

A New Antiherpetic Agent Produced by *Streptomyces* sp. Strain No. 758

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A new antiherpetic agent, AH-758, was isolated from the culture broth of *Streptomyces* sp. strain No. 758. The structure was determined by NMR spectral analyses to be a new antibiotic belonging to bafilomycin group containing (5-oxo-2-pyrroline-2-yl) methyl fumarate in its C-21.

In the course of our screening program for the antiherpetic agents from soil microorganisms, a new antiherpetic agent has been isolated from *Streptomyces* sp. strain No. 758 and was named AH-758. From the structural elucidation, it was concluded that AH-758 was a new 16-membered macrolide antibiotic belonging to bafilomycin group. However, it remarkably differed in a substituent in the C-21 side chain from bafilomycins. AH-758 inhibited viral proliferation in infected Vero cell culture, and 50% effective concentration (EC₅₀) of AH-758 was 0.09 µg/ml. While, 50% inhibitory concentration (IC₅₀) of the compound was 1.3 µg/ml against the cell growth. In addition, AH-758 showed strong antifungal activity and weak antibacterial activity. In this paper, we described the taxonomy, fermentation, purification, chemical structure, physical and biological properties of AH-758.

Materials and Methods

Microorganisms and Cells

The producing organism, strain No. 758 was isolated from a soil sample collected in Kumamoto City, Kumamoto, Japan. *Streptomyces rochei* IFO 12908 was used as type strain. Test organisms for antimicrobial activity were obtained from IFO.

Herpes simplex virus type 1 strain KOS and Vero cells were provided by the Chemo-Sero-Therapeutic Institute.

Taxonomic Studies

Cultural and physiological characteristics were determined by the methods of SHIRLING and GOTTLIEB¹⁾, and WAKSMAN²⁾. Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB³⁾.

Biological Assay

The antiviral and anticellular activities of AH-758 were measured by the plaque reduction assay⁴⁾ and cell growth inhibition test⁵⁾. Confluent monolayers of Vero cells

(1×10^6 cells) in 6-well plastic plates (35 mm diameter) were infected with 100 PFU of HSV-1 (KOS). After 1 hour adsorption period at 37°C, the cultures were overlaid with 2 ml of DULBECCO's modified Eagle minimum essential medium (DMEM) containing 2% heat-inactivated fetal calf serum and various concentrations of drugs. The cultures infected with HSV-1 were incubated in the CO₂ incubator, and fixed with formalin and stained with crystal violet in methanol at 3 days after infection.

Cell growth inhibition test was examined as described below. Vero cells were seeded in 6-well plastic plates at 1×10^6 cells per well. After 1 day, the cells were refed with DMEM containing 5% fetal calf serum and various concentrations of drugs. After incubation for 3 days, cells were dispersed by treatment with trypsin, and viable cell numbers were counted.

Waksman's agar dilution streak method was used for the determination of the antimicrobial spectrum of AH-758.

Fermentation Studies

Strain No. 758 was cultured for 2 days at 28°C in a medium (50 ml in a 200-ml Erlenmeyer flask with one intrusion) consisting of glucose 2.0%, starch 3.0%, C.S.L. 1.0%, S.B.F. 1.0%, peptone 0.5%, NaCl 0.3%, CaCO₃ 0.3%, pH 7.2. These cultures were used as inoculum for 10-liter fermentor and cultivated under the following cultural conditions: 4% inoculum was transferred to a 10-liter fermentor containing 5 liters medium consisting of glucose 5.0%, peptone 1.0%, meat extract 0.5%, NaCl 0.3%, CaCO₃ 0.3%, pH 7.2 and run at 28°C for 3 days with 350 rpm agitation and 4 liter/minute aeration.

Analytical Procedures

MP was determined with a Yanagimoto melting point apparatus. 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.

