Hydroxymycotrienins A and B, New Ansamycin Group Antibiotics

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New ansamycins designated hydroxymycotrienins A and B were isolated from culture broths of *Bacillus* sp. BMJ958-62F4. The two antibiotics inhibited more strongly the growth of human cervical cancer cell lines of human papilloma virus (HPV) positive than that of HPV negative cell lines. The structures, some biological and biochemical properties are reported.

There is much evidence that specific types of human papilloma virus (HPV) are responsible for 70% or more of human cervical cancers. The proteins encoded by E6 and E7 genes of the cancer-causing types of HPV inactivate respectively the protein products of p53 and Rb, the major tumor-suppressor genes[1].

Inhibitors of the E6 and/or E7 gene function could be effective against the cervical cancers that express those HPV genes. An *in vitro* screening was initiated to find microbial products that inhibited more strongly the growth of HPV positive human cervical cancer cell lines (HeLa, CaSki, SiHa) than that of HPV negative cell lines (C33A and HT-3). The study led us to find new members of the ansamycin family antibiotics designated hydroxymycotrienins A and B (Fig. 1). We report here the production, isolation, physico-chemical properties, the structures, some biological and biochemical properties of the two substances.

Production, Isolation and Structural Determination

Fermentation of the Antibiotic Producing Strain

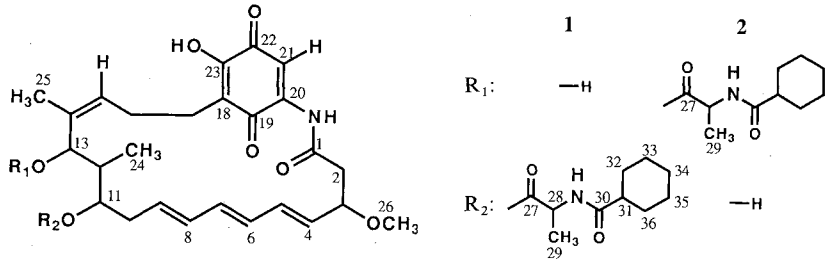
A loopful of a slant culture of *Bacillus* sp. BMJ958-62F4 was inoculated into two 500-ml Erlenmeyer flasks each containing 110 ml of a medium composed of 5.0% glucose, 0.4% Pepton (Nippon SEIYAKU Co. Ltd.), 0.1% yeast extract, 0.1% meat extract, 0.25% NaCl, 1.0% soybean meal and 0.5% $CaCO_3$. The pH was adjusted to 7.0 before sterilization by autoclaving.

After 2 days of fermentation at 27°C on a rotary shaker, about 2 ml portions of the seed culture were transferred to 90 flasks, each containing 110 ml of the same medium as described above. Fermentation was continued for two weeks under the same conditions as for the seed culture.

Isolation of the Antibiotics

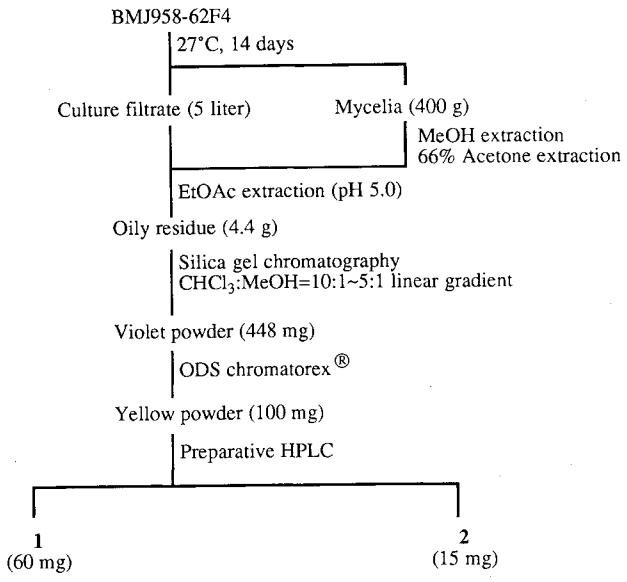
As shown in Fig. 2, 5-liter culture broth was filtered

Fig. 1. Structure of **1** and **2**.



