VICENISTATIN, A NOVEL 20-MEMBERED MACROCYCLIC LACTAM ANTITUMOR ANTIBIOTIC

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A new antitumor antibiotic vicenistatin was isolated from the culture broth of *Streptomyces* sp. HC34. The structure of vicenistatin was elucidated by NMR spectral analysis. Vicenistatin exhibited antitumor activity against human colon carcinoma Co-3 in the xenograft model.

In the couse of screening for new antitumor antibiotics, *Streptomyces* sp. HC34 was found to produce a novel antitumor antibiotic vicenistatin. Vicenistatin was recovered from the mycelium cake with acetone extraction and purified by chromatography. Structural studies revealed it to possess a novel 20-membered macrocyclic lactam ring and a novel amino sugar.

Vicenistatin showed growth inhibition against various tumor cells *in vitro*, and also showed antitumor activity against human colon carcinoma Co-3 in the xenograft model.

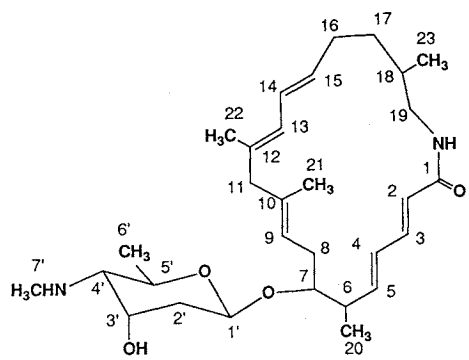
In this paper, we describe the production, isolation, structural elucidation and biological properties of vicenistatin.

Taxonomy of the Producing Strain

Culture HC34 was isolated from a soil sample collected at Kiryu, Gunma Prefecture, Japan. Characterization of the strain was carried out mainly by the methods described by SHIRLING and GOTTLIEB[1].

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 cylindrical or oval (0.5~0.7×0.7~1.6 μm) with smooth surfaces. The cultural and physiological properties of strain HC34 grown on various media at 27°C are shown in Tables 1 and 2, respectively. The whole-cell hydrolysate contained the L,L isomer of diaminopimelic acid which corresponds to cell-wall type 1. Based on these morphological and chemotaxonomic characteristics, it was concluded that the strain belongs to the genus *Streptomyces*.

Fig. 1. The total structure of vicenistatin.



Fermentation

A well grown agar slant of *Streptomyces* sp. HC34 was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of fermentation medium consisting of potato starch 3%, soya flake 1.5%, yeast extract 0.2%, corn steep liquor 0.5%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CoCl_2 \cdot 6H_2O$ 0.0005% and $CaCO_3$ 0.3%, the pH being adjusted

Table 1. Cultural characteristics of strain HC34.

Sucrose - nitrate agar	G:	Moderate	Tyrosine agar	G:	Poor
	R:	Greenish white		R:	Dark yellowish brown
	Am:	Moderate; bluish white		Am:	Poor; dark yellowish gray
	Sp:	None		Sp:	Pale red
Glucose - asparagine agar	G:	Good	Nutrient agar	G:	Moderate
	R:	Grayish yellow green		R:	Yellowish white
	Am:	Good; grayish		Am:	Poor; yellowish white
	Sp:	None		Sp:	None
Glycerol - asparagine agar	G:	Poor	Yeast extract - malt extract agar	G:	Good
	R:	Pinkish white		R:	Dark red
	Am:	Poor; pinkish white		Am:	Good; light greenish gray
	Sp:	None		Sp:	Pale red
Inorganic salts - starch agar	G:	Good	Oatmeal agar	G:	Good
	R:	Dull red purple		R:	Red purple
	Am:	Good; light olive gray		Am:	Good; light greenish gray
	Sp:	Pale red		Sp:	Pale red

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

Table 2. Physiological properties of strain HC34.

