ISOLATION AND STRUCTURAL ELUCIDATION OF NEW 18-MEMBERED MACROLIDE ANTIBIOTICS, VIRANAMYCINS A AND B

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Two cytotoxic antibiotics, designated viranamycins A and B, were isolated from the culture broth of *Streptomyces* sp. CH41. Their structures were elucidated as new 18-membered macrolides related to virustomycin A and concanamycin A from NMR spectral analysis. Viranamycins A and B inhibited the growth of P388 mouse leukemia and KB human squamous-cell-carcinoma cells.

In the course of our screening for new antitumor antibiotics, *Streptomyces* sp. CH41 was found to produce two cytotoxic antibiotics related to virustomycin A[1] and concanamycin A[2,3], whose structures had been determined to be novel 18-membered macrolides based on chemical degradations. Our compounds appeared to be new members of this family and were named viranamycins A and B. In this paper, we describe the fermentation, isolation, physico-chemical properties, structural elucidation including ^{1}H and ^{13}C NMR spectral assignments and biological activities of viranamycins A and B.

Fermentation

A stock culture of *Streptomyces* sp. CH41 was maintained on a BENNETT's agar slant at 4°C. The stock culture was inoculated into 50-ml tubes containing 15 ml of a seed medium and incubated by shaking at 27°C for 2 days. The medium consisted of soluble starch 1.0%, molasses 1.0%, Polypepton 1.0% and beef extract 1.0% (pH 7.2 before sterilization). The seed culture was transferred at 2% to 500-ml Erlenmeyer flasks containing 100 ml of a production medium consisting of glycerol 2.0%, molasses 3.0%, casein 1.0% and $CaCO_3$ 0.4% (pH 7.2 before sterilization). The flasks were cultivated on a rotary shaker at 27°C for 4 days.

Isolation and Purification

The fermentation broth (5 liters) was centrifuged to give a mycelial cake, which was extracted with 2.5 liters of acetone. The extract was concentrated to a small volume, and then extracted twice with 500 ml of ethyl acetate at pH 2.0. After evaporation, the residue was dissolved in chloroform and applied to a silica gel column (Wakogel C-200, 300 ml), which was eluted with chloroform-methanol stepwise to give two active fractions. One fraction eluted with chloroform-methanol (10:1) was concentrated to dryness and subjected to preparative TLC (Merck, Silica gel 60 F_{254}, layer thickness 0.5 mm). Development of the plates with chloroform-methanol-28% ammonia water (8:4:1) gave an active band, which was rechromatographed on silica gel TLC plates with chloroform-methanol (5:1). The active material thus

obtained was applied to a Sephadex LH-20 column (200 ml), and then eluted with chloroform - methanol (1 : 1). The active fraction was evaporated to dryness to yield a colorless powder of viranamycin A (2 mg).

The other fraction obtained by initial silica gel column chromatography with chloroform - methanol (5 : 1) was applied to a second silica gel column (100 ml), which was eluted with chloroform - methanol (10 : 1). After evaporation, the active eluate was chromatographed on preparative silica gel TLC plates with chloroform - methanol (10 : 1). The active material was subjected to Sephadex LH-20 column chromatography (200 ml). Development of the column with chloroform - methanol (1 : 1) gave an active fraction, which was concentrated to dryness to yield a colorless powder of viranamycin B (20 mg).

Physico-chemical Properties

The physico-chemical properties of the viranamycins are summarized in Table 1. The molecular formulae of viranamycins A and B were determined to be $C_{41}H_{64}O_{13}$ and $C_{44}H_{73}NO_{14}$ from HRFAB-MS, respectively. Their characteristic UV absorptions suggested that they contained the same chromophore as the 18-membered macrolide antibiotics such as virustomycin A[1] and concanamycin A[2,3]. The ^1H NMR spectra of viranamycins A and B are shown in Figs. 1 and 2, respectively. The ^1H NMR spectral analysis

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

	Viranamycin A	Viranamycin B
Nature	Colorless powder	Colorless powder
MP (°C)	89~90	108~109
$[\alpha]_D^{21}$	−12.6° (*c* 0.42, MeOH)	−25.2° (*c* 0.46, $CHCl_3$)
Formula	$C_{41}H_{64}O_{13}$	$C_{44}H_{73}NO_{14}$
FAB-MS ($(M+Na)^+$, *m/z*)		
Calcd:	787.4245	862.4929
Found:	787.4303	862.4920
UV λ_{max}^{MeOH} nm (ε)	241 (34,100), 284 (15,100)	245 (31,500), 284 (14,500)
IR ν_{max} cm^{-1} (KBr)	3430, 1710, 1690	3430, 1710, 1690

Fig. 1. ^1H NMR spectrum of viranamycin A in $CDCl_3$ at 22°C.

