A NEW MACROCYCLIC LACTAM ANTIBIOTIC, BE-14106

I. TAXONOMY, ISOLATION, BIOLOGICAL ACTIVITY AND STRUCTURAL ELUCIDATION

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A *Streptomyces* strain labeled A14106 was isolated and found to produce a novel 20-membered macrocyclic lactam derivative, BE-14106, which exhibited both cytotoxic activity against murine tumor cell lines and antimicrobial activity.

In the course of our screening program for new antitumor substances, a strain labeled A14106 isolated from a soil sample collected in Shiroyama-cho, Mie Prefecture, Japan, was found to produce an active principle. This strain was classified as *Streptomyces spheroides*. The active principle was extracted from the mycelium of the strain with methanol and was purified by silica gel column chromatography. BE-14106 inhibited the growth of a vincristine-resistant P388 murine leukemia cell line as well as its parent sensitive cell line. BE-14106 also displayed antimicrobial activity. This paper describes the taxonomy of the producing organism and the isolation, physico-chemical properties, biological activity and structure elucidation studies of BE-14106. The structure of BE-14106 is shown in Fig. 1.

Materials and Methods

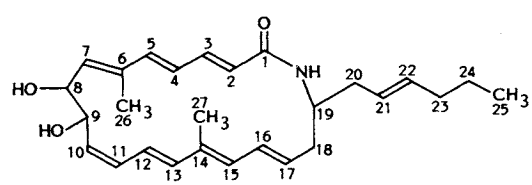
Taxonomic Study

Characterization of the producing strain followed the method of the International Streptomyces Project (ISP)[1], and several other tests were also used. Morphological observations were made with light and scanning electron microscopes. Cell wall analysis was performed by the method of BECKER *et al.*[2] and YAMAGUCHI[3]. Utilization of carbon sources was examined according to the method of PRIDHAM and GOTTLIEB[4].

Fermentation

Spores of strain A14106 grown on agar slant medium were inoculated into several 500-ml conical flasks, each containing 100 ml of a medium (pH 6.7) comprising glucose 0.1%, dextrin 2.0%, corn gluten meal 1.0%, fish meal 0.5%, yeast extract 0.1%, sodium chloride 0.1%, magnesium sulfate 0.05%, calcium chloride 0.05%, ferrous sulfate 0.0002%, cupric chloride 0.00004%, manganese chloride 0.00004%, cobalt chloride 0.00004%, zinc sulfate 0.00008%, sodium borate 0.00008%, ammonium molybdate 0.00024% and 3-(*N*-morpholino)propanesulfonic acid 0.5% and were cultured on a rotary shaker (180 rpm) at 28°C for 144 hours.

Fig. 1. Structure of BE-14106.



Assay of Cytotoxic Activity

In the *in vitro* cytotoxic assay using the P388 tumor cell lines P388/S and P388/VCR, BE-14106 was first dissolved in dimethyl sulfoxide (DMSO). The solution was serially diluted with a cell culture medium containing 20% DMSO (20% DMSO-RPMI-1640 medium) with 2.5×10^4 tumor cells and the mixture was incubated under 5% CO_2 at 37°C for 72 hours. The viable cells were then counted with a Coulter counter. The P388/S cell line is one of the mouse leukemia cell lines sensitive to antitumor agents, and the P388/VCR cell line is a subline of P388 leukemia that has acquired resistance to the antitumor agent vincristine[5].

Instrumental Analyses

IR and UV spectra were recorded on a Hitachi 270-30 spectrometer and a Shimadzu UV-265FW spectrometer, respectively. Mass spectrometry was performed on a Jeol JMX-DX300 spectrometer. ^1H and ^{13}C NMR spectra were obtained using a Varian VXR300 spectrometer at 300 MHz and 75 MHz, respectively. Chemical shifts were converted to values in ppm downfield from TMS as an internal standard.

