NEW ANTIVIRAL ANTIBIOTICS, CYCLOVIRACINS B_1 AND B_2[†]

I. PRODUCTION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITY

MITSUAKI TSUNAKAWA, NOBUJIRO KOMIYAMA, OSAMU TENMYO, KOJI TOMITA, KIMIO KAWANO, CHIKAKO KOTAKE, MASATAKA KONISHI and TOSHIKAZU OKI

Bristol-Myers Squibb Research Institute,
2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan

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Kibdelosporangium albatum No. R761-7 (ATCC 55061) produced new antiviral antibiotics, cycloviracins B_1 and B_2. They show weak activity against Gram-positive bacteria and potent antiviral activity against herpes simplex virus type 1. Unique acylsaccharide structures were established for cycloviracins B_1 and B_2 by degradation and spectroscopic analysis.

In our continuing efforts to discover novel bioactivities in microbial metabolites, a new actinomycete strain isolated from a soil sample collected in Mindanao Island, the Philippines was found to produce a complex of new antiviral antibiotics, cycloviracin. The antibiotics were extracted from the fermentation broth with 1-butanol and purified by column chromatography. Two major components cycloviracins B_1 and B_2 have been isolated from the complex. They showed weak antimicrobial activity against Gram-positive bacteria and also antiviral activity against herpes simplex virus type 1 by both the plaque reduction assay and the dye uptake assay. This paper reports the fermentation, isolation, physico-chemical and biological properties of the antibiotics. The structure determination is reported in a separate paper[1].

Production

A small agar piece of the slant culture of *Kibdelosporangium albatum* sp. nov., strain R761-7[2] was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium consisting of mashed potato 4%, corn steep liquor 2%, $CaCO_3$ 0.3% and NaCl 0.2% (the pH was adjusted to 8.0 before autoclaving). The seed culture was incubated at 28°C for 4 days on a rotary shaker (200 rpm) and 5 ml of the culture was transferred into a 500-ml Erlenmeyer flask containing 100 ml of the production medium having the same composition as the seed medium. The fermentation was carried out at 28°C for 6 days on a rotary shaker. The antibiotic production in the fermentation broth was monitored by the conventional cytopathic effect (CPE) assay using herpes simplex virus type 1 (KOS strain). The production reached a maximum after 4 to 5 days fermentation, which showed the antiviral activity up to 48-fold broth dilution in terms of IC_{50} value.

Extraction and Purification

The fermentation broth (20 liters, 50~100 μg/ml) was stirred vigorously with 1-butanol (8 liters) for 30 minutes. The 1-butanol extract was concentrated *in vacuo* to dryness and the residue (9.0 g) was mixed with silica gel (25 g) to adsorb the activity. This mixture was loaded on the top of a silica gel column

[†] Cycloviracins B_1 and B_2 were originally called BU-4224V B_1 and B_2, respectively.

(Wakogel C-200, 1.1 liters) which was developed with ethyl acetate - methanol - water (100 : 15 : 1) mixture. The eluate was collected in fractions (20 ml) and each fraction was monitored by the antiviral assay and TLC (SiO_2; EtOAc - MeOH - H_2O, 10 : 3 : 1, H_2SO_4 detection). The main active fractions were combined and concentrated *in vacuo* to give a crude mixture solid of cycloviracin (1.0 g).

The solid (1.0 g) was dissolved in methanol (5 ml) and applied on a reversed phase C_{18} column (YMC-ODS, AM type, Yamamura Chem. Lab. Co., Ltd., 800 ml). The column was developed with a 0.022 M phosphate buffer solution (pH 7.0) containing 45% acetonitrile (1.5 liters) and then with the buffer containing 50% acetonitrile (fr. 1~65). The presence of the antibiotics was detected by TLC (RP-18,

Table 1. Physico-chemical properties of cycloviracins B_1 and B_2.