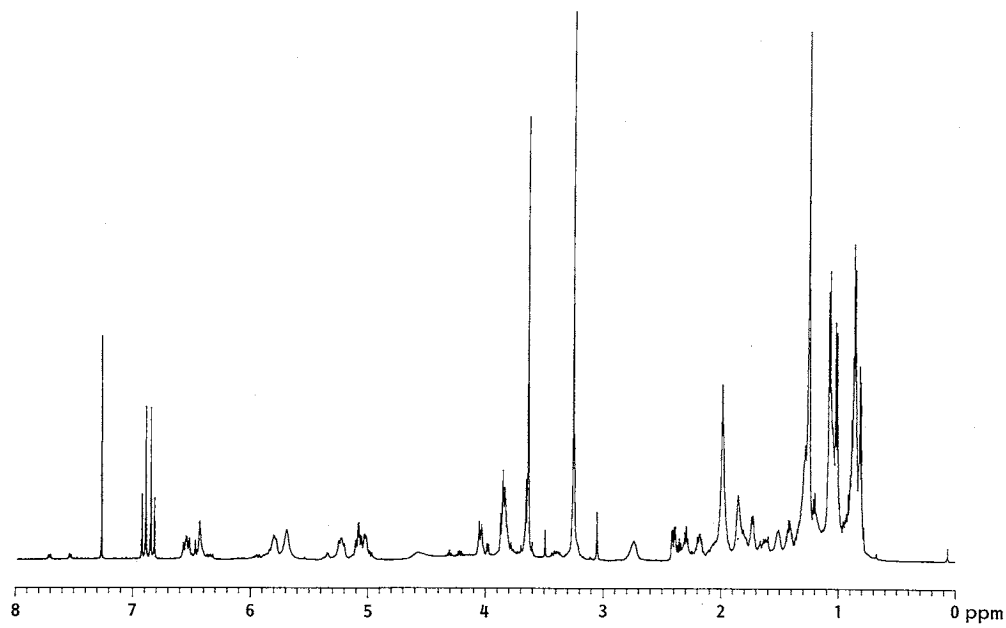


Fig. 2. ^1H NMR spectrum of viranamycin B in $CDCl_3$ at 22°C (bottom) and 60°C (top).

