Thiazinotrienomycins, New Ansamycin Group Antibiotics

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New triene-ansamycins designated thiazinotrienomycins A, B, C, D and E were isolated from culture broth of *Streptomyces* sp. MJ672-m3 for their activities against cervical cancer cell lines. The structures and some biological and biochemical properties of the antibiotics were determined.

Cancers are caused by complex combinations of somatic mutations in genes controlling cell growth and differentiation, *i.e.*, proto-oncogenes and tumor suppressor genes. They constitute most, if not all, of phenotypic diversities of the disease. It is unlikely therefore that a single drug can cure every type of cancers. As a primary screening system for new anticancer antibiotics, we have been using several cancer cell lines of human origins and selecting microbial products that are growth-inhibitory *in vitro* to certain cell lines, rather than to all the cell lines, to avoid compounds of non-specific toxicity.

Thiazinotrienomycins A, B, C, D and E, new members of the ansamycin family of antibiotics (Fig. 1), produced by a *Streptomyces* strain were isolated for their activities that are up to 10 times stronger against cell lines of cervical cancer than against cell lines of stomach, colon and breast cancers. We report here the production, isolation, physico-chemical properties, the structures and some biological and biochemical properties of these antibiotics.

Production, Isolation and Structural Determination

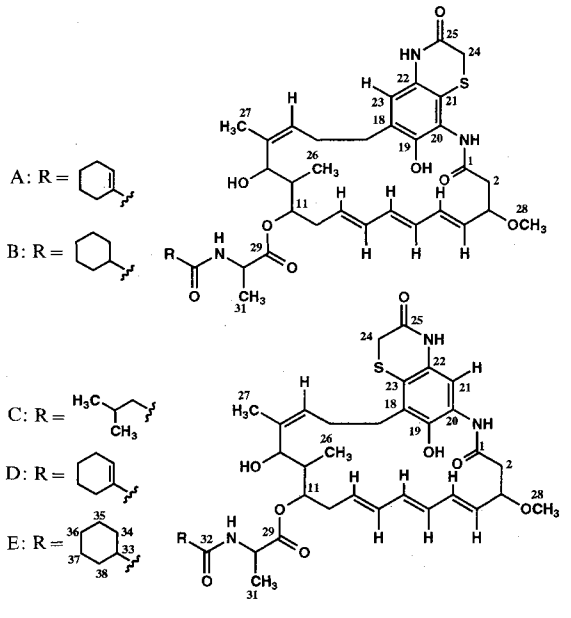
Fermentation of the Antibiotic Producing Strain

A loopful of a slant culture of *Streptomyces* sp. MJ672-m3 was inoculated into two 500-ml Erlenmeyer flasks each containing 110 ml of a medium composed of 5.0% glucose, 0.4% Pepton (Nippon Seisui Co. Ltd.), 0.1% yeast extract, 0.1% meat extract, 0.25% NaCl, 1.0% soybean meal, 0.5% $CaCO_3$ (pH 7.0 before sterilization). 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 5 days under the same conditions as for the seed culture.

Isolation of the Antibiotics

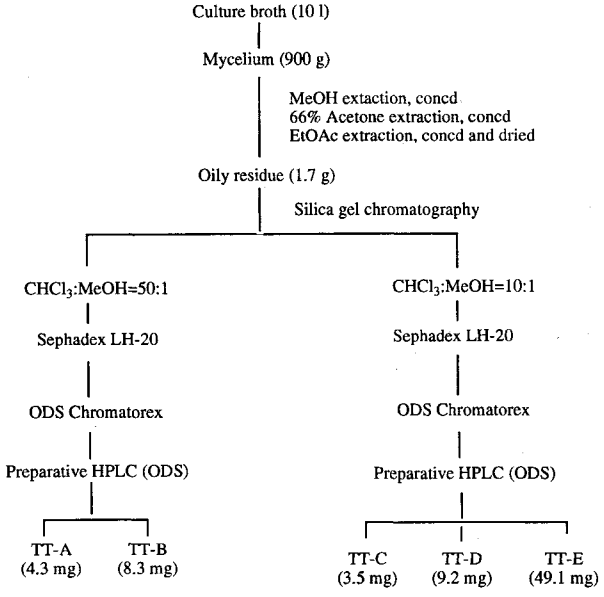
A preliminary test showed that the antibiotic activity was found more abundant in mycelia than in the culture filtrate. In a typical experiment, as shown in Fig. 2, a 10 liter fermentation gave 900 g (wet weight) of mycelia, which was extracted successively with 3 liters of MeOH and 3 liters of 66% aqueous acetone by stirring for 1 hour each. The combined extract was concentrated to

Fig. 1. Structures of thiazinotrienomycins A, B, C, D and E.



dryness *in vacuo*. The dried material (1.7 g) was chromatographed on a silica gel column (85 g, 5 × 20 cm), eluted with a linear gradient of $CHCl_3$-MeOH (100:1 ~ 5:1, 1.5 liters in total), and active fractions were purified using Sephadex LH-20 column (1.6 × 60 cm), developed with MeOH. Active fractions were collected and evaporated to give a sticky oil, from which the active substances were further purified by ODS column chromatography (Chromatorex, 2 × 30 cm, SSC Co. Ltd., Tokyo) developed with 65% MeOH into 10 ml fractions, and then by preparative HPLC using a μ-Bondasphere C_{18} column (5 μm-100 Å, 19 × 150 mm, Waters Co. Ltd.) developed with 65% MeOH at 9.9 ml/minute at 40°C and monitored at 260 nm. Five active substances were obtained and named thiazinotrienomycins (abbreviated as TT), A, B, C, D and E, whose purities were demonstrated by HPLC and TLC.