Temperature for growth	8~30°C
Production of melanoid pigments:	
Tyrosine agar	Negative
Peptone - yeast extract - iron agar	Negative
Tryptone - yeast extract agar	Negative
Hydrolysis of starch	Negative
Liquefaction of gelatin	Negative
Peptonization of milk	Negative
Coagulation of milk	Negative
Utilization of carbon source:	
Utilized	L-arabinose, D-xylose, D-glucose, D-fructose, sucrose, inositol, raffinose, D-mannitol, galactose, D-mannose, maltose
Not utilized	L-rhamnose, sorbitol

Table 3. Physico-chemical properties of vicenistatin.

Appearance	Colorless powder
MP	151~153°C (dec)
$[\alpha]_D^{22}$	−3° (*c* 0.1, MeOH)
Molecular formula	$C_{30}H_{48}O_4N_2$
HRFAB-MS Calcd:	501.3692
Found:	501.3697 $(M+H)^+$
UV λ_{max} (ε) (in MeOH)	235 (35,000), 240 (35,800), 268 (13,700)
IR ν (KBr) cm^{-1}	3400, 3300, 2920, 1655, 1625, 1540, 990

to 7.1 before sterilization. The fermentation was carried out at 27°C for 3 days with shaking on a rotary shaker. A 600 ml portion of the culture was inoculated into 50-liter jar fermenter containing 30 liters of a production medium having the same composition as the flask fermentation medium. The fermentation was run at 27°C for 5 days with agitation at 400 rpm and aeration rate of 30 liters per minute.

Isolation and Purification

The fermentation broth (60 liters) was centrifuged to give a mycelium cake. The mycelium cake was extracted with acetone (20 liters). The extract was filtered and concentrated *in vacuo* to an aqueous solution. The solution was extracted twice with 5 liters of ethyl acetate at pH 10. After evaporation, the residue was applied to a silica gel column (Wakogel, C-200, 12×30 cm) which was developed with chloroform - methanol (10 : 1). The active eluate was concentrated to dryness and then subjected to Sephadex LH-20 chromatography (3.5×50 cm) with chloroform - methanol (1 : 1). The active fractions were concentrated to dryness to give a colorless powder of vicenistatin (950 mg).

Structural Elucidation

The physico-chemical properties of vicenistatin (1) were summarized in Table 3. The molecular formula

Fig. 2. 500 MHz ^{1}H NMR spectrum of vicenistatin in pyridine-d_5.

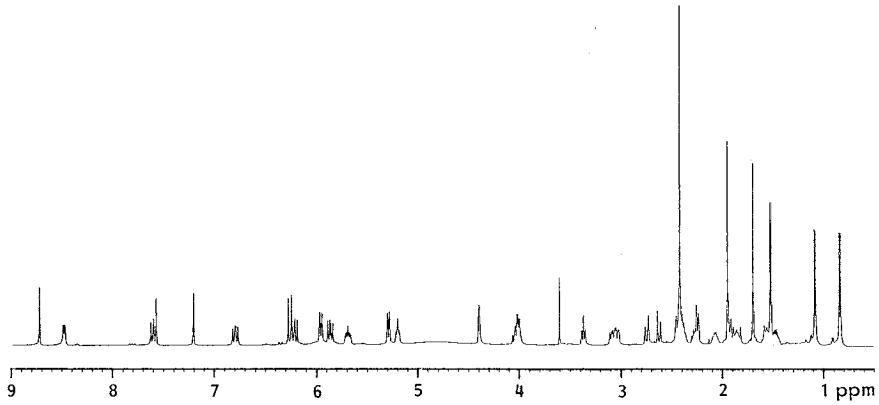


Table 4. 125 MHz ^{13}C NMR and 500 MHz ^{1}H NMR spectral data of vicenistatin[a].