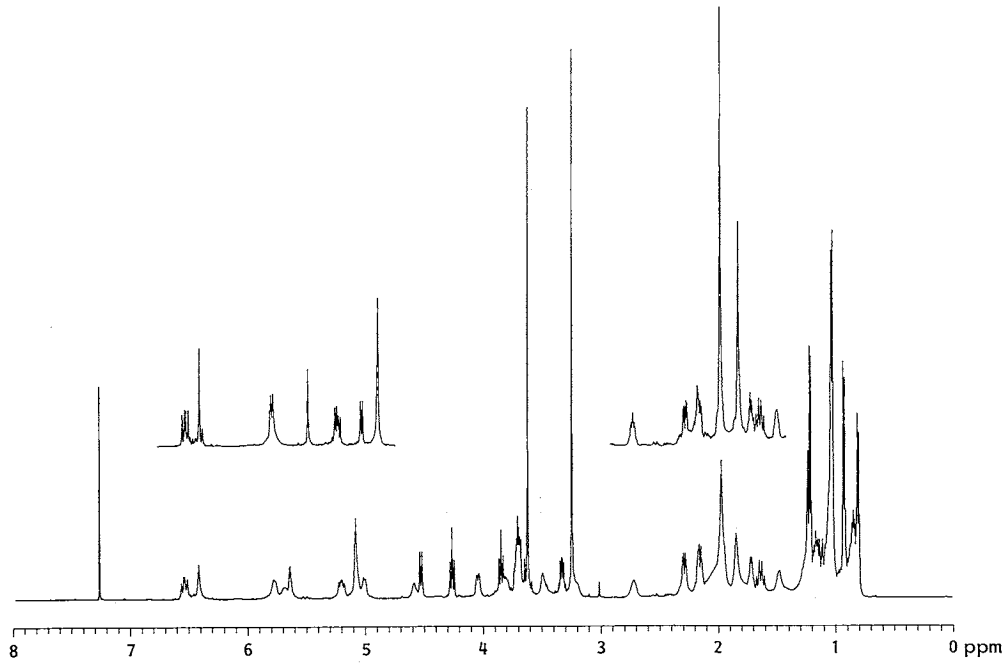


Table 2. ^{13}C and ^1H NMR data for viranamycins A and B in $CDCl_3$.

No.	Viranamycin A		Viranamycin B		No.	Viranamycin A		Viranamycin B	
	δ_C	δ_H	δ_C	δ_H		δ_C	δ_H	δ_C	δ_H
1	166.6		166.6		23	74.9	5.08	76.0	3.70
2	142.0		142.0		24	42.7	1.41	43.3	1.16
3	130.9	6.43	131.0	6.41	25	69.0	3.84	69.6	3.68
4	132.2		132.1		26	19.1	1.08	19.1	1.02
5	139.8	5.68	140.0	5.68	1′	164.1		96.6	4.52
6	34.8	2.74	34.7	2.71	2′	135.6[a]	6.90[a]	40.1	2.15,
7	74.5	3.83	74.3	3.79					1.63
8	43.6	1.51	43.6	1.47	3′	132.6[a]	6.83[a]	70.2	3.69
9	79.8	3.23	79.8	3.19	4′	168.2		79.8	4.25
10	36.4	2.05	36.3	2.29	5′			69.6	3.32
11	44.9	1.93	44.9	1.96	6′			17.6	1.21
12	142.0		142.0		4-CH_3	14.1	1.99	14.0	1.96
13	123.2	5.79	123.1	5.77	6-CH_3	16.8	1.07	16.8	1.02
14	133.1	6.54	133.2	6.53	8-$\underline{CH_2}CH_3$	23.0	1.17	22.8	1.15
15	127.4	5.22	127.2	5.20	8-$CH_2\underline{CH_3}$	11.6	0.87	11.6	0.84
16	81.4	3.84	81.4	3.84	10-CH_3	21.5	1.07	21.5	1.03
17	75.8	5.02	75.7	5.00	12-CH_3	16.4	1.85	16.3	1.84
18	37.0	2.18	36.9	2.16	18-CH_3	9.4	0.81	9.3	0.80
19	70.3	4.04	70.2	4.03	20-CH_3	7.0	1.02	7.0	1.02
20	41.5	1.73	41.5	1.71	24-CH_3	13.1	0.86	13.1	0.92
21	99.3		99.3		2-OCH_3	59.4	3.63	59.3	3.61
22	39.8	2.40,	40.0	2.28,	16-OCH_3	55.7	3.25	55.6	3.24
		1.25		1.11	4′-$OCONH_2$			157.6	

[a] Interchangeable.

Fig. 3. Proton spin systems derived from a COSY experiment for viranamycin B.

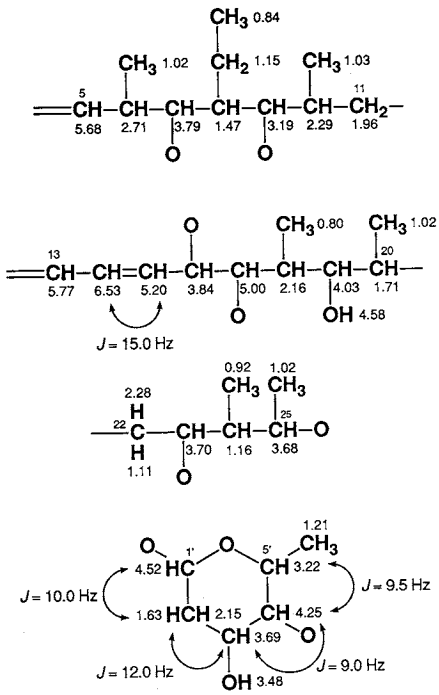
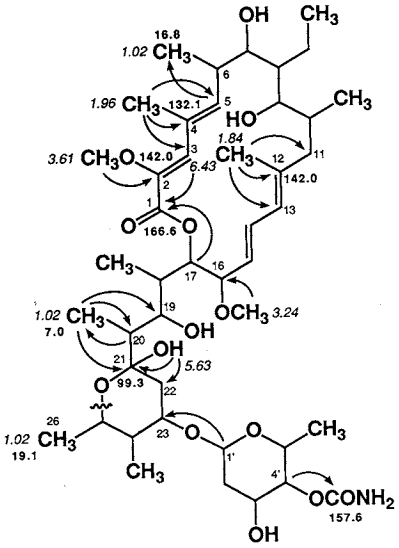


