A NEW TOPOISOMERASE-II INHIBITOR, BE-10988, PRODUCED BY A STREPTOMYCETE

I. TAXONOMY, FERMENTATION, ISOLATION AND CHARACTERIZATION

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A new topoisomerase inhibitor, BE-10988, was isolated from the culture broth of a strain of actinomycetes. The producing strain had a close resemblance to *Streptomyces fimicarius* and *Streptomyces xanthocidicus*. The active principle was extracted from the whole broth of strain BA10988 with ethyl acetate and purified by silica gel chromatography and by HPLC. BE-10988 increased DNA-topoisomerase complex formation and inhibited the growth of both doxorubicin-resistant and vincristine-resistant P388 murine leukemia cell lines, as well as sensitive P388 cell lines.

In the course of our screening program for a new topoisomerase inhibitor from culture broths, strain BA10988 was found to produce an active component, designated BE-10988. This compound was considered to be a new one, based on its physico-chemical properties. This paper describes the taxonomy of the producing strain and the fermentation, isolation and physico-chemical and biological properties of BE-10988. The structure of BE-10988 is shown in Fig. 1. The structure elucidation studies by spectral analyses, chemical derivation and total synthesis will be reported separately.

Taxonomy of the Producing Organism

Strain BA10988 was isolated from a soil sample collected in Takatsuki, Osaka Prefecture, Japan. Characterization of the strain followed the method adopted by the International Streptomyces Project (ISP)[1], and several other tests were also used.

Microscopic studies showed that long, straight

Fig. 1. The structure of BE-10988.

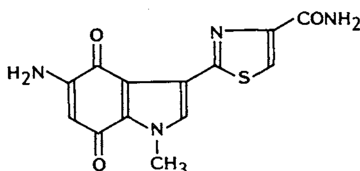


Fig. 2. Scanning electron microphotography of aerial mycelia of strain BA10988.

The bar represents 1.29 μm.

Table 1. Cultural characteristics of strain BA10988.

Agar medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Yeast extract - malt extract agar (ISP-2)	Good	Abundant Yellowish gray Powdery	Olive	None
Oatmeal agar (ISP-3)	Good	Abundant Yellowish gray Powdery	Light grayish olive	None
Inorganic salts - starch agar (ISP-4)	Good	Abundant Yellowish gray Powdery	Light grayish olive	None
Glycerol - asparagine agar (ISP-5)	Good	Abundant Grayish white Powdery	Pale yellowish green	None
Peptone - yeast extract - iron agar (ISP-6)	Good	Poor Grayish white Powdery	Pale yellowish green	None
Tyrosine agar (ISP-7)	Good	Abundant Grayish white Powdery	Yellowish green	None
Nutrient agar	Moderate	Poor Grayish white Powdery	Colorless	None
Sucrose - nitrate agar	Good	Abundant Grayish white Powdery	Pale yellowish green	None
Glucose - asparagine agar	Moderate	Abundant Grayish white Powdery	Pale yellowish green	None

and branching aerial hyphae developed from branched, non-fragmented substrate mycelia. The form of mature sporophores was *Rectiflexibiles* with 20 to 50 spores in each chain. The spores were cylindrical in shape, $0.8 \sim 1.0 \times 1.0 \sim 1.5\,\mu m$ in size and had smooth surfaces, as shown in Fig. 2. Cultural characteristics of strain BA10988 are shown in Table 1. Aerial mass color of the colony was grayish white to yellowish gray. Reverse-side growth was olive to yellowish green. Melanoid and other soluble pigments were not produced. The physiological properties and utilization of carbon sources of strain BA10988 are shown in Tables 2 and 3. The hydrolyzed cell wall of the strain contained L,L-diaminopimelic acid and glycine. Accordingly, the cell wall of the strain was classified as type I.

Table 2. Physiological properties of strain BA10988.