	Cycloviracin B_1	Cycloviracin B_2
Nature	White amorphous powder	White amorphous powder
MP	84~85°C	82~83°C
$[\alpha]_D^{26}$ (c 0.5, MeOH)	−15.6°C	−16.1°C
Negative FAB-MS (m/z)	1,675 $(M-1)^-$	1,673 $(M-1)^-$
MW	1,676	1,674
Elemental analysis	$C_{83}H_{152}O_{33}\cdot 2H_2O$ Calcd Found C 58.18 58.03 H 9.11 8.99	$C_{83}H_{150}O_{33}\cdot 2H_2O$ Calcd Found C 58.24 58.15 H 9.00 8.91
IR (KBr) cm^{-1}	3410, 2930, 2850, 1740, 1640, 1470, 1370, 1200~1000	3430, 2930, 2850, 1735, 1720 (sh), 1630, 1470, 1200~1000
TLC, SiO_2 (EtOAc - MeOH - H_2O = 10 : 3 : 1)	Rf 0.47	0.47
TLC, RP-18 (Merck: CH_3CN - 0.022 M phosphate buffer, pH 7.0, 70 : 30)	Rf 0.30	0.33
HPLC (YMC - Pack D-ODS-5, CH_3CN - 0.022 M phosphate buffer, pH 7.0, 60 : 40)	Rt 8.2 minutes	9.2 minutes

Fig. 1. IR spectrum of cycloviracin B_1 (KBr).

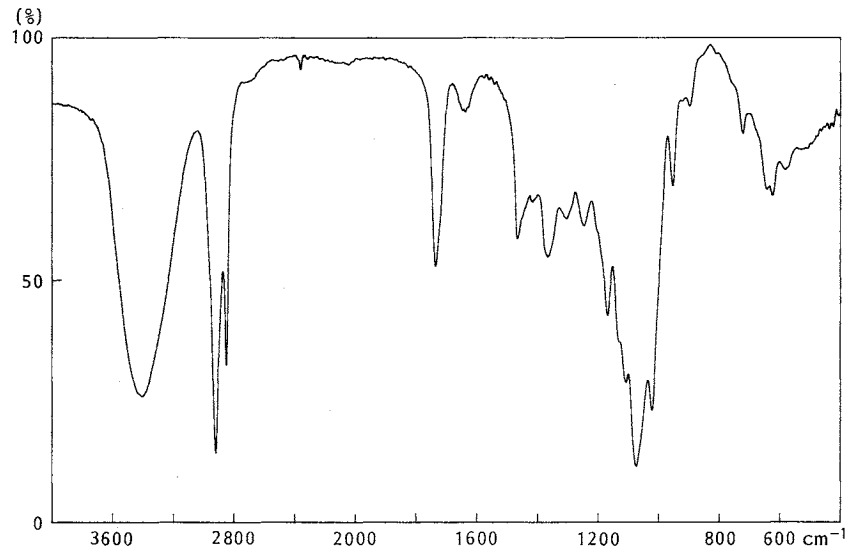


Fig. 2. ^{1}H NMR spectrum of cycloviracin B_1 (400 MHz, pyridine-d_5).