Results

Taxonomy of Strain A14106

The substrate mycelia of strain A14106 were well-developed and branched without fragmentation. The aerial mycelia branched monopodially and formed long spiral spore chains with more than 50 spores per chain. The spores, which were oblong in shape, were $0.4 \sim 0.5 \times 0.7 \sim 0.9$ μm in size and had a smooth surface. Sporangia, sclerotia and zoospores were not observed. The cultural characteristics of strain A14106 are summarized in Table 1. The aerial mass color was pale yellow to yellowish-white (yellow-color series). The reverse side of the growth was yellowish-brown to yellowish-white. Melanoid and other soluble pigments were not observed. The physiological properties and carbon source utilization of strain A14106 are summarized in Tables 2 and 3. Analysis of whole cell hydrolysates showed the presence of L,L-diaminopimelic acid. Accordingly, the cell wall of this strain is classified as type I.

Based on the above-mentioned taxonomic properties, strain A14106 is considered to belong to the genus *Streptomyces*. Accordingly, a comparison of this strain was made with published descriptions of

Table 1. Cultural characteristics of strain A14106.

Agar medium	Growth	Aerial mycerium	Reverse	Soluble pigment
Yeast extract - malt extract agar (ISP-2)	Good	Abundant Pale yellow	Light yellowish-brown	None
Oatmeal agar (ISP-3)	Good	Abundant Pale yellow	Light yellow	None
Inorganic salts - starch agar (ISP-4)	Good	Abundant Pale yellow	Dull yellow	None
Glycerol - asparagine agar (ISP-5)	Moderate	Moderate Yellowish-white	Pale yellow	None
Peptone - yeast extract - iron agar (ISP-6)	Poor	None	Colorless	None
Tyrosine agar (ISP-7)	Moderate	Moderate Yellowish-white	Pale yellow	None
Nutrient agar	Moderate	Moderate Yellowish-white	Yellowish-white	None
Sucrose - nitrate agar	Moderate	Moderate Pale yellow	Yellowish-white	None
Glucose - asparagine agar	Moderate	Moderate Yellowish-white	Yellowish-white	None

various *Streptomyces* species. Since strain A14106 was thought to closely resemble *Streptomyces spheroides*[6], this strain was identified as a strain of *S. spheroides* and was designated as *S. spheroides* A14106. The results of the comparison of strain A14106 and *S. spheroides* are summarized in Table 3. The strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the access No. FERM-P11378.

Isolation Procedure

The mycelium was obtained by filtration from the whole broth (*ca.* 6 liters) and washed with 2 liters of water. This mycelium was extracted with 2 liters of methanol, and the extract was concentrated under reduced pressure to yield 8.06 g of a crude substance containing BE-14106. This crude substance was chromatographed on a column of silica gel (3 × 35 cm) and developed with chloroform - methanol (50 : 1). Fractions containing BE-14106 were collected and concentrated *in vacuo*. The resulting precipitate was triturated with 10 ml of methanol and filtered to afford BE-14106 (186 mg) as a colorless amorphous solid.

Physico-chemical Properties of BE-14106

BE-14106 was scarcely soluble in water, sparingly soluble in methanol and soluble in dimethyl sulfoxide. Other properties are as follows: Rf 0.35 (chloroform - methanol (10 : 1), Silica gel 60, Merck), no obvious mp up to 270°C, HRFAB-MS *m/z* 424.2868 ($(M+H)^+$, Calcd: 424.2852 for $C_{27}H_{38}O_3N$), UV λ_{max}^{MeOH} nm (ε) 230 (sh, 11,800), 278 (91,800), 288 (110,800), 310 (sh, 22,000), 325 (sh, 16,100). The IR and ^1H NMR spectra are shown in Figs. 2 and 3, respectively.

Biological Activity

In the cytotoxic assay, the concentration of BE-14106 required to inhibit growth of the P388/S and P388/VCR cell lines by 50% (IC_{50}) was 1.65 and

Table 2. Physiological properties of strain A14106.