Fig. 2. Isolation procedure for thiazinotrienomycins.



Structure of TT-E

The physico-chemical properties of thiazinotrienomycins A, B, C, D and E are summarized in Tables 1 and 2. The structural study was started with TT-E, a major congener. The molecular weight of TT-E was shown to be 709 from of FAB-MS peaks at *m/z* 710 $(M+H^+)$, *m/z* 732 $(M+Na)^+$ and *m/z* 708 $(M-H)^-$. The high resolution measurement of the molecular ion peak in FAB-MS gave *m/z* 732.3320 [calcd. for $C_{38}H_{51}N_3O_8SNa$, *m/z* 732.3297, $(M+Na)^+$]. The existence of one sulfur atom was confirmed by the

Table 1. Physico-chemical properties of thiazinotrienomycins A and B.

	Thiazinotrienomycin A	Thiazinotrienomycin B
Appearance	White powder	Pale yellow powder
$[\alpha]_D^{22}$ (*c* 0.1, MeOH)	+61°	+121°
Molecular formula	$C_{38}H_{49}N_3O_8S$	$C_{38}H_{51}N_3O_8S$
FAB-MS (*m/z*, Pos.)	708 $(M+H)^+$	710 $(M+H)^+$
FAB-MS (*m/z*, Neg.)	706 $(M-H)^-$	708 $(M-H)^-$
HRFAB-MS (Pos., *m/z*)		
Calcd. for $C_{38}H_{51}N_3O_8SNa$:		732.3295
Found:		732.3295 $[(M+Na)^+]$
UV nm λ_{max}^{MeOH} ($\log\varepsilon$)	205 (4.40), 215 (sh) (4.38), 250 (4.34), 258 (4.32), 258 (4.32), 270 (4.18), 282 (4.08), 315 (3.51)	205 (4.46), 215 (sh) (4.38), 250 (4.48), 258 (4.48), 270 (4.38), 282 (4.28), 315 (3.51)
$\lambda_{max}^{MeOH-NaOH}$ ($\log\varepsilon$)	250 (4.32), 255 (4.32), 270 (4.15), 282 (4.08), 315 (3.62)	250 (4.36), 255 (4.46), 270 (4.40), 282 (4.28), 315 (3.62)
$\lambda_{max}^{MeOH-HCl}$ ($\log\varepsilon$)	210 (4.36), 250 (4.46), 258 (4.32), 270 (4.18), 282 (4.18), 315 (3.51)	210 (4.36), 250 (4.46), 258 (4.46), 270 (4.36), 282 (4.26), 315 (3.51)
IR ν_{max}^{KBr} cm^{-1}	3400, 2930, 2850, 1730, 1660, 1530, 1460, 1390, 1305, 1240, 1220, 1160, 1100, 1000	3400, 2940, 2850, 1740, 1735, 1670, 1540, 1480, 1380, 1300, 1220, 1160, 1100, 1000, 970
Rf value on TLC	1) 0.71 ($CHCl_3$-MeOH, 10:1 silica gel Art. 5715) 2) 0.46 (toluene-acetone, 3:2 silica gel Art. 5715) 3) 0.82 (benzene-$CHCl_3$-MeOH, 3:7:3 silica gel Art. 5715)	1) 0.62 ($CHCl_3$-MeOH, 10:1 silica gel Art. 5715) 2) 0.48 (toluene-acetone, 3:2 silica gel Art. 5715) 3) 0.82 (benzene-$CHCl_3$-MeOH, 3:7:3 silical gel Art. 5715)
Color reaction	Phosphomolybdate-H_2SO_4, $FeCl_3$	Phosphomolybdate-H_2SO_4, $FeCl_3$
HPLC retention time (min)[a]	6.5	7.5
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

[a] μ-Bondasphere 5 μ C_{18}-100 Å (3.9 mm × 15 cm), mobile phase: 65% MeOH (pH 5.0), flow rate: 1.0 ml/minute, detection: 260 nm, temperature: 40°C.

Table 2. Physico-chemical properties of thiazinotrienomycins C, D and E.