to separate the mycelial cake. The mycelial cake was stirred successively with 2 liters of MeOH and 2 liters of 66% aqueous acetone, one hour each, and filtered. The filtrates were combined and concentrated *in vacuo* to remove the organic solvents resulting in an aqueous solution. The condensed solution was combined with the culture filtrate and the antibiotics were extracted with an equal volume of EtOAc (5 liters). The EtOAc extract was concentrated *in vacuo* to an oily residue (4.4 g), which was chromatographed on a silica gel column (100 g, 5 × 20 cm) using a linear gradient elution of $CHCl_3$ - MeOH (10 : 1 ~ 5 : 1, 0.5 liters in total). Biologically active fractions (inhibitory against the growth of HeLa cells *in vitro*) were collected and evaporated to dryness yielding a violet powder, from which active substances were further purified by an ODS column chromatography (Chromatorex, 2 × 30 cm, SSC Co. Ltd., Tokyo) developed with 70% MeOH/12 mM phosphate buffer (pH 2.89), and then by a preparative HPLC (using a μ-Bondasphere C_{18} column, 5 μm-100 Å, 19 × 150 mm, Waters Co. Ltd.) developed with 70% MeOH/12 mM phosphate buffer (pH 2.89) at an elution rate of 9.9 ml/minute at 40°C under monitoring at 260 nm. Two active substances, whose purities were confirmed by HPLC and TLC, were named hydroxymycotrienins A (**1**) and B (**2**). The physico-chemical properties of **1** and **2** are shown in Table 1.

Fig. 2. Isolation procedure for **1** and **2**.



Structure of **1**

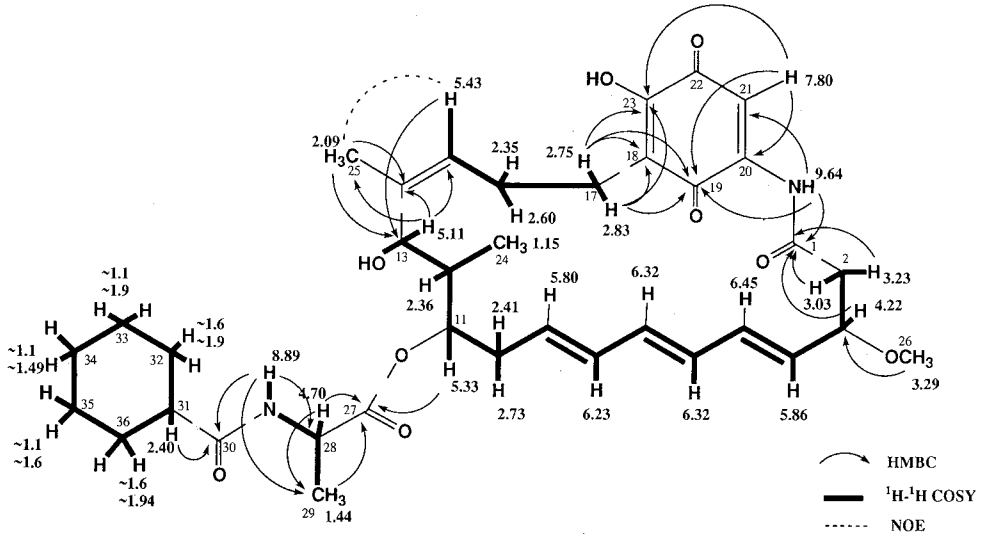
The molecular formula of **1** was established as $C_{36}H_{48}N_2O_9$ (MW 652) from HRFAB-MS and NMR

Table 1. Physico-chemical properties of **1** and **2**.

