STUDIES ON VIRIDENOMYCIN, A NOVEL 24-MEMBERED MACROCYCLIC POLYENE LACTAM ANTIBIOTIC

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A new antitumor antibiotic, designated AL081, was obtained from the culture filtrate of an actinomycete identified as *Streptomyces gannmycicus*, and found to be identical with viridenomycin by direct comparison. The structure of the antibiotic was determined by NMR spectral analysis including a variety of two-dimensional techniques to be a novel 24-membered macrocyclic polyene lactam. Viridenomycin prolonged the survival periods of mice bearing P388 leukemia and B16 melanoma cells.

During the course of our screening program for new antibiotics which prolonged the survival periods of mice bearing B16 melanoma cells, we isolated an antitumor antibiotic, designated AL081[1]. Recently, this antibiotic has been found to be identical with viridenomycin[2,3] by direct comparison. The structure of viridenomycin, however, has not been clarified because of its instability in various solvents. The structure assigned for viridenomycin possesses a novel 24-membered polyene lactam skeleton which is partially related to that of hitachimycin[4]. In this paper, we describe the production, isolation, structure elucidation and antitumor activities of viridenomycin as well as the taxonomy of its producing organism classified as *Streptomyces gannmycicus*.

Taxonomy of Strain AL081

Culture AL081 was isolated from a soil sample collected at Hiratsuka, Kanagawa Prefecture, Japan. Characterization of the strain was carried out mainly by the methods described by SHIRLING and GOTTLIEB[5].

The aerial mycelium of the strain monopodially branched on the long main stem and terminated in spirals forming spore chains with 10~50 spores per chain. The spores were oval (0.5~0.7 × 0.9~1.2 μm) with spiny surfaces (Fig. 1). The cultural and physiological properties of strain AL081 grown on various media at 27°C are shown in Tables 1 and 2, respectively. These properties can be summarized as follows: The aerial mass color was in the yellow

Fig. 1. Electron micrograph of spores of strain AL081.

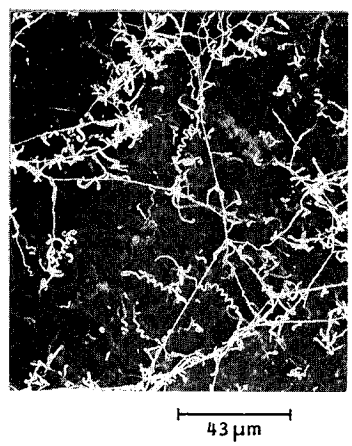


Table 1. Cultural characteristics of strain AL081.

Sucrose - nitrate agar	G:	Moderate
	R:	Yellowish white
	Am:	Poor; light olive gray
	Sp:	None
Glucose - asparagine agar	G:	Moderate
	R:	Yellowish brown
	Am:	Moderate; light olive gray
	Sp:	None
Glycerol - asparagine agar	G:	Good
	R:	Yellowish brown
	Am:	Abundant; yellowish white
	Sp:	None
Inorganic salts - starch agar	G:	Moderate
	R:	Yellowish brown
	Am:	Moderate; pale brown
	Sp:	None
Tyrosine agar	G:	Good
	R:	Dark yellowish brown
	Am:	Abundant; yellowish white
	Sp:	None
Nutrient agar	G:	Moderate
	R:	Dull yellow
	Am:	Poor; light olive gray
	Sp:	None
Yeast extract - malt extract agar	G:	Good
	R:	Dull yellow
	Am:	Abundant; light olive gray
	Sp:	None
Oatmeal agar	G:	Moderate
	R:	Yellowish brown
	Am:	Poor; light olive gray
	Sp:	None

G: Growth, R: reverse side of colony, Am: aerial mycelium, Sp: soluble pigment.

color series (yellowish white or light olive gray); the reverse side of colony showed no distinctive pigments; the strain produced melanoid pigments. Hydrolyzed whole cells contained LL-diaminopimelic acid. On the basis of these physiological and morphological features, strain AL081 was classified as the genus *Streptomyces*. Among the species of *Streptomyces* described in SHIRLING's report[6], the properties of strain AL081 were in good agreement with those of *Streptomyces gannmycicus*, except for slight differences between their utilization of carbon sources and temperature range for growth. Strain AL081 could not utilize sucrose and could grow at 45°C. Therefore, strain AL081 was identified as a strain of *Streptomyces gannmycicus*.