	Thiazinotrienomycin C	Thiazinotrienomycin D	Thiazinotrienomycin E
Appearance	Pale yellow powder	Pale yellow powder	White powder
$[\alpha]_D^{22}$ (c 0.1, MeOH)	+101°	+122°	+189°
Molecular formula	$C_{36}H_{49}N_3O_8S$	$C_{38}H_{49}N_3O_8S$	$C_{38}H_{51}N_3O_8S$
FAB-MS (m/z, Pos.)		708 $(M+H)^+$	710 $(M+H)^+$
FAB-MS (m/z, Neg.)	682 $(M-H)^-$	706 $(M-H)^-$	708 $(M-H)^-$
HRFAB-MS (Pos., m/z)			
Calcd. for $C_{38}H_{51}N_3O_8SNa$:			732.3297
Found:			732.3320 $(M+Na)^+$
Elemental analysis		$C_{38}H_{50}N_3O_8SNa$: Calcd.	Found
		C 62.38	62.08
		H 6.90	7.19
		N 5.74	5.93
		O 17.50	18.52
		S 4.37	4.13
UV nm λ_{max}^{MeOh} ($\log\varepsilon$)	225 (4.11), 250 (sh) (4.23), 260 (4.43), 270 (4.36), 282 (4.20), 320 (3.43)	225 (sh) (4.30), 250 (sh) (4.46), 260 (4.52), 270 (4.45), 282 (4.30), 320 (3.43)	225 (4.30), 250 (sh) (4.52), 260 (4.63), 270 (4.57), 282 (4.43), 320 (3.63)
$\lambda_{max}^{MeOH-NaOH}$ ($\log\varepsilon$)	225 (4.11), 260 (4.41), 270 (4.43), 282 (4.20), 340 (3.70)	225 (4.30), 260 (4.45), 270 (4.46), 282 (4.32), 340 (3.70)	225 (4.28), 260 (sh) (4.62), 270 (4.63), 282 (4.45), 340 (3.81)
$\lambda_{max}^{MeOH-HCl}$ ($\log\varepsilon$)	210 (4.08), 225 (4.11), 250 (sh) (4.23), 260 (4.43), 270 (4.36), 282 (4.20), 320 (3.43)	210 (4.28), 225 (sh) (4.46), 260 (4.52), 270 (4.45), 282 (4.32), 320 (3.43)	225 (4.30), 250 (sh) (4.52), 260 (4.63), 270 (4.57), 282 (4.43), 320 (3.63)
IR ν_{max}^{KBr} cm^{-1}	3400, 2950, 2920, 1740, 1660, 1600, 1540, 1460, 1380, 1200, 1150, 1140, 1100, 1000, 860	3450, 2950, 1740, 1670, 1540, 1470, 1400, 1230, 1180, 1100, 1010	3350, 2950, 2860, 2120, 1740, 1670, 1600, 1540, 1480, 1470, 1390, 1220, 1100, 1000, 760
Rf value on TLC	1) 0.39 ($CHCl_3$-MeOH, 10:1 silica gel Art. 5715) 2) 0.25 (toluene-acetone, 3:2 silica gel Art. 5715) 3) 0.76 (benzene-$CHCl_3$-MeOH, 3:7:3 silica gel Art 5715)	1) 0.39 ($CHCl_3$-MeOH, 10:1 silica gel Art. 5715) 2) 0.29 (toluene-acetone, 3:7:3 silica gel Art. 5715) 3) 0.77 (benzene-$CHCl_3$-MeOH, 3:7:3 silica gel Art 5715)	1) 0.43 ($CHCl_3$-MeOH, 10:1 silica gel Art. 5715) 2) 0.27 (toluene-acetone, 3:2 silica gel Art. 5715) 3) 0.78 (benzene-$CHCl_3$-MeOH, 3:7:3 silica gel Art. 5715)
Color reaction	Phosphomolybdate-H_2SO_4, $FeCl_3$	Phosphomolybdate-H_2SO_4, $FeCl_3$	Phosphomolybdate-H_2SO_4, $FeCl_3$
HPLC retention time (minute)[a]	6.6	8.0	9.4
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	Soluble: DMSO, $CHCl_3$, MeOH, pyridine, Me_2CO Insoluble: H_2O, n-hexane

[a] μ-Bondasphere 5 μ C_{18}-100 Å (3.9 mm × 15 cm), mobile phase: 65% MeOH (pH 5.0), flow rate: 1.0 ml/minute, detection: 260 nm, temperature: 40°C.

elemental analysis of the sodium salt (Table 2).

The UV absorption maxima in MeOH at 260 nm ($\log\varepsilon$ 4.52), 270 nm ($\log\varepsilon$ 4.63) and 280 nm ($\log\varepsilon$ 4.57) suggested the presence of a triene structure in the molecule. The IR spectrum of TT-E showed a strong absorption at 1000 cm^{-1} attributable to the triene structure. The UV absorption exhibited a bathochromic shift from 320 nm ($\log\varepsilon$ 3.63) in MeOH to 340 nm ($\log\varepsilon$ 3.81) in an alkaline MeOH solution, suggesting the presence of a phenol group. A positive color reaction with ferric chloride supported this result. Treatment of TT-E with diazomethane gave a methyl derivative in the phenolic chromophore part (δ_H 3.80, 3H, s; δ_C 62.0). The HMBC spectrum of the methyl derivative showed that the methoxy proton at δ_H 3.80 was coupled with C-19 (δ_C 145.0).

Analyses of ^1H-^1H COSY and HMBC spectra of TT-E indicated four partial structures in TT-E that were composed of the carbon numbers from 2 to 13, from 15 to 17, from 30 to 31 and from 33 to 38, respectively. The ^1H-^1H COSY spectra indicated the four partial structures, whose combination was indicated in the HMBC spectra, as shown in Fig. 3. The ^1H-^1H COSY spectrum also indicated that 13-OH (δ_H 5.56) was free because of its coupling with 13-H (δ_H 5.23). The HMBC spectrum showed that 20-NH (δ_H 10.83) linked to C-1 (δ_C 170.5) and to C-19 (δ_C 144.0). TT-E was therefore closely related to trienomycin A[1], a triene-ansamycin

Fig. 3. Structure of thiazinotrienomycin E elucidated by ^{1}H-^{1}H COSY, NOE and HMBC experiments.

