STRUCTURE-ACTIVITY RELATIONSHIP OF A NOVEL ANTITUMOR ANSAMYCIN ANTIBIOTIC TRIENOMYCIN A AND RELATED COMPOUNDS

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(Received for publication March 31, 1988)

Various derivatives of trienomycin A (**1**) were prepared and tested for cytocidal activities. All the derivatives except for 22-*O*-methyltrienomycin A (**5**) showed reduced cytotoxicity compared with **1**. It is concluded that the existence of a triene moiety, free 13-OH and an acyl group at C-11 owe important role for cytocidal activity.

Trienomycin A (**1**) was isolated as a cytocidal antibiotic from the fermentation broth of *Streptomyces* sp. No. 83-16 and subsequently trienomycins B (**2**) and C (**3**) were isolated as congeners of **1** produced by the same microorganism. In preceding papers, we reported the production, isolation and structural elucidation of **1**~**3**[1~3] and *in vivo* activity of **1** against various experimental murine tumors[4].

Trienomycins A (**1**)~C (**3**) are classified as benzenoid ansamycin antibiotics closely related to mycotrienins (ansatrienins)[5~13] but trienomycins are unique in that they do not have a *p*-quinone or *p*-hydroquinone moiety in the structure. Such a benzenoid moiety in the trienomycin group is rather similar to that of the maytansinoids which were originally isolated from higher plants[14]. It was reported that a *m*-C_7N unit derived from 3-amino-5-hydroxybenzoic acid was the precursor of the chromophore moiety[15] of the ansamycin antibiotics and it is noteworthy that the trienomycins contain a virtually unmodified 3-amino-5-hydroxybenzoic acid moiety.

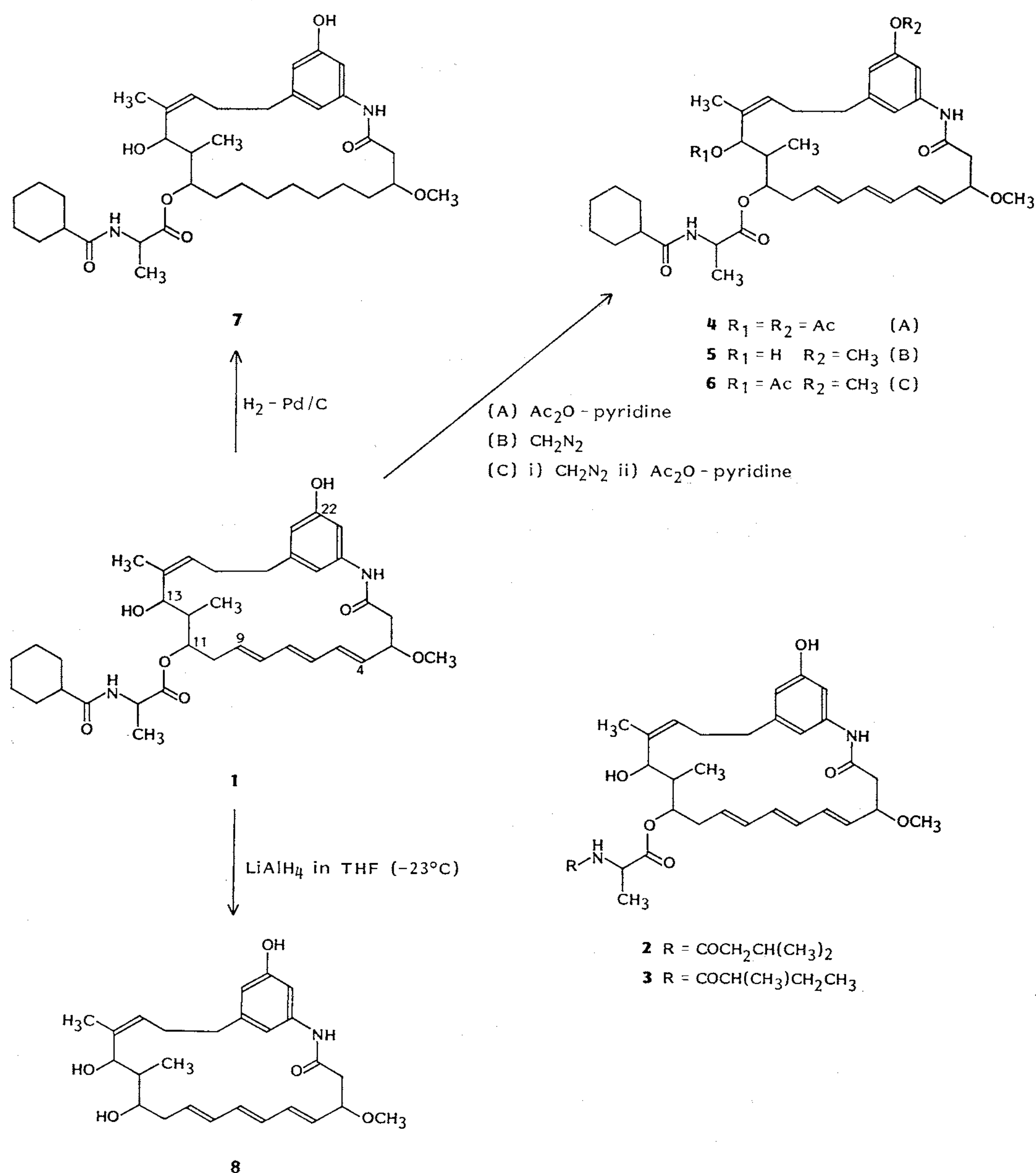
In the structure of trienomycin A (**1**), a hexahydrobenzoyl moiety is attached to the ansa moiety *via* D-alanine. Instead of the hexahydrobenzoyl moiety, a 3-methylbutyryl (isovaleryl) or 2-methylbutyryl moiety is present in the structure of trienomycins B (**2**) and C (**3**)[3], respectively (Fig. 1). Other partial structures including a triene moiety between C-4~C-9 and hydroxyl groups at C-13 and C-22 are common to **1**~**3**.

