ACTINOPLANONES C, D, E, F AND G, NEW CYTOTOXIC POLYCYCLIC XANTHONES FROM *ACTINOPLANES* SP.

KOJI KOBAYASHI and CHIKAO NISHINO

Mitsubishi-Kasei Institute of Life Sciences,
11 Minamiooya, Machida, Tokyo 194, Japan

JUNICHI OHYA, SHIGERU SATO and TAKASHI MIKAWA

Reseach Center, Mitsubishi Chemical Industries Limited,
1000 Kamoshida, Midori-ku, Yokohama 227, Japan

YOSHINORI SHIOBARA and MITSUAKI KODAMA

Faculty of Pharmaceutical Sciences, Tokushima Bunri University,
Yamashiro-cho, Tokushima 770, Japan

(Received for publication January 12, 1988)

Our previous finding of strong cytotoxic polycyclic xanthones, actinoplanones A (**1**) and B (**2**), in the culture broth of *Actinoplanes* sp. R-304 stimulated us to isolate further five new cytotoxic polycyclic xanthones which were named actinoplanones C (**3**), D (**4**), E (**5**), F (**6**) and G (**7**) from the broth. Actinoplanones C (**3**) and G (**7**) showed very strong cytotoxicity against HeLa cells at less than 0.00004 μg/ml dosage (IC_{50}). The structures of **3**~**7** were varieties of **1** for the N-2 and C-4 substituents. All or several actinoplanones showed strong antimicrobial activities against bacteria and the rice blast fungus. Actinoplanone A (**1**) was tested for cytotoxicity against various tumor cells and for inhibitory effect on HeLa cell macromolecular synthesis, and **1** exhibited strong cytotoxicity against the cells and inhibitory action on DNA synthesis.

In our study to discover antitumor antibiotics from microorganisms, polycyclic xanthone antibiotics, actinoplanones A (**1**) and B (**2**), have been found in the culture broth of *Actinoplanes* sp. R-304 (Actinoplanaceae) as strongly cytotoxic compounds against HeLa cells[1]. This study was undertaken to isolate further cytotoxic analogs of **1** from the broth. Actinoplanones C (**3**), D (**4**), E (**5**), F (**6**) and G (**7**) were consequently isolated.

This paper describes the isolation, structure determination and cytotoxicity against HeLa cells for **3**~**7**. All or several actinoplanones are also tested here for antimicrobial activities against bacteria and the rice blast fungus. Actinoplanone A (**1**) as representative of the actinoplanones is further tested for cytotoxicity against various tumor cells and investigated for inhibitory effect on macromolecular synthesis of HeLa cells. The results of these investigations are also involved in this report.

Materials and Methods

General

MP's, determined on a Yanagimoto micro melting apparatus, are uncorrected. Optical rotations were measured in $CHCl_3$ at 25°C with a Jasco DIP-360 digital polarimeter. UV spectra were recorded in EtOH at 25°C with a Cary 17 spectrometer. IR spectra were run in KBr on a Perkin-Elmer 1730 fourier transformation (FT)-IR spectrometer. ^1H and ^{13}C NMR spectra were taken in $CDCl_3$ on a Jeol JNM-GX400 or on a Bruker AM-500 spectrometer. Mass spectra were obtained with a Jeol JMS-HX100 spectrometer, and high-resolution fast atom bombardment mass spectra

(HRFAB-MS) with a Shimadzu GC-MS 9020-DF(FAB) spectrometer. Preparative TLC was carried out on pre-coated TLC plates of Silica gel 60 F_{254} (Merck) eluting with a solvent mixture of 4% MeOH in $CHCl_3$.

Biological Assays

The method to determine IC_{50} of cytotoxicity against HeLa-S_3 cells and other tumor cells was essentially according to MIRABELLI *et al.*[2]. Cytotoxicity against suspension cells (P-388, YAC-1 and Raji) was determined by counting cell numbers under a light microscope after staining with 0.4% Trypan Blue (Merck).

MIC values for antibacterial activity against Gram-positive and Gram-negative bacteria (Table 6) were estimated by the standard agar dilution method.

Antifungal activity against the rice blast fungus, *Pyricularia oryzae* F67-54, was evaluated according to AKATSUKA *et al.*[3].

Reference Antibiotics and Radioactive Precursors

Mitomycin C and doxorubicin were obtained commercially from Sigma, St. Louis, and used for the cytotoxicity test as the reference compounds. For incorporation experiments, the following radioactive compounds were purchased from Amersham International Inc., England: [*Methyl*-^{3}H]-thymidine (81 Ci/mM), [5,6-^{3}H]uridine (45 Ci/mM) and L-[4,5-^{3}H]leucine (158 Ci/mM).

Incorporation of Radioactive Precursors into HeLa Cell

Incorporation experiments with the radioactive precursors were carried out according to MORI *et al.*[4]. In each experiment, 1 μCi of a precursor was used, and radioactivity of the TCA-insoluble material was counted with a Beckmann LS-250 scintillation counter, after 1 hour incubation.

