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Novel Human Topoisomerase I Inhibitors, Topopyrones A, B, C and D

I. Producing Strain, Fermentation, Isolation, Physico-chemical Properties and Biological Activity

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In the course of a screening program for specific inhibitors of human topoisomerase I using a recombinant yeast, we have discovered four new active compounds. All four compounds were isolated from the culture broth of a fungus, *Phoma* sp. BAUA2861, and two of them were isolated from the culture broth of a fungus, *Penicillium* sp. BAUA4206. We designated these compounds as topopyrones A, B, C and D.

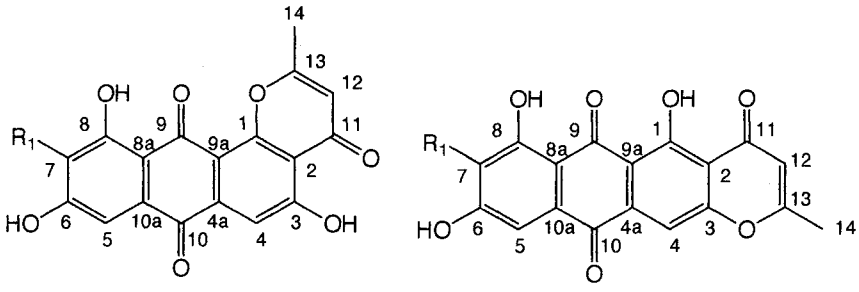
Topopyrones A, B, C and D selectively inhibited recombinant yeast growth dependent on expression of human topoisomerase I with IC_{50} values of 1.22, 0.15, 4.88 and 19.63 ng/ml, respectively. The activity and selectivity of topopyrone B were comparable to those of camptothecin. The relaxation of supercoiled pBR322 DNA by human DNA topoisomerase I was inhibited by these compounds, however they did not inhibit human DNA topoisomerase II. Topopyrones A, B, C and D were cytotoxic to all tumor cell lines when tested *in vitro*. Topopyrone B has potent inhibitory activity against herpesvirus, especially varicella zoster virus (VZV). It inhibited VZV growth with EC_{50} value of 0.038 μg/ml, which is 24-fold stronger than that of acyclovir (0.9 μg/ml). Topopyrones A, B, and C were inhibitory to Gram-positive bacteria.

DNA topoisomerases are nuclear enzymes that alter DNA conformation through a concerted breaking and rejoining of DNA strands, thereby controlling the DNA topology, required for replication, transcription and recombination[1].

These enzymes in cancer cells have lately attracted considerable attention as intracellular targets for cancer therapeutics, since many drugs with antitumor properties interact with them[2,3]. Although a number of clinically useful antitumor agents [*e.g.*, 4-(9-acridinylamino)-methanesulfonyl-*m*-anisidine (*m*-AMSA), adriamycin, etoposide (VP-16) and teniposide (VM-26)] have been shown to induce a cleavable complex with topoisomerase II[4~6], camptothecin derivatives are only examples which inhibit topoisomerase I[7,8].

WANG *et al.*[9] reported a human DNA topoisomerase I inhibition assay using recombinant yeast, in which a cleavable complex type human topoisomerase I inhibition can be detected by yeast growth inhibition. During our screening program for human topoisomerase I specific inhibitors using this method, we have discovered four new anthraquinone compounds having fused 1,4-pyrone rings, topopyrones A, B, C and D (Fig. 1). This paper describes taxonomy of the producing strain, fermentation, isolation, physico-chemical properties and biological activity of these compounds. Structure elucidation of topopyrones is described in an accompanying paper[10].

Fig. 1. Structures of topopyrones A, B, C and D.



	R_1
Topopyrone A (**1**)	Cl
Topopyrone C (**3**)	H

	R_1
Topopyrone B (**2**)	Cl
Topopyrone D (**4**)	H

Materials and Methods

Characterization of the Producing Strain

Strain BAUA2861 that produces topopyrones A, B, C and D, new topoisomerase I inhibitors, was isolated from an alimentary canal of *Thysanoptera* collected in Mt. Tsukuba, Ibaragi Prefecture, Japan. Strain BAUA4206 that produced topopyrones C and D was isolated from a sea-sediment sample collected in Okinawa Prefecture, Japan. For identification of the fungi, the following media were used; potato dextrose agar (PDA), cornmeal agar (CMA), Miura's medium (LCA). Morphology was observed under an optical microscope (Olympus BX50).

Fermentation

A slant culture of strain BAUA2861 grown on PDA was used to inoculate into a 24 mm i.d. test tube containing 10 ml of a sterile seed medium consisting of soluble starch 2.0%, glucose 1.0%, soybean flour 1.5%, malt extract 0.5%, $MgSO_4$ 0.05%, KH_2PO_4 0.05%, V8 vegetable juice 10%, potato dextrose 10%, with (for the preparation of topopyrone C) or without (for the preparation of topopyrone A) NaBr 0.01%. The test tube was shaken on a reciprocal shaker at 25°C for 72 hours. A half ml of the first seed culture was transferred to 30 ml of the same medium in a K1 flask, which was then fermented on a rotary shaker at 25°C for 120 hours.