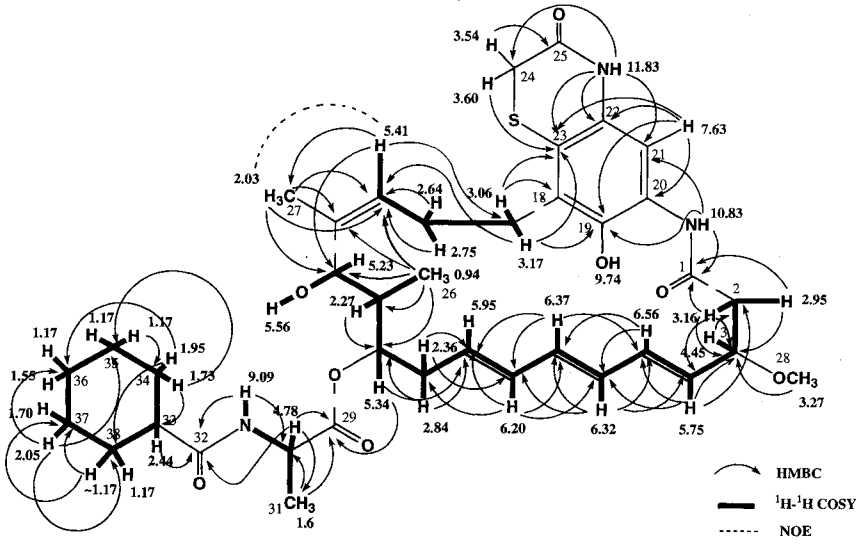
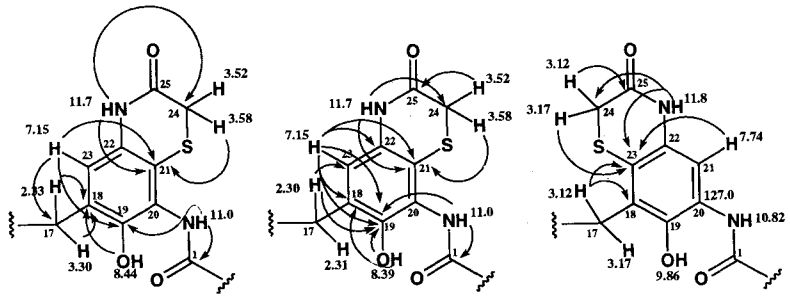
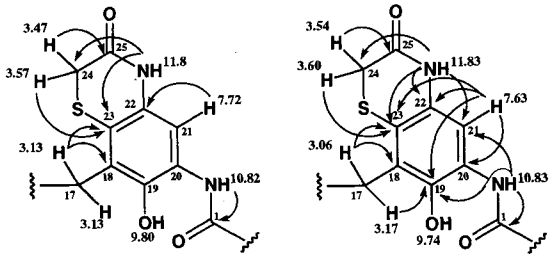


Fig. 4. ^{1}H-^{13}C correlations for partial structures of thiazinotrienomycins A, B, C, D and E by the HMBC experiments.



Thiazinotrienomycin A Thiazinotrienomycin B Thiazinotrienomycin C



Thiazinotrienomycin D Thiazinotrienomycin E

group antibiotic.

The ansa moiety in TT-E, from C-1 to C-17 and an *N*-(cyclohexyl carbonyl) alanine moiety, from C-29 to C-38 were the same as those of trienomycin A. TT-E was therefore structurally different from trienomycin A only in the phenolic chromophore moiety, and the phenolic chromophore of TT-E was deduced to have a composition of $C_8H_5NO_2S$. In addition, the HMBC spectrum of TT-E indicated that a 17-H proton at δ_H 3.06 was coupled to C-18 (δ_C 129.3) and to C-23 (δ_C 117.9), while the other 17-H proton at δ_H 3.17 was coupled to C-19 (δ_C 144.0). A 21-H at δ_H 7.63 was coupled to C-19, C-20 (δ_C 126.8), C-22 (δ_C 131.5) and C-23. In the phenolic chromophore moiety, the existence of another amide bond composed of 22-NH at δ_H 11.83 and C-25 at δ_C 165.9, and the signals of a methylene (24-H_2) at δ_H 3.54 and 3.60, δ_C 30.7, were shown by the ^{1}H and ^{13}C NMR spectra. The large spin coupling constant (14.6 Hz) between the methylene protons (24-H_2) suggested this methylene was involved in a cyclic structure. In the HMBC spectrum of TT-E, the methylene protons (24-H_2) were coupled to C-23 and C-25, while the amide NH (δ_H 11.83) was coupled to C-22 and C-24. Accordingly, it was evident that 24-H_2 were connected

to C-23 through a sulfide bond and the amide nitrogen atom linked to C-22 in the phenolic chromophore moiety. Thus, the structure of the chromophore was a benzothiazine nucleus consisted of $C_8H_5NO_2S$.

The geometry of the triene was all *E* in view of the coupling constants of $J_{4,5}$, $J_{6,7}$ and $J_{8,9}$, which were 15.6 Hz, 15.0 Hz and 15.3 Hz, respectively. NOE's were observed between 15-H at δ_H 5.41 and 27-H_3 at δ_H 2.03. This result was consistent with the geometry of the double bond that was 14*Z*. From the all spectroscopic experiments described above, the structure of TT-E was concluded as shown in Fig. 1. The stereochemistry at carbons 3, 11, 12, 13 and 30 were not defined.