Derivation of Actinoplanones E (**5**) and F (**6**) from Actinoplanone A (**1**)

To a solution of actinoplanone A (**1**) (2.0 mg) in EtOAc (200 μl), Me_2CO (100 μl) and AcOH (10 μl) were added. After stirring for 24 hours at room temp, the reactant was purified by preparative TLC (20×20 cm, 0.5 mm thickness) to give **5** (1.5 mg). Compound **6** (2.1 mg) was obtained when **1** (2.0 mg) was reacted with 2,3-butanedione (100 μl) for 6 hours at room temp. The physico-chemical and ^{1}H NMR data of **5** and **6** were respectively identical with those of actinoplanones E and F.

Results

Isolation

The isolation procedure[1] was monitored by cytotoxicity against HeLa cells. Extraction of the filtered culture broth (27 liters) of *Actinoplanes* sp. R-304 with EtOAc at pH 2.0 was followed by purification using silica gel column chromatography (eluted stepwisely with mixtures of $CHCl_3$ and MeOH containing 0.5% AcOH)[1] to give two active oily fractions.

The former active fraction (382 mg) was subjected to reversed-phase HPLC (column: 10.7× 250 mm packed with Unisil Q C8 (5 μm) (Gasukuro Kogyo, Tokyo); eluent: 55% MeCN; flow rate: 5 ml/minute; detection: RI detector), which afforded active fractions (100, 20, 7 and 48 mg) corresponding to retention times of 9.8, 11.0, 12.0 and 17.5 minutes, respectively. Each of the active fractions was finally purified by preparative TLC (20×20 cm, 1 mm thickness) to give yellowish solids of actinoplanones A (**1**) (95 mg), G (**7**) (14 mg), E (**5**) (6 mg) and F (**6**) (45 mg).

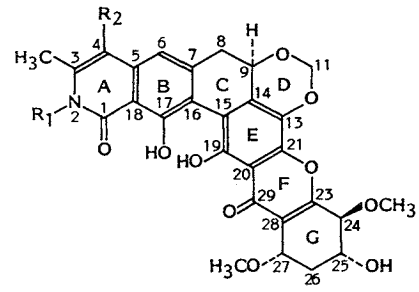
The latter active oily fraction (393 mg) was purified by HPLC (the same conditions as the above except that 50% MeCN was used as eluent). Fractions with retention times of 8.5, 9.5, 12.8 and 16.3 minutes gave crude actinoplanones D (21 mg), C (9 mg), B (73 mg) and A (15 mg), respectively, which were further purified by preparative TLC to give actinoplanones D (**4**) (18 mg), C (**3**) (8 mg), B (**2**) (62 mg) and A (**1**) (12 mg) as solids.

Structure

All actinoplanones C~G exhibited similar physico-chemical properties to those of actinoplanone A (**1**) as shown in Table 1. In the ^{1}H and ^{13}C NMR, similar spectral patterns were observed between the present actinoplanones and **1**, except for signals of protons and carbons on the A-ring (Tables 2 and 3). Thus, it was strongly suggested that the newly isolated actinoplanones possessed analogous structures to **1** and substitution patterns on their A-rings were different.

The electron impact mass spectra (EI-MS) of actinoplanones C and D showed molecular ions (M^+) at *m/z* 550 and 535, respectively, and no chlorine-containing ion clusters. These suggested the replacement of the chlorine atom at C-4 of **1** (M^+, *m/z* 584) by a hydrogen atom in

Fig. 1. Structures of actinoplanones.



Actinoplanone A (**1**) $R_1 = NH_2$ $R_2 = Cl$
Actinoplanone B (**2**) $R_1 = H$ $R_2 = Cl$
Actinoplanone C (**3**) $R_1 = NH_2$ $R_2 = H$
Actinoplanone D (**4**) $R_1 = R_2 = H$
Actinoplanone E (**5**) $R_1 = N{=}C(CH_3)CH_3$ $R_2 = Cl$
Actinoplanone F (**6**) $R_1 = N{=}C(CH_3)COCH_3$ $R_2 = Cl$
Actinoplanone G (**7**) $R_1 = N{=}C(CH_3)COCH_3$ $R_2 = H$

Table 1. Physico-chemical properties of actinoplanones A (**1**), C (**3**), D (**4**), E (**5**), F (**6**) and G (**7**).