	1	**2**
Appearance	Yellow powder	Brown powder
$[\alpha]_D^{22}$ (*c* 0.1, MeOH)	+190°	+150°
Molecular formula	$C_{36}H_{48}N_2O_9$	$C_{36}H_{48}N_2O_9$
FAB-MS (*m/z*, Neg.)	651 $(M-H)^-$	651 $(M-H)^-$
FAB-MS (*m/z*, Pos.)	675 $(M+Na)^+$	675 $(M+Na)^+$
HRFAB-MS (*m/z*, Neg.)		
Calcd.	652.3360 (as $C_{36}H_{48}N_2O_9$)	652.3360 (as $C_{36}H_{48}N_2O_9$)
Found	652.3373 M^-	652.3394 M^-
UV_{nm} λ_{max}^{MeOH} ($\log\varepsilon$)	245 (sh) (4.19), 250 (4.46), 260 (4.52), 272 (4.57), 280 (sh) (4.50), 315 (4.06)	245 (sh) (4.46), 250 (4.50),260 (4.55), 272 (4.59), 280 (sh) (4.52), 315 (4.07)
UV_{nm} $\lambda_{max}^{MeOH-NaOH}$ ($\log\varepsilon$)	240 (sh) (4.40), 250 (4.45), 260 (4.52), 272 (4.58), 280 (sh) (4.51), 320 (4.07)	240 (sh) (4.40), 250 (4.48), 260 (4.56), 272 (4.60), 280 (sh) (4.53), 320 (4.08)
UV_{nm} $\lambda_{max}^{MeOH-HCl}$ ($\log\varepsilon$)	208 (4.26), 250 (sh) (4.26), 260 (4.50), 272 (4.61), 280 (4.52), 305 (4.08)	208 (4.30), 250 (sh) (4.34), 260 (4.53), 270 (4.63), 280 (4.55), 305 (4.15)
IR ν_{max}^{KBr} cm^{-1}	3350 (NH, OH), 1210, 1730 (ester), 1530, 1660 (amide), 1000 (triene)	3350 (NH, OH), 1210, 1730 (ester), 1530, 1660 (amide), 1000 (triene)
Rf value on TLC	1) 0.54 ($CHCl_3$ - MeOH = 10 : 1 silica gel Art. 5715) 2) 0.28 (Toluene - Acetone = 3 : 2 silica gel Art. 5715) 3) 0.72 (Toluene - $CHCl_3$ - MeOH = 3 : 7 : 3 silica gel Art. 5715)	1) 0.61 ($CHCl_3$ - MeOH = 10 : 1 silica gel Art. 5715) 2) 0.34 (Toluene - Acetone = 3 : 2 silica gel Art. 5715) 3) 0.78 (Toluene - $CHCl_3$ - MeOH = 3 : 7 : 3 silica gel Art. 5715)
Color reaction	Phosphomolybdate-H_2SO_4 $FeCl_3$	Phosphomolybdate-H_2SO_4 $FeCl_3$
HPLC retention time (min)*	5.5	8.3
Solubility	Soluble: DMSO, $CHCl_3$, MeOH, pyridine, Me_2CO Insoluble: H_2O, *n*-Hexane	Soluble: DMSO, $CHCl_3$, MeOH, pyridine, Me_2CO Insoluble: H_2O, *n*-Hexane

* Column: μ-Bondaspere 5 μC_{18}-100 Å (3.9 mm × 150 mm). mobile phase: 70% MeOH/12 mM phosphate buffer (pH 2.89). flow rate: 1.0 ml/minute detection: 260 nm temperature: 40°C.

Fig. 3. Structure of **1** elucidated by ^1H-^1H COSY, NOE and HMBC experiments.



spectral data.

The UV absorption maxima in MeOH at 260 nm (log ε 4.52), 272 nm (log ε 4.57) and 280 nm (log ε 4.50) indicated the presence of a triene structure in the molecule. The IR spectrum showed a strong absorption at 1000 cm^{-1} arising from the triene structure.

Analysis of ^1H-^1H COSY spectrum gave four partial structures consisting of the following fragments: from C-2 to C-13, from C-15 to C-17, from C-28 to C-29 and from C-31 to C-36. The linkage between C-13 and C-14 was demonstrated by HMBC experiment, while that between C-25 and C-15 was shown by the observation of NOE between 25-H_3 and 15-H.

The HMBC spectrum of **1** revealed the structure which was the same as that of mycotrienin-I[2,3] except for the substitution of a hydroxyl group in the benzoquinone moiety as shown in Fig. 3. The difference in molecular weights between the two compounds is 16, suggesting the presence of an extra oxygen atom in **1**. The UV absorption profile which showed a bathochromic shift from 305 nm (log ε 4.08) in acidic MeOH to 320 nm (log ε 4.07) in alkaline MeOH solution and a positive color reaction with ferric chloride are consistent with the presence of the hydroxyl group in the benzoquinone chromophore. In addition, the HMBC spectrum showed that 21-H (δ_H 7.80) was coupled to C-19 (δ_C 182.68), C-20 (δ_C 140.23) and C-23 (δ_C 157.31), while 17-H_2 (δ_H 2.75, 2.83) were coupled to C-18 (δ_C 118.48), C-19 and C-23. The chemical shift of C-23 (δ_C 157.31) is assignable to an aromatic carbon bearing the hydroxyl group.

