Fattiviracin A1, a Novel Antiherpetic Agent Produced by *Streptomyces microflavus* Strain No. 2445

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Structure Elucidation

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A novel antiherpetic agent, fattiviracin A1, was isolated from the culture broth of strain No. 2445 identified as *Streptomyces microflavus*. It was purified through 1-butanol extraction, column chromatographies on Diaion HP-10 and silica gel and HPLC using a reverse phase column. The structure of fattiviracin A1 was determined by several spectroscopic experiments and chemical degradations. It is a new macrocyclic diester consisting of four D-glucose units and two (C_{24} and C_{33}) hydroxy fatty acids. It is closely related to cycloviracins B_1 and B_2, but differs from these known compounds in both the length of its side chain and the sugar moiety.

Antiviral drugs would be compounds that rapidly treat viral diseases without side-effects. Acyclovir (ACV)[1] was approved for the treatment of herpes simplex viruses (HSV) infections over 10 years ago, and it remains an important and reliable antiviral agent. However, the increasing prevalence of ACV-resistant mutants of HSV results in a need for development of new antiherpetic agents[2].

In the course of our screening program for the isolation of antiherpetic agents from soil microorganisms, we developed a rapid method for detection of antiherpetic agents using 96-well microtiter plates, and found new antiherpetic agents, AH-135Y[3], AH-758[4] and AH-1763 IIa[5]. More recently, among 660 *Streptomyces* strains tested, a potent strain for the production of an antiherpetic agent, strain No. 2445, was selected, which produced a new compound, named fattiviracin A1. (Fattiviracin A1 was originally named AH-2445 A1.)

In this paper we describe the taxonomy of the producing strain, as well as fermentation, isolation, physico-chemical properties and structure elucidation of fattiviracin A1.

Materials and Methods

Taxonomic Studies

The characterization and identification of strain No. 2445 were carried out mainly according to BERGEY's Manual of Systematic Bacteriology[6] and the International Streptomyces Project (ISP) report[7]. Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB[8]. For the evaluation of cultural characteristics, the strain was incubated for 14~28 days at 28°C. The culture has been deposited to the National Institute of Bioscience and Human-Technology in the Agency of Industrial Science and Technology under the accession No. FERM P-16524.

Fermentation Studies

Strain No. 2445 was cultured for 2 days at 28°C in S medium (50 ml in a 200 ml Erlenmeyer flask with one intrusion) consisting of glucose 2.0%, starch 3.0%, corn steep liquor 1.0%, soybean flour 1.0%, peptone 0.5%, NaCl 0.3%, $CaCO_3$ 0.3%. The pH was adjusted to 7.0 with NaOH before autoclaving. The seed culture was used as inoculum for the main culture which was cultivated under the following cultural conditions: 4% inoculum was transferred into a 200 ml Erlenmeyer flask

Table 1. Cultural characteristics of strain No. 2445.

Medium	Growth	Aerial mycelium	Soluble pigment
Yeast ext.-malt ext. agar (ISP No. 2)	Abundant	Abundant, gray	Brown
Oatmeal agar (ISP No. 3)	Moderate	Slightly, brownish gray	Brown
Sucrose-nitrate agar (Czapek's soln. agar)	Abundant	Light yellowish brown	None
Inorganic salts-starch agar (ISP No. 4)	Abundant	Moderate, brownish gray	Light grayish green
Glycerol-asparagine agar (ISP No. 5)	Abundant	Moderate, brownish gray	Light brown
Peptone-yeast ext. iron agar (ISP No. 6)	Moderate	None	Blackish brown
Tyrosine agar (ISP No. 7)	Abundant	Brownish gray	Brown
Nutrient agar	Moderate	None	None

containing 50 ml of a main culture medium consisting of glucose 5.0%, peptone 0.5%, corn steep liquor 1.0%, NaCl 0.3%, $CaCO_3$ 0.3%, pH 7.0. The main cultures were incubated at 28°C for 4 days on a rotary shaker set at 280 rpm.

Analytical Procedures

The melting point was determined with a Yanagimoto melting point apparatus. The UV absorption spectrum was measured in methanol with a Hitachi U-2000 spectrophotometer. Optical rotation was determined on a Jasco DIP-360 digital polarimeter. The IR spectrum was taken in KBr tablets on a Jeol JIR-6500W infrared spectrophotometer. Mass spectra were measured with a Jeol JMS-DX303HF MS spectrometer. ^1H NMR, ^{13}C NMR, ^1H-^1H COSY and ^1H-^{13}C COSY spectra with TMS as internal standard were taken in methanol-d_4 at 500 MHz on a Jeol JMN-GX500 spectrometer.