	1[a]	**3**	**4**
MP (°C)	276~278 (dec)	278~280 (dec)	222~224 (dec)
$[\alpha]_D^{25}$ ($CHCl_3$)	−619.8° (*c* 0.29)	−646.1° (*c* 0.35)	−624.1° (*c* 0.70)
Formula	$C_{28}H_{25}N_2O_{10}Cl$	$C_{28}H_{26}N_2O_{10}$	$C_{28}H_{25}NO_{10}$
FAB-MS (*m/z*)	585 $(M+H)^+$	551 $(M+H)^+$	536 $(M+H)^+$
HRFAB-MS (*m/z*)	585.1342 ($C_{28}H_{26}N_2O_{10}{}^{35}Cl$, Δmmu +6.8)		
UV λ_{max}^{EtOH} nm (log ε)	229 (4.49), 253 (4.52), 306 (4.13), 366 (4.36), 382 (4.40)	228 (4.28), 253 (4.31), 302 (4.15), 336 (sh), 360 (4.15), 375 (4.19)	221 (4.32), 249 (4.39), 298 (4.08), 312 (4.05), 328 (4.04), 358 (4.21), 372 (4.24)
IR ν_{max}^{KBr} cm^{-1}	3430, 1642, 1630, 1610, 1572, 1100	3450, 1653, 1627, 1599, 1075	3447, 1654, 1622, 1603, 1585, 1086

	5	**6**	**7**
MP (°C)	190~193	281~282 (dec)	173~176
$[\alpha]_D^{25}$ ($CHCl_3$)	−516.7° (*c* 0.21)	−569.1° (*c* 0.24)	−582.3° (*c* 0.44)
Formula	$C_{31}H_{29}N_2O_{10}Cl$	$C_{32}H_{29}N_2O_{11}Cl$	$C_{32}H_{30}N_2O_{11}$
FAB-MS (*m/z*)	625 $(M+H)^+$	653 $(M+H)^+$	619 $(M+H)^+$
HRFAB-MS (*m/z*)		653.1480 ($C_{32}H_{30}N_2O_{11}{}^{35}Cl$, Δmmu −5.6)	
UV λ_{max}^{EtOH} nm (log ε)	233 (4.32), 253 (4.42), 308 (4.03), 342 (sh), 370 (4.24), 386 (4.26)	231 (4.50), 254 (4.54), 308 (4.14), 346 (sh), 386 (4.26)	227 (4.49), 253 (4.50), 304 (4.16), 340 (4.10), 380 (4.22)
IR ν_{max}^{KBr} cm^{-1}	3445, 1652, 1600, 1587, 1075	3449, 1711, 1651, 1588, 1074	3449, 1710, 1655, 1628, 1598, 1075

[a] The data of **1** are from our previous report[1].

Table 2. ^{1}H NMR spectral data[a] of actinoplanones A (**1**), C (**3**), D (**4**), E (**5**), F (**6**) and G (**7**).

Proton	Chemical shift[b] (*J*, Hz)		
	1[c]	**3**	**4**
2-H	—	—	11.30 (s)
2-NH_2	4.92 (s)	4.88 (s)	—
3-CH_3	2.72 (s)	2.48 (s)	2.35 (s)
4-H	—	6.34 (s)	6.23 (s)
6-H	7.30 (s)	6.81 (s)	6.79 (s)
8-H_{ax}	2.97 (dd, 13.9, 12.8)	2.89 (dd, 13.8, 12.9)	2.88 (dd, 13.8, 12.9)
8-H_{eq}	3.32 (dd, 13.9, 4.7)	3.17 (dd, 13.8, 4.6)	3.19 (dd, 13.8, 4.6)
9-H	4.91 (dd, 12.8, 4.7)	4.88 (dd, 12.9, 4.6)	4.99 (dd, 12.9, 4.6)
11-H_{ax}	5.35 (d, 5.8)	5.32 (d, 5.8)	5.33 (d, 5.8)
11-H_{eq}	5.59 (d, 5.8)	5.55 (d, 5.8)	5.50 (d, 5.8)
17-OH	13.48 (s)	13.34 (s)	13.50 (s)
19-OH	12.92 (s)	12.86 (s)	12.86 (s)
24-H	4.19 (d, 2.1)	4.17 (d, 1.8)	4.19 (s)
24-OCH_3	3.71 (s)	3.69 (s)	3.69 (s)
25-H	4.31 (dddd, 8.6, 2.1, 2.0, 2.0)	4.29 (br d, 8.6)	4.32 (s)
25-OH	4.25 (d, 8.6)	4.26 (d, 8.6)	4.32 (s)
26-H_{ax}	2.00 (ddd, 15.0, 3.4, 2.0)	1.99 (ddd, 15.1, 3.4, 1.9)	2.01 (dd, 14.7, 3.1)
26-H_{eq}	2.43 (ddd, 15.0, 3.0, 2.0)	2.42 (ddd, 15.1, 3.0, 1.9)	2.44 (br d, 14.7)
27-H	4.77 (dd, 3.4, 3.0)	4.76 (dd, 3.4, 3.0)	4.77 (br d, 3.1)
27-OCH_3	3.59 (s)	3.58 (s)	3.61 (s)
2′-CH_3	—	—	—
3′-H_3	—	—	—
4′-H_3	—	—	—