Coagulation of milk	Negative
Peptonization of milk	Positive
Liquefaction of gelatin	Positive
Melanin formation	Negative
Hydrolysis of starch	Positive
NaCl tolerance	7%
Temperature range for growth	12~40°C

Based on the taxonomic properties described above, strain BA10988 is considered to belong to the genus *Streptomyces*. Among the known *Streptomyces* species, *S. fimicarius* and *S. xanthocidicus* are recognized to be similar to strain BA10988. The results of direct comparison of strain BA10988 and these two species are summarized in Table 3.

Strain BA10988 is related to *S. fimicarius* and *S. xanthocidicus* in microbiological properties, but it differs considerably from these two type strains in the utilization of carbon sources. The strain, *Streptomyces* sp. BA10988, has been deposited in the Fermentation Research Institute, Agency of Industrial

Table 3. Comparison of taxonomic characteristics of strain BA10988 with *Streptomyces fimicarius* and *Streptomyces xanthocidicus*.

	BA10988	*S. fimicarius* (JCM 4224)	*S. xanthocidicus* (JCM 4243)
Spore chain morphology	*Rectiflexibiles*	*Rectiflexibiles*	*Rectiflexibiles*
Spore number per chain	20 to 50	20 to 50	20 to 50
Spore surface	Smooth	Smooth	Smooth
Aerial mass color	Grayish white to yellowish gray	Grayish white to light gray	Grayish white to gray
Color of reverse	Olive to yellowish green	Yellowish brown to light brown	Pale yellow orange to yellowish gray
Soluble pigment	None	None	None to Yellow
Melanoid formation:			
ISP-medium 6	Negative	Negative	Negative
ISP-medium 7	Negative	Negative	Negative
Growth at 45°C	Negative	Negative	Negative
Growth on 7% NaCl	Good	Good	None
Hydrolysis of starch	Positive	Positive	Positive
Carbon utilization:			
D-Glucose	+	+	+
D-Xylose	−	+	+
L-Arabinose	−	+	+
L-Rhamnose	+	+	−
D-Fructose	+	+	+
D-Galactose	+	+	+
Raffinose	−	−	+
D-Mannitol	−	+	−
Inositol	−	−	−
Salicin	−	−	−
Sucrose	+	+	+

JCM: Japan Collection of Microorganisms, RIKEN (The Institute of Physical and Chemical Research).
+: Utilized, −: not utilized.

Science and Technology, Japan, with the accession No. FERM P-10490.

Topoisomerase Assay

The assay procedure (K/SDS assay) used for screening and detection of active fractions was based essentialy on the method of TRASK *et al.*[2] and modified as reported previously[3]. Briefly, P388 cells were prelabeled with [^{3}H]thymidine. After the incubation with samples P388 cells were lysed in SDS solution and the DNA was sheared by pipetting. Protein-linked DNA was co-precipitated with K/SDS and the radioactivity in the precipitates which represents the relative amount of DNA-topoisomerase complex was counted.

Fermentation

Spores of strain BA10988 were inoculated into several 500-ml Erlenmeyer flasks each containing 100 ml of a medium 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%, $CuCl_2 \cdot 2H_2O$ 0.0004%, $MnCl_2 \cdot 4H_2O$ 0.0004%, $CoCl_2 \cdot 6H_2O$ 0.0004%, $ZnSO_4 \cdot 7H_2O$ 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% and cultured at 28°C for 3 days on a rotary shaker. 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 at 28°C for 5 days on a rotary shaker.