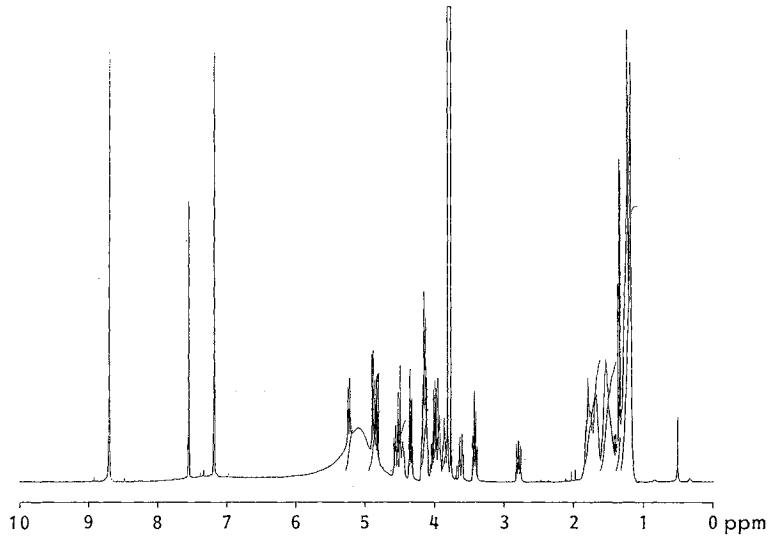
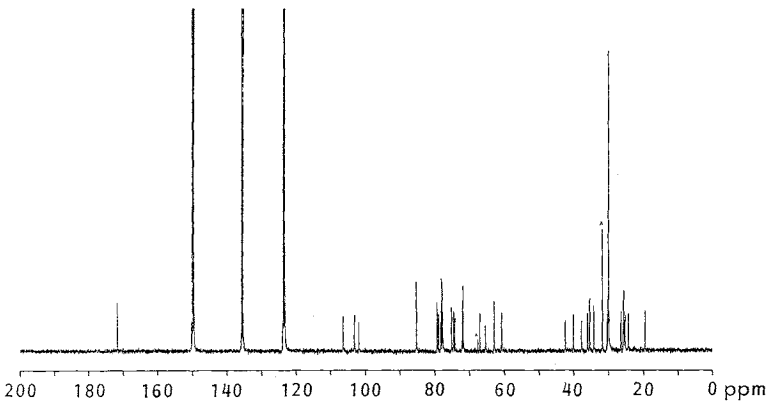


Fig. 3. ^{13}C NMR spectrum of cycloviracin B_1 (100 MHz, pyridine-d_5).



Merck: CH_3CN - 0.022 M phosphate buffer, pH 7.0, 70 : 30). Fraction Nos. 35 ~ 52 (Rf 0.32) were pooled, concentrated and extracted with 1-butanol. The 1-butanol extract was evaporated *in vacuo* to afford a white powder of cycloviracin B mixture (330 mg) which contained two main components, cycloviracins B_1 and B_2 by HPLC. Separation of the two components (140 mg) was carried out by preparative HPLC (Column: YMC-Pack D-ODS-5, Yamamura Chem. Lab. Co., Ltd., 20 i.d. × 250 mm, mobile phase: CH_3CN - 0.01 M phosphate buffer, pH 7.0, 54 : 46, detection: UV 210 nm). The first peak cuts containing cycloviracin B_1 were combined and concentrated. The aqueous concentrate was extracted with 1-butanol and the extract was evaporated to yield a white solid of cycloviracin B_1 (54 mg). This was chromatographed on a column of Sephadex LH-20 (200 ml) eluting with 90% aqueous methanol to afford a pure sample of cycloviracin B_1 (48 mg). The second peak cuts containing component B_2 were worked up by a similar manner to yield a pure solid of cycloviracin B_2 (49 mg).

Physico-chemical Properties

Cycloviracins B_1 and B_2 were soluble in methanol, pyridine and dimethyl sulfoxide, slightly soluble in ethyl acetate and acetone but practically insoluble in *n*-hexane, chloroform and water. They showed positive reactions to iodine and anthrone reagent but were negative to ninhydrin and Sakaguchi tests. The physico-chemical properties of cycloviracins B_1 and B_2 are summarized in Table 1. Both compounds showed no absorption maxima above 210 nm in the UV spectra. The molecular formulae of cycloviracins B_1 and B_2 were determined to be $C_{83}H_{152}O_{33}$ and $C_{83}H_{150}O_{33}$, respectively, based on the microanalysis and negative FAB-MS data. The IR spectra of cycloviracins B_1 (Fig. 1) and B_2 are similar, showing characteristic bands of hydroxyl at around 3400 and ester at 1740 cm^{-1}. The ^1H and ^{13}C NMR spectra of cycloviracin B_1 were shown in Figs. 2 and 3. The structures of cycloviracins B_1 and B_2 have been determined by chemical and spectroscopic methods[1].