Proton	Chemical shift[b] (*J*, Hz)		
	5	**6**	**7**
2-H	—	—	—
2-NH_2	—	—	—
3-CH_3	2.43 (s)	2.47 (s)	2.29 (d, 0.5)
4-H	—	—	6.45 (d, 0.5)
6-H	7.30 (s)	7.32 (s)	6.88 (s)
8-H_{ax}	2.96 (dd, 13.5, 13.0)	2.95 (dd, 13.9, 12.9)	2.91 (dd, 13.8, 12.9)
8-H_{eq}	3.28 (dd, 13.5, 4.4)	3.28 (dd, 13.9, 4.6)	3.20 (dd, 13.8, 4.6)
9-H	4.89 (dd, 13.0, 4.4)	4.89 (dd, 12.9, 4.6)	4.88 (dd, 12.9, 4.6)
11-H_{ax}	5.34 (d, 5.8)	5.31 (d, 5.9)	5.33 (d, 5.8)
11-H_{eq}	5.58 (d, 5.8)	5.56 (d, 5.9)	5.57 (d, 5.8)
17-OH	13.80 (s)	13.35 (s)	13.27 (s)
19-OH	12.96 (s)	12.92 (s)	12.91 (s)
24-H	4.17 (d, 1.5)	4.19 (d, 2.4)	4.17 (d, 1.7)
24-OCH_3	3.70 (s)	3.69 (s)	3.69 (s)
25-H	4.29 (br d, 8.4)	4.29 (br d, 8.3)	4.29 (br d, 8.5)
25-OH	4.25 (d, 8.4)	4.23 (d, 8.3)	4.25 (d, 8.5)
26-H_{ax}	1.99 (br d, 14.7)	1.99 (ddd, 14.8, 3.4, 2.0)	1.99 (ddd, 14.8, 3.4, 2.0)
26-H_{eq}	2.42[d]	2.42 (ddd, 14.8, 3.0, 2.0)	2.42 (ddd, 14.8, 3.0, 2.0)
27-H	4.76 (br s)	4.75 (dd, 3.4, 3.0)	4.76 (dd, 3.4, 3.0)
27-OCH_3	3.58 (s)	3.57 (s)	3.58 (s)
2′-CH_3	1.86[e] (s)	2.05 (s)	2.05 (s)
3′-H_3	2.38[e] (s)	—	—
4′-H_3	—	2.63 (s)	2.62 (s)

[a] Spectra were recorded on a 500-MHz instrument.
[b] Chemical shift (ppm) is given relative to TMS signal.
[c] The data of **1** are from our previous report[1].
[d] Overlap of other signals rendered the coupling constants of this peak unreadable.
[e] Assignments could be interchanged.
—: Denotes no signal.

Table 3. ^{13}C NMR spectral data[a] of actinoplanones A (1), C (3), D (4), E (5), F (6) and G (7).

Position	1[b]	3	4	5	6	7
C-1	163.30	164.87	167.58	158.74	158.23	158.65
C-3	138.57	140.91	139.45	135.94	135.40	138.21
3-CH_3	16.51	19.39	19.06	16.47	16.63	19.20
C-4	110.57	105.86	106.07	112.18	111.88	106.40
C-5	134.16	137.02	137.96	134.55	134.25	136.83
C-6	112.13	114.10	114.38	112.30	112.14	114.31
C-7	141.53	141.22	141.44	141.36	141.93	141.29
C-8	36.92	36.93	36.96	37.15	37.01	36.93
C-9	72.60	73.04	72.84	72.95	72.69	72.98
C-11	91.01	91.06	90.98	91.09	91.02	91.05
C-13	130.70	130.85	130.74	130.95	130.77	130.84
C-14	130.04	130.06	130.02	130.12	129.94	129.99
C-15	111.85[c]	112.47[d]	112.40[e]	112.07[f]	111.69[g]	111.04[h]
C-16	115.23	113.94	114.11	115.49	116.14	114.78
C-17	157.38	157.60	158.23	159.74	158.75	159.43
C-18	108.96	110.17	110.08	110.27	109.78	110.56
C-19	150.62	150.70	150.63	150.82	150.85	150.78
C-20	110.80[c]	111.02[d]	111.00[e]	111.06[f]	110.95[g]	112.26[h]
C-21	143.85	143.80	143.73	144.00	144.04	143.88
C-23	162.82	162.87	162.77	162.95	162.90	162.90
C-24	78.31	78.49	78.47	78.43	78.34	78.46
24-OCH_3	59.87	59.97	59.94	59.98	59.90	59.94
C-25	68.08	68.25	68.26	68.20	68.09	68.20
C-26	26.86	27.09	27.00	26.98	26.86	27.02
C-27	69.62	69.69	69.77	69.62	69.61	69.66
27-OCH_3	58.32	58.45	58.48	58.39	58.35	58.41
C-28	117.23	117.33	117.35	117.33	117.29	117.33
C-29	181.92	182.08	182.03	182.10	181.98	182.07
C-2′	—	—	—	180.92	174.45	174.09
2′-CH_3	—	—	—	20.47[i]	15.11	15.02
C-3′	—	—	—	24.94[i]	196.90	197.23
C-4′	—	—	—	—	25.73	25.69

[a] Chemical shifts (ppm) are given relative to $CDCl_3$ signal as internal reference (77.00 ppm) at 125 MHz.
[b] The date of **1** are from our previous report[1].
[c~i] Assignments bearing the same superscript could be interchanged.
—: Denotes no signal.

actinoplanones C and D. This was supported by their ^1H NMR spectra in which one proton signal respectively appeared at δ 6.34 and 6.23 in actinoplanones C and D. In the ^1H-^1H correlation spectroscopy (^1H-^1H COSY), the proton signals of both actinoplanones C and D indicated cross peaks with signals of 6-H and 3-methyl protons. These signals were thus assignable to 4-H. In the ^{13}C NMR spectra of actinoplanones C and D, an olefinic methine carbon signal assignable to C-4 appeared at δ 105.86 and 106.07, respectively, instead of the C-4 quaternary carbon signal of **1**. The structure of actinoplanone C was, therefore, determined to be **3**.

