FLUVIRUCINS A_1, A_2, B_1, B_2, B_3, B_4 AND B_5, NEW ANTIBIOTICS ACTIVE AGAINST INFLUENZA A VIRUS

I. PRODUCTION, ISOLATION, CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

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(Received for publication January 14, 1991)

Five unidentified actinomycete strains produced a series of novel antiviral antibiotics which have a unique 2,6-dialkyl-10-ethyl-3(or 9)-hydroxy-13-tridecanelactam nucleus substituted with 3-amino-3,6-dideoxy-L-talose or 3-amino-3,6-dideoxy-L-mannose(L-mycosamine). The antibiotic components exhibited potent inhibitory activity against influenza virus type A Victoria strain infection in Madin Darby canine kidney cells by the cytopathic effect reduction assay.

In our screening of fermentation broths for antiviral activity, we have discovered a series of new structurally related antibiotics named fluvirucins A_1, A_2, B_1, B_2, B_3, B_4 and B_5 which showed *in vitro* inhibitory activity against influenza virus type A strain in Madin Darby canine kidney (MDCK) cells.

All seven fluvirucin components are weakly basic to neutral lipophilic substances which could be recovered from the fermentation broth by solvent extraction and effectively purified by chromatography. They were obtained as colorless crystals with similar physico-chemical and spectral properties. The antibiotics have inhibitory activity against certain Gram-positive bacteria, anaerobic bacteria and fungi in addition to strong activity against influenza A virus. As detailed in the following papers[1,2], the antibiotics have novel structures with a similar 2,6-dialkyl-10-ethyl-3(or 9)-hydroxy-13-tridecanelactam nucleus to which is attached 3-amino-3,6-dideoxy-L-talose or its 4-*epi* analog. Herein are presented the production, isolation, physico-chemical properties and biological activities of fluvirucins A_1, A_2, B_1, B_2, B_3, B_4 and B_5.

Taxonomy

The preliminary taxonomical studies showed the fluvirucin-producing strains Q464-31, R869-90, R359-5, R516-16 and L407-5 to be actinomycetes, which may be classified as *Actinomadura* or more possibly "*Microtetraspora*"[3]. Detailed taxonomical studies are in progress and the results will be reported later.

Fermentation

Fluvirucin A_1

A well grown agar slant of strain No. Q464-31 was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of the seed medium shown in Table 1 with pH 7.0 before sterilization. The seed flask was incubated for 3 days at 32°C on a rotary shaker (200 rpm) and 5 ml portions of the culture were transferred into 500-ml Erlenmeyer flasks containing 100 ml of fermentation medium with the same

Table 1. Composition of fermentation media.

	Fluvirucin A_1	Fluvirucin A_2	Fluvirucin B_1	Fluvirucins B_2, B_3, B_4, B_5	Fluvirucin B_2
Strain	Q464-31	R869-90	R359-5	R516-16	L407-5
Seed medium	Soluble starch 2.0%, Pharmamedia 1.0%, $ZnSO_4 \cdot 7H_2O$ 0.003%, $CaCO_3$ 0.4%	Same as Q464-31	Mannitol 2.0%, fish meal 1.0%, $CaCO_3$ 0.5%	Glucose 3.0%, soybean meal 2.0%, distiller's solubles 1.5%, fish meat extract 0.2%, $CaCO_3$ 1.0%	Same as R516-16

composition as the seed medium. Fermentation was carried out for 6 days at 28°C on a rotary shaker at 200 rpm. For scale-up fermentation, a stir-jar fermenter was used; 500 ml of the seed culture prepared by flask fermentation was inoculated into a 20-liter stir-jar fermenter containing 12 liters of the fermentation medium. The fermenter was operated with agitation at 250 rpm and aeration rate 12 liters per minute at 28°C. Antibiotic production in the fermentation broth was monitored by bioassays using influenza A virus and *Candida albicans* A9540. After 113-hour of fermentation, antibiotic production reached maximum potency.

Fermentation of the other four strains was performed using the fermentation media shown in Table 1 and conditions as used for fluvirucin A_1.