Table 1. Cultural characteristics of strain No. 758.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar (Czapek's soln. agar)	Poor	None	None
Glucose-asparagine agar	Excellent	Excellent, birch	None
Glycerol-asparagine agar (ISP No. 5)	Excellent	Excellent, pinkish	None
Calcium malate agar	Good	Moderate, light gray	None
Nutrient agar	Good	None	None
Yeast ext.-malt ext. agar (ISP No. 2)	Excellent	Excellent, fawn to beaver	None
Oatmeal agar (ISP No. 3)	Moderate	Scant	None
Inorganic salts-starch agar (ISP No. 4)	Good	Good, birch	None
Tyrosine agar (ISP No. 7)	Excellent	Excellent, birch	None

Table 2. Comparison of taxonomic characteristics of strain No. 758 with *Streptomyces rochei*.

	No. 758	<i>Streptomyces rochei</i> IFO 12908
Spore chain morphology	Spiral	Spiral
Spore surface	Smooth	Smooth
Aerial mass color	Gray	Gray
Soluble pigment	-	-
Formation of melanoid pigment	-	-
Liquefaction of glucose peptone gelatin	-	-
Coagulation of milk	-	-
Peptonization of milk	+	+
Hydrolysis of starch	-	+
Utilization of		
L-Arabinose	+	+
D-Fructose	+	+
D-Glucose	+	+
Inositol	+	+
D-Mannitol	+	+
Raffinose	-	-
Rhamnose	±	± ~ (+)
Sucrose	-	-
D-Xylose	+	+

+, positive; -, negative; ±, doubtful.

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 400 MHz on a Jeol JMN-GX400 spectrometers.

Results and Discussion

Taxonomy

Strain No. 758 was isolated from a soil sample collected in Kumamoto City, Japan. Taxonomical characterization was carried out according to the method of International Streptomyces Project (ISP). The cultural characteristics of strain No. 758 grown on various media at 28°C for 14 days are shown in Table 1. The growth was good on various media, but poor on sucrose-nitrate agar. Soluble pigments were not produced in all agar media. Strain No. 758 grew well at the range of 28 to 37°C with optimum temperature at 37°C on glycerol-asparagine agar, but not below 14°C and over 50°C. Formation of melanoid pigment, liquefaction of gelatin, coagulation of milk, hydrolysis of starch, decomposition of cellulose were negative, but peptonization of milk was positive. Comparison of characteristics of strain No. 758 with those of *Streptomyces* species described by SHIRING¹⁾, WAKSMAN²⁾ and BERGEY⁶⁾ indicated that strain No. 758 closely resembled *Streptomyces rochei*. The comparison of both strains is summarized in Table 2. The physiological properties of *Streptomyces rochei* were similar to the strain No. 758 except for hydrolysis of starch. Therefore strain No. 758 was identified as a strain belonging to *Streptomyces rochei*.

Isolation

The production of AH-758 started in the logarithmic growing phase and increased with the growth of the mycelium. The antiherpetic activity reached maximum after 3 days of cultivation and gradually decreased thereafter.

Isolation of AH-758 was carried out by monitoring the antiherpetic activity. Culture filtrate (ca. 5 liters) of strain No. 758 was adsorbed batchwisely on Diaion HP-10 for 12 hours. After washing with 80% MeOH, AH-758 was eluted with 100% MeOH. The active fractions were pooled and concentrated *in vacuo* to form an oily material, which was dissolved in a small volume of CHCl_3 , and applied to a silica gel column. AH-758 was eluted with a mixture of CHCl_3 - Me_2CO -MeOH (8:1:0.5). The fractions containing AH-758 were pooled and concentrated *in vacuo* to form crude yellow powder, which was dissolved in a small volume of CHCl_3

and applied to a Sephadex LH-20 column. The chromatography was developed with CHCl_3 and the active fractions were pooled and concentrated *in vacuo*. Yield of AH-758 was 10 mg from 5 liters of the culture filtrate.