The following differences were observed between the ^1H NMR spectra of **3** and actinoplanone D. Actinoplanone C (**3**) exhibited an NH_2 proton signal at δ 4.88, whereas in the spectrum of actinoplanone D no signal was seen in this region. In addition, the appearance of a D_2O exchangable amide proton signal at δ 11.30 in actinoplanone D suggested that this compound corresponded to the deamino derivative of **3**. Hence, the structure **4** was assigned to actinoplanone D.

The MH^+ of actinoplanone E appeared at *m/z* 625 in its FAB-MS. This implies the presence of two nitrogen atoms in this compound whose EI-MS indicated the presence of a chlorine atom. The ^1H NMR spectrum of actinoplanone E showed singlet signals due to two methyl groups (δ 1.86 and 2.38), but no NH_2 proton signal. Analysis of the ^{13}C distortionless enhancement by polarization transfer (DEPT) spectrum of actinoplanone E indicated the presence of two methyl carbons and a quaternary carbon at δ 20.47, 24.94 and 180.92, respectively, whereas similar carbon signals were not observed in the ^{13}C NMR spectrum of **1**. These ^1H NMR and DEPT spectral data were interpreted by the methyl and quaternary carbons attached to the nitrogen atom on N-2 to form the grouping, $>N(2)N=C(CH_3)_2$. Thus, the structure **5** was postulated for actinoplanone E, and confirmed by the synthesis of **5** from **1** and acetone. The physico-chemical and ^1H NMR data of **5**, prepared from **1**, were identical with those of actinoplanone E.

The molecular formula of actinoplanone F was determined to be $C_{32}H_{29}N_2O_{11}Cl$ (*m/z* 653.1480 observed for $C_{32}H_{30}N_2O_{11}{}^{35}Cl$ $(M+H)^+$, mmu error -5.6) by the HRFAB-MS. The fragment ion at *m/z* 609.1312 ($C_{30}H_{26}N_2O_{10}{}^{35}Cl$ $(M-CH_3CO)^+$, mmu error $+3.7$) indicated the presence of an acetyl group in the molecule. In the ^1H NMR spectrum of actinoplanone F, protons of three methyl groups resonated at δ 2.05, 2.47 and 2.63, but signals due to NH_2 protons were not detected. These three methyl carbon signals were recognized at δ 15.11, 16.63 and 25.73 by analyzing the ^{13}C-^1H COSY spectrum. The singlet signal of the methyl protons at δ 2.47 showed cross peaks with C-3 (δ 135.40) and C-4 (δ 111.88) signals in the long-range ^{13}C-^1H COSY. This indicates that this methyl group is located on C-3. The other methyl protons (δ 2.05 and 2.63) were correlated with the quaternary carbons at δ 174.45 and 196.90, respectively, indicating the presence of the groupings, $CH_3\overset{|}{C}=$ and $CH_3\overset{|}{C}=O$, the latter of which was also suggested by the HRFAB-MS. From these results and the detailed comparison of ^1H NMR spectra between actinoplanone F and **1**, the above two groups should be connected for the substituent on N-2. However, two plausible moieties, $CH_3COC(CH_3)=N$ and $CH_3CON=C(CH_3)$, were still considered. In order to confirm the structure of actinoplanone F, **6** was prepared from **1** and 2,3-butanedione. The identity of the physico-chemical and ^1H NMR data for synthetic **6** and actinoplanone F established the structure of the latter.

The MH^+ of actinoplanone G was observed at *m/z* 619 in the FAB-MS and the absence of chlorine atom in the molecule was verified by its EI-MS. Accordingly, $C_{32}H_{30}N_2O_{11}$ was assigned to the molecular formula of this compound. The ^1H NMR spectra of actinoplanone G and **6** were extremely similar except in the following resonance region. Actinoplanone G exhibited a doublet signal ($J=$ 0.5 Hz) at δ 6.45, but **6** gave no such signal. Since this signal had a coupling with the 3-methyl proton signal (δ 2.29), the signal at δ 6.45 was assigned to 4-H. Therefore, the structure **7** could be assigned to actinoplanone G.

Biological Activities

Biological activities of actinoplanones are listed in Tables 4, 5 and 6.