Structure of TT-B

The molecular weight of TT-B was found to be 709 [*m/z* 710 $(M+H)^+$, *m/z* 708 $(M-H)^-$] which was the same as that of TT-E whose molecular formula was $C_{38}H_{51}N_3O_8S$. The UV spectra of TT-B and TT-E resembled to each other at a neutral pH. The ^1H-^1H COSY and HMBC spectra indicated that the structure of TT-B differed from that of TT-E only in the aromatic moiety. The structure of TT-B was revealed from the HMBC spectral analyses: a 23-H in the aromatic portion (δ_H 7.15) was coupled to C-18 (δ_C 131.5), C-19 (δ_C 147.0), C-21 (δ_C 114.9) and C-22 (δ_C 131.4), while a 17-H proton at δ_H 2.30 was coupled to C-23 (δ_C 118.5) and C-19. It was concluded that TT-B differed from TT-E on the

Table 3. ^{13}C and ^1H NMR data of thiazinotrienomycins A and B in pyridine-d_5.

Carbon No.	Thiazinotrienomycin A		Thiazinotrienomycin B	
	δ_C ppm (125 MHz)	δ_H ppm (*J* in Hz, 500 MHz)	δ_C ppm (125 MHz)	δ_H ppm (*J* in Hz, 500 MHz)
1	171.5 (s)	—	171.5 (s)	—
2	42.7 (t)	3.18, 3.28 (2H, m)	42.6 (t)	3.17, 3.25 (2H, m)
3	80.7 (d)	4.54 (1H,, m)	80.7 (d)	4.52 (1H, m)
4	131.5 (d)	5.73 (1H, dd, 8.6, 15.3)	131.4 (d)	5.72 (1H, dd, 8.9, 15.3)
5	135.7 (d)	6.67 (1H, dd, 10.7, 15.3)	135.7 (d)	6.66 (1H, dd, 10.9, 15.3)
6	129.9 (d)	6.42 (1H, dd, 10.7, 15.0)	129.9 (d)	6.42 (1H, dd, 10.9, 15.3)
7	135.2 (d)	6.57 (1H, dd, 10.4, 15.0)	135.8 (d)	6.60 (1H, dd, 10.9, 15.3)
8	134.0 (d)	6.33 (1H, dd, 10.4, 15.3)	134.0 (d)	6.33 (1H, dd, 10.9, 15.3)
9	130.8 (d)	6.03 (1H, m)	130.8 (d)	6.10 (1H, m)
10	33.8 (t)	2.40, 2.85 (2H, m)	33.9 (t)	2.42, 2.86 (2H, m)
11	75.4 (d)	5.37 (1H, m)	75.4 (d)	5.37 (1H, m)
12	39.2 (d)	2.26 (1H, m)	39.2 (d)	2.30 (1H, m)
13	68.1 (d)	5.24 (1H, br s)	68.2 (d)	5.29 (1H, br)
	—	5.50 (13-OH, br, 6.0)	—	5.61 (13-OH, br, 5.0)
14	140.4 (s)	—	140.3 (s)	—
15	123.1 (d)	5.36 (1H, m)	123.1 (d)	5.37 (1H, m)
16	26.5 (t)	2.71 (2H, m)	26.5 (t)	2.72 (2H, m)
17	32.1 (t)	2.33, 3.30 (2H, m)	32.1 (t)	2.30, 2.31 (2H, m)
18	131.5 (s)	—	131.5 (s)	—
19	147.0 (s)	—	147.0 (s)	—
	—	8.44 (19-OH, br)	—	8.39 (19-OH, s)
20	124.5 (s)	—	124.5 (s)	—
	—	11.0 (20-NH, s)	—	11.0 (20-NH, s)
21	115.0 (s)	—	114.9 (s)	—
22	130.9 (s)	—	131.4 (s)	—
	—	11.7 (22-NH, s)	—	11.7 (22-NH, s)
23	118.5 (d)	7.15 (1H, s)	118.5 (d)	7.15 (1H, s)
24	30.6 (t)	3.52 (1H, d, 14.9)	30.6 (t)	3.52 (1H, d, 14.6)
	—	3.58 (1H, d, 14.6)	—	3.58 (1H, d, 14.6)
25	165.9 (s)	—	165.8 (s)	—
26	9.9 (q)	0.87 (3H, d, 7.0)	10.0 (q)	0.89 (3H, d, 6.7)
27	21.2 (q)	1.93 (3H, s)	21.2 (q)	1.93 (3H, s)
28	56.2 (q)	3.30 (3H, s)	56.3 (q)	3.30 (3H, s)
29	173.2 (s)	—	173.4 (s)	—
30	50.0 (d)	4.84 (1H, m)	49.6 (d)	4.82 (1H, m)
	—	8.86 (30-NH, d, 6.4)	—	9.01 (30-NH, d, 6.1)
31	17.3 (q)	1.64 (3H, d, 7.3)	17.4 (q)	1.59 (3H, d, 7.3)
32	169.7 (s)	—	176.9 (s)	—
33	131.5 (s)	—	45.0 (d)	2.42 (1H, m)
34	133.9 (d)	6.86 (1H, m)	30.1 (t)	1.72 (2H, m)
35	25.5 (t)	2.0 (2H, m)	26.0 (t)	1.16, 1.55 (2H, m)
36	21.9 (t)	1.44 (2H, m)	26.1 (t)	1.15 (2H, m)
37	22.5 (t)	1.53 (2H, m)	26.2 (t)	1.70 (2H, m)
38	24.7 (t)	2.44 (2H, m)	30.1 (t)	2.06 (2H, m)

Table 4. ^{13}C and ^{1}H NMR data of thiazinotrienomycins C, D and E in pyridine-d_5.

