STRUCTURES OF TRIENOMYCINS A, B AND C, NOVEL CYTOCIDAL ANSAMYCIN ANTIBIOTICS

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The structures of novel antibiotics, trienomycins A, B and C produced by *Streptomyces* sp. No. 83-16, have been determined on the basis of their spectroscopical and chemical properties. Trienomycins are unique ansamycin antibiotics with a triene and an 1,3,5-trisubstituted benzene moieties in the molecule. The antibiotics possess potent cytocidal activity against HeLa S_3 cells at concentrations of 0.005 (trienomycin A), 0.1 (trienomycin C) and 0.2 μg/ml (trienomycin B) (IC_{50} value), respectively. However, trienomycins showed no antimicrobial activity against the bacteria, fungi and yeasts examined with the exception of weak activity *versus Piricularia oryzae* at a concentration of 1,000 μg/ml.

In the preceding paper, we reported the production, isolation, physico-chemical properties and biological activities of trienomycin A†, together with the taxonomy of the producing organism, *Streptomyces* sp. No. 83-16[1]. Through careful fractionation of the fermentation broth from which trienomycin A was isolated, two minor components, designated as trienomycins B and C, were obtained.

This paper deals with the isolation and structural elucidation of trienomycins A, B and C in detail and the preliminary biological activities of these compounds; a preliminary communication concerning our work on the structural elucidation of trienomycin A[2] has been presented.

Isolation of Trienomycins A, B and C

The isolation, characterization and structural elucidation of trienomycin A were reported in the preceding papers[1,2].

The same fermentation procedure employed for trienomycin A was used for the production of trienomycins B and C. Separation of trienomycins B and C was performed by silica gel column chromatography using a gradient solvent system with chloroform - methanol as solvent, followed by preparative HPLC using methanol - water (13: 7) as solvent.

Rf values in TLC of trienomycins A, B and C are given in Table 1.

Physico-chemical Properties of Trienomycins A, B and C

The physico-chemical properties of trienomycins A, B and C are summarized in Table 2. The UV and IR spectra of these compounds were quite similar and it was shown through UV and IR spectral analysis that triene, amide and ester functions are common to these three compounds.

The molecular formula and molecular weight of trienomycin A are $C_{36}H_{50}N_2O_7$ and 622, while the molecular formula and molecular weight of both trienomycins B and C are $C_{34}H_{48}N_2O_7$ and 596.

† 19-Deoxymycotrienin II isolated from the culture broth of *Streptomyces rishiriensis* T-23[20] is identical with trienomycin A.

Table 1. Chromatographic behavior of trienomycins A, B and C and mycotrienin II on TLC (Kieselgel 60 F_{254} DC-Fertigplatten, Merck).

Solvent system	Rf value			
	Trienomycin A	Trienomycin B	Trienomycin C	Mycotrienin II
$CHCl_3$ - MeOH (19: 1)	0.41	0.36	0.38	0.32
Toluene - acetone (3: 2)	0.39	0.35	0.36	0.38

Table 2. Physico-chemical properties of trienomycins A, B and C.

	Trienomycin A	Trienomycin B	Trienomycin C
Appearance	Colorless powder	Colorless powder	Colorless powder
MP (°C)	128~132	124~126	119.5~121.5
Molecular formula	$C_{36}H_{50}N_2O_7$	$C_{34}H_{48}N_2O_7$	$C_{34}H_{48}N_2O_7$
Molecular weight	622	596	596
UV λ_{max}^{MeOH} nm	250, 260, 271 and 282	250, 260, 271 and 282	250, 260, 271 and 282
IR ν_{max}^{KBr} cm^{-1}	3400 (NH, OH), 1730, 1205 (ester), 1650, 1540 (amide), 1000 (triene)	3400 (NH, OH), 1730, 1205 (ester), 1650, 1540 (amide), 1000 (triene)	3400 (NH, OH), 1730, 1210 (ester), 1660, 1550 (amide), 1000 (triene)
$[\alpha]_D^{20}$ (c 0.1, MeOH)	+174°	+170°	+186°

