A NEW TOPOISOMERASE II INHIBITOR, BE-22179, PRODUCED BY A STREPTOMYCETE

I. PRODUCING STRAIN, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

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(Received for publication August 5, 1993)

A new topoisomerase II inhibitor, designated BE-22179, was isolated from the culture broth of *Streptomyces* sp. A22179, which resembles "*Streptomyces gangtokensis*". The inhibitor was extracted from the mycelial cake of the culture broth with organic solvent and successively purified by silica gel chromatography. BE-22179 inhibited topoisomerase II strongly but not topoisomerase I and showed potent antitumor activity against various tumor cell lines both *in vitro* and *in vivo*.

In the course of our screening program for new topoisomerase inhibitors, a strain, A22179, isolated from a soil sample collected in Gumma prefecture, Japan, was found to produce a potent topoisomerase II inhibitor. This compound, BE-22179, was isolated from the mycelial cake of culture broth and the compound had a unique structure including a cyclic octapeptide with two thioester bonds and two 3-hydroxyquinoline-2-carboxylic acid chromophores. BE-22179 inhibited topoisomerase II but not topoisomerase I, and it had a potent antitumor activity against various tumor cell lines. In this paper we describe taxonomy of the producing strain, fermentation, isolation, physico-chemical and biological properties of BE-22179.

Materials and Methods

Taxonomic Study

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

Assay of Topoisomerase Activity

Topoisomerases I and II were prepared from L1210 mouse leukemia cells by hydroxyapatite (Seikagaku Corporation, Tokyo, Japan), Mono Q and Mono S (Pharmacia LKB, Biotechnology, Uppsala, Sweden) chromatography, according to the method of DRAKE *et al.*[5]. Topoisomerase activity was measured by DNA relaxation assay, according to the method reported previously[6].

Assay of Cytotoxic Activity

L1210 or P388 mouse leukemia cell line was cultured with RPMI 1640 medium containing 10% fetal bovine serum. Additionally, 20 μM 2-mercaptoethanol was added for the culture of the P388 cell line. Each cell line (3×10^4 cells/ml) was cultured with test samples for 3 days, and then the cell number of L1210 and P388 was counted with a Coulter Counter. The medium used for the culture of the MKN-45 cell line, a human stomach adenocarcinoma, was DULBECCO's modified EAGLE's medium (DMEM) containing 10% fetal bovine serum. MKN-45 cell line (1×10^5 cells/ml) was pre-cultured for one day before addition of test samples. After 3 days-culture with samples, the number of MKN-45 cell was counted by the sulforhodamine B method[7].

Assay of Antitumor Activity *In Vivo*

L1210 leukemia cells (1×10^6) were implanted ip into CDF_1 female mice (five weeks old) on day 0 and BE-22179 was administered ip once daily for 10 consecutive days from day 1. The number of survivors was recorded everyday. The antitumor activity (T/C %) was calculated from the mean survival days of the test (T) and that of control (C) groups.

Instrumental Analyses

IR and UV spectra were recorded on a Hitachi 270-30 spectrometer and a Shimadzu UV-265 FW spectrometer, respectively. Mass spectrometry was performed on a JEOL JMX-DX300 spectrometer. ^{1}H NMR spectra were obtained using a Varian VXR 300 spectrometer at 300 MHz. Optical rotation was measured with a Horiba SEPA-200 high-sensitivity polarimeter.

Results

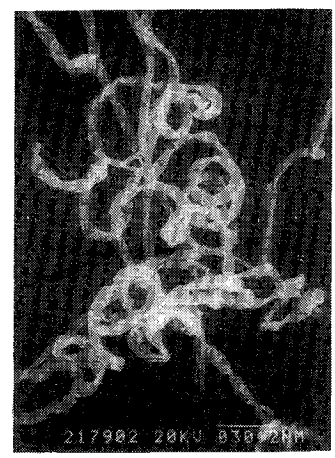
Taxonomy

The BE-22179-producing organism, strain A22179, was isolated from a soil sample collected at Mt. Myogi, Gumma prefecture, Japan.

Strain A22179 formed well-developed, branching substrate mycelia without fragmentation. The aerial mycelia branched monopodially and formed long spiral spore chains with hygroscopic disintegration. The spores, surfaces of which were smooth, had oblong shape and $1.0 \sim 0.5 \times 0.7 \sim 0.4$ μm in size (Fig. 1). Sporangia, sclerotia and zoospores were not observed.

Fig. 1. Scanning electron micrograph of spore chains of strain A22179 grown on ISP 4 medium at 28°C for 14 days.

Bar represents 3 μm.