Trienomycins showed potent cytocidal activities against HeLa S_3 cells *in vitro* whereas these compounds were devoid of activity against microorganisms except for *Piricularia oryzae*[1,3]. Consequently, we are interested in elucidating the structural features of the trienomycins that are required for their cytocidal activity.

This paper deals with structure-activity relationships of trienomycin A (**1**) and related compounds.

Because trienomycin A (**1**) possesses hydroxyl groups at C-13 and C-22, these moieties were modified by acetylation and/or methylation. Thus, when **1** was treated with Ac_2O - pyridine, the diacetyl derivative (**4**) was obtained, whereas the C-22 monomethylether (**5**) was obtained by treating **1** with CH_2N_2. A 22-*O*-methyl and 13-*O*-acetyl derivative (**6**) was prepared by acetylation of **5** (Fig. 1).

Fig. 1. Preparation of compounds **1**~**8**.



On the other hand, a hexahydrogenated compound **7** was obtained through the catalytic reduction of **1** and the structure confirmed by spectroscopic analysis. Interestingly, only the triene moiety was hydrogenated in this case. In addition, by treating **1** with $LiAlH_4$ at −23°C[16], C-11 deacylated compound (trienomycinol) was obtained and the structure defined as **8** by spectroscopic analysis (Fig. 1).

^{13}C NMR spectral data of the compounds obtained herein are summarized in Table 1.

Cytocidal activities against HeLa S_3 and P388 leukemia cells of **1**~**8** were compared and the results are summarized in Table 2. As shown in Table 2, all the compounds except for **5** showed much weaker

Table 1. ^{13}C NMR assignments of trienomycin A (1) and related compounds.