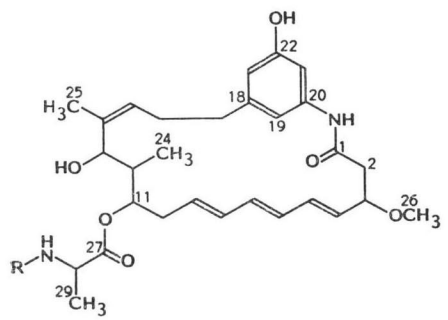
The differences in molecular formula and molecular weight between trienomycin A and trienomycins B and C result from the fact that the latter two compounds lack a C_2H_2 unit and corresponding have 26 less mass units than trienomycin A.

The ^{13}C NMR spectral data of these three compounds are summarized in Table 3. In the ^{13}C NMR spectrum of trienomycin A, 36 signals were observed whereas 34 signals were observed in the ^{13}C NMR spectra of trienomycins B and C.

Structural Elucidation of Trienomycins A, B and C

The structural elucidation procedure of trienomycin A was presented in the preceding communication[2] and the structure of trienomycin A was established as **1** (Fig. 1).

Fig. 1. Structures of trienomycins A, B and C.



1 R = $-C(=O)-C_6H_{11}$

2 R = $-C(=O)CH_2CH(CH_3)_2$

3 R = $-C(=O)CH(CH_3)CH_2CH_3$

Because the absorption maxima at around λ_{max} (MeOH) 260, 270 and 280 nm in the UV spectrum and ν_{max} (KBr) 1000 cm^{-1} in the IR spectrum are common among trienomycins A, B and C, it was suggested that the triene moiety is common among these three compounds.

In the ^1H NMR spectra of trienomycins B and C, a set of three aromatic protons which were coupled (dd, J=1.5~2.0 Hz, respectively) and had been assigned to the trisubstituted phenol moiety of trienomycin A[2], were also observed.

On the other hand, in the MS of trienomycins A, B and C, m/z 423 ($C_{26}H_{33}NO_4$) was common among these three compounds.

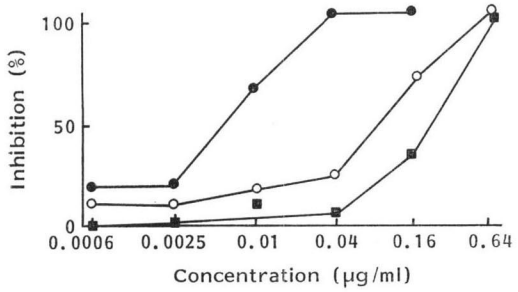
Table 3. ^{13}C NMR spectral data for trienomycins A, B and C.

		Trienomycin A	Trienomycin B	Trienomycin C
Ansa moiety	1	170.8 s	168.9 s	169.0 s
	2	44.8 t	43.6 t	43.7 t
	3	81.6 d	79.1 d	79.2 d
	4	132.5 d[a]	130.7 d[a]	129.5 d[a]
	5	135.2 d[b]	134.0 d[b]	134.2 d[b]
	6	131.0 d[a]	129.4 d[a]	129.6 d[a]
	7	135.0 d[b]	133.8 d[b]	133.9 d[b]
	8	134.6 d[b]	133.4 d[b]	133.4 d[b]
	9	130.5 d[a]	129.4 d[a]	130.8 d[a]
	10	33.7 t	33.3 t	33.3 t
	11	76.4 d	75.6 d	75.6 d
	12	40.4 d	39.6 d	39.5 d
	13	69.7 d	68.5 d	68.5 d
	14	139.7 s[e]	138.3 s[e]	138.4 s[e]
	15	125.9 d	125.0 d	124.7 d
	16	30.8 t	29.3 t	29.7 t[e]
	17	37.3 t	36.3 t	36.3 t
Aromatic	18	144.9 s	144.0 s	144.1 s
	19	112.9 d[d]	111.2 d[d]	111.3 d[d]
	20	140.2 s[e]	138.3 s[e]	138.6 s[e]
	21	107.2 d	106.1 d	106.1 d[d]
	22	158.6 s	157.3 s	157.4 s
	23	113.4 d[d]	112.3 d[d]	112.3 d[d]
Me	24	10.2 q	9.9 q	10.0 q
	25	20.8 q	20.3 q	20.5 q
	26	56.6 q	56.7 q	56.7 q
Ala	27	173.3 s	172.9 s	173.2 s
	28	50.5 d	48.7 d	48.7 d
	29	17.2 q	17.6 q	17.6 q
Acyl	30	179.2 s	173.3 s	167.4 s
	31	45.9 d	45.4 t	42.8 d
	32	30.5 t	26.1 d	29.5 t[e]
	33	26.7 t	22.4 q	20.0 q
	34	26.9 t	22.4 q	27.3 q
	35	26.7 t		
	36	30.5 t		