Position	δ_C	δ_H	Position	δ_C	δ_H
1	166.4 s		17	33.0 t	1.48[c], 1.57[c]
2	124.7 d	6.26 (d, 15.0[b])	18	33.6 d	1.86[c]
3	140.2 d	7.59 (dd, 11.5, 15.0)	19	43.5 t	3.03 (ddd, 3.0, 5.0, 13.5),
4	128.4 d	6.20 (dd, 11.5, 15.5)			4.00 (m)
5	143.2 d	5.86 (dd, 10.0, 15.5)	19-NH		8.47 (br d, 6.2)
6	46.1 d	2.40[c]	20	18.7 q	1.08 (d, 6.5)
7	85.9 d	3.36 (ddd, 2.8, 9.0, 9.0)	21	18.0 q	1.68 (s)
8	36.5 t	2.27[c], 3.08 (ddd, 2.8, 7.5, 9.0)	22	17.3 q	1.94 (s)
9	122.3 d	5.20 (dd, 7.5, 7.5)	23	17.8 q	0.84 (d, 6.5)
10	134.8 s		1′	100.7 d	5.29 (dd, 3.0, 9.5)
11	49.5 t	2.62 (d, 15.0), 2.74 (d, 15.0)	2′	39.5 t	1.90 (ddd, 2.8, 9.5, 14.5), 2.43[c]
12	134.1 s		3′	63.4 d	4.39 (ddd, 2.8, 3.0, 4.2)
13	127.9 d	5.95 (d, 11.5)	4′	65.3 d	2.24 (dd, 3.0, 9.8)
14	128.4 d	6.79 (dd, 11.5, 15.0)	5′	71.5 d	4.02 (dq, 6.5, 9.8)
15	132.6 d	5.68 (ddd, 5.8, 6.0, 15.0)	6′	19.6 q	1.52 (d, 6.5)
16	27.9 t	2.07 (m), 2.39[c]	7′	34.0 q	2.42 (s)

[a] Taken in pyridin-d_5.
[b] Coupling constants in J=Hz.
[c] Resonance in one-dimentional spectra obscured by overlapping signals.

of **1** was established as $C_{30}H_{48}O_4N_2$ on the basis of HRFAB-MS data. In the IR spectrum, **1** showed strong bands at 3400 (OH and/or NH) and 1655 cm^{-1} (amide). The ^{1}H NMR spectrum taken in pyridine-d_5 (Fig. 2) revealed the presence of six methyl resonances (δ 0.84, 1.08 and 1.52, 1.68, 1.94 and 2.42), eight olefinic methine protons (δ 5.20, 5.68, 5.86, 5.95, 6.20, 6.26, 6.79 and 7.59) and one amide proton (δ 8.47, br d) in addition to 19 other methylene or methine protons at around δ 1.4~5.3. The ^{13}C NMR spectrum demonstrated 30 signals which were assigned to six methyls, six methylenes, fifteen methines and three quaternary carbons by DEPT experiment. The ^{1}H and ^{13}C NMR spectral data for **1** are summarized in Table 4.

Detailed analysis of the ^{1}H-^{1}H COSY and the decoupling experiments proved the partial structures A, B and C as shown in Fig. 3. And the remaining carbons of **1** were two singlet methyls (C-21, δ 18.0 and C-22, δ 17.3), one NCH_3 (C-7′, δ 34.0), one isolated methylene (C-11, δ 49.5) and one amide carbonyl carbon (C-1, δ 166.4). Further structural elucidation was performed by the observations of the long range

Fig. 3. Partial structures of vicenistatin.