No hydroxyl protons at C-13 and C-23 were observed in the ^1H NMR spectrum (pyridine-d_5). However, the presence of the two hydroxyl groups in **1** was confirmed by the NMR analysis of a diacetyl derivative which was obtained by a treatment of **1** with pyridine and acetic anhydride at room temperature for 3 hours. The result of NMR analysis is shown in Fig. 4. On the basis of these results, the planar structure of **1** was concluded as shown in Fig. 1.

Structure of 2

The molecular formula of **2** is the same as that of **1**, $C_{36}H_{48}N_2O_9$. The UV spectra of **2** were almost the same as those of **1**. The comparisons for ^1H and ^{13}C NMR data of **2** with those of **1** revealed that the structure of **2** was different from that of **1** in the ansa-ring moiety. The ^1H and ^{13}C NMR signals of C-11 were observed at δ_H 3.99 and δ_C 70.85 for **2** and δ_H 5.33 and δ_C 75.37 for **1**, while those of C-13 were observed at δ_H 6.47 and δ_C 74.37 for **2** and δ_H 5.11 and δ_C 68.69 for **1**. In the HMBC spectra of **2**, the proton signal of 13-H (δ_H 6.47) was coupled to C-27 (δ_C 173.31). The ^1H-^{13}C long range cross peak indicates that *N*-(cyclohexylcarbonyl)alanine moiety is linked to C-13.

Though the two hydroxyl protons at C-11 and C-23 were not observed in pyridine-d_5, their existence were confirmed by the NMR analysis of the diacetyl derivative of **2** which was obtained by the same procedure for the aceteyl derivative of **1**, as shown in Fig. 4. Thus, the planar structure of **2** was established as shown in Fig. 1.

Table 2. ^{13}C and ^{1}H NMR data of **1** and **2**.

Carbon No.	**1**		**2**	
	^{13}C[a]	^{1}H[b]	^{13}C[a]	^{1}H[b]
1	170.50 (s)	—	170.48 (s)	—
2	44.76 (t)	3.03 (1H, dd, 3.6, 12.6), 3.23 (1H, dd, 10.0, 12.6)	44.87 (t)	3.04 (1H, dd, 3.6, 12.4), 3.19 (1H, dd, 10.0, 12.4)
3	79.79 (d)	4.22 (ddd, 3.6, 7.6, 10.0)	79.83 (d)	4.22 (1H, ddd, 3.6, 7.6, 10.0)
4	132.24 (d)	5.86 (1H, dd, 7.6, 15.6)	131.80 (d)	5.84 (1H, dd, 7.6,15.6)
5	134.35 (d)	6.45 (1H, m)	134.46 (d)	6.47 (1H, dd, 9.5, 15.6)
6	130.13 (d)	6.32 (1H, m)	129.44 (d)	~6.3 (1H, m)
7	134.09 (d)	6.32 (1H, m)	135.00 (d)	~6.3 (1H, m)
8	133.55 (d)	6.23 (1H, m)	132.83 (d)	~6.3 (1H, m)
9	130.63 (d)	5.80 (ddd, 6.4, 8.5, 15.0)	132.88 (d)	6.08 (1H, ddd, 5.8, 8.9, 15.0)
10	33.00 (t)	2.41, 2.73 (2H, m)	36.82 (t)	~2.4, 2.60 (2H, m)
11	75.37 (d)	5.33 (1H, m)	70.85 (d)	3.99 (1H, ddd, 2.4, 5.2, 8.0)
12	39.52 (d)	2.36 (1H, m)	40.16 (d)	2.37 (1H, m)
13	68.69 (d)	5.11 (1H, br, 4.6)	74.37 (d)	6.47 (1H, m)
14	140.03 (s)	—	135.00 (s)	—
15	125.51 (d)	5.43 (1H, br d, 8.5)	127.48 (d)	5.46 (1H, m)
16	27.04 (t)	2.35, 2.60 (2H, m)	26.96 (t)	~2.3, ~2.7 (2H, m)
17	24.12 (t)	2.75, 2.83 (2H, m)	23.89 (t)	~2.7, ~2.8 (2H, m)
18	118.48 (s)	—	118.14 (s)	—
19	182.68 (s)	—	182.55 (s)	—
20	140.23 (s)	—	140.25 (s)	—
	—	9.64 (20-NH, s)	—	9.58 (20-NH, s)
21	111.19 (d)	7.80 (1H, s)	111.19 (d)	7.85 (1H, s)
22	185.19 (s)	—	185.32 (s)	—
23	157.31 (s)	—	157.00 (s)	—
24	10.63 (q)	1.15 (3H, d, 7.0)	11.21 (q)	1.08 (3H, d, 6.8)
25	20.86 (q)	2.09 (3H, br s)	20.85 (q)	1.84 (3H, br s)
26	56.29 (q)	3.29 (3H, s)	56.23 (q)	3.28 (3H, s)
27	173.18 (s)	—	173.31 (s)	—
28	49.18 (d)	4.70 (1H, quintet, 7.0)	48.97 (d)	4.87 (1H, quintet, 7.0)
	—	8.89 (28-NH, d, 6.6)	—	8.84 (28-NH, d, 7.0)
29	17.35 (q)	1.44 (3H, d, 7.3)	17.64 (q)	1.54 (3H, d, 7.4)
30	176.74 (s)	—	176.64 (s)	—
31	44.87 (d)	2.40 (1H, m)	44.95 (d)	~2.35 (1H, m)
32	30.12 (t)	~1.6, ~1.9 (2H, m)	30.02 (t)	~1.6, 1.93 (2H, m)
33	25.89 (t)	~1.1, ~1.6 (2H, m)	25.96 (t)	~1.1, ~1.6 (2H, m)
34	25.96 (t)	~1.1, ~1.49 (2H, m)	26.10 (t)	~1.1, 1.49 (2H, m)
35	26.09 (t)	~1.1, ~1.6 (2H, m)	25.94 (t)	~1.1, ~1.6 (2H, m)
36	29.78 (t)	~1.6, ~1.94 (2H, m)	29.98 (t)	~1.6, 1.93 (2H, m)