The culture characteristics of strain A22179 are summarized in Table 1. The whole cell hydrolysate contained L,L-diaminopimelic acid. This suggests the cell wall of this strain belongs to type I. The physiological properties and carbon utilization of strain A22179 are shown in Table 2.

The above-mentioned characteristics of strain A22179 revealed that it belonged to the genus *Streptomyces*. According to the published description of *Streptomyces* species, the taxonomic features of strain A22179 resembled those of "*S. gangtokensis*"[8] except for coagulation of milk and several carbon utilization (Table 2). Therefore this strain was identified as a strain of "*S. gangtokensis*".

Table 1. Culture characteristics of strain A22179.

Agar medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP-2)	Good	Moderate Brownish gray Hygroscopic	Deep yellowish brown	None
Oatmeal agar (ISP-3)	Good	Moderate Dark grayish brown Hygroscopic	Deep brown	Grayish brown
Inorganic salts-starch agar (ISP-4)	Good	Moderate Brownish gray Hygroscopic	Dark grayish brown	None
Glycerol-asparagine agar (ISP-5)	Moderate	Moderate Light brownish gray Hygroscopic	Light brown	None
Peptone-yeast extract-iron agar (ISP-6)	Moderate	None	Grayish brown	Brownish black
Tyrosine agar (ISP-7)	Moderate	Poor Yellowish white	Light yellowish brown	None
Nutrient agar	Moderate	None	Yellowish pink	None
Sucrose-nitrate agar	Poor	None	Pinkish gray	None
Glucose-asparagine agar	Moderate	Trace	Yellowish white	None

Table 2. Comparison of taxonomic characteristics of strain A22179 with "*S. gangtokensis*".

	A22179	"*S. gangtokensis*"
Spore chain morphology	Spirals	Spirals
Spore surface	Smooth	Smooth
Aerial mass color	Gray color-series Hygroscopic	Gray color-series Hygroscopic
Melanoid formation		
Tryptone-yeast broth (ISP-1)	−	N.D.
Peptone-yeast extract-iron agar (ISP-6)	+	N.D.
Tyrosine agar (ISP-7)	−	−
Coagulation of milk	−	+
Peptonization of milk	+	+
Liquefaction of gelatin	+	+
Melanoid production	+	+
Hydrolysis of starch	+	+
NaCl tolerance	≤7%	N.D.
Temperature range for growth	17~29°C	N.D.
Carbon utilization		
D-Glucose	+	+
D-Xylose	+	−
L-Arabinose	−	−
L-Rhamnose	−	−
D-Fructose	+	+
Raffinose	−	−
D-Mannitol	+	−
i-Inositol	+	−
Sucrose	−	−
D-Galactose	+	+
Salicin	−	−

Data of "*S. gangtokensis*" are cited from ref 8.
N.D.; Not described.

Since a scientific name "*S. gangtokensis*" had not been recognized as the nomenclature along Bacteriological Code 1990 Revision, this strain was designated as *Streptomyces* sp. A22179.

The strain A22179 was deposited in the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology, Japan, with the name of *Streptomyces* sp. A22179 under the accession No. FERM P-12078.

Fermentation

Spores of strain A22179 were inoculated into several 500-ml Erlenmeyer flasks each containing 100 ml of a medium (pH 6.8) composed of glucose 0.1%, dextrin 2.0%, corn gluten meal 1.0%, fish meal 0.5%, yeast extract 0.1%, NaCl 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.0002%, $CuCl_2 \cdot 2H_2O$ 0.00004%, $MnCl_2 \cdot 4H_2O$ 0.00004%, $CoCl_2 \cdot 6H_2O$ 0.00004%, $ZnSO_4 \cdot 7H_2O$ 0.00008%, $Na_2B_4O_7 \cdot 10H_2O$ 0.00008%, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.00024% and 3-(*N*-morpholino)propanesulfonic acid 0.5%. The flasks were shaken on a rotary shaker (180 rpm) at 28°C for 72 hours. Two ml of the seed culture were inoculated into each of one hundred of 500-ml Erlenmeyer flasks containing 100 ml of the above medium and cultured on a rotary shaker at 28°C for 96 hours.

Isolation

The mycelial cake obtained by the filtration of culture broth (*ca.* 10 liters) was extracted three times with 2 liters of methanol. After evaporation to remove methanol, the extract was dissolved in 2 liters of 2% sodium chloride solution. This solution was extracted twice with 2 liters of ethyl acetate and dried *in vacuo*. The crude residue was dissolved in 2 liters of 70% aqueous ethanol and washed with 2 liters of *n*-hexane. After evaporation to remove ethanol, the residual 200 ml of water solution was extracted three times with 200 ml of chloroform. Chloroform layer was dried up *in vacuo* and the residue was applied on a silica gel column (4.5 × 40 cm). The column was eluted with chloroform - ethyl acetate - acetic acid (100 : 200 : 3). The fractions which contained BE-22179 were dried up *in vacuo* and 126 mg of pure BE-22179 was obtained by crystallization from methanol.