All actinoplanones showed strong cytotoxicity against HeLa cells, in particular actinoplanones C (**3**) and G (**7**) exhibited IC_{50} values (concentration causing 50% inhibition of cell growth in an *in vitro* assay) at less than 0.00004 μg/ml (Table 4). For assays with other tumor cells, only actinoplanone A (**1**) was used because of the quantities isolated[1]. Actinoplanone A (**1**) exhibited $10^2 \sim 10^3$ times stronger cytotoxicity than the reference antibiotics, mitomycin C and doxorubicin, in all the test cells (Table 5).

Table 4. Cytotoxic and antifungal activity of actinoplanones A~G (**1**~**7**) in *in vitro* assay.

Compound	Activity (IC_{50}[a], μg/ml)	
	HeLa-S_3	*Pyricularia oryzae* F67-54
1	0.00004	0.0016
2	0.005	0.106
3	<0.00004	0.046
4	0.011	0.082
5	0.00013	0.0056
6	0.002	0.056
7	<0.00004	0.037

[a] IC_{50} values against *P. oryzae* were evaluated as concentrations causing 50% inhibition of the spore germination.

Table 5. Cytotoxicity of actinoplanone A (**1**) against various tumor cells in *in vitro* assay.

Cell line	Cytotoxicity (IC_{50}[a], μg/ml)		
	1	Mitomycin C[b]	Doxorubicin[b]
P-388	0.00008	0.018	0.007
YAC-1	0.00016	0.067	0.009
Raji	0.000009	0.024	0.013
L-929(1)	0.00054	>1.0	0.73
CHO	0.0004	0.98	0.31
VERO	0.00009	0.69	>1.0
KB	0.0013	0.97	0.10
HeLa-S_3	0.00004	0.02	0.02
HLC	0.0004	0.03	0.09
PLC	0.0001	0.11	0.19
KN	0.0004	0.06	>1.0

[a] IC_{50} values were evaluated at less than 1.0 μg/ml.
[b] Reference antibiotics.

Table 6. Antibacterial activity of actinoplanones A (**1**), B (**2**) and F (**6**) in *in vitro* assay.

Organism	Activity (MIC[a], μg/ml)		
	1	**2**	**6**
Staphylococcus aureus FDA 209P JC-1	<0.0007	0.049	1.56
S. aureus Terajima	<0.0007	0.049	1.56
S. aureus MS353	<0.0007	0.049	0.78
Bacillus subtilis ATCC 6633	<0.0007	0.098	1.56
Micrococcus luteus ATCC 9341	<0.0007	0.098	6.25
Escherichia coli NIHJ JC-2	3.12	>50	>50
E. coli K-12 C-600	0.39	50	>50
Klebsiella pneumoniae PCI 602	0.19	>50	>50
Morganella morganii IFO 3848	0.05	>50	>50
Salmonella typhimurium IID-971	3.12	>50	>50
S. schottmuelleri 8006	0.78	>50	>50
S. enteritidis G 14	0.39	>50	>50
Serratia marcescens IAM 1184	6.25	>50	>50
Pseudomonas aeruginosa IFO 3445	12.5	>50	>50
P. aeruginosa NCTC 10490	6.25	>50	>50
P. aeruginosa PAO-1	12.5	>50	>50
Proteus vulgaris OX 19	0.19	>50	>50
P. vulgaris HX 19	3.12	>50	>50
P. rettgeri IFO 3850	0.39	>50	>50
Enterobacter aerogenes ATCC 13048	12.5	>50	>50
E. cloacae 936	0.78	>50	>50

[a] MIC values were determined at a range of concentrations of 0.0007~50 μg/ml.

Antifungal activity of actinoplanones against the rice blast fungus, *P. oryzae*, was significantly strong. In this case, the concentration causing 50% inhibition of the spore germination of the fungus was expressed as IC_{50} which was recorded in the range of 0.0016~0.106 μg/ml (Table 4).

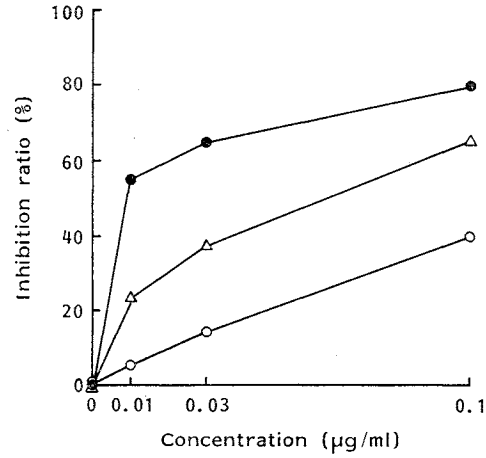
For the antibacterial assay, actinoplanones A (**1**), B (**2**) and F (**6**) were used. Actinoplanone A (**1**) was highly active against Gram-positive bacteria at less than 0.0007 μg/ml (MIC), and was rather effective even against Gram-negative bacteria at 0.05~12.5 μg/ml (MIC) (Table 6). However, actinoplanones B (**2**) and F (**6**) were inactive against Gram-negative species (>50 μg/ml).