Coagulation of milk	Negative
Peptonization of milk	Positive
Liquefaction of gelatin	Positive
Melanoid production	Negative
Hydrolysis of starch	Positive
NaCl tolerance	≤4%
Temperature range for growth	13~30°C

Table 3. Comparison of taxonomic characteristics of A14106 with those of *Streptomyces spheroides*.

	A14106	*S. spheroides*
Spore chain morphology	Spirals	Spirals
Spore number per chain	More than 50	Often more than 50
Spore surface	Smooth	Smooth
Aerial mass color	Yellow color-series	Yellow color-series
Color of reverse	Colorless to dull yellow	Colorless to light yellow
Soluble pigment	None	None
Melanoid formation	Negative	Negative
Carbon utilization:		
D-Glucose	Positive	Positive
D-Xylose	Positive	Positive
L-Arabinose	Positive	Positive
L-Rhamnose	Positive	Positive
D-Fructose	Positive	Positive
Raffinose	Negative	Negative
D-Mannitol	Positive	Positive
i-Inositol	Negative	Negative
Sucrose	Positive	Positive
D-Galactose	Positive	
Salicin	Positive	

Fig. 2. IR spectrum of BE-14106 in KBr.

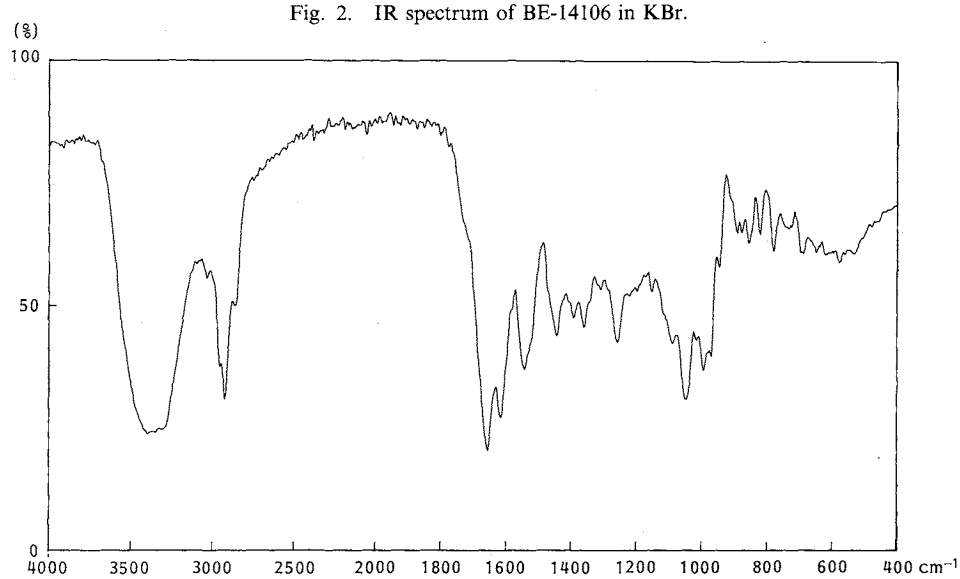
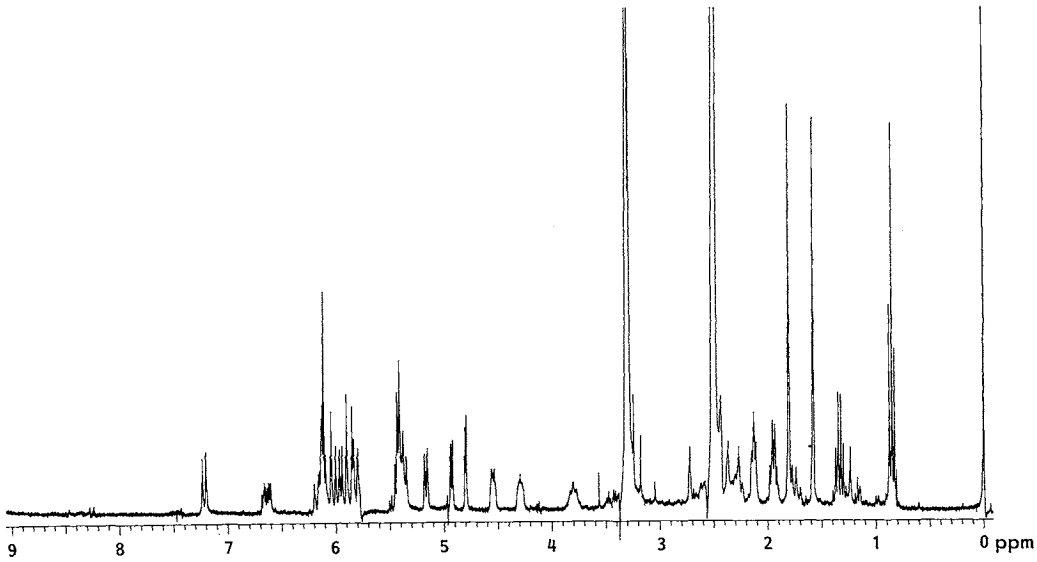