Carbon No.	1	4	5	6	7	8
C-1	170.8	170.6	168.0	170.5	170.7	168.8
C-2	44.8	43.6	43.8	43.9	41.7	44.9
C-3	81.6	78.9	78.8	78.9	79.2	80.9
C-4	131.0[a]	131.2[a]	130.9[a]	131.5[a]	24.4[a]	132.3
C-5	135.0[a]	133.8[a]	133.3[a]	134.0[a]	26.2[a]	134.3
C-6	135.2[a]	133.9[a]	134.0[a]	134.1[a]	29.0[a]	134.9
C-7	132.5[a]	133.5[a]	129.7[a]	133.4[a]	29.7[a]	132.6[a]
C-8	130.5[a]	129.8[a]	129.5[a]	129.8[a]	30.0[a]	129.6[a]
C-9	134.6[a]	133.7[a]	133.7	133.6	30.0[a]	133.0
C-10	33.7	33.3	33.4	33.3	33.5	38.0
C-11	76.4	74.0	75.3	73.0	77.5	72.6
C-12	40.4	37.7	39.3	37.7	40.9	42.1
C-13	69.7	71.5	68.4	71.6	70.6	69.5
C-14	139.7[b]	138.4[b]	138.5[b]	138.5[b]	137.5[b]	140.4[b]
C-15	125.9	129.0	124.6	129.1	127.4	125.0
C-16	30.8	29.0	29.3	29.1	30.1	31.0
C-17	37.3	35.6	36.5	36.0	36.7	37.1
C-18	144.9	143.5	144.0	143.4	144.8	144.6
C-19	112.9[c]	117.4[c]	111.2[c]	111.3[c]	112.6[c]	111.9[c]
C-20	140.2[b]	138.5[b]	138.6[b]	138.5[b]	139.2[b]	141.1[b]
C-21	107.2	111.9	104.1	104.0	106.5	106.9
C-22	158.6	157.2	160.0	160.1	157.7	159.4
C-23	113.4[c]	117.9[c]	112.2[c]	112.3[c]	112.2[c]	112.6[c]
C-24	10.2	10.3	9.9	10.3	10.7	11.5
C-25	20.8	20.3	20.4	20.4	18.6	21.2
C-27	173.3	172.1	172.9	172.2	173.7	—
C-28	50.5	47.9	48.6	47.9	48.9	—
C-29	17.2	18.1	17.6	18.2	18.1	—
C-30	179.2	175.6	176.6	175.5	177.7	—
C-31	45.9	45.1	45.0	45.2	45.5	—
C-32	30.5	29.5	29.5	29.6	29.8	—
C-33	26.7	25.6	25.6	25.6	26.2	—
C-34	26.9	25.7	25.6	25.7	26.5	—
C-35	26.7	25.6	25.6	25.6	26.2	—
C-36	30.5	29.5	29.5	29.6	29.8	—
3-OCH_3	56.6	56.6	56.7	56.6	57.0	56.6
13-$OCOCH_3$	—	167.9[d]	—	167.8	—	—
	—	20.8	—	20.8	—	—
22-OCH_3	—	—	55.3	55.3	—	—
22-$OCOCH_3$	—	169.6[d]	—	—	—	—
	—	21.1	—	—	—	—

[a~d] Assignments in a vertical column may be interchanged.

cytocidal activities than **1** against HeLa S_3 and P388 cells *in vitro*. It is especially noteworthy that in the case of compound **8**, the cytocidal activity was reduced to 1/25,000 of compound **1**. On the other hand, in compound **7** in which the triene moiety was reduced, the cytocidal activity was reduced to *ca*. 1/5,000 of that of **1**. Furthermore, cytocidal activities were reduced considerably in compounds **4** and **6** in which both 13-OH and 22-OH were replaced with OAc$\times$2 or OAc and OCH_3, respectively.

From all of the observations described above, it was concluded that the hexahydrobenzoyl-D-alanyl moiety of **1** played very important role in the cytocidal activity and the existence of a triene

moiety between C-4～C-9 and a free 13-OH were also important. However, it appeared that the 22-OH moiety did not affect the cytocidal activity because **5**, in which only 22-OH was masked, showed almost the same cytocidal activities as the original compound (Fig. 2 and Table 2). In addition, because cytocidal activities of trienomycins B (**2**) and C (**3**) are 1/40 and 1/20 of those of **1**, it can be said that as an acyl group attached to the D-alanyl moiety, hexahydrobenzoyl moiety is superior to the isovaleryl or 2-methylbutyryl moiety for cytotoxic activity.

Because 22-*O*-methyltrienomycin A (**5**) showed almost the same cytocidal activity against P388 leukemia cells *in vitro*, an investigation of the *in vivo* activity of compound **5** was performed. As a result, **5** showed similar antitumor activity to **1** against P388 leukemia *in vivo* (Table 3).