Carbon No.	Thiazinotrienomycin C		Thiazinotrienomycin D		Thiazinotrienomycin E	
	δ_C ppm (125 MHz)	δ_H ppm (J in Hz, 500 MHz)	δ_C ppm (125 MHz)	δ_H ppm (J in Hz, 500 MHz)	δ_C ppm (125 MHz)	δ_H ppm (J in Hz, 500 MHz)
1	170.2 (s)	—	170.5 (s)	—	170.5 (s)	—
2	43.8 (t)	2.98 (1H, t, 11.6)	43.8 (t)	2.98 (1H, t, 11.9)	43.6 (t)	2.95 (1H, dd, 10.7, 12.2)
	—	3.17 (1H, dd, 4.3, 11.6)	—	3.18 (1H, dd, 3.1, 12.5)	—	3.16 (1H, dd, 4.3, 12.2)
3	80.8 (d)	4.45 (1H, m)	80.9 (d)	4.49 (1H, m)	80.8 (d)	4.45 (1H, m)
4	131.8 (d)	5.78 (1H, m)	131.9 (d)	5.82 (1H, m)	131.7 (d)	5.75 (1H, dd, 8.9, 15.6)
5	135.2 (d)	6.55 (1H, dd, 9.8, 15.3)	135.2 (d)	6.85 (1H, dd, 10.0, 15.3)	135.5 (d)	6.56 (1H, dd, 10.1, 15.6)
6	130.0 (d)	6.34 (1H, m)	134.7 (d)	6.33 (1H, m)	129.9 (d)	6.32 (1H, dd, 10.0, 15.0)
7	134.8 (d)	6.34 (1H, m)	130.0 (d)	6.32 (1H, m)	134.8 (d)	6.37 (1H, dd, 10.0, 15.0)
8	133.8 (d)	6.21 (1H, m)	133.8 (d)	6.23 (1H, m)	133.7 (d)	6.20 (1H, dd, 10.1, 15.3)
9	130.7 (d)	5.93 (1H, m)	130.7 (d)	5.84 (1H, m)	130.8 (d)	5.95 (1H, m)
10	33.5 (t)	2.38, 2.82 (2H, m)	33.3 (t)	2.35, 2.85 (2H, m)	33.5 (t)	2.36, 2.84 (2H, m)
11	75.5 (d)	5.36 (1H, m)	75.5 (d)	5.33 (1H, m)	75.4 (d)	5.34 (1H, m)
12	39.0 (d)	2.30 (1H, m)	38.9 (d)	2.30 (1H, m)	38.9 (d)	2.27 (1H, m)
13	68.4 (d)	5.25 (1H, br s)	68.2 (d)	5.16 (1H, br s)	68.3 (d)	5.23 (1H, br s)
	—	5.59 (13-OH, br)	—	5.38 (13-OH, br s)	—	5.56 (13-OH, br, 4.6)
14	140.5 (s)	—	140.6 (s)	—	140.6 (s)	—
15	124.0 (d)	5.42 (1H, d, 7.3)	124.0 (d)	5.40 (1H, m)	124.0 (d)	5.41 (1H, m)
16	27.3 (t)	2.63, 2.75 (2H, m)	27.2 (t)	2.65 (2H, m)	27.2 (t)	2.64, 2.75 (2H, m)
17	30.0 (t)	3.12 (1H, m), 3.17 (1H, m)	30.0 (t)	3.13 (2H, m)	29.8 (t)	3.06, 3.17 (2H, m)
18	130.0 (s)	—	129.3 (s)	—	129.6 (s)	—
19	149.5 (s)	—	149.5 (s)	—	144.0 (s)	—
	—	9.86 (19-OH, br s)	—	9.80 (19-OH, br s)	—	9.74 (19-OH, br)
20	127.0 (s)	—	127.0 (s)	—	126.8 (s)	—
	—	10.82 (20-NH, s)	—	10.82 (20-NH, s)	—	10.83 (20-NH, s)
21	109.5 (d)	7.74 (1H, br s)	109.6 (d)	7.72 (1H, s)	109.5 (d)	7.63 (1H, s)
22	135.9 (s)	—	136.0 (s)	—	131.5 (s)	—
	—	11.8 (22-NH, s)	—	11.8 (22-NH, s)	—	11.8 (22-NH, s)
23	117.7 (s)	—	119.1 (s)	—	117.9 (s)	—
24	30.7 (t)	3.59 (1H, d, 14.6)	30.6 (t)	3.47 (1H, br)	30.7 (t)	3.54 (1H, d, 14.6)
	—	3.52 (1H, d, 14.6)	—	3.57 (1H, d, 14.6)	—	3.60 (1H, d, 14.6)
25	166.0 (s)	—	165.9 (s)	—	165.9 (s)	—
26	10.4 (q)	0.97 (3H, d, 6.1)	10.2 (q)	0.94 (3H, d, 5.7)	10.2 (q)	0.94 (3H, d, 6.7)
27	21.3 (q)	2.04 (3H, s)	21.4 (q)	2.02 (3H, s)	21.4 (q)	2.03 (3H, s)
28	56.2 (q)	3.28 (3H, s)	56.2 (q)	3.29 (3H, s)	56.2 (q)	3.27 (3H, s)
29	173.4 (s)	—	172.9 (s)	—	173.2 (s)	—
30	49.7 (d)	4.80 (1H, m)	50.0 (d)	4.73 (1H, m)	49.6 (d)	4.78 (1H, m)
	—	9.30 (30-NH, d, 5.8)	—	9.02 (30-NH, br s)	—	9.09 (30-NH, d, 6.1)
31	17.2 (q)	1.56 (3H, d, 7.3)	17.1 (q)	1.64 (3H, d, 7.0)	17.3 (q)	1.58 (3H, d, 7.3)
32	173.3 (s)	—	169.9 (s)	—	177.0 (s)	—
33	45.3 (t)	2.30, 2.35 (2H, m)	133.8 (s)	—	44.9 (d)	2.44 (1H, m)
34	26.5 (d)	2.35 (1H, m)	134.2 (d)	6.87 (1H, m)	29.2 (t)	1.95, 1.73 (2H, m)
35	22.6 (q)	0.98 (3H, d, 6.4)	25.6 (t)	2.0 (2H, m)	26.1 (t)	1.17 (2H, m)
36	22.8 (q)	0.98 (3H, d, 6.4)	21.9 (t)	1.48 (2H, m)	26.2 (t)	1.17, 1.55 (2H, m)
37	—	—	22.5 (t)	1.55 (2H, m)	26.0 (t)	1.70, 2.05 (2H, m)
38	—	—	24.6 (t)	2.30, 2.40 (2H, m)	29.9 (t)	~1.17 (2H, m)