[a~e] Assignments may be exchangeable.

Fig. 2. Effect of trienomycins A, B and C on colony formation of HeLa S_3 cells.

● Trienomycin A, ■ trienomycin B, ○ trienomycin C.



From the accumulated observations described above, it was found that trienomycins A, B and C possess common ansa and phenol moieties. This was confirmed by comparison of the ^{13}C NMR spectral data of these regions (Table 3).

The existence of an alanine moiety in the structures of trienomycins A, B and C was inferred from the ^{1}H NMR and ^{13}C NMR spectra of these compounds, and when trienomycins A, B or C was treated with 6 N HCl for 16 hours at 120°C, alanine was obtained in each case. The *N*-hexahydrobenzoylalanine moiety in the struc-

ture of trienomycin A was also confirmed by the presence of *m/z* 154.1226 (154.1231 Calcd for $C_9H_{16}NO$) in the HR-MS analysis of this compound[2].

In the HR-MS of trienomycin A, other than the peaks described above, *m/z* 111.0821 (111.0809 Calcd for $C_7H_{11}O$) and 83.0889 (83.0860 Calcd for C_6H_{11}) were observed and were explained by the presence of the hexahydrobenzoyl moiety in the structure[2]. On the other hand, instead of the peaks attributed to the hexahydrobenzoyl moiety described above, *m/z* 85 (attributed to C_5H_9O) and 57 (attributed to C_4H_9) were observed in the MS of both of trienomycins B and C.

From these results, it was shown that the difference in the molecular formula (C_2H_2, 26 mass) between trienomycin A and trienomycins B and C is that the $C_7H_{11}O$ unit of the former compound is substituted by the C_5H_9O unit in the latter two compounds.

The ^{13}C NMR spectra of trienomycins B and C were quite similar to that of trienomycin A (**1**) except for the signals attributed to the acyl groups attached to the alanyl moiety (Table 3). The ^{13}C NMR signals attributed to the acyl moieties of trienomycins A, B and C were as follows; trienomycin A [δ 26.7 (t, 2×C), 26.9 (t), 30.5 (t, 2×C), 45.9 (d) and 179.2 (s)], trienomycin B [δ 22.4 (q, 2×C), 26.1 (d), 45.4 (t) and 173.3 (s)] and trienomycin C [δ 20.0 (q), 27.3 (q), 29.5 (t), 42.8 (d) and 167.4 (s)]. These observations indicated that instead of the hexahydrobenzoyl moiety of trienomycin A (**1**), the 3-methylbutyryl (isovaleryl) moiety is attached to the alanyl moiety in trienomycin B and the 2-methylbutyryl moiety is attached to the alanyl moiety in trienomycin C.

From all of the accumulated data described above, structures of trienomycins A, B and C were concluded to be **1**, **2** and **3** (Fig. 1), respectively.