It is interesting that mycotrienins I (**9**) and II (**10**) showed potent antifungal activities (MIC 2～10 μg/ml) against such organisms as *Candida albicans*, *Saccharomyces* sp. and *Aspergillus niger*[5] whereas trienomycins showed no activity up to 1,000 μg/ml against G(+) and G(−) bacteria, fungi, yeasts except for trienomycins A (**1**), B (**2**) and C (**3**) and 22-*O*-methyltrienomycin A (**5**)

Table 2. Cytocidal activities (IC_{50}) of trienomycin A (1) and related compounds.

Compound	HeLa S_3 (μg/ml)	P388 (μg/ml)
1	0.005	0.02
2	0.2	1.6
3	0.1	0.1
4	25.0	1.6
5	0.005	0.02
6	5.0	1.6
7	25.0	6.3
8	125.0	6.3

Fig. 2. Structural requirements for cytocidal and antifungal activities for trienomycin A (1) and related compounds.

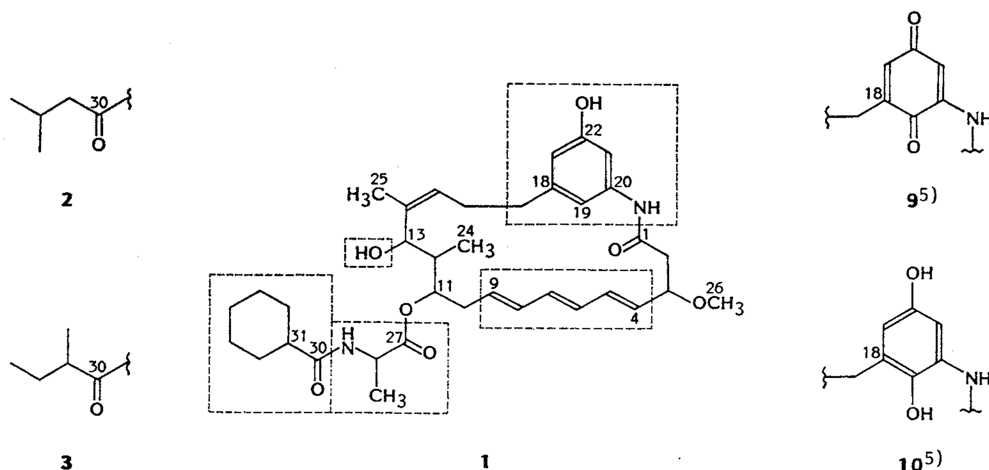


Table 3. Antitumor activity of trienomycin A (1) and 22-*O*-methyltrienomycin A (5) on P388 leukemia.

Sample	Dose (mg/kg)	Survival days					MSD	ILS (%)	Day 7 B.W.[a]
Saline	—	11	11	11	11	11	11.0	—	23.9
1	200 (40×5[b])	17	18	18	19	23	19.0	72.7	22.3
	50 (10×5)	11	11	15	15	15	13.4	21.8	21.8
5	160 (32×5)	15	15	15	15	15	15.0	36.4	22.2
	40 (8×5)	11	15	15	15	15	14.2	29.1	22.8

[a] Body weight (g). [b] Treatment: days 1～5 (ip).

which showed weak anti-*P. oryzae* activity. In addition, cytocidal and antitumor activities of **9** and **10** are weaker than those of trienomycins and the LD_{50}s of **9** (56 mg/kg) and **10** (80 mg/kg)[9] are quite different from that of **1** (*ca.* 400 mg/kg). Because the only structural difference between the trienomycins and mycotrienins[7,8] is the 19-oxygen atom (Fig. 2), this feature must also determine the differences in biological activity.

Experimental

General Experimental Procedures

MP's were determined using a Yanagimoto MP-3 hot stage microscope and are uncorrected. UV spectra were recorded on a Shimadzu model UV-200S spectrophotometer and IR spectra on a Jasco model A-102 interferometer. Mass spectra were obtained with a Jasco model D-100 and DX-3000 mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Varian XL-400 instrument. Kieselgel 60 (Merck) was used for column chromatography and DC-Fertigplatten Kieselgel 60 (Merck) was used for TLC analysis and for preparative TLC.