[a] Measured in pyridine-d_5 at 125 MHz; chemical shifts in ppm from TMS.
[b] Measured in pyridine-d_5 at 500 MHz; chemical shifts in ppm from TMS.

Although J values of the triene portion in pyridine-d_5 solution of **1** were not fully obtained, the geometries of the triene portion were determined by the ^{1}H NMR spectral analysis of a 13-monoacetyl derivative of **1** in pyridine-d_5. The observed values of $J_{4,5}$, $J_{6,7}$ and $J_{8,9}$ in the monoacetate were 15.0 Hz, 15.8 Hz and 15.8 Hz, respectively, indicating the geometries in the triene moiety of **1** are 4*E*, 6*E*, and 8*E*. The coupling constants of 6-H in **2** was not completely analyzed on the ^{1}H NMR spectrum in pyridine-d_5, either. However, the molar absorption coefficient (ε) values at 260 nm, 272 nm and 280 nm, arising from the triene portion are almost the same as those of **1**. The geometry of C-6 in **2** is therefore estimated to be *E* as in **1**. In the ^{1}H NMR spectra of **1**, NOE's were observed between 15-H at δ_H 5.43 and 25-H_3 at δ_H 2.09. NOE's in **2** were also observed between 15-H at δ_H 5.46 and 25-H_3 at δ_H 1.84. Thus, the geometries of the both double bonds in **1** and **2** are 14*Z*. From the all spectral data, the structures of **1** and **2** were determined as shown in Fig. 1. The configurations of C-3, C-11, C-12, C-13 and C-28 remain to be determined.[4,5]

Biological Activity

Both **1** and **2** were 3 ~ 10 times more growth-inhibitory *in vitro* against human cervical cancer cell lines of HeLa, CaSki and SiHa, all of which are HPV gene positive, than against C33A and HT-3, both of which are HPV

Fig. 4. Partial structures of 13,23-diacetyl derivative of **1** and 11,23-diacetyl derivative of **2** by ^{1}H-^{1}H COSY, HMQC and HMBC experiments (pyridine-d_5).