Fig. 4. HMBC data summary for viranamycin B.



for the viranamycins at room temperature was hampered by several broad signals, which were sharpened to aid the structural elucidation by measurement at 60°C as shown in Fig. 2.

Structural Elucidation

The ^{13}C and ^{1}H NMR data for the viranamycins are summarized in Table 2. All one-bond ^{1}H-^{13}C correlations were established by heteronuclear multiple-quantum coherency (HMQC)[4] experiments.

The COSY spectrum of viranamycin B indicated the presence of four separate spin systems as shown in Fig. 3. The partial structure representing C-1′ to C-6′ was required to be in a six-membered ring with all equatorial substituents by virtue of the large vicinal coupling constants between 1′-H, 2′-H_{ax}, 3′-H and 4′-H, forming *β*-2,6-dideoxy-*arabino*-hexapyranoside.

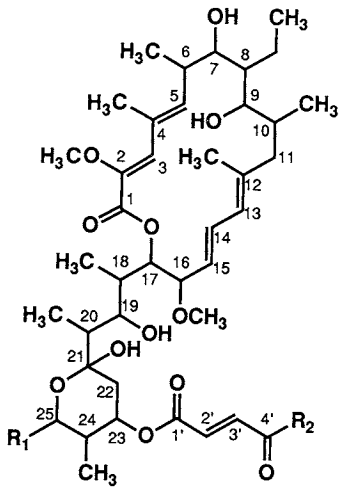
The heteronuclear multiple-bond correlation (HMBC)[5] spectrum revealed ^{1}H-^{13}C long-range couplings from an allylic methyl (δ 1.84, 12-CH_3) to C-11, C-12 and C-13, from a secondary methyl (20-CH_3) to C-19, C-20 and C-21, from a hydroxyl (δ 5.63, 21-OH) to C-21 and C-22, and from an anomeric proton (1′-H) to C-23, indicating that these partial structures were connected as shown in Fig. 4 except for the ether linkage with the hemiketal carbon (C-21).

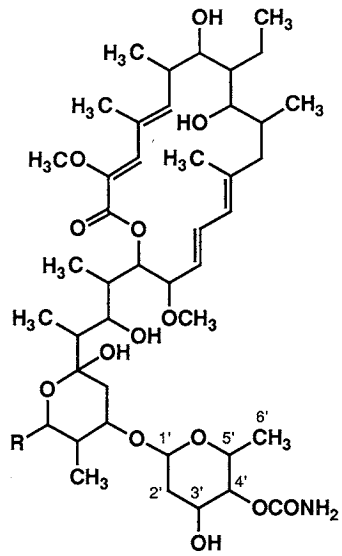
^{1}H-^{13}C long-range correlations from an allylic methyl (δ 1.96, 4-CH_3) to three olefinic carbons (C-3, C-4 and C-5), and from 3-H (δ 6.43) to an ester carbonyl (δ 166.6, C-1) suggested that these units formed a conjugated diene-carbonyl system causing the characteristic UV absorption of viranamycin B.

The ester linkage of C-1 with C-17 was established by a long-range coupling between 17-H and C-1. Two methoxy groups (δ 3.61 and 3.24) were elucidated to be located on C-2 and C-16 by their three-bond ^{1}H-^{13}C correlations.