Preparation of Trienomycins A (1), B (2) and C (3)

The taxonomy, fermentation, isolation and physico-chemical and biological characteristics of trienomycins A (**1**), B (**2**) and C (**3**) were described previously[1~3].

Preparation of Diacetyltrienomycin A (4)

Trienomycin A (**1**, 150 mg) was dissolved in pyridine (3.0 ml) and Ac_2O (3.0 ml) was added and the mixture kept at room temp overnight. The reaction mixture was poured into cold water (30 ml) and then extracted with $CHCl_3$ (2×30 ml). The combined $CHCl_3$ layer was washed with water (30 ml) and then dried (anhydrous Na_2SO_4), filtered and concentrated *in vacuo* to yield diacetyltrienomycin A (**4**, 134.1 mg) as a colorless powder: MP 123~130°C; UV $\lambda_{max}^{CH_3OH}$ nm 211, 249, 258, 270, 282; IR ν_{max}^{KBr} cm^{-1} 3275, 1755, 1710, 1604, 1520, 1429, 1180, 990; MS *m*/*z* (% relative intensity) 707 ($M^+ +1$, 3), 646 (5), 447 (50), 443 (60), 415 (29), 391 (12), 299 (17), 199 (16), 83 (100); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.241 (3H, s, 13-$OCOCH_3$), 2.719 (3H, s, 22-$OCOCH_3$); ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1.

Preparation of 22-*O*-Methyltrienomycin A (5)

Trienomycin A (**1**, 105.2 mg) was dissolved in C_2H_5OH (5.0 ml) and ethyl ether solution of CH_2N_2 was added and the reactants stirred for 24 hours at room temp. The reaction mixture was concentrated *in vacuo* to afford a pale yellow powder which was purified by preparative TLC on silica gel developed with $CHCl_3$ - MeOH (19 : 1) to afford 22-*O*-methyltrienomycin A (**5**, 68.29 mg) as a colorless powder: MP 98~103°C; UV $\lambda_{max}^{CH_3OH}$ nm 224, 259, 268, 280, 292; IR ν_{max}^{KBr} cm^{-1} 3290, 1724, 1642, 1597, 1530, 1441, 1185, 991; MS *m*/*z* (% relative intensity) 636 (M^+, 2), 619 (2), 618 (4), 604 (4), 531 (2), 438 (28), 437 (87), 419 (21), 406 (36), 405 (100), 387 (28), 230 (30), 137 (36), 83 (37); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.803 (3H, s, 22-OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1.

Preparation of 13-*O*-Acetyl-22-*O*-methyltrienomycin A (6)

22-*O*-Methyltrienomycin (**5**, 68.3 mg) was dissolved in pyridine (1.0 ml) and to this solution was added Ac_2O (1.0 ml) and left at room temp overnight. The reaction mixture was poured into cold water (10 ml) and the mixture extracted with $CHCl_3$ (2×10 ml). The combined $CHCl_3$ layers were washed with water (10 ml) and the solution dried (anhydrous Na_2SO_4), filtered and concentrated *in vacuo* to yield pale brown powder (75.8 mg) which was subjected on preparative TLC to give 13-*O*-acetyl-22-*O*-methyltrienomycin A (**6**, 38.7 mg) as colorless powder: MP 123~130°C; UV $\lambda_{max}^{CH_3OH}$ nm 217, 250, 259, 270, 282; IR ν_{max}^{KBr} cm^{-1} 3285, 1729, 1647, 1599, 1534, 1433, 1231, 994; MS *m*/*z* (% relative intensity) 678 (M^+, 34), 618 (17), 479 (66), 419 (100), 405 (18), 404 (18), 387 (36); ^{1}H NMR (400 MHz, $CDCl_3$ - CD_3OD) δ 2.019 (3H, s, 13-$OCOCH_3$), 3.792 (3H, s, 22-OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$ - CD_3OD) see Table 1.