Fig. 3. ^{1}H NMR spectrum of BE-14106 in DMSO-d_6 (300 MHz).



1.89 μM, respectively. The antimicrobial activity of BE-14106 is shown in Table 4. With regard to the acute toxicity of BE-14106 in female ICR mice, no deaths were observed on the 5th day after 100 mg/kg was intraperitoneally administered.

Structural Elucidation

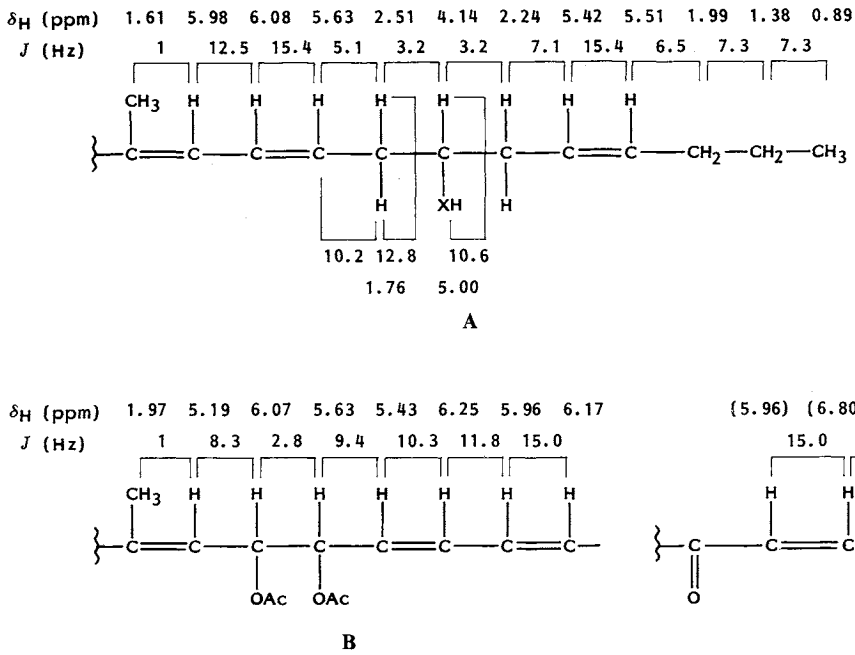
The molecular formula of BE-14106 (**1**) was established as $C_{27}H_{37}O_3N$ from the results of the HRFAB-MS and NMR spectral analyses. In accordance with the UV spectrum, the ^{1}H NMR spectrum of **1** in DMSO-d_6 displayed fourteen olefinic protons in the range between 5.1 and 6.7 ppm. Besides two olefinic methyl groups at 1.6 and 1.8 ppm, *n*-propyl, $-CH_2-CH(XH)-CH_2-$ and $-CH(OH)-CH(OH)-$

Table 4. Antimicrobial activity of BE-14106.

