A New Antiherpetic Agent, AH-1763 IIa, Produced by *Streptomyces cyaneus* Strain No. 1763

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A new antiherpetic agent, AH-1763 IIa, was isolated from the culture broth of strain No. 1763 identified as *Streptomyces cyaneus*. It was purified through column chromatographies of Diaion HP-10 and silica gel. The structure was determined to be 11-hydroxy-5-methyl-2-(2-hydroxy-1-methylpropyl)-4*H*-anthraceno[1,2-*b*]pyran-4,7,12-trione by several spectroscopic experiments, that is a new antibiotic belonging to pluramycin-group.

In the course of our screening program for the antiherpetic agents from soil microorganisms, two antiherpetic agents have been isolated from the culture broth of strain No. 1763 identified as *Streptomyces cyaneus* and were named AH-1763 Ia and IIa. From the structural elucidation, AH-1763 Ia was thought to be tetracenomycin C[1] (1,4,4a,5,12,12a-hexahydro-4,4a,11,12a-tetrahydroxy-3,8-dimethoxy-9-methoxycarbonyl-10-methyl-1,5,12-trioxonaphthacene), whereas AH-1763 IIa was thought to be a new antibiotic belonging to pluramycin-group including pluramycin A[2], neopluramycin[2], SF-2330[3], hydramycin[4], saptomycins[5] and espicufolin[6]. AH-1763 IIa inhibited viral proliferation in infected Vero cell culture, and 50% effective concentration (EC_{50}) of AH-1763 IIa was 2.1 μg/ml. While, 50% inhibitory concentration (IC_{50}) of the compound was 15.2 μg/ml against the cell growth. In addition, AH-1763 IIa showed antibacterial activity against Gram-positive bacteria. In this paper, we described the fermentation, purification, chemical structure, physical and biological properties of AH-1763 IIa.

Materials and Methods

Microorganisms and Cells

The producing organism, strain No. 1763 was isolated from a soil sample collected in Kumamoto City, Kumamoto, Japan. 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

The characterization and identification of strain No. 1763 were carried out mainly according to BERGEY's Manual of Systematic Bacteriology[7], the International Streptomyces Project (ISP) report[8]. Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB[9]. For the evaluation of cultural characteristics, the strain was incubated for 14~28 days at 28°C.

Biological Assay

The antiviral and anticellular activities of AH-1763 IIa were measured by the plaque reduction assay[10] and cell growth inhibition test[11]. 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 the drug. The cultures infected with HSV-1 were incubated in the CO_2 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 the drug . After incubation for 3 days, cells were dispersed by treatment with trypsin, and viable cell numbers were counted.

WAKSMAN's agar dilution streak method[12] was used for the determination of the antimicrobial spectrum of

AH-1763 IIa.

Fermentation Studies

Strain No. 1763 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%, $CaCO_3$ 0.3%, pH 7.0. These cultures were used as inoculum for main culture and cultivated under the following cultural conditions: 4% inoculum was transferred to a main culture containing 50 ml medium consisting of glucose 5.0%, peptone 0.5%, corn steep liquor 1.0%, NaCl 0.3%, $CaCO_3$ 0.3%, pH 7.0 and run at 28°C for 6 days with 180 rpm agitation.

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. Mass spectra were measured with a Jeol JMS-DX303HF MS spectrometer. ^1H NMR, ^{13}C NMR, ^1H-^1H COSY, ^1H-^{13}C COSY and HMBC spectra with TMS as internal standard were taken in chloroform-d at 500 MHz on a Jeol JMN-GX500 spectrometer.

Results and Discussion

Taxonomy

Strain No. 1763 was isolated from a soil sample collected in Kumamoto, Japan. The cultural characteristics of strain No. 1763 grown on various media at 28°C for 28 days are shown in Table 1. The growth was good 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 strain grew well at the range of 28 to 37°C with optimum temperature at 37°C on yeast extract-malt extract agar, but not below 14°C and over 50°C. Liquefaction of gelatin and decomposition of cellulose were negative, but hydrolysis of starch, peptonization of milk were positive. 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 cyaneus*[13]. Therefore, it was called *Streptomyces cyaneus* strain No. 1763, hereafter.

Isolation

The production of AH-1763 IIa started in the logarithmic growing phase and increased with the growth of the mycelium. The antiherpetic activity reached maxinum after 6 days of cultivation and decreased thereafter.