Table 2. Physiological properties of strain AL081.

Temperature for growth	20~45°C
Production of melanoid pigments:	
Tyrosine agar	Positive
Peptone - yeast extract - iron agar	Positive
Tryptone - yeast extract agar	Positive
Hydrolysis of starch	Negative
Liquefaction of gelatin	Negative
Peptonization of milk	Negative
Coagulation of milk	Weakly positive
Utilization of carbon sources:	
Utilized	L-Arabinose, D-xylose, D-glucose, D-fructose, inositol, L-rhamnose, raffinose, D-mannitol
Not utilized	Sucrose, sorbitol

Production and Isolation of Viridenomycin

Strain AL081 (FERM BP-1998) was cultured at 27°C for 4 days with shaking in 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of glucose 2.5%, soybean meal 1.5%, dry yeast 0.2% and $CaCO_3$ 0.4% (pH 7.0) on a rotary shaker. The mycelial cake collected by centrifugation of the culture broth (1 liter) was extracted with acetone. The extract was concentrated to a small volume and then extracted with ethyl acetate at pH 2.0. The solvent layer was concentrated and applied to a silica gel column (2 × 30 cm), which was washed with $CHCl_3$ and eluted with $CHCl_3$ - methanol (40 : 1). Further purification was achieved by Sephadex LH-20 column chromatography (2 × 60 cm) developed with $CHCl_3$ - methanol (1 : 1). The active eluate was evaporated to dryness to give a colorless powder of viridenomycin (25 mg). The above isolation had to be finished in a few hours to avoid degradation of viridenomycin.

Structural Elucidation

Physico-chemical properties of AL081 and viridenomycin were summarized as shown in Table 3. The molecular formula of viridenomycin was established as $C_{34}H_{37}NO_6$ by FD-MS (m/z 556, $(M+H)^+$) and elemental analysis (calcd: C 73.49, H 6.71, N 2.52, O 17.28; found: C 73.58, H 6.56, N 2.42, O 17.44). The 500 MHz ^1H NMR spectrum of viridenomycin taken in $CDCl_3$ (Fig. 2) revealed the presence of 5 aromatic protons, 15 olefinic protons, a methoxy (δ 3.65), an allylic methyl (δ 2.14) and a tertiary methyl group (δ 1.33). The ^{13}C NMR spectrum showed signals due to all 34 carbons, which were assigned to 7 quaternary carbons, 23 methines, 1 methylene and 3 methyls by DEPT experiments. The ^{13}C and ^1H NMR spectral data were summarized as shown in Table 4.

All one-bond ^1H-^{13}C connectivities were established by a heteronuclear multiple-quantum coherence (HMQC)[7] experiment. Partial structures including a tetraene system, a phenyl group and a diol moiety as shown in Fig. 3 were determined by a ^1H-^1H COSY experiment. The remaining olefinic methine (C-21) which could not be assigned due to overlapping of three olefinic protons (20-H, 21-H and 22-H) at δ 6.22

Table 3. Physico-chemical properties of viridenomycin and AL081.