Test organism	MIC (μg/ml)	Test organism	MIC (μg/ml)
Bacillus subtilis ATCC 6633	3.13	*Pseudomonas aeruginosa* IFO 3445	100
B. cereus IFO 3001	3.13	*Flavobacterium meningosepticum* IFO 12535	100
Staphylococcus aureus FDA 209P	6.25		
S. aureus Smith	3.13	*Wicherhamia fluorescens* IFO 1116	12.5
Streptococcus thermophilus IFO 3535	3.13	*Saccharomyces cerevisiae* IFO 0283	6.25
Escherichia coli NIHJ JC-2	>100	*Schizosaccharomyces pombe* IAM 4863	3.13
Klebsiella pneumoniae ATCC 10031	>100	*Candida albicans* IFO 1270	25
Enterobacter cloacae IFO 13535	>100	*Endomyces ovetensis* IFO 1201	100

Fig. 4. Partial structures **A**, **B** and **C** for **2**.

Chemical shifts are indicated as ppm from TMS in $CDCl_3$, and the values in parenthesis are those in CD_3OD.



moieties were deduced from the ^1H-^1H COSY spectrum and homonuclear decoupling experiments. The ^{13}C NMR spectrum in DMSO-d_6 revealed the presence of twenty-six carbons at 12.0 (q), 12.4 (q), 13.4 (q), 22.1 (t), 34.1 (t), 38.0 (t), 49.3 (d), 69.6 (d), 72.1 (d), 123.7 (d), 123.8 (d), 124.8 (d), 127.0 (d), 128.4 (d), 129.8 (d), 130.2 (d), 130.5 (d), 131.1 (d), 131.4 (s), 132.8 (s), 136.1 (d), 138.5 (d), 139.4 (d), 142.4 (d) and 166.4 (s) ppm; one carbon overlapped in the solvent peak.

Acetylation of **1** with acetic anhydride and pyridine afforded a diacetyl derivative of **1** (**2**, FAB-MS *m/z* 508 $(M+H)^+$), which exhibited improved solubility in chloroform and methanol. Analyses of the ^1H-^1H COSY spectra of **2** in $CDCl_3$ and CD_3OD, together with the decoupling experiments of **2**, revealed three partial structures **A**, **B** and **C** as shown in Fig. 4. The partial structure **C** was deduced from the decoupling experiments of **2** in CD_3OD, because two protons overlapped at 6.19 ppm in $CDCl_3$.

The ^1H and ^{13}C NMR data for **2** in $CDCl_3$ are shown in Table 5. The assignments of the carbon

Table 5. ^{1}H and ^{13}C NMR data for **2**.

	^{1}H NMR in CD_3OD	^{1}H NMR in $CDCl_3$	^{13}C NMR in $CDCl_3$
1	—	—	167.2
2	5.96 (d, 15.0)[a]	5.76 (d, 15.0)	122.8
3	6.80 (dd, 10.5, 15.0)	6.93 (ddd, 3.5, 7.2, 15.0)	141.6
4	6.33 (dd, 10.5, 15.3)	6.19[b]	125.6
5	6.21 (d, 15.3)	6.19[b]	143.0
6	—	—	135.4
7	5.22 (br d, 8.3)	5.19 (br d, 8.3)	131.3
8	6.09 (dd, 2.9, 8.3)	6.07 (dd, 2.8, 8.3)	71.4
9	5.65 (ddd, 0.9, 2.9, 9.4)	5.63 (dd, 2.8, 9.4)	70.7
10	5.42 (dd, 9.4, 10.4)	5.43 (dd, 9.4, 10.3)	122.4
11	6.27 (br dd, 10.4, 10.8)	6.25 (dd, 10.3, 11.8)	132.5
12	6.02 (dd, 10.8, 15.0)	5.96 (dd, 11.8, 15.0)	122.8
13	6.21 (d, 15.0)	6.17 (d, 15.0)	138.5
14	—	—	132.9
15	5.93 (br d, 10.9)	5.98 (br d, 12.5)	131.3
16	6.19 (dd, 10.9, 15.0)	6.08 (dd, 12.5, 15.4)	130.2
17	5.55 (m)	5.63 (ddd, 5.1, 10.2, 15.4)	130.9
18	1.82 (m)	1.76 (m)	41.2
	2.45 (m)	2.51 (ddd, 3.2, 5.1, 12.8)	
19	3.92 (m)	4.14 (m)	49.7
20	2.21 (m)	2.24 (m)	38.2
21	5.45 (m)	5.42 (dt, 7.1, 15.4)	125.3
22	5.51 (m)	5.51 (dt, 6.5, 15.4)	133.8
23	1.99 (m)	1.99 (dt, 6.5, 7.3)	34.6
24	1.38 (m)	1.38 (tq, 7.3, 7.3)	22.5
25	0.89 (t, 7.3)	0.89 (t, 7.3)	13.6
26	1.99 (br s)	1.97 (br s)	12.7
27	1.67 (br s)	1.61 (br s)	12.4
NH	—	5.00 (d, 10.6)	—
CH_3CO	2.03 (s)	2.07 (s)	21.0, 170.2
CH_3CO	2.09 (s)	2.11 (s)	21.0, 170.0