Physico-chemical Properties

The physico-chemical properties of BE-22179 are summarized in Table 3. BE-22179 was obtained as a pale yellow crystalline powder. It is soluble in chloroform, ethyl acetate and dimethyl sulfoxide, but insoluble in methanol and water. It shows positive color reactions to iodine, $KMnO_4$ and $FeCl_3$ reagents.

The molecular formula of BE-22179 was established to be $C_{46}H_{48}O_{12}N_{10}S_4$ on the basis of HRFAB-MS and the other spectral data.

^1H NMR spectra of native BE-22179 showed a complicated pattern (Fig. 2). The structure of BE-22179 was elucidated by the spectral analysis of mono- and di-acetylated derivatives, since the NMR spectra of acetylated BE-22179 were less

Table 3. Physico-chemical properties of BE-22179.

Appearance	Pale yellow crystalline powder
$[\alpha]_D^{20}$	−96.6° (*c* 0.47, $CHCl_3$)
Molecular formula	$C_{46}H_{48}O_{12}N_{10}S_4$
HRFAB-MS (*m/z*)	
Found:	1061.2410
Calcd:	1061.2414 $(M+H)^+$
UV λ [MeOH, max] nm (ε)	218 (87,500), 226 (84,600), 290 (12,800), 360 (12,000)
IR (KBr) cm^{-1}	3382, 1662, 1524, 1449, 1401, 1338, 1170, 999
TLC (Rf)[a]	0.35
HPLC (Rt, minutes)[b]	10.3

[a] Silica gel 60 (F_{254}), Merck; solvent: $CHCl_3$ - EtOAc - AcOH (100 : 200 : 3).

[b] Column: Inersil ODS (250 × 4.6 mm i.d.); mobile phase: 20 mM AcONa (pH 6.5) - MeCN (35 : 65); flow rate: 1.0 ml/minute; detection: 280 nm.

Fig. 2. ^{1}H NMR spectrum of BE-22179 in d-$CHCl_3$ (300 MHz).

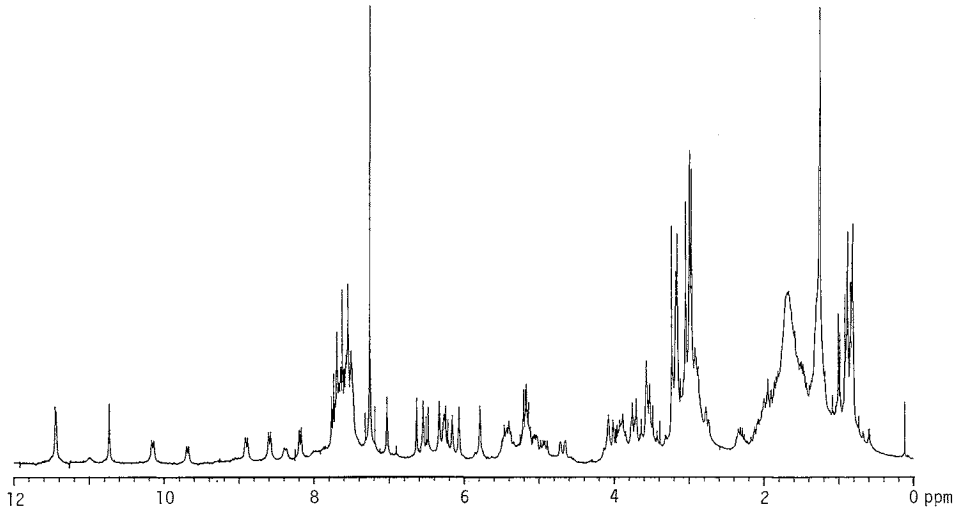
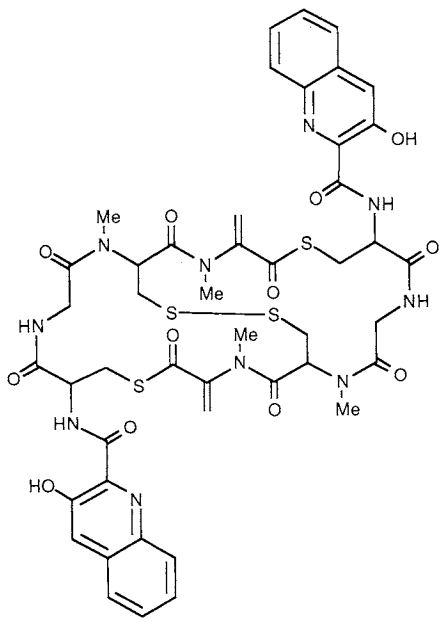


Fig. 3. Structure of BE-22179.



complicated than that of BE-22179 (data not shown). The structure of BE-22179 is shown in Fig. 3. The structural elucidation studies will be described in a separate paper[9].