Physico-chemical Properties

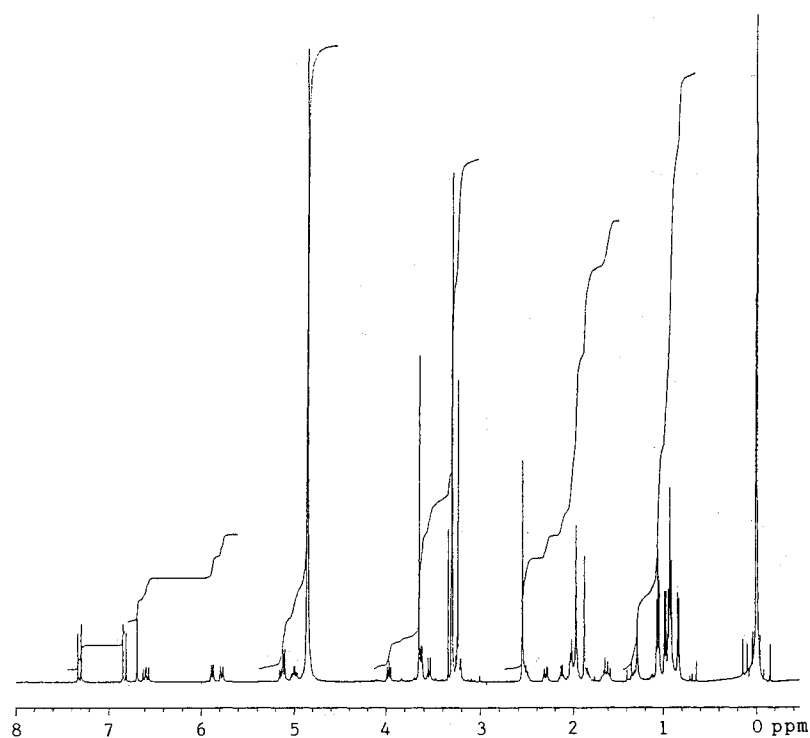
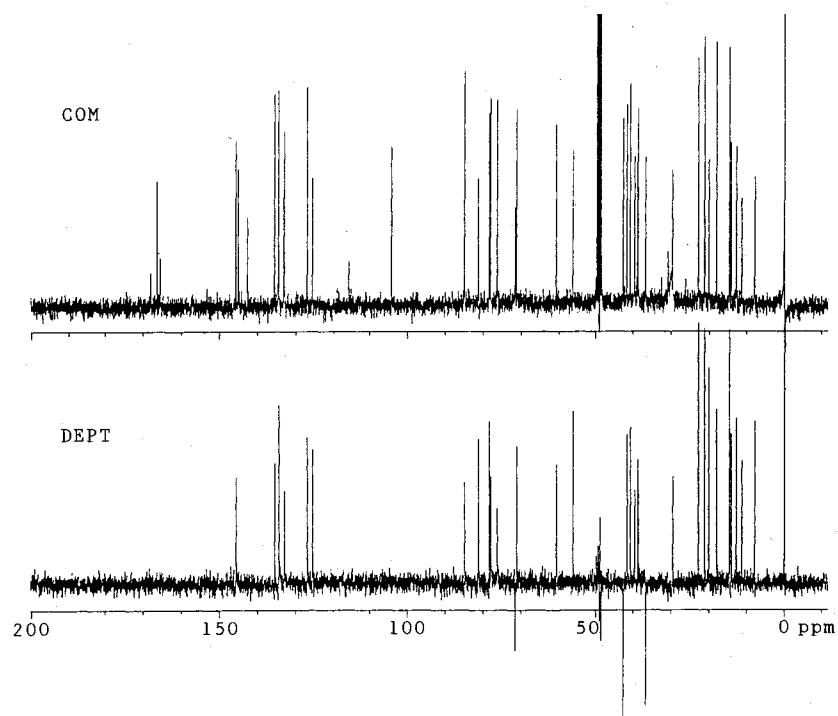
Physico-chemical properties of AH-758 are shown in Table 3. AH-758 was obtained as yellow green powder with MP at 128~130°C. It was readily soluble in Me_2CO , MeOH and CHCl_3 but insoluble in water. The UV absorption maxima of AH-758 in MeOH were observed at 249 (ϵ 38,100), 285 (shoulder; ϵ 16,300) and 350 nm (shoulder; ϵ 3,200). It showed IR absorptions at 3425, 3251, 2873 and 1722 cm^{-1} due to hydroxyl, amino, methoxy and carbonyl groups, respectively. The EI-MS of AH-758 showed several intensive fragment ion peaks up to m/z 568, but no molecular ion peak. The elementary analysis of AH-758 afforded $\text{C}_{45}\text{H}_{67}\text{NO}_{13}$ as molecular formula, which agreed with the m/z 828 ($\text{M}-\text{H}$)⁻ as negative ion peak and m/z 852 ($\text{M}+\text{Na}$)⁺ as positive ion peak on the FAB-MS. The molecular formula was also supported by the ^1H NMR (Fig. 1) and ^{13}C NMR (Fig. 2) spectral data, which were summarized in Table 4. The ^{13}C NMR spectrum of AH-758 showed the 45 carbons. The analysis of DEPT spectrum indicated that AH-758 consisted of the following functional groups: $\text{CH}_3 \times 9$, $\text{CH}_2 \times 3$, $\text{CH} \times 6$, $\text{OCH}_3 \times 3$, $\text{CH}_2\text{-O} \times 1$, $\text{CH-O} \times 6$, $\text{O-C-O} \times 1$, CH= $\times 8$, C= $\times 4$, $\text{C=O} \times 4$.