mode of substitution in the benzothiazine ring as shown in Fig. 4.

Structures of TT-A and TT-D

The structural difference between TT-A and TT-D was similar to that between TT-B and TT-E. The molecular weight of TT-A and TT-D were both found to be 707 with the same molecular formular of $C_{38}H_{49}N_3O_8S$. The ^{1}H, ^{13}C NMR and HMBC spectra of TT-A and TT-D indicated their structural differences (Table 3 and Fig. 4). The cyclohexane moiety that was common to TT-B and E was substituted for a cyclohexene moieties in both TT-A and D (Fig. 5).

Structure of TT-C

The structure of TT-C was determined by comparing of its physico-chemical data with those of TT-E. The results of ^{1}H, ^{13}C NMR, ^{1}H-^{1}H COSY and HMBC spectra indicated that the cyclohexane moiety in TT-E was replaced by an isobutyl group in TT-C.

Fig. 5. HMBC and ^1H-^1H COSY experiments of thiazinotrienomycins A, B, C, D and E.

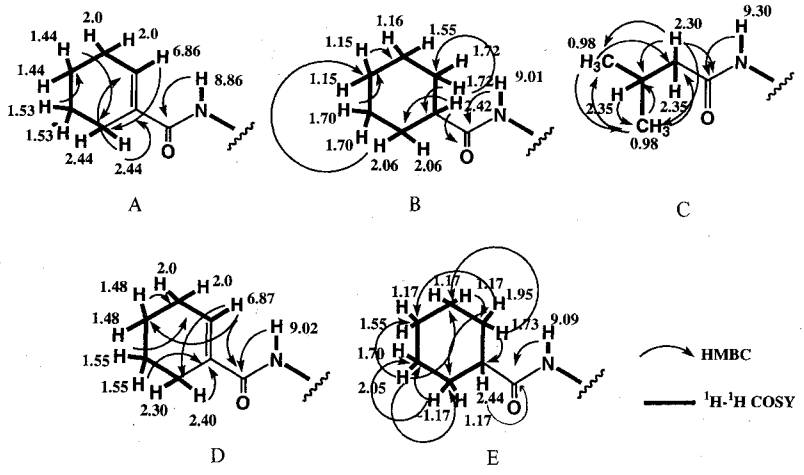


Table 5. Growth-inhibitory activities of thiazinotrienomycins against human cancer cell lines.

	Growth-inhibitory activities *in vitro*: IC_{50}, ng/ml				
	Cervix		Somach	Colon	Breast
	HeLa	C33A	MKN28	COLO201	MCF7
A	5.0	2.2	60	40	40
B	1.5	0.6	8.4	30	90
C	200	100	1,330	1,200	1,000
D	25	20	399	250	450
E	13	23	725	200	550

Biological Activity

TT-A and B were about 10 times stronger than TT-D and E in the growth inhibitory activities against, for example, HeLa, as shown in Table 5. Among the cell lines tested, TT-A and B showed about 10 times stronger activities against cell lines of cervical cancers than against cell lines of stomach, colon and breast cancers. The acute toxicity (LD_{50}) of TT-B, the most active congener, was 125 mg/kg, in mice (ip). TT-B did not inhibit the growth of any bacteria, fungi, and yeast at 100 μg/ml *in vitro*. A preliminary study on the mode of action of TT-B in HeLa cells showed that the antibiotic inhibited the membrane transport of thymidine and uridine but not leucine at concentrations where the cell growth was partially inhibited.