	Viridenomycin	AL081
Appearance	Colorless fine plates	White powder
Melting point	168~170°C (dec)	185~190°C (dec)
$[\alpha]_D$	+893° (32°C, c 0.5 in $CHCl_3$)	+195° (25°C, c 0.5 in $CHCl_3$)
UV λ_{max} nm (in MeOH)	310	309
MW (m/z)	566 (vapor pressure osmometry)	556 (FD-MS; MH^+)
Elemental analysis		
Found:	C 72.09, H 7.01, N 2.42, O 18.48	C 73.58, H 6.56, N 2.42, O 17.44
Calcd:		C 73.49, H 6.71, N 2.52, O 17.28
Molecular formula	$C_{34\sim36}H_{35\sim41}NO_{6\sim7}$	$C_{34}H_{37}NO_6$

Fig. 2. The 500 MHz ^1H NMR spectrum of viridenomycin in $CDCl_3$.

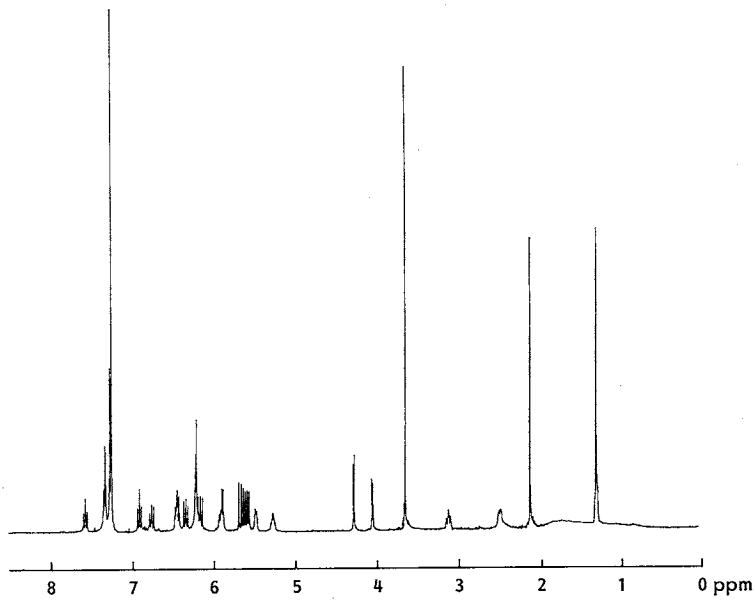
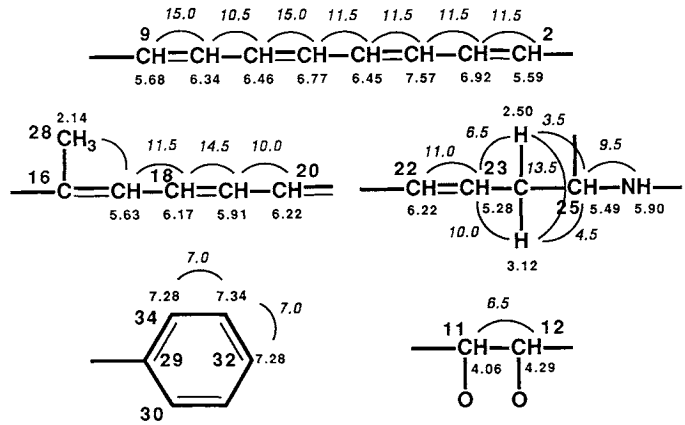


Table 4. ^{13}C and ^{1}H NMR assignments for viridenomycin in $CDCl_3$.