Biological Activities of Trienomycins A, B and C

The antimicrobial activity of trienomycins were determined on various microorganisms such as *Bacillus subtilis* PCI 219, *B. cereus* IFO 3001, *Micrococcus luteus* ATCC 9341, *Staphylococcus aureus* FDA 209 P, *Salmonella typhimurium* KB 20, *Shigella flexneri* E 20, *S. sonnei* E 33, *Escherichia coli* NIHJ, *Klebsiella pneumoniae* PCI 602, *Enterobacter aerogenes* IAM 1183, *Proteus vulgaris* IFO 3167, *Candida albicans* KF 1, *Saccharomyces sake* FK 26, *Shizosaccharomyces pombe* IAM 4863, *Rhizopus javanicus* IAM 6241, *Aspergillus niger* ATCC 6275, *Alternaria kikuchiana* KF 185, *Mucor racemosus* IFO 5403 and *Piricularia oryzae* KF 180. The antibiotics show no antimicrobial activities even at a concentration of 1,000 μg/ml except a slight activity on *Piricularia oryzae*. Incomplete inhibitory zone of trienomycins A, B and C at 1,000 μg/ml to *Piricularia oryzae* were 20, 15 and 12 mm,respectively.

On the other hand, trienomycins exhibit cytocidal activities against HeLa S_3 cells *in vitro*. As shown in Fig. 2, trienomycin A (**1**) exhibited 20~40 fold stronger activity than trienomycins B (**2**) and C (**3**).

Discussion

Trienomycins B (**2**) and C (**3**) are new trienomycin group ansamycin antibiotics isolated from the culture broth of *Streptomyces* sp No. 83-16, a trienomycin A (**1**) producing strain[1,2].

The trienomycin group is closely related to mycotrienins or ansatrienins* in its structure[3~10,20]. However, trienomycins are unique among the benzenoid ansamycin antibiotics in that trienomycins A (**1**), B (**2**) and C (**3**) do not have a *p*-quinone or *p*-hydroquinone moiety in the structure. Such a benzenoid moiety of the trienomycin group is rather similar to those of maytansinoids (higher plant

* It was reported that mycotrienins I and II have the same structures as ansatrienins A and B, respectively, except for the stereochemistry of alanine[6~8].

origin)[11] or ansamitocins (microbial origin)[12]. As in the case of mycotrienins I and II and ansatrienins A and B, the hexahydrobenzoyl moiety has also been found in the structure of trienomycin A (**1**). Hexahydrobenzoic acid had been reported as a metabolite of *Curtobacterium pusillum*[13] and as a component of asukamycin[14,15]. Instead of the hexahydrobenzoyl moiety, the 3-methylbutyryl (isovaleryl) and 2-methylbutyryl moieties are involved in the structures of trienomycins B and C respectively. Such moieties were found previously in the structures of ansatrienins A_2 and A_3[10]. Naphthomycins A, B and C[16,17] and actamycin[18] are known as ansamycin antibiotics with a triene moiety in their structures other than the trienomycin, mycotrienin and ansatrienin groups.

Among the benzenoid ansamycin antibiotics possessing cytotoxic activity, mycotrienins I and II and geldanamycin were reported to have antifungal (mycotrienins I and II)[6] and antimicrobial (geldanamycin)[19] activities respectively. It is noteworthy that trienomycins A (**1**), B (**2**) and C (**3**) exhibited considerable cytotoxic activity against HeLa S_3 cells (Fig. 2) without showing activity on microorganisms including bacteria, fungi and yeast tested (only a weak activity against *Piricularia oryzae* was observed at doses of up to 1,000 μg/ml). Structure activity relationships, especially between the trienomycin group and mycotrienin group, will be of interest.