Isolation of AH-1763 IIa was carried out by monitoring the antiherpetic activity. Culture filtrate (7 liters) of strain No. 1763 was adsorbed batchwisely on Diaion HP-10 for 12 hours. After washing with 80% MeOH, AH-1763 IIa was eluted with 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-1763 IIa was eluted with $CHCl_3$. The fractions containing AH-1763 IIa were pooled and concentrated *in vacuo* to form crude yellow powder, which was dissolved in a small volume of benzene and applied to a silica gel column. The chromatography was developed with benzene-ethyl-

Table 1. Cultural characteristics of strain No. 1763.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar (CZAPEK's soln. agar)	Abundant	Abundant, pinkish grey	Light reddish brown
Peptone-yeast ext. iron agar (ISP No. 6)	Abundant	None	Blackish brown
Glycerol-asparagine agar (ISP No. 5)	Abundant	Moderate, brownish grey	None
Nutrient agar	Moderate	None	Light brown
Yeast ext.-malt ext. agar (ISP No. 2)	Abundant	Pinkish grey to brownish grey	Brown
Oatmeal agar (ISP No. 3)	Abundant	Dark greyish brown	Light yellowish brown
Inorganic salts-starch agar (ISP No. 4)	Abundant	Brownish grey	None
Tyrosine agar (ISP No. 7)	Abundant	Abundant, pinkish grey to brownhish grey	Light brown

Table 2. Taxonomic characteristics of strain No. 1763.

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.

Table 3. Physico-chemical properties of AH-1763 IIa.

Nature	Yellow needle
MP (°C)	224~226
$[\alpha]_D^{25}$ (c 0.1, $CHCl_3$)	+6.63°
Analysis	Calcd for $C_{22}H_{18}O_6$: C 69.83, H 4.79 Found: C 69.35, H 4.85
EI-MS (m/z)	378 $(M)^+$
IR (KBr) cm^{-1}	3500, 1674, 1639, 1581
UV λ_{max}^{MeOH} nm (ε)	239.5 (22,600), 267 (12,000), 287 (sh, 7,000), 417 (6,400)

acetate (10 : 1) and the active fractions were pooled and concentrated *in vacuo*. AH-1763 IIa was recrystallized from MeOH to give pure yellow needle. Yield of AH-1763 IIa was 3 mg from 7 liters of the culture filtrate.

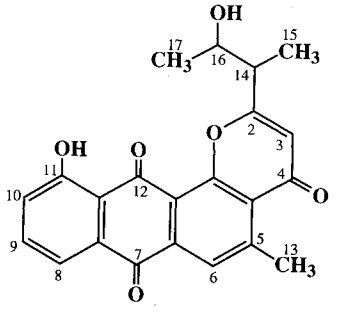
Physico-chemical Properties

Physico-chemical properties of AH-1763 IIa are shown in Table 3. AH-1763 IIa was obtained as yellow needle with MP at 224~226°C. It was readily soluble in Me_2CO, MeOH and $CHCl_3$ but insoluble in water. The UV absorption maxima of AH-1763 IIa in MeOH were observed at 239.5 (ε 22,600), 267 (ε 12,000), 287 (shoulder; ε 7,000) and 417 nm (ε 6,400). It showed IR absorptions at 3500 and 1639 cm^{-1} due to hydroxyl and carbonyl group, respectively. The EI-MS of AH-1763 IIa showed an ion peak at m/z 378. The elementary analysis of AH-1763 IIa afforded $C_{22}H_{18}O_6$ as molecular formula, which agreed with the m/z 378 $(M)^+$ as ion peak on the

Table 4. NMR spectral data for AH-1763 IIa in $CDCl_3$.

Position	δ_C	δ_H
2	171.4 (s)	—
3	112.1 (d)	6.28 (s, 1H)
4	178.8 (s)	—
4a	126.5 (s)	—
5	150.4 (s)	—
6	126.1 (d)	8.09 (s, 1H)
6a	136.1 (s)	—
7	181.7 (s)	—
7a	132.3 (s)	—
8	119.7 (d)	7.82 (dd, J=1.2, 7.3, 1H)
9	136.8 (d)	7.69 (t, J=7.3, 1H)
10	125.6 (d)	7.36 (dd, J=1.2, 7.3, 1H)
11	162.9 (s)	—
11a	116.7 (s)	—
12	187.8 (s)	—
12a	119.7 (s)	—
12b	156.6 (s)	—
13	24.3 (q)	3.02 (s, 3H)
14	45.5 (d)	2.88 (dq, J=3.3, 7.3, 1H)
15	12.6 (q)	1.43 (d, J=7.3, 3H)
16	68.8 (d)	4.32 (ddq, J=3.3, 4.3, 6.1, 1H)
17	20.6 (q)	1.30 (d, J=6.1, 3H)
11-OH	—	12.64 (s, 1H)
16-OH	—	3.88 (d, J=4.3, 1H)

Fig. 1. Deduced structure of AH-1763 IIa.