Isolation

The cultured whole broth (*ca.* 10 liters) was extracted with 10 liters (5 liters × 2) of EtOAc, the EtOAc layer was concentrated *in vacuo* and 13.7 g of oily crude material was obtained. This crude material was subjected to silica gel chromatography (3 × 30 cm) and the column was developed by a solvent ($CHCl_3$ - EtOAc from 1 : 1 to 1 : 3) stepwise, and active material-containing fractions were obtained. From the active fractions, 374 mg of crude material was obtained by evaporation *in vacuo*. This crude material was dissolved in 200 ml of *N,N*-dimethylformamide - MeOH (1 : 1) and subjected to HPLC (Develosil ODS-10/20, 5 × 50 cm, eluting solvent: 24% CH_3CN, flow rate: 100 ml/minute). The BE-10988-containing fraction (*ca.* 2.2 liters) obtained by HPLC was kept at 0°C for 3 days and the resulting dark-red crystalline precipitates of BE-10988 were collected (45 mg).

Physico-chemical Properties

BE-10988 is obtained as a dark-red crystalline powder. The inhibitor is soluble in dimethyl sulfoxide, sparingly soluble in MeOH and hardly soluble in water. The IR and ^1H NMR spectra of BE-10988 are shown in Figs. 3 and 4, respectively. BE-10988 is stable in the pH range 2.0 ~ 9.0.

BE-10988 possessed the following physical constants: UV λ_{max}^{MeOH} nm (ε) 213 (43,200), 235 (sh, 24,200), 278 (22,300), 305 (sh, 13,300), 318 (sh, 12,100), 385 (7,300), 525 (2,400); EI-MS *m/z* 302 $(M)^+$.

Biological Properties

BE-10988 produced DNA-topoisomerase complex in L1210 cells in a dose dependent manner to the extent of 3 μg/ml. This effect was about three times stronger than that of VP-16, a nonintercalative topoisomerase-II inhibitor. At concentrations over 3 μg/ml the complex formation was gradually reduced, as in the case of intercalative topoisomerase-II inhibitors such as doxorubicin, ellipticine and mitoxantrone.

The growth inhibitory activity of BE-10988 against P388 mouse tumor cells was evaluated. Doxorubicin-resistant P388 cells (P388/ADR), vincristine-resistant cells (P388/VCR) and their parental cells (P388/S)[4] were suspended in RPMI medium containing 10% FBS at a density of 2.5×10^4 cells/ml. BE-10988 was dissolved in dimethyl sulfoxide and 2 μl of serially diluted solution was incubated with 200 μl

Fig. 3. IR spectrum of BE-10988 in KBr.