No.	δ_C	δ_H	(J, Hz)	No.	δ_C	δ_H	(J, Hz)
1	166.2 s*			18	126.3 d	6.17	(dd, 14.5, 11.5)
2	120.8 d	5.59	(d, 11.5)	19	133.7 d	5.91	(dd, 14.5, 10.0)
3	134.9 d	6.92	(t, 11.5)	20	134.3 d	6.22	(br s)
4	124.6 d	7.57	(t, 11.5)	21	128.1 d	6.22	(br s)
5	135.8 d	6.45	(t, 11.5)	22	133.7 d	6.22	(br s)
6	126.3 d	6.77	(dd, 15.0, 11.5)	23	125.7 d	5.28	(ddd, 11.0, 10.0, 6.5)
7	136.3 d	6.46	(dd, 15.0, 10.5)	24	33.7 t	3.12	(ddd, 13.5, 10.0, 4.5),
8	131.4 d	6.34	(dd, 15.0, 10.5)			2.50	(ddd, 13.5, 6.5, 3.5)
9	141.7 d	5.68	(d, 15.0)	25	51.5 d	5.49	(ddd, 9.5, 4.5, 3.5)
10	46.8 s			25-NH		5.90	(d, 9.5)
11	83.4 d	4.06	(d, 6.5)	26	17.0 q	1.33	(s)
12	85.3 d	4.29	(d, 6.5)	27	59.1 q	3.65	(s)
13	171.1 s			28	15.9 q	2.14	(s)
14	105.9 s			29	139.5 s		
15	167.1 s			30, 34	126.3 d	7.28	(d, 7.0)
16	147.7 s			31, 33	128.5 d	7.34	(t, 7.0)
17	119.1 d	5.63	(d, 11.5)	32	127.3 d	7.28	(d, 7.0)

* s: Singlet, d: doublet, t: triplet, q: quartet.

Fig. 3. Partial structures of viridenomycin.



was assumed to form another tetraene system together with C-16 ~ C-20, C-22 and C-23 from their chemical shifts. This tetraene moiety was confirmed by ^{1}H-^{13}C long range couplings in the heteronuclear multiple-bond correlation (HMBC)[8] spectrum. As shown in Fig. 4, long range couplings were observed between 20-H (or 21-H) and the carbon at δ 133.7, and 24-H and the carbon at δ 133.7. Therefore carbon signals at δ 134.3, 128.1 and 133.7 were assigned to C-20, C-21 and C-22, respectively.

Fig. 4. Partial structures of viridenomycin.

The solid-line arrows indicate ^{1}H-^{13}C long range couplings detected by HMBC.

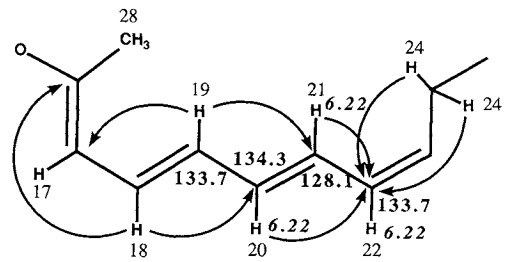


Fig. 5. Partial structures of viridenomycin.

The solid line arrows are the same as those in Fig. 4.

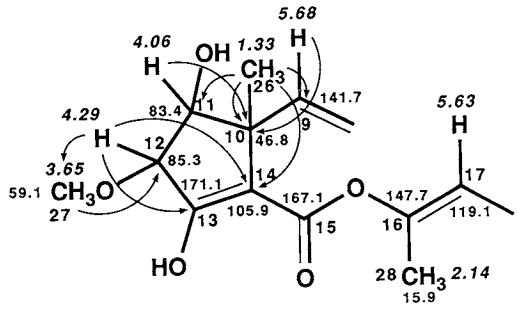
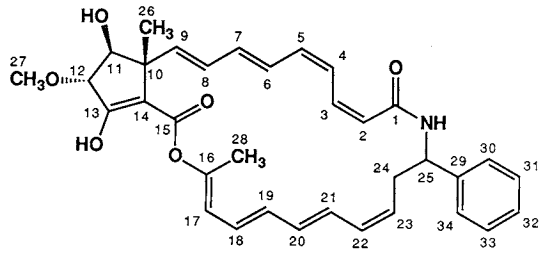


Fig. 6. The structure of viridenomycin.



The connectivities of the partial structures thus obtained were elucidated by observation of the ^{1}H-^{13}C long range correlations from 2-H, 3-H and 25-NH to a carbonyl carbon (C-1, δ 166.2), and from 30-H and 34-H to a methine carbon (C-25, δ 51.5), thereby showing that the tetraene moiety consisting of C-2 to C-9 was attached to C-25 through an amide linkage and the phenyl group to C-25 directly.