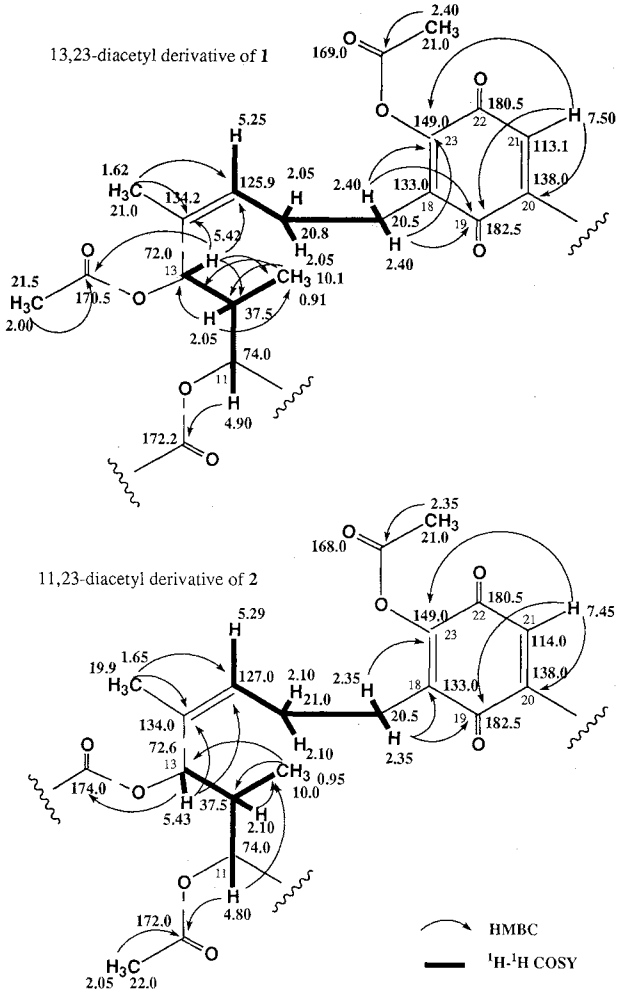
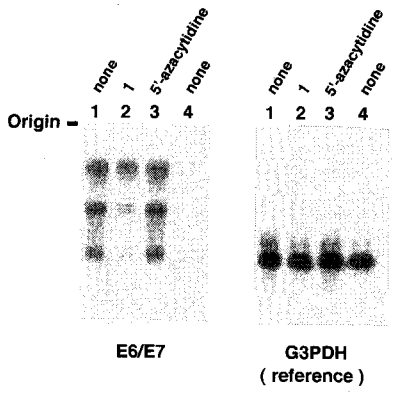


Fig. 5. Effect of **1** on the expression of HPV-18 E6/E7 genes in HeLa cells.



Lanes 1, 2 and 3: HeLa cells, Lane 4: C-33A cells.

Lane 1 ~ 3: HeLa cells (HPV positive), 4: C33A cells (HPV negative). 1: Control (not treated), 2: Cells were treated with 1 μg/ml of **1** for 24 hours. 3: Cells were treated with 0.04 μg/ml of 5-azacytidine for 24 hours.

The probes were PCR products by using the genomic DNA from HeLa cells as the template and the following primers; 5-GGCTGCAGAAGGGAGTAACCAA-3 (E6 sense), 5-GGGAATTCAGTTCCGTGCACAG-3 (E6 anti-sense), 5-GGCTGCAGATGGACCTAAGGCAA-3 (E7 sense) and 5-GGGAATTCCTGCTGGGAT-3 (E7 anti-sense). A PCR (total volume of 100 μl) was conducted in a buffer (20 mM Tris - HCl, pH 8.3, 25 mM KCl, 1.5 mM $MgCl_2$, 0.05% Tween 20) containing 0.5 to 1 μg of the template DNA, 20 p moles of each primer, 50 μM each of the four deoxynucleoside triphosphates and 2.5 U of AmpliTaqR DNA polymerase (Perkin Elmer, N 801-0060). Thirty successive cycles of denaturation at 93°C for 30 s and annealing/extention at 50°C for 1 minute. Human glyceraldehyde-3 phosphate dehydrogenase cDNA (CO-LONTECH, 9805-1) was used as control for loading. Each probe was labeled by nick-translation (Takara Nick Translation Kit, 6040A).