Extraction and Purification

Fluvirucin A_1

The harvested broth (Q464-31, 50 liters) was stirred with butanol (20 liters) for 30 minutes. The organic layer was separated from the aqueous layer and mycelial mass by use of a Sharples centrifuge and concentrated to 0.5 liter under reduced pressure. This was added dropwise to 3 liters of *n*-hexane to deposit the active precipitate which was collected by centrifugation. The brown solid obtained (11 g) was charged on a column of silica gel (E. Merck, Darmstadt, Silica gel 60, No. 9385, 40 × 380 mm) which was developed with ethyl acetate-methanol (7:3) and then with a 4:6 mixture of methanol and the lower phase of a methylene chloride-28% ammonia (10:1) mixture. The eluate was collected in fractions and examined by bioassay against influenza A virus and TLC on silica gel (E. Merck, Silica gel 60 F_{254}, No. 5715, 3:7 mixture of methanol and the lower phase of chloroform-28% ammonia (10:1)). The appropriate fractions (TLC, Rf 0.42) were pooled, concentrated *in vacuo* and then applied to a silica gel column (Wako Pure Chemical Industries, Ltd., Wakogel C-300, 40 × 320 mm). Elution was carried out first with a mixture of lower phase of methylene chloride-28% ammonia (9:1) mixed in 19:1 ratio with methanol, and then with the mixture of 9:1 ratio. The colorless needles deposited in several of the most bio-active fractions were collected by filtration (146 mg). The combined mother liquor and the side fractions containing homogeneous fluvirucin A_1 were concentrated *in vacuo* and the concentrate was added dropwise into methanol. The pale yellow precipitate deposited (2.24 g) was collected by filtration and crystallized from methanol as colorless needles.

Fluvirucin A_2

The activity was recovered from the fermentation broth (R869-90, 2.5 liters) by butanol extraction (1.5 liters). The crude solid (0.9 g) obtained was chromatographed on silica gel followed by Sephadex

LH-20 (Pharmacia Fine Chemical, 22 × 720 mm, methanol) to afford pure solid fluvirucin A_2 (65 mg). This was crystallized from methanol to give colorless needles (46 mg).

Fluvirucin B_1

Isolation and purification of fluvirucin B_1 were performed by a similar procedure as used for fluvirucin A_1. The whole broth (R359-5, 2.6 liters) was extracted with 1.7 liters of butanol and the crude active solid (1.60 g) was purified twice by the silica gel column chromatography using the same solvent as used of fluvirucin A_1. The final purification was carried out by Sephadex LH-20 column (22 × 680 mm, methanol) and crystallization from methanol (86 mg).

Fluvirucins B_2, B_3, B_4 and B_5

Fermentation was carried out using strain R516-16 (2.5 liters). The crude extract (2.25 g) was chromatographed on a silica gel column (30 × 420 mm) developed with mixtures of the lower phase of methylene chloride - 28% ammonia (10 : 1) and methanol with stepwise increases in methanol concentration. After TLC examination of the active eluate (SiO_2, 9 parts of the lower layer of chloroform - 28% ammonia (9 : 1) to 1 part-methanol, fluvirucins B_2, Rf 0.12; B_3, 0.20; B_4, 0.46 and B_5, 0.53), the appropriate fractions were pooled and concentrated to yield four components (fluvirucins B_2, 342 mg; B_3, 67 mg; B_4, 138 mg and $B_4 + B_5$ mixture, 857 mg). Fluvirucin B_2 fraction (340 mg) was further chromatographed on silica gel with the above solvent mixture (methanol 5%) and then on Sephadex LH-20 with methanol to afford homogeneous fluvirucin B_2 (244 mg) as a white solid. Subsequent crystallization from methanol gave colorless needles (48 mg). The crude fluvirucin B_3 (65 mg) was purified by a similar chromatographic procedure (silica gel chromatography, methanol content 3%) and crystallized from methanol to give colorless needles (10 mg). Purification of fluvirucin B_4 (137 mg) was carried out by silica gel (20 × 460 mm, benzene - methanol (95 : 5)) and subsequent Sephadex LH-20 (methanol) chromatography. Crystallization of the purified solid (75 mg) from methanol yielded colorless needles (34 mg). Homogeneous fluvirucin B_5 (204 mg) was obtained by silica gel (benzene - methanol, 95 : 5) purification of the above fluvirucins B_4 and B_5 mixture (795 mg). A part of the sample (27 mg) was further purified by Sephadex LH-20 chromatography and crystallization (7 mg). Strain L407-5 was found to produce only fluvirucin B_2. Thus, from 20 liters of the fermentation broth, colorless needles of pure fluvirucin B_2 (650 mg) were isolated by the work-up described above.