Results

Taxonomy

Strain No. 2445 was isolated from a soil sample collected at a riverside of River Tuboi in Kumamoto, Japan. The cultural characteristics of strain No. 2445 grown on various media at 28°C for 28 days are shown in Table 1. Good growth was observed on various media. Melanoid pigments were produced on peptone-yeast extract iron agar (ISP-6) and WAKSMAN's melanin formation medium, but not on tyrosine agar (ISP-7). The strain was determined to be chromogenicity-positive. The

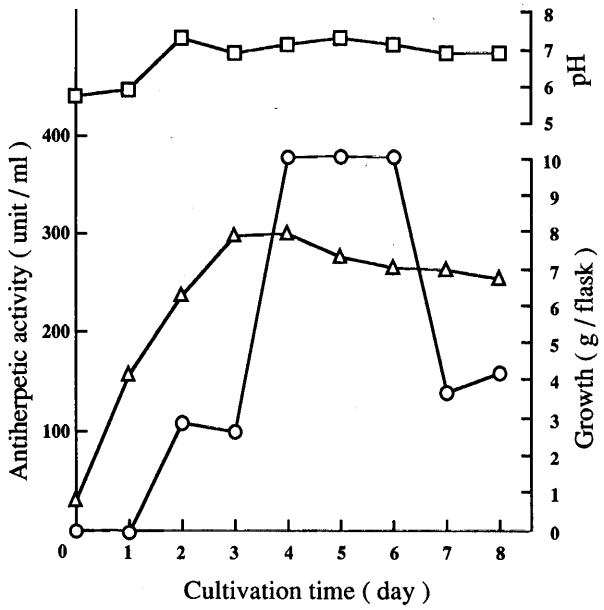
Table 2. Taxonomic characteristics of strain No. 2445.

Spore chain morphology	Spiral
Spore surface	Smooth
Aerial mass color	Gray
Formation of melanoid pigment	+
Liquefaction of gelatin	−
Coagulation of milk	−
Peptonization of milk	+
Hydrolysis of starch	+
Decomposition of cellulose	−
Utilization of	
L- Arabinose	+
D- Xylose	+
D- Glucose	+
D- Fructose	+
Rhamnose	+
Sucrose	+
Raffinose	+
i-Inositol	+
D- Mannitol	+
Salicin	±
Cellulose	−
Starch	+

+, Positive; −, negative.

Fig. 1. Time course of *Streptomyces microflavus* No. 2445 culture.

○ Antiherpetic activity, △ growth, □ pH.



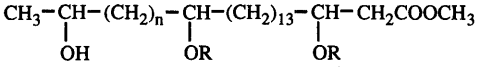
strain grew well in the range of 28 to 37°C but not below 14°C or over 50°C with optimum temperature of 37°C on yeast extract-malt extract agar. Liquefaction of gelatin and decomposition of cellulose were negative, but hydrolysis of starch and peptonization of milk were positive. By microscopic observation, the strain showed characteristic morphology of aerial mycelium. From the key characters based on (GY; S; C+; SM), that is, gray series of spore mass color; spiral aerial mycelium; chromogenicity positive; smooth spore surface, this strain was classified as a strain belonging to *Streptomyces microflavus*. Therefore, it was called *Streptomyces microflavus* strain No. 2445, hereafter.

Fermentation and Isolation

A typical fermentation time course of *Streptomyces microflavus* strain No. 2445 is shown in Fig. 1. The antiherpetic activity in the broth started after 1 day of cultivation and reached a maximum after 4 days fermentation.

All the purification steps were monitored by measuring the inhibitory activity of plaque formation by HSV-1[9]. Culture filtrate (7.1 liters) of strain No. 2445 was extracted with an equal volume of 1-butanol. The organic layer was concentrated *in vacuo* to dryness and applied to Diaion HP-10 column (28 × 200 mm, Mitsubishi Chemical Industries, Ltd.). The column was washed with

Fig. 2. Deduced structures of degradation products **1**, **2**, **3** and **4**.