Effect on Macromolecular Synthesis

In studying the effect of actinoplanones on macromolecular (DNA, RNA and protein) synthesis in HeLa cells, actinoplanone A (**1**) was used as representative of the actinoplanones. Incorporation of the radioactive precursors (^{3}H-labeled thymidine, uridine and leucine for DNA, RNA and protein synthesis, respectively) into the cell was evaluated after 1 hour incubation of the cell with or without **1** (control). Based on the incorporation data at this incubation period, inhibition ratio (%) of **1** depending upon its quantities (0.01, 0.03 and 0.1 μg/ml) was calculated for each synthesis, and is shown in Fig. 2.

Fig. 2. Inhibitory effect of actinoplanone A (**1**) on incorporation of labeled precursors in HeLa-S_3 cells.

● [^{3}H]Thymidine, △ [^{3}H]leucine, ○ [^{3}H]uridine.



The inhibitory effect of **1** was observed at 0.03 and 0.1 μg/ml. However, DNA synthesis was predominantly inhibited even at 0.01 μg/ml. Protein and RNA syntheses were inhibited by **1** moderately and weakly, respectively. Thus, the primary target of actinoplanone A (**1**) in HeLa cells was DNA synthesis. Other actinoplanones are expected to behave similarly.

Discussion

The structures of actinoplanones C~G (**3**~**7**) isolated in study differ from the previously described[1] actinoplanones A (**1**) and B (**2**) by a variety of substituents at the N-2 and C-4 positions. They are formulated as having the same absolute stereochemistry as actinoplanone A (**1**)[1]; namely 9*R*, 24*S*, 25*R* and 27*S*. This is supported by the near coincidence throughout the series of the chemical shifts of analogous atoms, both the protons and carbons, in the two regions with chiral centers and by the essentially identical molecular rotations of all members of the series.

Actinoplanone A (**1**) could be converted into actinoplanones E (**5**) and F (**6**) on condensation with acetone and 2,3-butanedione, respectively, in the presence of a catalytic quantity of acetic acid. This chemical reaction suggests a possibility of artificial formation of **5** and **6** from **1** in the fermentation and the subsequent extraction processes, because both acetone and 2,3-butanedione (diacetyl) can be derived from acetyl-CoA[5] in the microorganism. Especially, considering the acidic condition (pH 2) of the extraction process, the endogeneous acetone and 2,3-butanedione are assumed to react with **1** as condensation reagents. A possibility of artificial conversion of actinoplanone C (**3**) to actinoplanone G (**7**) is also probable. Although **5**~**7** could be artifacts, their very strong biological activities are of interest.

Among the known polycyclic xanthone antibiotics, albofungin[6], chloroalbofungin[6], lysolipin I[7], cervinomycins A_1 and A_2[8] and LL-D42067α and β[9], only albofungin (P-42-1) has been reported to possess cytotoxicity against HeLa cells and to prolong the life of mice into which Ehrlich ascites tumor cells have been transplanted[10]. The cytotoxicity of most actinoplanones (except **4**) against HeLa cells was much stronger than that of albofungin whose cytotoxicity was reported to be 0.005~0.01 μg/ml[10].

From the present results, structure-activity relationships for the N-2 and C-4 region of the actinoplanones may be described. No specific substitution at the N-2 and C-4 seems to be essential for cytotoxic potency (see cytotoxicity of **4** despite weak activity in Table 4). The NH_2 substituent on N-2, as in **3**, and its 2,3-butanedione derivative, as in **7**, considerably enhance the activity. The

chlorine atom at C-4 (see **2**) slightly elevates the activity (compare the activity of **4** and **2**) in the absence of an N-2 substituent. However, the chlorine atom becomes detrimental to activity in the presence of the NH_2 substituent at N-2 or its derivative as seen by comparison of the following pairs of compounds: **1** and **3**, and **6** and **7**.

The antifungal activity of actinoplanones against the rice blast fungus (Table 4) is ordered as follows: A (**1**)>E (**5**)>G (**7**)>C (**3**)>F (**6**)>D (**4**)>B (**2**). The NH_2 and its derivatives at the N-2 are preferred for activity; the actinoplanones without such groups (**2** and **4**) have only weak activity. The role of the C-4 chlorine atom in antifungal activity is unclear from the present assay results.

Albofungin[10], lysolipin I[11] and cervinomycin[12] are reported to be active against Gram-positive bacteria, but almost inactive against Gram-negative bacteria. However, actinoplanone A (**1**) has activity against both Gram-negative (MIC: 0.05~12.5 μg/ml) and Gram-positive bacteria (Table 6). The presence of an unsubstituted NH_2 at the N-2 seems to enhance antibacterial activity as demonstrated by the lower activity of **2** and **6** than that of **1**. The effect of the chlorine atom at C-4 can not be delineated.

In the present incorporation experiments, DNA synthesis was inhibited by **1** more than other macromolecular syntheses in HeLa cell, which implies some interaction between **1** and DNA. An X-ray analysis of daunorubicin-d(CGTACG) complex has indicated the intercalating action of the antibiotic with DNA base pairs[13]. In the analysis, the planars B~D ring system is shown to intercalate into DNA base pairs, and a hydrogen bonding interaction of the O-9 of daunorubicin was also demonstrated with the N-2 and N-3 of a guanine moiety of DNA, as well as the hydrogen bond between the O-13 of the antibiotic and the O-2 of cytosine *via* a water molecule.