Physico-chemical Properties

Fluvirucins A_1, A_2, B_1, B_2, B_3 and B_4 were obtained as colorless needles and fluvirucin B_5 as colorless rods by crystallization from methanol. Fluvirucins A_1, A_2, B_1, B_2 and B_3 are soluble in chloroform - methanol (1 : 1) mixture, slightly soluble in lower alcohols, chloroform, dimethyl sulfoxide, ethyl acetate, pyridine and acidic water, but insoluble in *n*-hexane and water. Fluvirucins B_4 and B_5 are soluble in lower alcohols, pyridine, ethyl acetate, but insoluble in *n*-hexane and water. All these components gave positive responses to iodine vapor, sulfuric acid, anthrone and ninhydrin (except fluvirucins B_4 and B_5) tests, but negative responses to ferric chloride and Sakaguchi tests. Their molecular formulae were assigned as shown in Table 2 based on their FAB-MS and elemental analyses. Weak UV absorption maxima were observed at 246, 252, 258, 264 and 268 nm for fluvirucins B_4 and B_5, while the other compounds showed only end absorption. The IR and ^1H NMR spectra of fluvirucins A_1 and B_1 are

Fig. 1. IR spectrum of fluvirucin A_1 (in KBr).

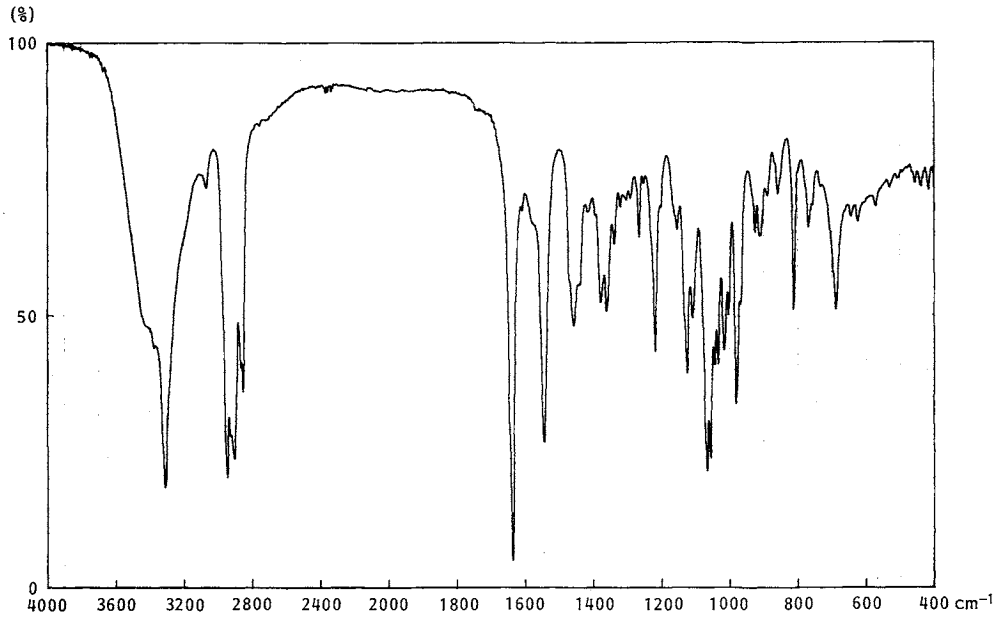
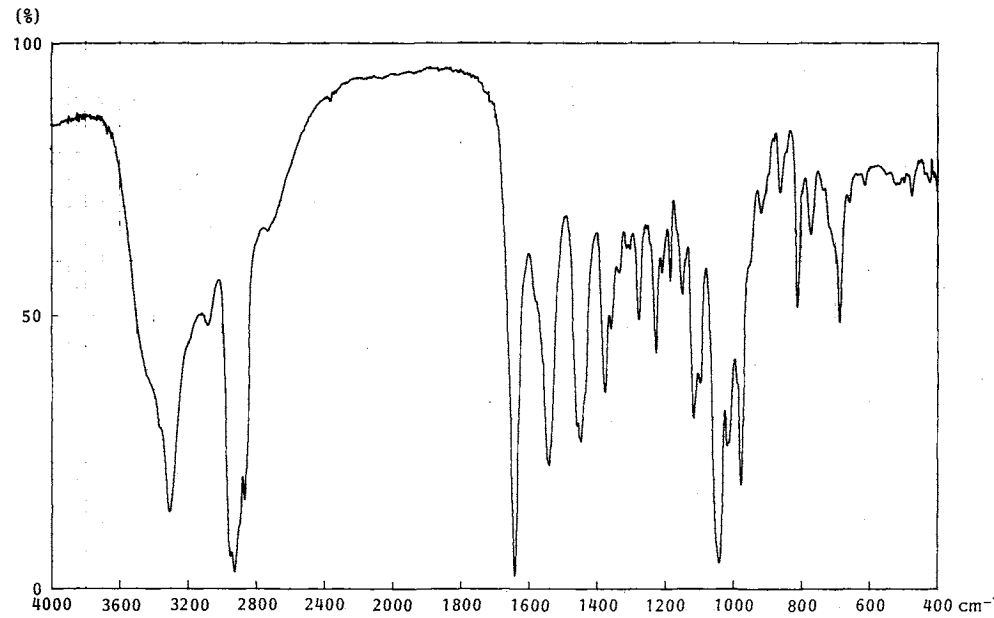