	R	n
1	β-D-glucopyranosyl	14
2	β-D-glucopyranosyl	5
3	H	14
4	H	5

80% MeOH, and eluted with MeOH. The active fractions were pooled and concentrated *in vacuo* to form an oily material (2.8 g). This active fraction was applied to a silica gel column (28 × 230 mm) and eluted with $CHCl_3$-MeOH-H_2O (14:6:1). The presence of fattiviracin A1 was detected by heating at 110°C after spraying 10% H_2SO_4 after TLC on silica gel ($CHCl_3$-MeOH-H_2O, 14:6:1 as developing solvent system). The fractions containing fattiviracin A1 (Rf 0.23) were pooled and applied to another silica gel column (20 × 500 mm) and eluted with 1-BuOH-AcOH-H_2O (8:1:1), pooling fractions on the basis of TLC. Further purification was carried out by preparative HPLC (column: μBondapak C_{18}, Waters Associates, 19 i.d. × 300 mm, mobile phase: 90% MeOH, flow rate: 5 ml/minute, detection: UV 210 nm). The peak cuts (retention time, 28.01 minutes) containing fattiviracin A1 were collected and concentrated to yield 7.9 mg of purified fattiviracin A1 from 7.1 liters of the culture filtrate.

Physico-chemical Properties

Physico-chemical properties of fattiviracin A1 are shown in Table 3. Fattiviracin A1 was obtained as oily material. It was readily soluble in water, pyridine, dimethyl sulfoxide and MeOH but insoluble in acetone, ethyl acetate and $CHCl_3$. It showed no absorption maxima above 210 nm in the UV spectrum. It showed IR absorptions at 3500 and 1639 cm^{-1} due to hydroxyl and carbonyl groups, respectively. The negative ion FAB-MS of fattiviracin A1 showed an ion peak at *m/z* 1569 $(M-H)^-$. The molecular formula of fattiviracin A1 was determined to be $C_{81}H_{150}O_{28}$ by the negative ion FAB-MS, ^1H NMR and ^{13}C NMR data.

Structure Elucidation

The ^1H NMR spectrum of fattiviracin A1 revealed the

presence of two methyls (δ 1.14 × 2), forty-five methylenes (δ 1.18~1.54), two methylenes adjacent to carbonyl groups (δ 2.50~2.54, 2.60, 2.80 × 2) and four anomeric protons (δ 4.24, 4.32, 4.34, 4.35). The large coupling constant (J=7.33, 7.33, 7.33, 7.94 Hz) of the four anomeric protons allowed the assignment of β-configuration to the sugars. The ^{13}C NMR spectrum of fattiviracin A1 revealed the presence of two carbonyls (δ 172.50, 173.41), two methylenes adjacent to carbonyl groups (δ 42.21, 42.33) and four anomeric carbons (δ 103.41, 103.67, 103.97, 104.38). The ^{1}H-^{13}C and ^{1}H-^{1}H COSY spectra revealed that the methyl groups (δ 23.56 × 2) were connected to O-methines (δ 68.58 × 2). These results suggested that fattiviracin A1 was composed of four sugars and two fatty acids. From the ^{1}H and ^{13}C NMR spectra, the structure of fattiviracin A1 was found to be closely related to those of cycloviracins B_1 and B_2[10,11]. So, the actual structure assignment could be made on the basis of analogy to the original cycloviracins work.

Table 3. Physico-chemical properties of fattiviracin A1.

Appearance	colorless oily compound
$[\alpha]_D^{19}$ (c 0.1, MeOH)	-19.1 °
FAB-MS (m/z)	1569 (M-H)$^-$
Molecular formula	$C_{81}H_{150}O_{28}$
UV λ_{max}^{MeOH} nm	end absorption
IR ν_{max} (ATR) cm^{-1}	3388, 2923, 2854, 1731, 1459, 1369, 1164, 1078, 1022
Solubility : soluble	H_2O, MeOH
insoluble	acetone, $CHCl_3$
TLC, SiO_2	
($CHCl_3$: MeOH : H_2O = 14:6:1)	Rf 0.23
(1-BuOH : AcOH : H_2O = 4:1:5)	Rf 0.22
HPLC	Rt 6.11 minutes
(Mightysil RP-18, 90% MeOH, 0.8 ml/min)	