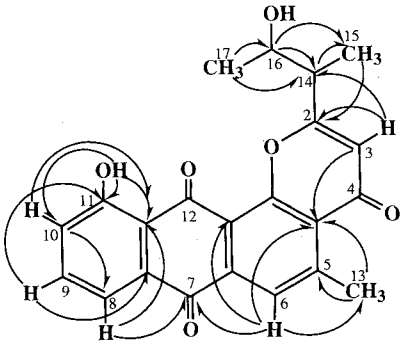


EI-MS. The molecular formula was also supported by the ^1H NMR and ^{13}C NMR spectral data, which were summarized in Table 4. The ^{13}C NMR spectrum of AH-1763 IIa showed the 22 carbons. The analysis of DEPT spectrum indicated that AH-1763 IIa consisted of the following functional groups: $CH_3 \times 3$, $CH \times 1$, $CH{-}O \times 1$, $CH{=} \times 5$, $C{=} \times 6$, $O{-}C{=} \times 3$, $C{=}O \times 3$.

Structural Elucidation

In the ^1H NMR spectrum of AH-1763 IIa a series of three coupled aromatic protons was observed at δ_H 7.82 (dd), δ_H 7.69 (t) and δ_H 7.36 (dd), and two singlet signals were observed at δ_H 8.09 and δ_H 6.28. Also observed were resonances for a C-methyl (δ_H 3.02) singlet, two C-methyl (δ_H 1.43, 1.30) doublets, two methine protons (δ_H 4.32,

Fig. 2. ^{1}H-^{13}C connectivities of AH-1763 IIa as revealed by HMBC experiments.



2.88), a phenolic hydroxyl (δ_H 12.64) and an alcoholic hydroxyl (δ_H 3.88). The ^{1}H NMR spectrum could account for eighteen protons.

In the ^{13}C NMR spectrum of AH-1763 IIa resonances for three carbonyl carbons (δ_C 187.8, 181.7, 178.8), fourteen resonances for aromatic-type carbons, one of which was phenol-bearing carbon (δ_C 162.9), five of which were substituted by proton (δ_C 136.8, 126.1, 125.6, 119.7, 112.1), nine of which were without proton (δ_C 171.4, 162.9, 156.6, 150.4, 136.1, 132.3, 126.5, 119.7, 116.7), and a methyl carbon (δ_C 24.3) indicated the chromophore moiety of the pluramycin group antibiotics[2~6], also supported by the UV spectrum of AH-1763 IIa. Two methyl carbon (δ_C 20.6, 12.5), a methine carbon (δ_C 45.2) and an oxygen bearing methine carbon (δ_C 68.8) accounted for the remaining resonances.

In the HMBC experiments (Fig. 2) the aromatic proton signal at δ_H 6.28 (H-3) was correlated with δ_C 45.2 (C-14). The methyl proton signal at δ_H 1.43 (H-15) was correlated with δ_C 171.4 (C-2), δ_C 45.5 (C-14) and δ_C 68.8 (C-16). From these results the structure of AH-1763 IIa was deduced to be 11-hydroxy-5-methyl-2-(2-hydroxy-1-methylpropyl)-4*H*-anthraceno[1,2-*b*]pyran-4,7,12-trione as shown in Fig. 1. AH-1763 IIa differs from SF-2330[3], hydramycin[4], and saptomycins A, F[5] in the alkyl side chain at C-2. The stereochemistry of the chiral centers remains to be determined.

Antiviral Activities

AH-1763 IIa showed the antiherpetic activity of 2.1 μg/ml as EC_{50} against HSV-1, and cytotoxicity of 15.2 μg/ml as IC_{50} against Vero cells. Therefore the selectivity (the ratio of IC_{50} to EC_{50}) of AH-1763 IIa was calculated as 7.2. Pluramycin group of antibiotics were reported to show the antitumor activity[14,15]. However, there has been no report on antiherpetic activity of these antibiotics.

Table 5. Antimicrobial activities of AH-1763 IIa.