Structural Elucidation

Partial structures I, II, III, IV, V and VI (Fig. 3) were deduced from the ^1H - ^1H COSY and ^1H - ^{13}C COSY spectra. The partial structure I was deduced by the correlation from the signal at δ_{H} 3.26 (H-7) through δ_{H} 2.50 (H-6) to δ_{H} 5.88 (H-5), 1.07 (27-H), and through δ_{H} 1.84 (H-8) to δ_{H} 2.02 (H-9), 0.92 (28-H) in the ^1H - ^1H

Table 3. Physico-chemical properties of AH-758.

Nature	Yellow green powder
MP (°C)	128 ~ 130
$[\alpha]_{\text{D}}^{25}$ (c 0.1, MeOH)	+ 24.4°
FAB-MS (m/z)	852($\text{M}+\text{Na}$) ⁺ , 828($\text{M}-\text{H}$) ⁻
Molecular formula	$\text{C}_{45}\text{H}_{67}\text{NO}_{13}$
UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (ϵ)	249 (38,100) 285 (sh, 16,300) 350 (sh, 3,200)
IR ν_{max} (KBr) cm^{-1}	3425, 3251, 2873, 1722, 1689
Solubility	
Soluble	Me_2CO , MeOH, CHCl_3
Insoluble	H_2O

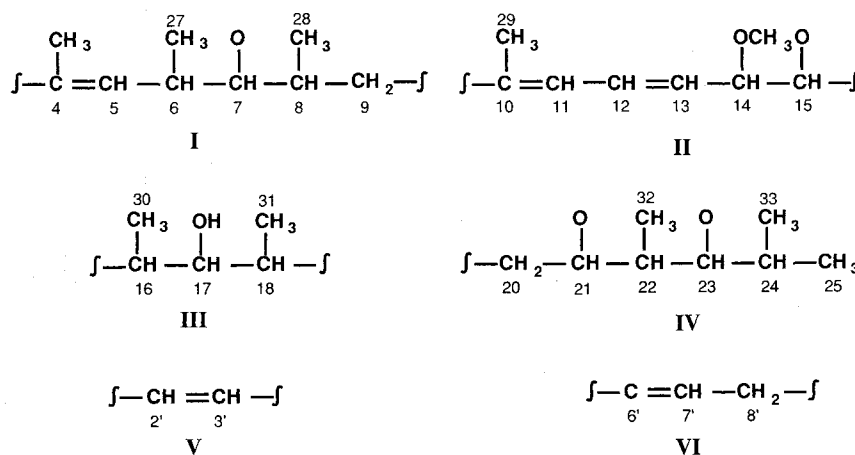
Fig. 1. ^1H NMR spectrum of AH-758 in CD_3OD (400 MHz).Fig. 2. ^{13}C NMR spectrum of AH-758 in CD_3OD (400 MHz).