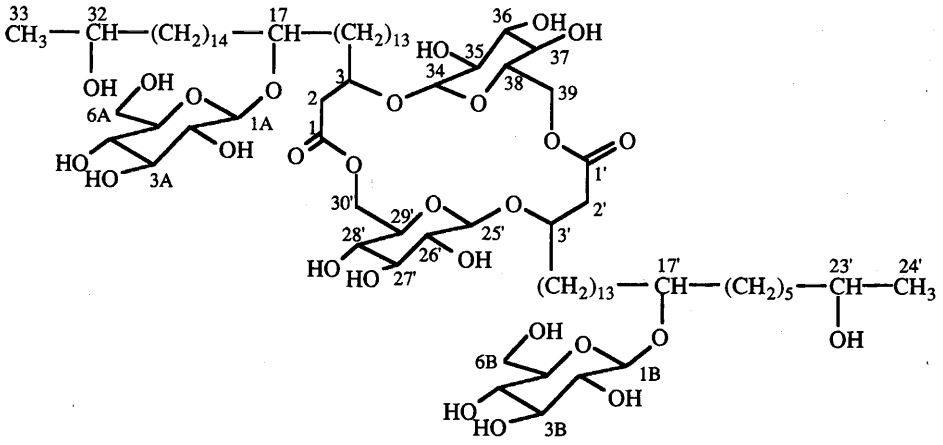
Degradation of fattiviracin A1 was examined to determine the four sugar molecules and the length of two alkyl chains. Fattiviracin A1 was reacted in CH_2N_2/ether at room temperature for 3 hours. Two degradation products (**1**, **2**) were purified by ODS column chromatography. Compounds **1** and **2** were reacted with 1 N HCl at 60°C for 1 hour, then extracted with $CHCl_3$. The sugar molecules in each aqueous layer (sugar fraction) were identified as D-glucose by spectroscopy and by comparison with an authenthic sample. After the $CHCl_3$ layers (lipid fractions) were reacted in CH_2N_2/ether at room temperature for 3 hours, compounds **3** and **4** were obtained.

The negative ion FAB-MS of **3** ($C_{34}H_{68}O_5$) showed

Table 4. ^{13}C and ^{1}H NMR data of fattiviracin A1 (500 MHz, in CD_3OD).

position	^{13}C (ppm)	^{1}H (ppm)	position	^{13}C (ppm)	^{1}H (ppm)
1, 1'	172.50, 173.41	—	1A, 1B	103.41, 103.67	4.29 (d, J=7.94, 1H)
2, 2'	42.21, 42.33	2.50-2.54 (m, 1H)			4.32 (d, J=7.33, 1H)
		2.60 (dt, J=15.27, 5.50, 1H)	2A, 2B	75.16, 75.20	3.13-3.19 (m, 2H)
		2.80 (td, J=15.26, 6.71, 2H)	3A, 3B	77.72, 77.90	3.31-3.34 (m, 2H)
3, 3'	78.21, 78.27	4.06-4.14 (m, 2H)	4A, 4B	71.62, 71.62	3.24-3.30 (m, 2H)
17, 17'	80.48, 80.96	3.60-3.64 (m, 2H)	5A, 5B	78.06, 78.06	3.44 (m, 2H)
32, 23'	68.58, 68.58	3.69-3.73 (m, 2H)	6A, 6B	62.88, 62.97	3.64-3.68 (m, 2H)
33, 24'	23.56, 23.56	1.14 (d, J=6.10, 6H)			3.82-3.86 (m, 2H)
34, 25'	103.97, 104.38	4.34 (d, J=7.33, 1H)	methylenes of fatty acid	40.19, 36.10	1.18-1.54 (m, 90H)
		4.35 (d, J=7.33, 1H)		36.01, 35.90	
35, 26'	75.27, 75.31	3.13-3.19 (m, 2H)		35.63, 35.34	
36, 27'	77.72, 77.90	3.31-3.34 (m, 2H)		34.91, 31.05	
37, 28'	71.82, 71.95	3.24-3.30 (m, 2H)		30.91, 30.86	
38, 29'	78.21, 78.28	4.16-4.20 (m, 2H)		30.50, 30.30	
39, 30'	65.20, 65.20	4.42-4.48 (m, 2H)		30.22, 30.15	
		4.06-4.14 (m, 2H)		30.01, 29.85	
				26.86, 26.38	
				26.28, 26.02	
				25.82, 23.56	