Strain used		MIC (μg/ml)
Bacillus subtilis	IFO 3007	10
Staphylococcus aureus	IFO 3060	2
Micrococcus luteus	IFO 3232	100
Escherichia coli	IFO 3301	>100
Pseudomonas aeruginosa	IFO 3167	>100
Proteus vulgaris	IFO 3448	100
Saccharomyces cerevisiae	IFO 0305	100
Candida albicans	IFO 0583	100
Aspergillus niger	IFO 4066	>100
A. oryzae	IFO 4075	>100

Antimicrobial Activities

As described in Table 5, AH-1763 IIa indicated the inhibitory activities against Gram-positive bacteria such as *Bacillis subtilis* and *Staphylococcus aureus*. Gram-negative bacteria, yeast and fungi seemed to be insensitive. The antimicrobial activity of AH-1763 IIa was similar to those of neopluramycin, hydramycin and saptomycins.

During purification procedure we found the strain produces more than 5 components of antiherpetic agents. The structures of the other components than AH-1763 Ia and IIa will be reported in the future.

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References

1) Egert, E.; M. Noltemeyer, J. Siebers, J. Rohr & A. Zeeck: The structure of tetracenomycin C. J. Antibiotics 45: 1190~1192, 1992
2) Kondo, S.; M. Miyamoto, H. Naganawa, T. Takeuchi & H. Umezawa: Structures of pluramycin A and neopluramycin. J. Antibiotics 30: 1143~1145, 1977
3) Ito, J.; T. Tsuyuki, K. Fujita & M. Sezaki: Studies on a new antibiotic SF-2330. II. The structural elucidation. J. Antibiotics 39: 780~783, 1986
4) Hanada, M.; K. Kaneta, Y. Nishiyama, Y. Hoshino, M. Konishi & T. Oki: Hydramycin, a new antitumor antibiotic. Taxonomy, isolation, physico-chemical properties, structure and biological activity. J. Antibiotics 44: 824~831, 1991
5) Abe, N.; Y. Nakakita, T. Nakamura, N. Enoki, H. Uchida & M. Munekata: Novel antitumor antibiotics, saptomycins. II. Isolation, physico-chemical properties

and structure elucidation. J. Antibiotics 46: 1536～1549, 1993

6) KIM, J.-S.; K. SHIN-YA, J. EISHIMA, K. FURIHATA & H. SETO: A novel neuronal cell protecting substance, espicufolin, produced by *Streptomyces* sp. cu39. J. Antibiotics 49: 947～948, 1996

7) LOCCI, R.; BERGEY'S Manual of Systematic Bacteriology, *Ed.*, S. T. WILLIAMS, M. E. SHARPE & J. G. HOLT, pp. 2451～2503, Williams & Wilkins Co., Baltimore, 1989

8) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313～340, 1966

9) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bacteriol. 56: 107～114, 1948

10) SCHINAZI, R. F.; J. PETERS, C. C. WILLIAMS, D. CHANCE & A. J. NAHMIAS: Effect of combinations of acyclovir with vidarabine or its 5'-monophosphate on herpes simplex viruses in cell culture and in mice. Antimicrob. Agent. Chemother. 22: 499～507, 1982

11) BOYD, M. R.; T. H. BACON, D. SUTTON & M. COLE: Antiherpesvirus activity of 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine (BRL39123) in cell culture. Antimicrob. Agents Chemother. 31: 1238～1242, 1987

12) WAKSMAN, S. A. & H. C. REILLY: Agar-streak method for assaying antibiotic substance. Ind. Eng. Chem., Anal. Ed. 17: 556～557, 1945

13) LOCCI, R.: BERGEY'S Manual of Systematic Bacteriology. *Ed.*, S. T. WILLIAMS, M. E. SHARPE & J. G. HOLT, p. 2474, Williams & Wilkins Co., Baltimore, 1989

14) TAKEUCHI, T.; K. NITTA & H. UMEZAWA: Antitumor effect of pluramycin crude powder on EHRLICH carcinoma of mice. J. Antibiotics, Ser. A 9: 22～30, 1956

15) ABE, N.; Y. NAKAKITA, T. NAKAMURA, N. ENOKI, H. UCHIDA & M. MUNEKATA: Novel antitumor antibiotics, saptomycins. I. Taxonomy of the producing organism, fermentation, HPLC analysis and biological activities. J. Antibiotics 46: 1530～1535, 1993