Fig. 2. IR spectrum of fluvirucin B_1 (in KBr).



illustrated in Figs. 1~4. Their IR spectra exhibited in common the presence of amide bands at around 1640 and 1550 cm^{-1}, and of sugar absorptions at 1130~980 cm^{-1}.

The physico-chemical properties of the antibiotics are summarized in Table 2. The physico-chemical and spectral data of fluvirucin B_2 are nearly identical with those reported for antibiotic AB-85[4]. A direct comparison has not been performed because an authentic sample of AB-85 was not available.

Fig. 3. ^1H NMR spectrum of fluvirucin A_1 (400 MHz, in $CDCl_3$-CD_3OD, 1:1).

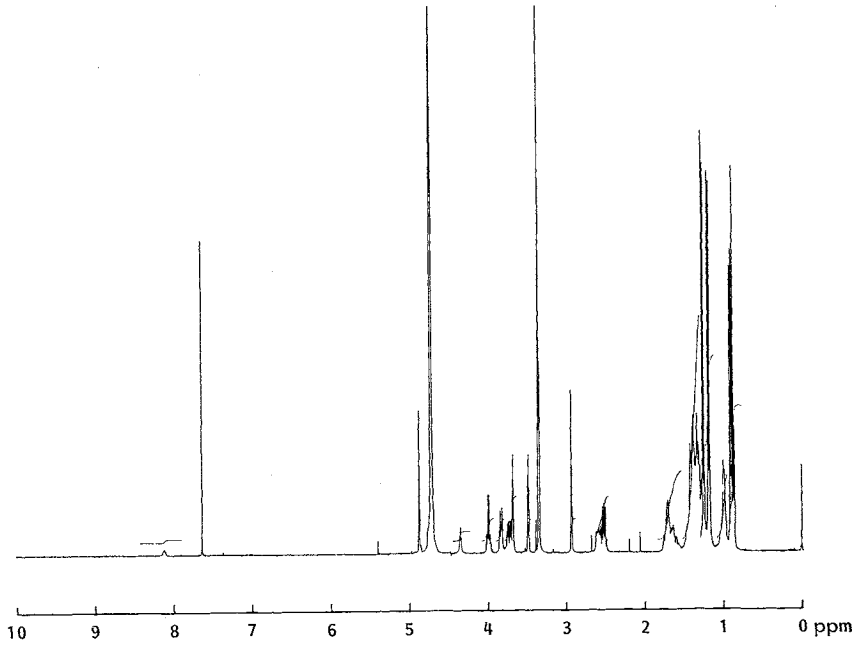
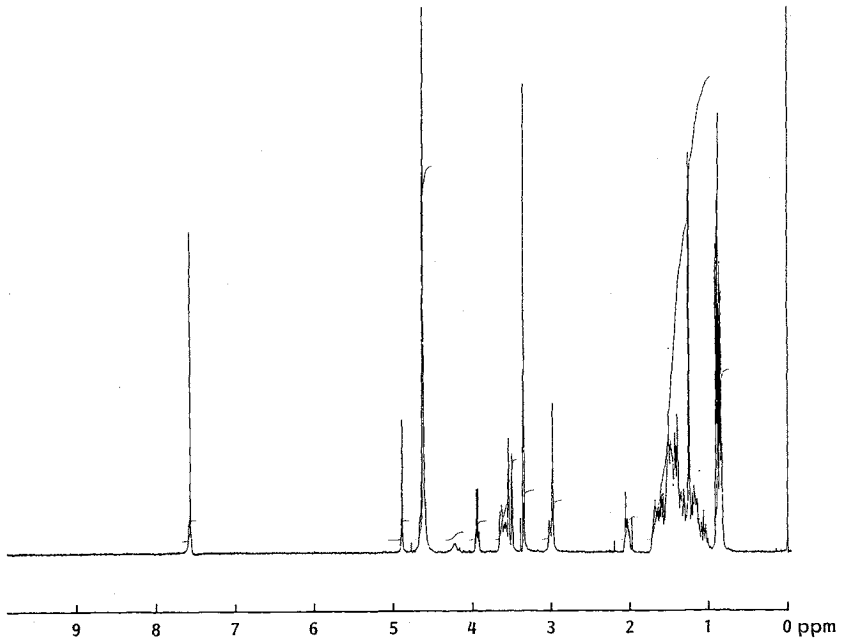