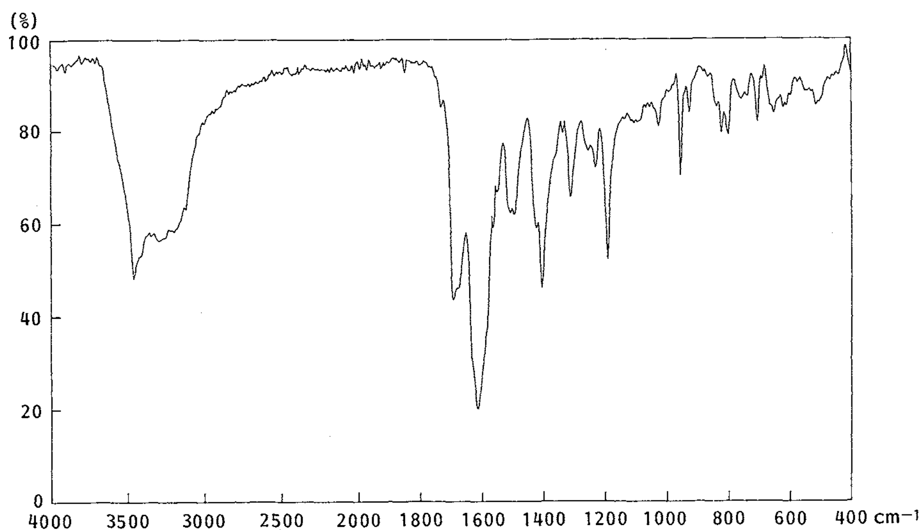
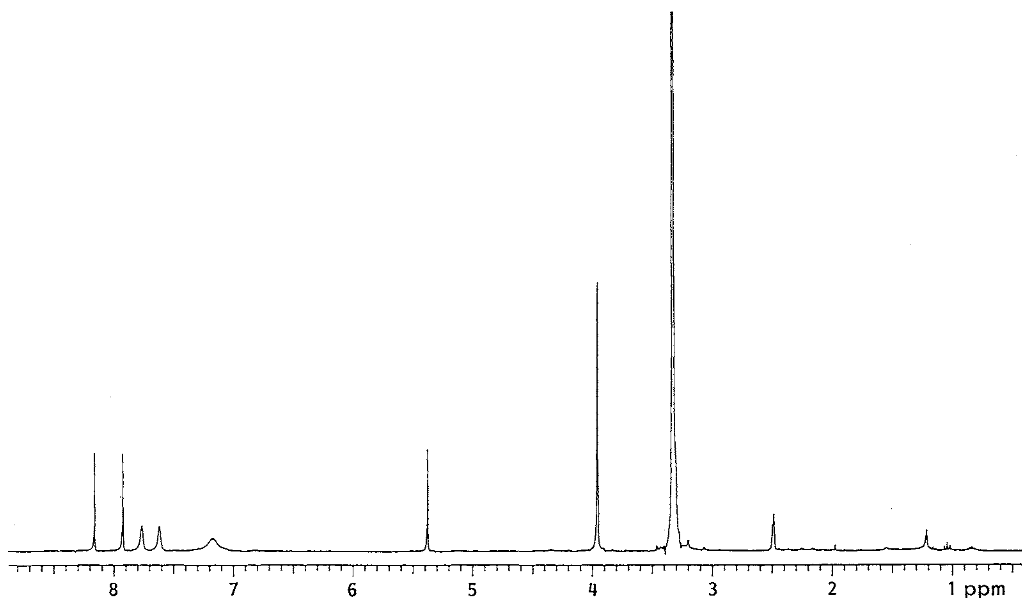


Fig. 4. ^{1}H NMR spectrum of BE-10988 in DMSO-d_6.



of cell suspension under 5% CO_2 at 37°C for 72 hours. The number of cells was counted by a Coulter Counter and the IC_{50} value (concentration required for 50% inhibition of cell growth) was calculated. BE-10988 markedly inhibited the growth of not only P388/S cells but also P388/VCR and P388/ADR cells. The IC_{50} values were 0.5, 0.4 and 2.0 μM for P388/S, P388/VCR and P388/ADR cells, respectively.

Discussion

An antitumor substance having a new structure is always desired as a new lead for chemotherapeutic agents. It is known that the topoisomerases are the target enzymes of antitumor drugs, since DNA topoisomerases-I and -II are critical nuclear enzymes which regulate the three dimensional structure of DNA. Several cytotoxic agents such as amsacrine, doxorubicin and epipodophyllotoxins induce topoisomerase-II mediated cleavage of DNA *in vitro*[5~12]. Camptothecin, a potent cytotoxic plant alkaloid induces topoisomerase-I mediated single strand cleavage of DNA *in vitro*[13,14]. These clinically useful drugs appear to stabilize topoisomerase-DNA covalent complexes, which can be trapped as cleavable complexes upon denaturation. Thus we screened for inhibitors of topoisomerase by using the K/SDS assay and succeeded in finding a new compound, BE-10988. BE-10988 inhibited the relaxation of pBR322 plasmid DNA by topoisomerase-II, as will be reported elsewhere. It should be pointed out that this topoisomerase inhibitor inhibits the growth of vincristine-resistant doxorubicin-resistant P388 cell lines that are known to contain and express an amplified gene, *mdrl*, which is transcribed into a 4.5-kilobase mRNA and translated into a 170-kilodaltone membrane glycoprotein that participates in increased drug efflux[15]. Thus, we hope that BE-10988 might be a new lead for antitumor agents. Further biochemical and biological studies are in progress.

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