The remaining carbon (δ 157.6) was assignable to a carbamoyl group based on its chemical shift and the presence of a nitrogen atom in viranamycin B. This residue was determined to connect to the C-4′ oxygen from a long-range coupling between the carbamoyl carbon and an oxymethine proton (4′-H).

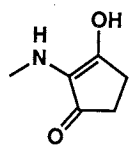
Fig. 5. Structures of viranamycins A and B, virustomycin A, and concanamycin A.





Viranamycin A $R_1=CH_3$ $R_2=OH$

Virustomycin A $R_1=CH=CH-CH_3$ $R_2=$



Viranamycin B $R=CH_3$

Concanamycin A $R=CH=CH-CH_3$

The geometrical configuration at C-14 was established to be *E* by $J_{14-15}=15.0$ Hz. These data and the structural similarity to concanamycin A indicated the planar structure of viranamycin B as shown in Fig. 5.

Three secondary methyls (C-26, 6-CH_3, 20-CH_3) with the same proton chemical shifts (δ 1.02) could be distinguished by using ^1H-^{13}C long-range correlations from 5-H to 6-CH_3 and from 20-H to 20-CH_3 to complete ^{13}C NMR assignments for viranamycin B.

The ^{13}C NMR data for the aglycone part of viranamycin A were almost identical with those of viranamycin B except for slight upfield shifts for C-23, C-24 and C-25 as shown in Table 2. In the ^1H and ^{13}C NMR spectra of viranamycin A, however, the signals for the sugar moiety of viranamycin B were replaced by signals due to two ester and/or carboxylic acid carbonyls (δ 168.2 and 164.1) and *trans* olefinic methines (δ_H 6.90, δ_C 135.6; δ_H 6.83, δ_C 132.6; $J=16.0$ Hz), thereby indicating that viranamycin A contained a fumaric acid moiety in place of the sugar residue. The chemical shift difference of 23-H (δ 5.08 and 3.70) between viranamycins A and B revealed that the fumaric acid residue was connected to the C-23 oxygen with an ester linkage.

The protons on C-22 to C-25 showed large vicinal coupling constants ($J_{22ax-23}=11.0$ Hz, $J_{23-24}=11.0$ Hz and $J_{24-25}=10.0$ Hz) and were required to be in a six-membered ring with all equatorial substituents, thereby showing that the remaining ether linkage was formed between C-21 and C-25.

The structures of viranamycins A and B thus established are shown in Fig. 5. The viranamycins appeared to be 25-depropenyl-25-methyl dlerivatives of virustomycin A or concanamycin A.

Biological Activities

Viranamycins A and B inhibited the growth of P388 mouse leukemia cells at very low concentrations. The IC_{50} values were 5.8 ng/ml and 2.8 ng/ml, respectively. Viranamycins A and B were also cytotoxic to KB human squamous-cell-carcinoma cells at the IC_{50}s of 6.4 ng/ml and 1.9 ng/ml, respectively.

Further biological evaluations are in progress.

Experimental

General

Specific rotations were obtained on a Jasco DIP-140 spectropolarimeter at 589.6 nm and 21°C. Mass spectra were measured on a Jeol HX-110 spectrometer in the FAB mode using *m*-nitrobenzyl alcohol-sodium iodide matrix. UV spectra were recorded on a Shimadzu UV-160 spectrophotometer. NMR spectra in $CDCl_3$ were obtained on a Jeol JNM-GSX500 spectrometer with ^1H NMR at 500 MHz and ^{13}C NMR at 125 MHz. Chemical shifts are given in ppm using TMS as internal standard.

Microorganism

A culture designated CH41 was isolated from a soil sample collected at Fuji-yoshida, Yamanashi Prefecture. The culture contained LL-diaminopimelic acid and could be classified as belonging to the genus *Streptomyces*. Further taxonomic studies are in progress.

Cells and Cell Culture

P388 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and 10 μM 2-mercaptoethanol. The cells at 2×10^4 cells/ml were incubated with various concentrations of testing agents for 2 days. KB cells were cultured in EAGLE's minimum essential medium supplemented with 10% heat-inactivated fetal calf serum and 1% Bacto-peptone. The cells at 8×10^4 cells/ml were incubated with various concentrations of samples for 2 days. Both of the cells were cultured in humidified atmosphere of 5% CO_2 in air at 37°C.

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