Preparation of Hexahydrotrienomycin A (7)

Trienomycin A (**1**, 21.6 mg) was dissolved in EtOAc (1.0 ml) and 2.0 mg of Pd - C (10%) and a

drop of glacial acetic acid were added. Then hydrogen gas was passed into this mixture, and stirring was continued 40 hours. The reaction mixture was filtered and concentrated *in vacuo* to afford a pale yellow powder (19.7 mg) which was purified by preparative TLC on silica gel using $CHCl_3$ - MeOH (19:1) as solvent to afford hexahydrotrienomycin A (**7**, 12.3 mg) as colorless needles: MP 185~187°C; UV $\lambda_{max}^{CH_3OH}$ nm 218, 251; IR ν_{max}^{KBr} cm^{-1} 3249, 1729, 1706, 1634, 1600, 1519, 1432, 1282, 1191, 1150, 998; MS *m/z* (% relative intensity) 629 (M^++1, 5), 628 (M^+, 13), 610 (1), 447 (7), 430 (43), 429 (100), 411 (21), 401 (14), 372 (11), 360 (13), 216 (15), 205 (17), 204 (20), 188 (15), 154 (6), 148 (23), 123 (20), 111 (7), 95 (14), 83 (25); ^{1}H NMR (400 MHz, $CDCl_3$ - CD_3OD) δ 0.999 (3H, d, J=7 Hz, 24-H_3), 1.2~1.9 (22H, m, 4-, 5-, 6-, 7-, 8-, 9-, 32-, 33-, 34-, 35- and 36-H_2), 1.349 (3H, d, J=8 Hz, 29-H_3), 1.610 (2H, dt, J=14 and 8 Hz, 10-H_2), 1.801 (3H, s, 25-H_3), 1.975 (1H, m, 12-H), 2.163 (1H, m, 31-H), 2.17 and 2.40 (1H, each m, 16-H_2), 2.502 (1H, d, J=14 Hz, 2-*H*H), 2.62 (2H, m, 17-H_2), 2.673 (1H, dd, J=4 and 14 Hz, 2-H*H*), 3.420 (3H, s, 3-OCH_3), 3.630 (1H, m, 3-H), 4.308 (1H, d, J=4 Hz, 13-H), 4.367 (1H, q, J=7 Hz, 28-H), 4.707 (1H, ddd, J=2, 4 and 9 Hz, 11-H), 5.347 (1H, dd, J=6 and 6 Hz, 15-H), 6.457 (1H, dd, J=2 and 2 Hz, 23- or 19-H), 6.790 (1H, dd, J=2 and 2 Hz, 19- or 23-H), 6.810 (1H, dd, J=2 and 2 Hz, 21-H), 7.462 (1H, s, 20-NH); ^{13}C NMR (100 MHz, $CDCl_3$ - CD_3OD) see Table 1.

Preparation of Deacylated Trienomycin A (Trienomycinol) (**8**)

Trienomycin A (**1**, 72.1 mg) was dissolved in dry THF (7 ml) and was kept at −23°C (CCl_4-dry ice bath). To this solution $LiAlH_4$ (20 mg) was added with stirring. After 2 hours, the reaction mixture was diluted with 20 ml of ethyl acetate and washed with water. The solution was dried over Na_2SO_4 and evaporated *in vacuo*. The resulting reaction mixture was purified by preparative TLC to afford **8** (28 mg) as pale yellow powder: MP 137~142°C; UV $\lambda_{max}^{CH_3OH}$ nm 215, 251, 261, 272, 283; IR ν_{max}^{KBr} cm^{-1} 3351, 2900, 1656, 1600, 1541, 1431, 1292, 1068, 992; MS *m/z* (% relative intensity) 441 (M^+, 8), 423 (17), 408 (84), 391 (82), 316 (59), 298 (100); ^{1}H NMR (400 MHz, pyridine-d_5) δ 1.140 (3H, d, J=7 Hz, 24-H_3), 1.953 (3H, s, 25-H_3), 2.116 (1H, dqd, J=5, 7 and 9 Hz, 12-H), 2.234 (1H, m, 16-*H*H), 2.462 (1H, m, 10-*H*H), 2.474 (2H, m, 17-H_2), 2.507 (1H, m, 16-H*H*), 2.640 (1H, ddd, J=7, 7 and 14 Hz, 10-H*H*), 2.684 (1H, dd, J=10 and 12 Hz, 2-*H*H), 2.960 (1H, dd, J=4 and 12 Hz, 2-H*H*), 3.484 (3H, s, 3-OCH_3), 4.059 (1H, ddd, J=2, 7 and 9 Hz, 11-H), 4.302 (1H, ddd, J=4, 8 and 10 Hz, 3-H), 5.210 (1H, m, 15-H), 5.250 (1H, d, J=5 Hz, 13-H), 5.715 (1H, dd, J=8 and 16 Hz, 4-H), 5.957 (1H, ddd, J=6, 7 and 15 Hz, 9-H), 6.022 (1H, dd, J=11 and 15 Hz, 6-H), 6.137 (1H, dd, J=4 and 15 Hz, 7- or 8-H), 6.167 (1H, dd, J=4 and 15 Hz, 8- or 7-H), 6.349 (1H, dd, J=11 and 16 Hz, 5-H), 6.698 (1H, br s, 23- or 19-H), 6.930 (1H, br s, 19- or 23-H), 7.805 (1H, dd like, J=1 and 1 Hz, 21-H); ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1.