COSY spectrum. The partial structure II was deduced by the correlation from the olefinic proton at δ_{H} 5.15 (H-13) through δ_{H} 3.97 (H-14) to δ_{H} 5.11 (H-15), and through the olefinic proton at δ_{H} 6.59 (H-12) to δ_{H} 5.77 (H-11). The partial structure III was deduced by the

correlation from the signal at δ_{H} 3.21 (H-17) through δ_{H} 2.13 (H-16) to δ_{H} 0.98 (30-H), and through δ_{H} 1.60 (H-18) to δ_{H} 0.84 (31-H). The partial structure IV was deduced by the correlation from the signal at δ_{H} 3.55 (H-23) through δ_{H} 2.02 (H-22) to δ_{H} 0.94 (32-H), 4.98 (21-H)

Table 4. NMR spectral data for AH-758 in CD₃OD.

Position	δ_C	δ_H	Position	δ_C	δ_H
1	168.0 (s)	-	23	71.1 (d)	3.55 (d, J= 10.3, 1H)
2	142.5 (s)	-	24	29.6 (d)	2.02 (m, 1H)
3	134.6 (d)	6.69 (s, 1H)	25	11.3 (q)	0.92 (d, J= 6.96, 3H)
4	133.2 (s)	-	26	14.1 (q)	1.97 (d, J= 0.73, 3H)
5	145.5 (d)	5.88 (d, J= 9.16, 1H)	27	18.0 (q)	1.07 (d, J= 5.50, 3H)
6	38.6 (d)	2.50 (m, 1H)	28	14.5 (q)	0.92 (d, J= 5.13, 3H)
7	81.2 (d)	3.26 (m, 1H)	29	20.0 (q)	1.88 (s, 3H)
8	41.6 (d)	1.84 (m, 1H)	30	7.8 (q)	0.98 (d, J= 6.96, 3H)
9	42.6 (t)	2.02 (m, 2H)	31	12.7 (q)	0.84 (d, J= 6.60, 3H)
10	144.9 (s)	-	32	22.7 (q)	0.94 (d, J= 7.33, 3H)
11	125.6 (d)	5.77 (d, J= 10.6, 1H)	33	21.1 (q)	1.05 (d, J= 6.96, 3H)
12	135.6 (d)	6.59 (dd, J= 10.6, 15.6, 1H)	1'	166.2 (s)	-
13	127.0 (d)	5.15 (d, J= 7.69, 1H)	2'	133.0 (d)	6.82 (d, J= 15.4, 1H)
14	84.9 (d)	3.97 (dd, J= 7.70, 8.42, 1H)	3'	135.6 (d)	7.31 (d, J= 15.4, 1H)
15	78.0 (d)	5.11 (d, J= 8.43, 1H)	4'	166.2 (s)	-
16	39.6 (d)	2.13 (m, 1H)	5'	71.5 (t)	3.63 (m, 2H)
17	78.4 (d)	3.21 (m, 1H)	6'	116.0 (s)	-
18	38.8 (d)	1.60 (m, 1H)	7'	127.0 (d)	5.11 (d, J= 7.69, 1H)
19	104.2 (s)	-	8'	30.8 (t)	1.29 (m, 2H)
20	36.7 (t)	1.59 (m, 1H), 2.30 (dd, J= 4.77, 13.2, 1H)	9'	165.4 (s)	-
21	76.3 (d)	4.98 (td, J= 5.87, 10.6, 1H)	2-OMe	60.6 (q)	3.65 (s, 3H)
22	40.7 (d)	2.02 (m, 1H)	14-OMe	56.0 (q)	3.24 (s, 3H)
			19-OMe	56.0 (q)	3.31 (s, 3H)

Fig. 3. Partial structure of AH-758 deduced from ¹H-¹H COSY and ¹³C-¹H COSY experiments.

which coupled to the unequivocal methylene protons at δ_H 1.59 and 2.30 (20-H), and through δ_H 2.02 (H-24) to δ_H 0.92 (25-H), 1.05 (33-H). The signals at H-22 and H-24 showed same chemical shifts in the ¹H NMR spectrum. They showed correlation with the signal at δ_C 40.7 and 29.6, respectively, in the ¹H-¹³C COSY spectrum. The partial structures V and VI were deduced in the same way.