The remaining functional groups including a tertiary methyl, a methoxy, a diol moiety and three quaternary carbons were assembled as shown in Fig. 5 by analysis of the HMBC spectral data, which revealed the ^{1}H-^{13}C long range couplings from the tertiary methyl (26-H) to C-9, C-10, C-11 and C-14, from the oxymethine (12-H) to C-13 and C-14, and from the methoxy (27-H) to C-12. These correlations established a cyclopentene ring structure (C-10$\sim$C-14) substituted with a methoxy group at C-12 and a tetraene moiety at C-9. The only remaining carbon (C-15) was assignable to an ester group from its chemical shift (δ 167.1) and an IR absorption at 1700 cm^{-1}. In order to explain the chemical shifts of C-13 (δ 171.1) and C-14 (δ 105.9), and a positive ferric chloride reaction for viridenomycin, C-13 and C-14 must form an enol group conjugated to the ester carbonyl (C-15).

The ester linkage between C-15 and C-16 was determined by the chemical shift of C-16 (δ 147.7) and long range couplings from the allylic methyl (28-H) to only two carbons, C-16 and C-17.

Six of the eight geometries of the two tetraene systems were established to be 2*Z*, 4*Z*, 6*E*, 8*E*, 18*E*, and 22*Z* by $J_{2\text{-}3}=11.5$ Hz, $J_{4\text{-}5}=11.5$ Hz, $J_{6\text{-}7}=15.0$ Hz, $J_{18\text{-}19}=14.5$ Hz and $J_{22\text{-}23}=11.0$ Hz. An upfield chemical shift of C-28 (δ 15.9) and no NOE between 17-H and 28-H showed the *E* configuration for C-16. The remaining stereochemistry at C-20 proved to be 20*E* by the chemical shifts of C-19 (δ 133.7) and C-22 (δ 133.7) observed at a low-field region free from the γ effects[9] in comparison with C-21 (δ 128.1) and C-23 (δ 125.7).

NOEs observed from 26-H to 12-H but not to 11-H indicated that the relative configuration of the cyclopentene ring was as shown in Fig. 6. The stereochemistry at C-25 remains to be determined.

Thus, the structure of viridenomycin was established as shown in Fig. 6 except for the absolute configuration. This antibiotic is partially related to hitachimycin[4], which is a 19-membered lactam antibiotic possessing a phenyl group and a cyclopentene ring, but devoid of the tetraene systems and ester linkage.

Antitumor Activities

Although viridenomycin was reported to be active against Gram-positive bacteria and some fungi, no activities were described with regard to animal tumors. We found that viridenomycin showed antitumor activity against murine tumors as shown in Table 5. Viridenomycin prolonged the survival periods of CDF_1 mice bearing P388 leukemia (ILS 23.0%) and BDF_1 mice bearing B16 melanoma (ILS 37.0%)

Table 5. Antitumor activities of viridenomycin.

Dose (mg/kg/day)	P388		B16	
	Survival days	ILS (%)	Survival days	ILS (%)
0	9.2±0.42		14.7±0.75	
5	11.3±1.37	23.0	20.1±1.15	37.0
10	Toxic		Toxic	

Treatment schedule: Day 1 (ip). Tumor cells were inoculated intraperitoneally at 10^6 cells/mouse.

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Experimental

Specific rotation was obtained on a Jasco DIP-140 spectropolarimeter. The mass spectra were measured on a Hitachi M80 spectrometer, and UV and visible spectra were taken by a Shimadzu UV-300 spectrophotometer. NMR spectra were obtained on a Jeol GX-500 spectrometer with ^1H NMR at 500 MHz and ^{13}C NMR at 125 MHz in $CDCl_3$. Chemical shifts are given in ppm using TMS as an internal standard.

Acknowledgments

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