Experimental

Melting points were determined using a Kofler hot plate and are uncorrected. IR spectra were recorded with an A-102 (Jasco) spectrophotometer with polystyrene calibration at 1601 cm^{-1}; absorption bands were recorded in wave numbers (cm^{-1}). UV spectra were measured with a Shimadzu UV 200S double beam spectrophotometer. ^1H and ^{13}C NMR spectra were obtained in CD_3OD (trienomycin A) or $CDCl_3$ (trienomycins B and C) with Jeol FX-200 instruments. Tetramethylsilane (TMS) was used as an internal standard and chemical shifts were recorded in δ ppm units. Mass spectra were obtained on D-100 and DX-3000 (Jasco) spectrometers. Optical rotations were measured with a DIP-181 (Jasco) polarimeter. Kieselgel 60 F_{254} DC-Fertigplatten (Merck) were used for TLC analysis, and Kieselgel 60 (Merck) was used for column chromatography. TRI Rotar-V (Jasco) and Uvidec-100 (Jasco) instruments were used for HPLC with a column of YMC A-303 (Yamamura Chemical Lab.; 4.6 $\phi \times$ 250 mm) eluted with MeOH - H_2O (13: 7); flow rate, 1.0 ml/minute; detection, UV at 220 nm.

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

The taxonomy, fermentation, isolation and physico-chemical and biological characteristics of trienomycin A (**1**) were described in the preceding paper[1].

Trienomycins B (**2**) and C (**3**) were isolated from the culture broth of *Streptomyces* sp. No. 83-16, a trienomycin A producing strain, as minor components.

The fermentation broth (400 liters) was adjusted to pH 5.0 and extracted with EtOAc (400 liters). The EtOAc layer was dried over Na_2SO_4 (anhydrous) and concentrated *in vacuo* to give a brown residue (23.5 g) which was chromatographed over silica gel using $CHCl_3$ - MeOH (gradient) or benzene - acetone (gradient) to give an oily residue (1.05 g) containing trienomycins A (**1**), B (**2**) and C (**3**). Further purification of the trienomycin complex was achieved through preparative HPLC using MeOH - H_2O (13: 7) as solvent to give trienomycins A (**1**, 412 mg), B (**2**, 43 mg) and C (**3**, 47 mg) respectively.

The chromatographic behaviors on TLC of trienomycins A (**1**), B (**2**) and C (**3**) are shown on Table 1.

Acid Hydrolysis of Trienomycin A (1)

Trienomycin A (**1**, 50 mg) was suspended in 6 N HCl (5.0 ml) and kept in a sealed tube at 120°C for 16 hours. The reaction mixture was diluted with H_2O (5.0 ml) and filtered. The filtrate was passed through a column of Amberlite CG-120 (H^+ type, 15 ml) and washed with H_2O (30 ml), and the fractionation was carried out using 1% NH_4OH as solvent. Fractions containing ninhydrin positive compound were collected and concentrated *in vacuo* to give D-alanine. Silica gel TLC: Rf 0.23, BuOH - AcOH - H_2O (4 : 1 : 2) and Rf 0.56, PrOH - H_2O (3: 2), $[\alpha]_D^{20}$ −14.6° (*c* 0.2, 1 N HCl).

Acid Hydrolysis of Trienomycins B (**2**) and C (**3**)

Trienomycins B (**2**) and C (**3**) (2.0 mg each) were suspended in 6 N HCl (0.5 ml) and kept in a sealed tube at 120°C for 16 hours. The filtrate was treated in the same manner as the hydrolyzate of trienomycin A (**1**) to afford alanine.

Biological Activities

The antimicrobial spectra of trienomycins 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.

To determine the effects of the antibiotics on the growth of mammalian cells, a single cell suspension of HeLa S_3 cells (200 cells per 5 ml of minimum essential medium supplemented with 10% calf serum) was placed in a 5-cm Petri dish (Nunclon, Nunc Co.). After preincubation for 24 hours, trienomycins dissolved in MeOH were added to the medium (final concentration of MeOH was 1.0%) and incubated for 8 days. The colonies were then fixed with MeOH, stained with Giemsa solution, and counted.

Acknowledgments

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