Therefore the structural analogy of actinoplanones to daunorubicin was examined using Dreiding models. This revealed that the planars C~F ring system of actinoplanones is almost superimposable on the B~D rings of daunorubicin and that the substituents (OH and OCH_3) with axial configuration on the G ring of actinoplanones orient similarly to the oxygen atoms (O-9 and O-13) on the A ring of daunorubicin. Accordingly, the following speculations are possible in the actinoplanone structure: (i) The intercalation of the C~F ring system into DNA base pairs, and (ii) the hydrogen bonding interaction of the substituents on the G ring with oxygen and nitrogen atoms of DNA bases.

Although detailed investigation on the mode of action is required, the intercalative interaction of actinoplanones with DNA base pairs may be a plausible cause for their inhibitory action on DNA synthesis.

Acknowledgment

We thank Dr. SEIJI TAKAHASHI, Shimadzu Seisakusho Co., Ltd., for measurement of the HRFAB-MS.

References

1) KOBAYASHI, K.; C. NISHINO, J. OHYA, S. SATO, T. MIKAWA, Y. SHIOBARA & M. KODAMA: Actinoplanones A and B, new cytotoxic polycyclic xanthones from *Actinoplanes* sp. J. Antibiotics 41: 502~511, 1988
2) MIRABELLI, C. K.; H. BARTUS, J. O. L. BARTUS, R. JOHNSON, S. M. MONG, C. P. SUNG & S. T. CROOKE: Application of a tissue culture microtiter test for the detection of cytotoxic agents from natural products. J. Antibiotics 38: 758~766, 1985
3) AKATSUKA, T.; O. KODAMA, H. SEKIDO, Y. KONO & S. TAKEUCHI: Novel phytoalexins (oryzalexins A, B and C) isolated from rice blast leaves infected with *Pyricularia oryzae*. Part I: Isolation, characterization and biological activities of oryzalexins. Agric. Biol. Chem. 49: 1689~1694, 1985
4) MORI, A.; C. NISHINO, N. ENOKI & S. TAWATA: Cytotoxicity of plant flavonoids against HeLa cells. Phytochemistry 27: 1017~1020, 1988
5) METZLER, D. E.: Biochemistry. *Ed.* D. E. METZLER, p. 442, 554, Academic Press, New York, 1977
6) ONOPRIENKO, V. V.; Y. P. KOZ'MIN & M. N. KOLOSOV: Chemistry of albofungin. XVI. Revised structure of albofungin and chloroalbofungin. Bioorg. Khim. 4: 1418~1422, 1978
7) DOBLER, M. & W. KELLER-SCHIERLEIN: Metabolites of microorganisms. 162nd communication. The crystal and molecular structure of lysolipin I. Helv. Chim. Acta 60: 178~185, 1977
8) NAKAGAWA, A.; S. ŌMURA, K. KUSHIDA, H. SHIMIZU & G. LUKACS: Structure of cervinomycin, a novel

xantone antibiotic active against anaerobe and mycoplasma. J. Antibiotics 40: 301~308, 1987

9) LEE, T. M.; D. B. BORDERS, G. T. CARTER, M. HERTZ & J. P. KIRBY: LL-D42067α and β, novel antibacterial and antiprotozoal agents: Isolation, characterization and structure determination. Program and Abstracts of the 26th Intersci. Conf. on Antimicrob. Agents Chemother., No. 222, p. 136, New Orleans, Sept. 28~Oct. 1, 1986

10) FUKUSHIMA, K.; K. ISHIWATA, S. KURODA & T. ARAI: Identity of antibiotic P-42-1 elaborated by *Actinomyces tumemacerans* with kanchanomycin and albofungin. J. Antibiotics 26: 65~69, 1973

11) DRAUTZ, H.; W. KELLER-SCHIERLEIN & H. ZÄHNER: Stoffwechselproducte von Mikroorganismen. 149. Mitteilung. Lysolipin I, ein neues Antibioticum aus *Streptomyces violaceoniger*. Arch. Microbiol. 106: 175~190, 1975

12) ŌMURA, S.; Y. IWAI, K. HINOTOZAWA, Y. TAKAHASHI, J. KATO, A. NAKAGAWA, A. HIRANO, H. SHIMIZU & K. HANEDA: Cervinomycin A_1 and A_2, new antibiotics active against anaerobes, produced by *Streptomyces cervinus* sp. nov. J. Antibiotics 35: 645~652, 1982

13) QUIGLEY, G. J.; A. H.-J. WANG, G. UGHETTO, G. VAN DER MAREL, J. H. VAN BOOM & A. RICH: Molecular structure of an anticancer drug-DNA complex: Daunomycin plus d(CpGpTpApCpG). Proc. Natl. Acad. Sci. U.S.A. 77: 7204~7208, 1980