Discussion

Natural products containing the benzothiazine ring have been reported[2~4]. Studies on the biosynthesis of the ansa moiety[5] and the cyclohexene residue[6] in mycotrienin were reported, while no report on the benzothiazine portion was available.

Biological activities of TT-A and B were much stronger than those of TT-D and E, indicating the importance of the mode of substitution in the benzothiazine ring. Activities of TT-D and E were stronger than that of TT-C indicating the importance of a bulky side chain[7]. The acute toxicity of TT-B, the most active member of TT's was lower than those of triene-ansamycin antibiotics reported so far, such as mycotrienin[8].

Studies are in progress on the molecular basis for the biological activities of TT's.

Experimental

Human Cancer Cell Lines and Culture Conditions

HeLa S3 (ATCC CCL 2.2, cervix), C33A (ATCC HTB31, cervix), MKN28 (JCRBO 223, stomach), COLO 201 (JCRBO 226, colon), and MCF7 (ATCC HTB 22, breast). These cells were grown in EAGLE's minimum essential medium (MEM, Gibco) supplemented with 2% v/v fetal bovine serum (FBS, Hyclone) and 1 mM Na-pyruvate, 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 3. Cell growth inhibition caused by samples was determined as reported[9].

General

UV spectra were recorded on a Hitachi U-3210 spectrophotometer and IR spectra on a Hitachi 285-spectrophotometer. NMR spectra were recorded on a JEOL JNM-A500 NMR spectrometer at 500 MHz for ^1H NMR and at 125 MHz for ^{13}C NMR. Mass spectra were measured on a JEOL SX102 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 65% MeOH at 1 ml/minute at 40°C and 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 benzene - $CHCl_3$ - MeOH (3 : 7 : 3), and the spots on TLC were detected with phosphomolybdate - H_2SO_4.

Preparation of 19-*O*-Methyl TT-E

TT-E (10 mg) was dissolved in methanol (1 ml) and then mixed with an excess of diazomethane in diethylether. After the reaction mixture was kept at 22°C for 2 hours, the solution was concentrated *in vacuo* and the residue was dissolved in MeOH (0.5 ml). The solution was subjected to a preparative HPLC. The 19-*O*-methyl TT-E was eluted at a retention time of 35 minutes and finally obtained as colorless powder (4.3 mg). Found: FAB-MS *m/z* 724 (MH^+), *m/z* 722 (M − H); δ_H (C_5D_5N) (500 MHz) 3.80 (3H, s); δ_C (C_5D_5N) (125 MHz) 62.0 ($-OCH_3$), 145.0 (C-19).

References

1) FUNAYAMA, S.; K. OKADA, K. IWASAKI, K. KOMIYAMA & I. UMEZAWA: Structures of trienomycins A, B and C, novel cytocidal ansamycin antibiotics. J. Antibiotics 38: 1677 ~ 1683, 1985
2) BEUTLER, J. A.; G. N. CHMURNY, P. CLARK, C. J. METRAL, J. ROMAN & S. FORENZA: Two novel phenolics from *Actinomadura verrucosospora*. J. Antibiotics 43: 107 ~ 109, 1990
3) TANIDA, S.; S. SHINAGAWA, M. TAKIZAWA, T. TAKAHASHI, S. HARADA & T. HASEGAWA: Two sulfur-containing ansamycin antibiotics from *Streptomyces albolongus*. Experientia 42: 1167 ~ 1170, 1986
4) HORI, Y.; S. YAMAGISHI, N. SHIGEMATSU, K. SAKAMOTO, H. TERANO & M. OKUHARA: Japan Kokai 6-239749, Aug. 30, 1994
5) WU, T. S.; J. DUNCAN, S. W. TSAO, C. J. CHANG, P. J. KELLER & H. G. FLOSS: Biosynthesis of the ansamycin antibiotic ansatrienin (Mycotrienin) by *Streptomyces collinus*. J. Natural Products 50: 108 ~ 118, 1987
6) REYNOLDS, K. A.; P. WANG, K. M. FOX & H. G. FLOSS: Biosynthesis of ansatrienin by *Streptomyces collinus*: cell-free tansformations of cyclohexene and cyclohexadienecarboxylic acids. J. Antibiotics 45: 411 ~ 419, 1992
7) NOMOTO, H.; S. KATSUMATA, K. TAKAHASHI, K. S. FUNAYAMA, K. KOMIYAMA, I. UMEZAWA & S. OMURA: Structural studies on minor components of trienomycin group antibiotics trienomycins D and E. J. Antibiotics 42: 479 ~ 481, 1989
8) SUGITA, M.; Y. NATORI, T. SASAKI, K. FURIHATA, A. SHIMAZU, H. SETO & N. OTAKE: Studies on mycotrienin antibiotics, a novel class of ansamycins. J. Antibiotics 35: 1460 ~ 1479, 1982
9) SUZUKAKE-TSUCHIYA, K.; Y. MORIYA, K. YAMAZAKI, M. HORI, N. HOSOKAWA, T. SAWA, H. IINUMA, H. NAGANAWA, C. IMADA & M. HAMADA: Screening of antibiotics preferentially active against ras oncogene-expressed cells. J. Antibiotics 43: 1489 ~ 1496, 1990