[a] Multiplicity, J in Hz.
[b] Overlapping signals.

signals was accomplished by the ^{1}H-^{13}C COSY and long-range ^{1}H-^{13}C COSY spectral analyses. The connectivity of partial structures **A** and **B** was confirmed by the observation of ^{1}H-^{13}C long-range couplings between 13-H (6.17 ppm) and C-27 (12.4 ppm) and also between 15-H (5.98 ppm) and C-13 (138.5 ppm). The carbonyl carbon at 167.2 ppm was assigned to be an amide carbon from the result of the ^{1}H-^{13}C long-range coupling of this carbon to the D_2O-exchangeable proton at 5.00 ppm and 2-H (5.76 ppm). These observations show the connectivity of the partial structures of **A** and **C**. The remaining connectivity of the partial structures **B** and **C** was confirmed from the observation of ^{1}H-^{13}C long-range coupling of 7-H (5.19 ppm) with C-5 (143.0 ppm).

The *trans*-configuration of the C-6 and C-7 double bond was confirmed by the NOE observed with 8-H on irradiation of 26-H. Also, the *trans*-configuration of C-14 and C-15 was confirmed by the NOE observed with 16-H on irradiation of 27-H.

Based on the above results, the structure of BE-14106 was determined to be the novel 20-membered macrocyclic lactam shown in Fig. 1.

Stubomycin (hitachimycin)[7] has a similar structure to BE-14106, but stubomycin is a 19-membered lactam and has a phenyl group.

Discussion

Diploid pairs of regulatory genes in adult cells are known to suppress multiple structural genes capable of coding for transforming factors that can release the cell from its normal growth constraints[8]. The deletion or double mutation of these tumor suppressor genes and the mutation of cellular oncogenes predisposes the cells to cancer[9]. Thus, it is understood that cancer is a genetic disorder[10]. So, in addition to the screening of anti-oncogene product substances[11] or substances that may be able to replace the function of suppressor gene products, the screening of cytotoxic substances and substances that indirectly suppress tumor growth[12] must be performed until we are able to repair to functionally replace the damaged parts of the genes in tumor cells. In this context, biological target-oriented screening, cellular level screening and *in vivo* level screening must be performed in concert to find new lead substances for antitumor agents, along with further studies of currently available clinically useful drugs[13~15]. In the present study, we found a new macrocyclic lactam antibiotic that had cytotoxic activity against P388/S and P388/VCR cell lines. BE-14106 also showed antimicrobial activity comparable to that of stubomycin[7], although its *in vivo* antitumor activity against murine experimental systems such as Ehrlich ascites tumor was not strong (data not shown). Chemical modification will be necessary to further evaluate this compound as an antitumor lead.

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