Fig. 3. Deduced structure of fattiviracin A1.



ion peaks at m/z 555 $(M-H)^-$. The ^1H NMR spectrum of **3** showed one methyl (δ 1.19), one *O*-methyl (δ 3.71), three *O*-methines (δ 3.58, 3.80, 4.00), one methylene (δ 2.41, 2.51) adjacent to a carbonyl group and twenty-seven methylenes (δ 1.18~1.54). The ^{13}C NMR spectrum of **3** showed the presence of one ester carbonyl (δ 173.5) in addition to the above assigned groups. The ^1H-^1H NMR spectrum of **3** showed that one of the *O*-methines (δ 3.80) was connected to the methyl (δ 1.19), and the other *O*-methine (δ 4.00) was connected to the methylene (δ 2.41, 2.51) adjacent to a carbonyl group. The position of the third *O*-methine (δ 3.58) was established by EI-MS. Compound **3** showed fragment ion peaks at m/z 315, 103 and 45. The ions at m/z 103, 315 and 45 indicated that three hydroxyl groups were located at C-3, C-17 and C-32, respectively. Thus, **3** is methyl 3,17,32-trihydroxytritriacontanoate. The positive ion FAB-MS of **4** ($C_{25}H_{50}O_5$) showed ion peaks at m/z 431 $(M+H)^+$. The ^1H NMR spectrum of **4** showed eighteen methylenes (δ 1.18~1.54) and the other signals were very similar to those of **3**. The fragment ions were observed at m/z 315, 103 and 45 in the EI-MS. Thus, **4** is methyl 3,17,23-trihydroxytetracosanoate.

The negative ion FAB-MS of **1** ($C_{46}H_{88}O_{15}$) showed ion peaks at m/z 879 $(M-H)^-$. Its ^1H NMR spectrum showed one methyl (δ 1.14), twenty-seven methylenes (δ 1.18~1.54), one methylene adjacent to a carbonyl (δ 2.51, 2.72) and two anomeric protons (δ 4.31, 4.32), suggesting that **1** was a derivative of the compound **3** containing two β-D-glucose units. The sugar molecules were determined to be attached to to the C-17 and C-32 hydroxyls based on the ^{13}C NMR data of fattiviracin A1. The negative ion FAB-MS of **2** showed ion peaks at m/z 753 $(M-H)^-$. Similarly, **2** ($C_{37}H_{70}O_{15}$) was determined to be a derivative of the compound **4** containing two β-D-glucose units at C-17 and C-32. Thus, **1** and **2** are methyl 3,17-di-(β-D-glucopyranosyl)-32-hydroxytritriacontanoate and methyl 3,17-di-(β-D-glucopyranosyl)-23-hydroxytetracosanoate, respectively.

Taking into consideration of the molecular formulae of **1** ($C_{46}H_{88}O_{15}$), **2** ($C_{37}H_{70}O_{15}$) and fattiviracin A1 ($C_{81}H_{150}O_{28}$), it is thought that fattiviracin A1 was cleaved at two ester bonds (C-1 and C-1′) to give **1** and **2**. The differences of carbon chemical shifts were observed between C-39 and C-30′ (δ 65.20 and 65.20), and 6A and 6B (δ 62.88 and 62.97) in fattiviracin A1. These differences might depend on the acylation shift by the bonds between C-1 and C-30′, and C-1′ and C-39.

Thus, the structure of fattiviracin A1 was proposed as described in Fig. 3.

Discussion

The structure of fattiviracin A1 has been deduced by spectral analyses and chemical degradation. It is macrocyclic dilactone consisting of four D-glucose units and two trihydroxylated fatty acids. There have been reported two antiviral agents, cycloviracins B_1 and B_2, which are macrocyclic dilactones consisting of two D-glucose, three 2-*O*-methyl-D-glucose and two polyhydroxylated fatty acids. Fattiviracin A1 has the same macrocyclic diester structure of cycloviracins B_1 and B_2, but differs from these known compounds in both the length of its side chain and the sugar moiety.

Streptomyces microfluvas No. 2445 has produced several antiherpetic agents in addition to fattiviracin A1.

The other compounds will be reported in the future. Recently we found that fattiviracin A1 has antiviral activity against human immunodeficiency virus. The biological properties of fattiviracin A1 including the antiviral activities will be reported in the next paper.

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