Table 3. Growth-inhibitory activities of **1**, **2** and other triene-ansamycins against cervical cancer cell lines.

	HPV (+)			HPV (−)	
	SiHa	CaSKi	HeLa	C33A	HT-3
1	0.18	0.15	0.07	0.9	2.0
2	0.45	0.75	0.85	3.5	5.0
Mycotrienin I	0.025	0.10	0.02	0.005	0.03
Mycotrienin II	0.025	0.016	0.005	0.008	0.01
Trienomycin A	0.008	0.009	0.008	0.005	0.01

IC_{50}: μg/ml.

gene negative, as shown in Table 3. The activity of **1** was about 5 times stronger than that of **2** when tested. No sign of acute toxicity of **1** was detectable at 100 mg/kg, in mice (ip). 1 did not inhibit the *in vitro* growth of any bacteria, fungi or yeast at 100 μg/ml. As shown in Fig. 5, **1** inhibited the expression of HPV18 E6 and E7 genes that had been integrated into the HeLa cell genome (left, lane 3 *vs.* 4), under conditions where the expression of the control gene was little altered (right, lane 3 *vs.* 4). 5-Azacytidine, an inhibitor of DNA methylation, showed no or little effect on the expression of etiher the viral genes (left, lane 2 *vs.* 4) or the control gene (right, lane 1 *vs.* 4), contrary to our expectation. The absence of hybridization with the RNA from C33A (left, lane 1) proved the specificity of our E6 and E7 probes.

Discussion

1 and **2** are rare examples of triene-ansamycins produced by *Bacillus* strains; so far only aurantinin[6] (KM-214) has been known of the *Bacillus* origin. Under the fermentation conditions employed, **1** and **2** were obtained as violet powders of iron complexes[7], which turned out to be as active as iron-free preparations. The hydroxyl groups in the benzoquinone portions of both

Table 4. Growth-inhibitory activities of clinically used antitumor drugs against cervical cancer cell lines.

	HPV (+)			HPV (−)	
	SiHa	CaSKi	HeLa	C33A	HT-3
5FU	0.4	10	9.0	0.1	0.5
Bleomycin	3.5	5.0	10	0.4	1.0
Cisplatin	1.0	5.0	2.5	0.4	—
Adriamycin	0.03	0.1	0.2	0.05	0.1

IC_{50}: μg/ml.

1 and **2** seem to be important for the selective effect against the HPV gene positive cell lines, because mycotrienin-I, which lacked the hydroxyl group, did not show this selectivity. The other ansamycins, for example, thiazinotrienomycins[8] showed little selectivity against these cell lines.

Anticancer drugs clinically used against cervical cancers, such as cisplatin, bleomycin, 5-Fu and adriamycin are all more inhibitory against the cell lines that are HPV negative than against those that are HPV positive. This suggests that both **1** and **2** act somewhat differently from the conventional anticancer agents against cervical cancers. Retinoic acid[9] and 5-azacitidine[10] also showed some selectivity against the HPV positive cell lines, though less significantly (data not shown).

The growth inhibitory activity against HeLa cells of **1** was generally 5 times stronger than that of **2**, suggesting the importance of the location of the bulky side chain. It is an interesting finding that **1** inhibits the expression of the virus-originated gene in HeLa cells, while leaving the expression of acontrol gene almost unaffected. This may explain the selectivity of **1** and probably of **2** against the cervical cell lines harboring the viral genes; inhibition of the expression of viral genes will lower the intracellular concentrations of their gene products, releasing p53 and Rb proteins to work as cell growth suppressors. How **1** inhibits the viral genes remains to be studied.

Experimental

Human Cancer Cell Lines and Culture Conditions

HeLa, CaSki (ATCC CRL 1550, cervix), SiHa (ATCC CCL HTB35, cervix), C33A (ATCC HTB31) and HT-3 (ATCC HTB32) cells were grown in EAGLE's MEM (NISSUI Pharmaceutical Co. Ltd.) supplemented with 10% v/v fetal bovine serum (FBS, serum), pH 7.4 (adjusted with $NaHCO_3$) at 37°C, in 5% CO_2-containing humidified air. Cells were seeded at a density of 1×10^4 cells/ml/well in Coster 24-well tissue culture clusters (day 0). The cells received test samples on day 1 and were incubated further until day 4. Cell growth was determined using the MTT method and the inhibition caused by the samples was calculated as reported[11].