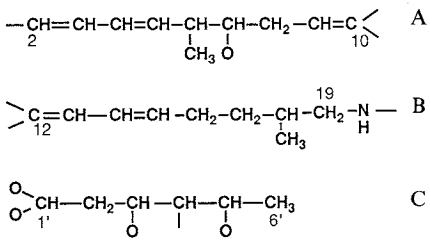
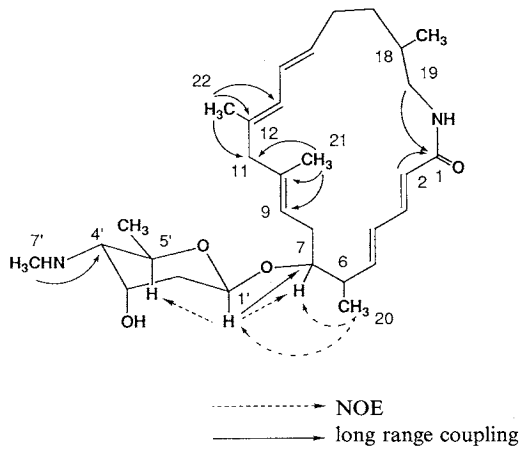


Fig. 4. ^{1}H-^{13}C long range couplings and NOEs.

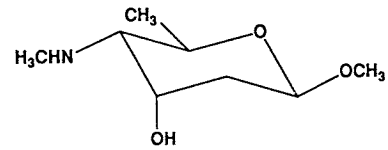


^{1}H-^{13}C connectivities which were detected by heteronuclear multiple-bond correlation (HMBC)[2] experiment and NOE effects.

The HMBC experiment on **1** showed the long range couplings of 21-CH_3 to C-9 (δ 122.3), C-10 (δ 134.8) and C-11 (isolated methylene) and 22-CH_3 to C-11, C-12 (δ 134.1) and C-13 (δ 127.9). Furthermore, the HMBC experiment also showed the long range couplings of 2-H (CH) and 19-H (CH_2) to C-1 (amide carbonyl carbon). These correlations confirmed the linkages of the partial structures A and B through C-11 and C-1, respectively. And the presence of 20-membered macrocyclic lactam moiety in **1** was established (Fig. 4).

In the partial structure C, NOE was observed between 1′-H and 5′-H (Fig. 4). Therefore, the partial structure C was confirmed to constitute a hexopyranose. And the long range coupling of 1′-H (anomeric proton) to C-7 (δ 85.9) in the HMBC experiment showed the linkage of this hexopyranose to the macrocyclic lactam ring at C-7. A NCH_3 group located at C-4′ (δ 65.3) was also proved by the observation of long range coupling in the HMBC experiment (Fig. 4). The relative stereochemistry for this amino sugar was determined by the coupling constants. Large coupling constants of $J_{1',2'ax}$ = 9.5 Hz and $J_{4',5'}$ = 9.8 Hz indicated that 1′-H, 4′-H and 5′-H were axially oriented. 3′-H was determined to be the equatorial orientation because of its small coupling constants ($J_{2'ax,3'}$ = 2.8 Hz, $J_{2'eq,3'}$ = 4.2 Hz and $J_{3',4'}$ = 3.0 Hz). Methanolysis of **1** yielded a methyl β-glycoside (**2**). The absolute configuration of **2** was determined by TACu method[3]. The negative contribution ($\Delta[M]_{435(TACu)}$ −872°) of **2** suggested that this sugar belonged to the D-series (Table 5). Therefore, the structure of this amino sugar was established to be 4-methylamino-2,4,6-trideoxy-β-D-ribohexopyranose. As far as we know, this amino sugar was found for the first time and the name vicenisamine was given. From all these findings, the total structure of **1**

Table 5. Physico-chemical properties of **2** (methyl β-D-vicenisaminide)[a].