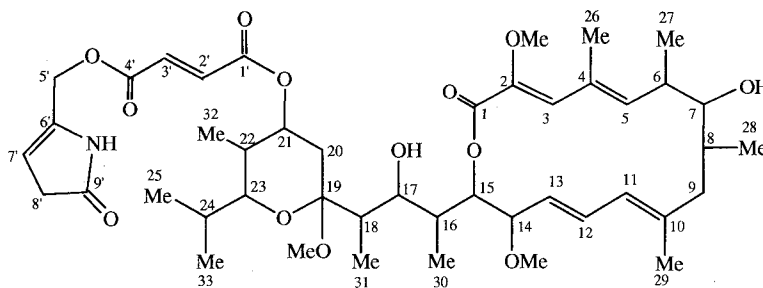
The EI-MS of AH-758 showed fragment ion peaks similar to those of bafilomycins⁷⁾ and in the ¹³C NMR spectrum the signals of AH-758 from C-1 to C-33 were

assigned by comparing with those of bafilomycins derived from 16-membered lactone ring and 6-membered hemiketal ring⁸⁾.

The bafilomycins consisted of ten types derivatives differing from the substituents at C-7, C-19 or C-21⁷⁾. AH-758 was deduced as a C-21 substituted derivative of bafilomycin B₂.

The residual carbon signals were assigned as follows. In the ¹³C NMR spectrum carbonyl signal at δ_C 166.2 showed higher intensity than that of δ_C 165.4. This fact may be explained by the presence of two carbons having

Fig. 4. Proposed structure of AH-758.



same structure. The carbon signals at δ_C 166.2 (C-1'), 133.0 (C-2'), 135.6 (C-3') and 166.2 (C-4') were assigned to fumarylester, which supported by the partial structure V (Fig. 3). An oxymethylene signal at δ_H 3.63 (H-5'), which showed the correlation with a signal at δ_C 71.5 (C-5') in the 1H - ^{13}C COSY spectrum, showed no correlation with the other signals in 1H - 1H COSY spectrum. Taking into consideration of the partial structure VI (Fig. 3), a carbon signal at δ_C 165.4 (C-9') and a residual formula -NH, the C-21 substituent was elucidated as 3'-(5''-oxo-2''-pyrrolin-2''-yl)methoxycarbonyl acryloyl, commonly named as (5-oxo-2-pyrrolin-2-yl)methyl fumarate, which supported by the fragment ion peak at m/z 211 in the EI-MS.

From the results described above, the structure of AH-758 was determined as shown in Fig. 4.

Antiviral Activities

AH-758 showed the antiherpetic activity of 0.09 $\mu g/ml$ as EC_{50} against HSV-1, and cytotoxicity of 1.3 $\mu g/ml$ as IC_{50} against Vero cells. Therefore the selectivity (the ratio of IC_{50} to EC_{50}) of AH-758 was calculated as 14.4. The effects of AH-758 on other viruses such as human cytomegalovirus, influenza virus and respiratory syncytial virus were examined. AH-758 strongly inhibited proliferation of human cytomegalovirus at the lowest concentration of the range tested (0.5 ng/ml) without cytotoxicity. AH-758 indicated 50% inhibition against influenza virus and respiratory syncytial virus at 1.6 and 2.8 ng/ml, respectively. Interestingly, AH-758 inhibited both DNA virus and RNA virus without viral selectivity.

Antimicrobial Activities

AH-758 indicated the weakly inhibitory activities (20 $\mu g/ml$ as MIC) against Gram-positive bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus*

luteus. Gram-negative bacteria seemed to be insensitive. It showed rather strong antifungal activity against *Saccharomyces cerevisiae* (MIC, <0.1 $\mu g/ml$) and *Aspergillus niger* (MIC, 0.5 $\mu g/ml$). The antimicrobial activities of AH-758 were similar to those of bafilomycins⁷⁾.

Acknowledgment

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