Antiviral Activity

Antiviral activity of cycloviracins B_1 and B_2 was evaluated by the dye uptake[3] and the plaque reduction assay using herpes simplex virus (HSV) type 1 (KOS strain) infection in Vero cells. In the dye uptake assay, 200 μl of the Vero cell suspension containing 1.6×10^4 cells was poured into each well of 96-well microplates, and then 50 μl of medium containing a test compound at various concentrations was added to each well. The viral suspension (50 μl) containing approximately $30 \times TCID_{50}$ was inoculated to each well. For cytotoxicity tests, the same set of wells without viruses was prepared. After incubation at 37°C for 72 hours under the humidified 5% CO_2-95% air environment, the degree of inhibition of the virus-induced cytopathic effect and

Table 2. Anti-HSV activity of cycloviracins B_1 and B_2.

	HSV-Vero cells			
	Dye uptake assay		Plaque reduction assay	
	ID_{50} (μg/ml)	TD_{50} (μg/ml)	ID_{50} (μg/ml)	MTD[a] (μg/ml)
Cycloviracin B_1	4.9	>400	10.9	>200
Cycloviracin B_2	5.0	>400	9.3	>200
Acyclovir	0.09	>100	0.27	>100

[a] MTD: Minimal toxic dose.

Table 3. Antibacterial activity of cycloviracins B_1 and B_2.

Organism		MIC (μg/ml)	
		Cycloviracin B_1	Cycloviracin B_2
Escherichia coli	NIHJ	>100	>100
Klebsiella pneumoniae	D11	>100	>100
Pseudomonas aeruginosa	A9930	>100	>100
Proteus vulgaris	A9436	>100	>100
Staphylococcus aureus	FDA 209P	12.5	50
S. aureus	Smith	12.5	100
S. aureus	D136	25	>100
S. aureus	A15097	25	>100
S. epidermidis	D153	3.1	6.3
Streptococcus faecalis	A9612	>100	>100
Micrococcus luteus	1001	>100	>100
Bacillus subtilis	PCI219	3.1	6.3
Candida albicans	IAM 4888	>100	>100
Cryptococcus neoformans	IAM 4514	>100	>100
Aspergillus fumigatus	IAM 2034	>100	>100
Trichophyton mentagrophytes	D155	>100	>100

the drug-induced cytotoxicity were determined by means of the uptake of neutral red. ID_{50} was expressed as the concentration showing the 50% inhibition of the cytopathic effect of control, and TD_{50} was the concentration exhibiting the 50% cytotoxicity against Vero cells without viral infection. Acyclovir was used as a reference compound of anti-HSV activity. The antiviral activity of cycloviracins B_1 and B_2 was also evaluated by the conventional plaque reduction assay method using a 24-well microplate.

The results are shown in Table 2. Both components demonstrated a potent antiviral activity against HSV-1 with ID_{50} values of 5 μg/ml by the dye uptake assay, but its antiviral activity was less potent than that of acyclovir. In the plaque reduction assay, cycloviracins showed somewhat weaker antiviral activity than that in the dye uptake assay.

Antimicrobial Activity

The antimicrobial spectra of cycloviracins B_1 and B_2 against various bacteria and fungi are shown in Table 3. MICs were determined by the agar dilution method using nutrient agar medium (Eiken) for aerobic bacteria and Sabouraud dextrose agar for fungi. The inoculum size was adjusted to $10^3 \sim 10^4$ cfu/ml for aerobic bacteria and 10^6 cfu/ml for fungi. Cycloviracins B_1 and B_2 exhibited weak antibacterial activity against aerobic Gram-positive bacteria but did not show activity against aerobic Gram-negative bacteria and fungi.

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