methanol - 17% ammonium hydroxide (2: 1: 1).

KE were not separated on the paper chromatography tested in these studies (Table 2), on silica gel thin-layer plates developed with isopropyl alcohol - methanol - 17% ammonium hydroxide (2: 1: 1) or solvent (B) (see Materials and Methods) fortimicins A, B, C, D and KE had different Rf values.

Antibacterial Properties of Fortimicins C, D and KE

Table 4 shows the *in vitro* antibacterial activities of fortimicins C, D and KE compared with fortimicin A and kanamycin. Fortimicins C and D exhibit potent, broad spectrum antibacterial activity against Gram-positive and negative bacteria, while fortimicin KE is rather weakly active. Fortimicin C is less active than fortimicin A and kanamycin. Fortimicin D is equal in activity to fortimicin A and slightly superior to kanamycin.

As shown in the succeeding paper, fortimicin D is a 6′-demethylated derivative of fortimicin A, fortimicin KE is a 6′-demethylated derivative of fortimicin B and fortimicin C is a carbamoyl derivative of fortimicin A. These results confirm that glycine is important to the antibacterial activity of fortimicins[1], and suggest that the removal of a 6′-methyl residue from fortimicin A does not reduce the antibacterial activities.

Table 4. Antimicrobial spectra of fortimicins C, D and KE.

	MIC (mcg/ml)				
	FM-C	FM-D	FM-KE	FM-A	KM
Staphylococcus aureus ATCC6538P	0.33	0.02	1.65	0.04	0.08
Streptococcus faecalis ATCC10541	25	12.5	>100	12.5	50
Bacillus subtilis KY4273	0.16	0.02	>100	0.02	0.02
Escherichia coli ATCC26	1.3	0.32	25	0.16	0.32
Pseudomonas aeruginosa KY4276	5	5	>100	5	>100
Klebsiella pneumoniae ATCC10031	0.66	0.08	25	0.08	0.08
Salmonella typhosa ATCC9992	0.66	0.08	12.5	0.16	0.16
Serratia marcescens 177VA	0.16	0.08	>100	0.08	1.28
Providencia stuartii ATCC25826	0.64	0.32	>100	0.16	0.32
Shigella sonnei ATCC9290	2.6	0.16	25	0.32	0.32
Proteus vulgaris ATCC6897	0.32	0.16	25	0.16	0.32

Assay at pH 8.0, FM; Fortimicin, KM; Kanamycin.

Table 5. *In vitro* activities of fortimicins C and D against aminoglycoside-resistant organisms.

Test organisms	Inactivating enzymes	MIC (mcg/ml)			
		FM-C	FM-D	FM-A	KM
Escherichia coli KY8332	AAC(6′)-I	0.08	0.04	0.04	1.3
E. coli KY8348	AAC(3)-I	>20.8	>20.8	>20.8	0.04
E. coli KY8302	APH(3′)-1	0.64	0.32	0.16	>20.8
E. coli KY8327	ANT(2″)	1.28	0.64	0.32	>20.8
E. coli KY8321	ANT(2″) APH(3′)-I	0.32	0.16	0.08	>20.8
Serratia marcescens KY4248	AAC(6′)-I	1.28	0.64	0.32	>20.8
Providencia sp. KY8464	AAC(2′)-I	2.56	1.28	0.63	>20.8
Klebsiella pneumoniae KY4261	ANT(2″)	6.25*	3.12*	3.12*	>100*
Pseudomonas aeruginosa KY8510	AAC(6′)-IV	50*	50*	12.5*	>100*
Staphylococcus aureus KY8976	ANT(4′)	0.78*	0.39*	0.39*	50*
S. epidermidis KY4149	ANT(4′)	1.56*	0.78*	0.78*	50*

Assay at pH 8.0, * assay at pH 7.2.

Table 5 compares the activities of fortimicin C, fortimicin D, fortimicin A and kanamycin *in vitro* against resistant organisms known to possess various aminoglycoside inactivating enzymes. Both fortimicins C and D are found to exhibit the same antibacterial spectrum as fortimicin A against aminoglycoside-resistant clinical isolates. Although fortimicins C and D are inactivated by aminoglycoside 3-N-acetyltransferase Type I (AAC(3)-I), as reported for fortimicin A[9], they are not inactivated by the other aminoglycoside inactivating enzymes. It is interesting that fortimicins A, C and D are active against *Staphylococcus* possessing aminoglycoside 4′-nucleotidylating enzyme, such as *Staphylococcus aureus* KY8976 and *Staphylococcus epidermidis* KY4149, which are frequently isolated in clinics.

The LD_{50} in *dd*-mouse, weighing 20 ± 1 g and injected intravenously with 0.2 ml of fortimicin D sulfate solution, was 159 mg activity per kg body weight. The acute toxicity of fortimicin D is lower than other aminoglycoside antibiotics.

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