Antimicrobial Activity Tests of Trienomycin A (**1**) and Related Compounds

The antimicrobial spectra of trienomycin derivatives were determined using 8-mm paper discs (Toyo Seisakusho Co., Ltd.) and Mueller-Hinton agar medium (Difco) for bacteria and potato broth agar medium for fungi or yeasts. Antimicrobial activity was observed after 24 hours incubation at 37°C for bacteria or longer incubation at 27°C for fungi or yeasts.

The antimicrobial activity of **1** and related compounds were determined on various microorganisms such as *Staphylococcus aureus* KB34 (FDA 209P), *Bacillus subtilis* KB27 (PCI 219), *Micrococcus luteus* KB40 (PCI 1001), *Mycobacterium smegmatis* KB42 (ATCC 607), *Escherichia coli* KB8 (NIHJ), *E. coli* KB176 (NIHJ JC-2), *Pseudomonas aeruginosa* KB105 (P-3), *Xanthomonas oryzae* KB88, *Bacteroides fragilis* KB169, *Acholeplasma laidlawii* PG 8 (KB174), *C. albicans* KF1, *Saccharomyces sake* KF26, *Mucor racemosus* KF223 (IFO 4581), *Piricularia oryzae* KF180, *A. niger* KF103 (ATCC 6275) and *Rhizopus javanicus* IAM 6241. Compounds **1**~**8** showed no antimicrobial activities against these microorganisms even at a concentration of 1,000 µg/ml except for trienomycin A (**1**) and its natural congeners **2** and **3** and 22-*O*-methyltrienomycin A (**5**) showed slight activities against *P. oryzae* KF180 at a concentration of 1,000 µg/ml.

Cytocidal Activity Tests of Trienomycin A (**1**) and Related Compounds

HeLa S_3 and P388 leukemia cells were cultured in EAGLE's MEM (for HeLa S_3) and RPMI 1640 medium (for P388 leukemia) supplemented with 10% bovine serum and 60 µg/ml of kanamycin, respectively.

To determine the effects of the antibiotics on the growth of mammalian cells, 200 μl of a single cell suspension of HeLa S_3 cells (2×10^4/ml) or P388 cells (2×10^4/ml) were plated in 96-well microplate and incubated at 37°C in a 5% CO_2 - 95% air atmosphere. After preincubation for 24 hours, each well was added with 5 μl of methanol solution containing a different concentration of antibiotics. After further 72 hours of incubation, the growth ratio of cultured cells were determined by cell count.

Antitumor Activity Test of Trienomycin A (**1**) and 22-*O*-Methyltrienomycin A (**5**)

P388 leukemia cells were inoculated (1×10^5/mouse, ip) in CDF_1 mice (6-week old, female). Samples were dissolved in a small amount of methanol and Tween 80, diluted with saline and injected ip into tumor bearing mice on days 1~5. Antitumor activity was evaluated by the increase in life span (ILS): $(T/C-1)\times100$ (%), where "T" is the mean survival days (MSD) of the treated group and "C" is the MSD of the control group.

Acknowledgments

This work was supported, in part, by Grant-in-Aid from the Ministry of Health and Welfare, the Ministry of Education, Science and Culture, Japan, and by funds from Japan Keirin Association. One of the authors (SF) is indebted to a Grant-in-Aid for Cancer Research for the Encouragement of Young Scientists, from the Ministry of Education, Science and Culture.

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