	δ_C	δ_H in CD_3OD
1	100.4	4.75 (dd, 2.5, 9.5)[b]
2	38.8	1.67 (ddd, 2.8, 9.5, 14.5), 2.03 (ddd, 2.5, 4.2, 14.5)
3	62.4	4.35 (ddd, 2.8, 3.0, 4.2)
4	63.3	2.91 (dd, 2.8, 9.0)
5	68.0	4.01 (dq, 6.5, 9.0)
6	18.7	1.36 (d, 6.5)
4-NCH_3	31.3	2.74 (s)
1-OCH_3	56.7	3.44 (s)

[a] MP 183~185°C (dec), MW 175 [FD-MS, *m/z* 175 (M^+)], $[\alpha]_D^{24}$ (*c* 0.4, H_2O) −47°, $[\alpha]_{435}^{24}$ (*c* 0.04, H_2O) −5°, $[\alpha]_{435}^{24}$ (*c* 0.04, tetraamminecopper (II) sulfate) −503.5°, $\Delta[M]_{TACu}$ −872°.

[b] Multiplicity and coupling constant (Hz) are in parentheses.

was deduced as shown in Fig. 1.

The geometries of C-2, C-4 and C-14 were proved to be all *E* by the coupling constants of $J_{2,3}=15.0$ Hz, $J_{4,5}=15.5$ Hz and $J_{14,15}=15.0$ Hz, respectively. Upfield chemical shifts of C-21 (δ 18.0) and C-22 (δ 17.3) and no NOEs between 9-H and 21-H, and 13-H and 22-H showed *E* configurations for C-10 and C-12. The *cis* relation between 20-CH_3 and 7-H was established based on NOE networks as shown in Fig. 4 and a large coupling constant ($J_{6,7}=9.0$ Hz), but the stereochemistry at C-18 remains to be determined.

Some other macrocyclic lactam antibiotics are known such as hitachimycin[4], fluvirucins[5] and BE-14106[6]. Especially, BE-14106 also possess a 20-membered macrocyclic lactam ring. However, **1** is different from BE-14106 in the point that **1** contains a novel amino sugar (vicenisamine).

Table 6. Antitumor activity of vicenistatin against Co-3 human colon carcinoma.

Compound	Dose (mg/kg/day)	Treatment schedule	TGIR (%)[a]
Vecenistatin	8	Day 1~5	65
	2	Day 1~5	52
Mitomycin C	6.7	Day 1	67

Tumor fragment of Co-3 was implanted sc into 6~8-week-old female nude mice (BALB/c nu/nu Slc) and when tumor size reached 100~300 mm^3, vicenistatin or mitomycin was given iv. From the start of the treatment, tumor growth inhibition rate (TGIR) of each compound was calculated for 3 weeks as follows; TGIR = (1 − mean tumor volume of treatment group/mean tumor volume of control group) × 100.

[a] Maximum TGIR value through 3 weeks.

Biological Activity

1 was tested for its *in vitro* cytotoxicity. IC_{50} (μg/ml) values against HL-60 (human leukemia) and COLO205 (human colon carcinoma) were 0.12 and 0.19, respectively. Additionally, the antitumor activity of **1** was determined in Co-3 (human colon carcinoma) implanted nude mice[7]. The results are shown in Table 6. **1** exhibited antitumor activity against Co-3. The unique structural feature of **1** makes it an interesting lead compound for antitumor agents. Further biological studies are in progress.

Experimental

General

Specific rotation was obtained on a Jasco DIP-140 spectropolarimeter. Mass spectra were measured on a JEOL JMS-SX102A in the FAB mode using glycerol matrix. UV and IR spectra were recorded on a Hitachi U-3200 spectrophotometer and a Jasco A-3 spectrophotometer, respectively. NMR spectra were obtained on a JEOL JNM-GX500 spectrophotometer with ^1H NMR at 500 MHz and ^{13}C NMR at 125 MHz. Chemical shifts are given in ppm using TMS as internal standard.

Methanolysis of 1

A solution of 50 mg of **1** in 10 ml of 5% HCl-MeOH was boiled under reflux for 40 minutes. The solution was concentrated, and the residue was chromatographed on a silica gel using chroloform-methanol (5:1). The eluate was concentrated to yield 9.2 mg of methyl β-D-vicenisaminide (**2**). Physico-chemical properties of **2**: see Table 5.

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