Fig. 4. ^1H NMR spectrum of fluvirucin B_1 (400 MHz, in $CDCl_3$-CD_3OD, 1:1).



Antimicrobial Activity

The MICs of fluvirucins A_1, A_2, B_1, B_2, B_3, B_4 and B_5 against various microorganisms were determined by the serial 2-fold dilution method with overnight incubation at 37°C. Nutrient agar medium was used for Gram-positive and Gram-negative bacteria, GAM agar medium for anaerobic bacteria and Sabouraud

Table 2. Physico-chemical properties.

	Fluvirucin A_1	Fluvirucin A_2	Fluvirucin B_1	Fluvirucin B_2
Nature	Colorless needles	Colorless needles	Colorless needles	Colorless needles
MP (°C)	275~277	261~263	262~263	261~263
Microanalysis				
Calcd for	$C_{23}H_{44}N_2O_5$: C 64.45, H 10.35, N 6.54	$C_{24}H_{46}N_2O_6 \cdot \frac{1}{2}H_2O$: C 61.60, H 10.13, N 5.99	$C_{24}H_{46}N_2O_5$: C 65.12, H 10.48, N 6.33	$C_{25}H_{48}N_2O_5$: C 65.75, H 10.59, N 6.13
Found:	C 64.12, H 10.37, N 6.42	C 61.80, H 10.11, N 5.93	C 64.95, H 10.51, N 6.54	C 65.49, H 10.67, N 6.01
FAB-MS (m/z) $(M+H)^+$	429	459	443	457
UV λ_{max}^{MeOH} nm (ε)	End absorption	End absorption	End absorption	End absorption
TLC[a] (Rf)				
I	0.42	0.30	0.57	0.51
II	0.08	0.05	0.19	0.12

Table 2. (Continued)

	Fluvirucin B_3	Fluvirucin B_4	Fluvirucin B_5
Nature	Colorless needles	Colorless needles	Colorless rods
MP (°C)	263~266	>245 (dec)	>210 (dec)
Microanalysis			
Calcd for	$C_{25}H_{48}N_2O_5$: C 65.75, H 10.59, N 6.13	$C_{34}H_{57}N_3O_6$: C 67.63, H 9.51, N 6.96	$C_{34}H_{57}N_3O_6$: C 67.63, H 9.51, N 6.96
Found:	C 65.60, H 10.72, N 5.99	C 67.72, H 9.63, N 6.91	C 67.33, H 9.56, N 6.68
FAB-MS (m/z) $(M+H)^+$	457	604	604
UV λ_{max}^{MeOH} nm (ε)	End absorption	246 (810), 252 (810), 258 (800), 264 (sh, 680), 268 (sh, 610)	246 (800), 252 (810), 258 (800), 264 (sh, 680), 268 (sh, 600)
TLC[a] (Rf)			
I	0.59	0.80	0.80
II	0.20	0.46	0.53

[a] SiO_2. I: Lower layer of $CHCl_3$-28% NH_4OH (9:1)-MeOH (7:3), II: lower layer of $CHCl_3$-28% NH_4OH (9:1)-MeOH (9:1).

dextrose agar medium for fungi. As shown in Table 3, fluvirucins B_1, B_2 and B_3 showed good inhibitory activity against Gram-positive bacteria, *Bacteroides fragilis*, and certain yeasts, while fluvirucins A_1 and A_2 showed considerably lower activity and fluvirucins B_4 and B_5 no activity to these organisms. They did not exhibit activity against Gram-negative bacteria, Gram-positive anaerobic bacteria and filamentous fungi.