Table 4. Antitumor effect of BE-22179 on L1210 leukemia.

	Dose (mg/kg/injection)	Survival time in days Mean±SD (T/C %)
Control	0	15.7± 2.54 (100)
BE-22179	0.016	20.2± 7.29 (129)
	0.031	28.2± 8.23 (180)
	0.063	38.0± 7.31 (242)
	0.125	46.2± 6.26 (294)
	0.25	>45.2±14.8 (>282)
	0.5	25.4± 3.21 (162)
	1	5.2± 0.45 (33)

$P<0.05$ by t-test.

Table 5. Antibacterial activity of BE-22179.

Organism	MIC (μg/ml)
Bacillus subtilis ATCC 6633	0.2
Staphylococcus aureus FDA 209P	0.2
S. aureus Smith	0.2
Enterococcus faecalis IFO 12580	0.39
Escherichia coli NIHJ JC-2	>100
Klebsiella pneumoniae ATCC 10031	>100
Serratia marcescens IFO 3736	>100
Pseudomonas aeruginosa IFO 3445	>100
Saccharomyces cerevisiae IFO 0283	>100
Schizosaccharomyces pombe IAM 4863	>100
Candida albicans IFO 1270	>100

Biological Properties

The topoisomerase inhibitory activity of BE-22179 was measured by the relaxation assay of supercoiled pBR322 plasmid DNA. BE-22179 inhibited the topoisomerase II of L1210 murine leukemia cell

line quite strongly compared with the topoisomerase II inhibitor, etoposide. The IC_{50} value of BE-22179 was 0.03 μM, while that of etoposide was 9.2 μM. BE-22179 did not inhibit the topoisomerase I of L1210 cell line at a dose of 10 μM.

BE-22179 showed potent cytocidal activity *in vitro* against various tumor cell lines. The IC_{50} values of BE-22179 on P388 and L1210 murine leukemia, and MKN-45 human stomach adenocarcinoma cell lines were 0.003, 0.0058 and 0.0062 μM, respectively. The *in vivo* antitumor activity was also examined in tumor bearing mice. As shown in Table 4, BE-22179 induced a prolongation of survival time of mice transplanted with L1210 leukemic cells. The maximum effect was shown at a dose of 0.25 mg/kg. Toxicity was detected by the administration of 1.0 mg/kg of BE-22179 in this experiment.

The minimum inhibitory concentration (MIC) of BE-22179, determined by the agar dilution method, is shown in Table 5. BE-22179 was strongly active against Gram-positive bacteria but inactive against Gram-negative bacteria and fungi.

Discussion

Topoisomerases I and II play important roles in DNA metabolism including relief of torsional stress during DNA replication and transcription, suppression of recombination in ribosomal DNA, and chromosome condensation at mitosis[10~12]. The topoisomerases have been recognized as key targets for many antitumor drugs, such as camptothecin (inhibitor of topoisomerase I) and etoposide, doxorubicin and m-AMSA (inhibitors of topoisomerase II)[13]. Accordingly, novel topoisomerase inhibitors having unique structures or mechanisms might be useful for the therapeutic treatment of tumors. For this reason we are carrying out screening studies with topoisomerases as targets.

BE-22179 is a structurally unique cyclic octapeptide having two thioester bonds, two *N*-methyldehydroalanines, a *N,N'*-dimethyl-cystine and two 3-hydroxyquinoline-2-carboxylic acid chromophores per molecule[9]. Although the structure of BE-22179 is partly related to quinoxaline antibiotics, such as triostin[14] and echinomycin[15,16], BE-22179 was 200 times more active against topoisomerase II than echinomycin in this assay condition (data not shown). Moreover, inhibitory activity of BE-22179 on topoisomerase II was much stronger than that on topoisomerase I. On the basis of these results, BE-22179 is thought to represent a new class of topoisomerase II inhibitors. Detailed studies on topoisomerase inhibiting activity will be described elsewhere[17].

BE-22179 is effective not only on mouse leukemia (L1210 and P388) cell lines but also on human stomach adenocarcinoma (MKN-45) cell lines *in vitro*. The antitumor activity of BE-22179 was quite strong *in vivo*. These results suggest that BE-22179 might be a lead compound to new antitumor agents.

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