Northern Blot Analysis of HPV18 E6 and E7 Gene Expression

HeLa cells were treated with 1 μg/ml of **1** and 0.04 μg/ml of 5-azacytidine, which were partially inhibitory concentrations against the cell growth, and RNA was extracted after 24 hours of incubation.

Total RNAs were extracted from the HeLa cells according to the procedure as described[12]. Ten μg of each RNA sample was separated by electrophoresis in 1.2% agarose gels containing 2% formaldehyde and transferred to nitrocellulose filter (Schleicher & Schuell, BA-S 85) by capillary blotting, baked at 80°C for 3 hours, and hybridized with ^{32}P-labeled 158 bp and 105 bp PCR products specific for HPV18 E6 and E7 genes, respectively (see legend to Fig. 5) under stringent conditions (50% formamide, 50 mM sodium phosphate pH 6.5, 5 × SSC, 10 × Denhardt solution) at 42°C for 15 hours. Human glyceraldehyde 3 phosphate dehydrogenase cDNA (CLONTECH, 9805-1) was used as a control probe for loading. The filters were washed with 2 × SSC at 65°C for 30 minutes and then with 0.2 × SSC containing 0.1% SDS at 65°C for 2 hours and were exposed to XRP-5 film (Kodack, 161-8438) with an enhancing screen at −80°C for 3.5 hours.

General

UV spectra were recorded on a Hitachi U-3210 spectrophotometer and IR spectra on a Hitachi 260-10 spectrophotometer. NMR spectra were recorded on a JEOL JNM-A500 NMR spectrometer at 500 MHz for ^1H and at 125 MHz for ^{13}C NMR. Mass spectra were measured on a JEOL JMS-SX 102 mass spectrometer. Optical rotation was measured with a Perkin-Elmer 241 polarimeter.

Analytical Procedures

An HPLC system (SSC Co. Ltd., 3520) using a μ-Bondasphere (C_{18}, 5 μm-100 Å) column (3.9 mm × 150 mm, Waters Co. Ltd.) was developed with 70% MeOH/12 mM phosphate buffer (pH 2.89) at a flow rate of 1 ml/minute at 40°C, and the eluate was monitored at 260 nm. Silica gel TLC (Kieselgel 60 F_{254} Art. 5715, Merck) was developed with either $CHCl_3$-MeOH (10:1), toluene-acetone (3:2), or toluene-$CHCl_3$-MeOH (3:7:3), and spots on the TLC were detected with phosphomolybdate-H_2SO_4.

Preparation of 13,23-Diacetyl Derivative of 1

1 (6.5 mg, 0.01 mmol) was dissolved in pyridine of 0.7 ml. The solution was mixed with 0.35 ml of acetic anhydride and stirred at room temperature for 3 hours. Without drying, the solution was subjected to a preparative HPLC. The HPLC conditions were according to the method in the analytical procedure described above. Fractions eluted at a retention time of 30 minutes were combined and dried *in vacuo*, yielding 4.5 mg of the diacetyl derivative as yellow powders. FAB-MS *m/z* 737

$(M+H)^+$; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 2.00 (3H, s), 2.40 (3H, s).

Preparation of 11,23-Diacetyl Derivative of **2**

2 (6.5 mg, 0.01 mmol) was dissolved in 0.7 ml of pyridine. The solution was mixed with 0.35 ml of acetic anhydride and stirred at room temperature for 3 hours. Without drying, each solution was subjected to a preparative HPLC. Fractions eluted at the retention time of 40 minutes were combined and dried *in vacuo*, yielding 3.5 mg yellow powder of the diacetyl derivative. FAB-MS *m/z* 737 $(M+H)^+$; ^1H NMR ($CDCl_3$, 500 MHz); δ_H 2.05 (3H, s), 2.34 (3H, s).

Preparation of 13-Monoacetyl Derivative of **1**

Acetylation of **1** was performed under the same conditions as the procedure of the 13,23-diacetyl derivative of **1** except for drying the reaction mixture before the preparative HPLC. The monoacetyl derivative was eluted at a retention time of 32 minutes, and finally obtained as yellow powders (5.8 mg). FAB-MS *m/z* 695 $(M+H)^+$; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 2.14 (3H, s).

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