Antiviral Activity

In vitro antiviral activities of fluvirucins A_1, A_2, B_1, B_2, B_3, B_4 and B_5 were assessed by the dye uptake assay method using the influenza virus type A (Victoria strain)-MDCK cell system[5]. The cell suspension (200 μl) containing 2×10^4 cells was inoculated to each well of 96-well microplates and cultured at 37°C for 48~72 hours under humidified 5% CO_2-95% air environment. The growth medium in each well was replaced with 250 μl of fresh medium (Eagle minimum essential medium without serum) containing a test compound at various doses, and then 50 μl medium containing approximately $10 \times 50\%$ tissue culture infectious dose ($TCID_{50}$) of virus was added to each well. For cytotoxicity tests, the same set of wells

Table 3. Antibacterial activity.

Test organism	MIC (μg/ml)						
	Fluvirucin						
	A_1	A_2	B_1	B_2	B_3	B_4	B_5
Staphylococcus aureus FDA 209P	50	50	3.1	3.1	3.1	>100	>100
S. aureus Smith	100	100	3.1	3.1	3.1	>100	>100
S. epidermidis D153	12.5	3.1	0.8	1.6	0.8	>100	>100
Streptococcus faecalis A9612	>100	>100	>100	>100	>100	>100	>100
Micrococcus luteus No. 1001	6.3	3.1	0.8	3.1	1.6	>100	>100
Bacillus subtilis PCI 219	6.3	12.5	0.8	3.1	3.1	>100	>100
Escherichia coli NIHJ	>100	>100	>100	>100	>100	>100	>100
Enterobacter cloacae A9659	>100	>100	>100	>100	>100	>100	>100
Klebsiella pneumoniae D11	>100	>100	>100	>100	>100	>100	>100
Pseudomonas aeruginosa A9930	>100	>100	>100	>100	>100	>100	>100
Clostridium difficile A21675	>100	>100	>100	>100	>100	>100	>100
C. perfringens A9635	>100	>100	>100	>100	>100	>100	>100
Bacteroides fragilis A22693	6.3	6.3	3.1	1.6	0.8	>100	>100
Candida albicans IAM 4888	>100	12.5	1.6	1.6	>100	>100	>100
Cryptococcus neoformans D49	25	50	1.6	1.6	100	>100	>100
Aspergillus fumigatus IAM 2530	>100	>100	>100	>100	>100	>100	>100
Trichophyton mentagrophytes D155	>100	>100	>100	>100	>100	>100	>100

without virus were prepared. After 72 hours of incubation, the degrees of inhibition of the virus-induced cytopathic effect and the drug-induced cytotoxicity were determined. ID_{50} was expressed as the concentration showing 50% inhibition of the cytopathic effect of control, and 50% toxic dose (TD_{50}) the concentration exhibiting 50% cytotoxicity against MDCK cells without viral infection. Ribavirin was used as a reference compound.

Table 4. Anti-influenza virus activity of fluvirucins components.

Compound	Influenza virus-MDCK cells	
	ID_{50} (μg/ml)	TD_{50} (μg/ml)
Fluvirucin A_1	4.6	33.1
Fluvirucin A_2	4.3	31.6
Fluvirucin B_1	2.3	17.9
Fluvirucin B_2	9.9	31.5
Fluvirucin B_3	4.6	38.6
Fluvirucin B_4	19.8	36.4
Fluvirucin B_5	>50	>50
Ribavirin	10	>100

The results are shown in Table 4. Fluvirucins A_1, A_2, B_1, B_2 and B_3 exhibited antiviral activity against influenza virus with ID_{50} values of 2~10 μg/ml by the assay. Fluvirucin B_4 showed very weak activity and fluvirucin B_5 no antiviral activity. Fluvirucin B_1 was twice as potent against the influenza virus (ID_{50} 2.3 μg/ml) as the best of the other four compounds, but it also showed 2-fold stronger cytotoxicity. Fluvirucins A_1, A_2 and B_3 exhibited almost the same antiviral activity, while the antiviral activity of fluvirucin B_2 was somewhat weaker.

Acknowledgments

The authors wish to express their thanks to Dr. H. KAWAGUCHI for his valuable suggestions and encouragement. Their thanks are also due to the members of the fermentation and evaluation groups for their excellent technical

assistance.

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