The slant culture of strain BAUA4206 was inoculated into a 24 mm i.d. test tube containing 10 ml of a sterile seed medium consisting of soluble starch 2.0%, glucose 1.0%, soybean flour 1.5%, malt extract 0.5%, $MgSO_4$ 0.05%, KH_2PO_4 0.05%, V8 vegetable juice 10%, potato dextrose 10% and synthetic sea salts; Jamarin S. The test tube was shaken on a reciprocal shaker at 25°C for 72 hours. A half ml of the first seed culture was transferred to 30 ml of the same medium in a K1 flask, which was then fermented on a rotary shaker at 25°C for 96 hours.

Isolation of Topopyrone A

A culture broth (2580 ml) was extracted with BuOH (946 ml), and the organic layer was concentrated. The residue was dissolved in DMSO and was applied to preparative HPLC (Senshu Pak PEGASIL ODS 20× 250 mm). The mobile phase was MeCN - 50 mM AcOH (pH 3.1) with a linear gradient from 35% MeCN to 75% MeCN for 65 minutes at a flow rate of 10 ml/minute. The fraction containing topopyrone A (Rt: 38.5 minutes) was concentrated, giving topopyrone A as orange powder (0.65 mg).

Isolation of Topopyrone B

A culture broth (2580 ml) was extracted with BuOH (946 ml), and the organic layer was concentrated. The residue was dissolved in DMSO and was applied to preparative HPLC (Senshu Pak PEGASIL ODS 20× 250 mm). The mobile phase was MeCN - 50 mM AcOH (pH 3.1) with a linear gradient from 35% MeCN to 75% MeCN for 65 minutes at a flow rate of 10 ml/minute. The fraction containing topopyrone B (Rt: 27.6 minutes) was concentrated, giving topopyrone B (1.8 mg) as yellow powder.

Conversion of Topopyrone A to Topopyrone B

Purified topopyrone A (1.0 mg) was dissolved in 1% NaOH/MeOH solution (2 ml). The mixture was kept for 3 days at room temperature, and then evaporated. The residue was dissolved in water and the solution was adjusted to pH 3.0. The precipitates were collected by centrifugation (2200×*g*, 10 minutes). Precipitates which were dissolved in DMSO and applied to analytical HPLC (Shiseido CAPCELL PAK UG120 4.6×100 mm). The mobile phase was MeCN - 10 mM sodium phosphate buffer (pH 2.6) with a linear gradient from 30% MeCN to 60% MeCN for 23 minutes at a flow rate of 1 ml/minute. Topopyrone B (0.5 mg) was isolated as yellow powder.

Isolation of Topopyrone C from Strain BAUA2861

A culture broth (1050 ml) was extracted with BuOH (1155 ml). The organic layer was concentrated and the resulting solution was adjusted to pH 3.0 and extracted with EtOAc. After EtOAc layer was washed with water, the organic layer was concentrated. The residue was dissolved in DMSO and applied to preparative HPLC (Senshu Pak PEGASIL ODS 20×250 mm). Topopyrone C was purified by elution isocratically with MeCN - 50 mM AcOH (45 : 55) at a flow rate of 10 ml/minute. The fractions containing topopyrone C (Rt: 29.6 minutes) were concentrated, giving topopyrone C (23.0 mg) as orange powder.

Isolation of Topopyrone C from Strain BAUA4206

A culture broth (1200 ml) was extracted with BuOH (1320 ml). The organic layer was concentrated and the resulting solution was adjusted to pH 3.0 and extracted with EtOAc. After washed with water, the organic layer was concentrated. The residue was dissolved in DMSO and applied to preparative HPLC. Topopyrone C was purified as orange powder (10.3 mg).

Isolation of Topopyrone D from Strain BAUA2861 and Strain BAUA4206

Since the amount of topopyrone D was very small in the culture broths, it was difficult to purify topopyrone D. However, it was found that topopyrone D was converted from topopyrone C by alkali treatment. Therefore topopyrone D was prepared by chemical conversion of topopyrone C as described in the next part.

Conversion of Topopyrone C to Topopyrone D

Purified topopyrone C (20.6 mg) was dissolved in 1% NaOH/MeOH solution (20 ml). The mixture was kept for 4 days at room temperature, and then evaporated. The residue was dissolved in water and the solution was adjusted to pH 3.0. Precipitates were collected by centrifugation (2200×*g*, 10 minutes). Precipitates were dissolved in DMSO and applied to preparative HPLC (Senshu Pak PEGASIL ODS2 20×250 mm). A mobile phase was MeCN - 50 mM AcOH (pH 3.1) with a linear gradient from 35% MeCN to 75% MeCN for 65 minutes at a flowrate of 10 ml/minute. Topopyrone D (7.2 mg) was isolated as orange powder.

Topoisomerase I Inhibition Assay using Recombinant Yeasts

A standard procedure described by WANG *et al.* was pursued. JN2-134hTOP1 was cultured in a basal medium (Yeast Nitrogen Base w/o a.a. 0.17%, L-tryptophane 0.002%, L-histidine 0.002%, L-leucine 0.003%, L-lysine monohydrochloride 0.003%) and galactose 2.0% on hTop1 induction condition, glucose 2.0% on hTop1 non-induction condition. For the Top1 deficient condition, JN2-134 was cultured in a basal medium containing 2.0% galactose and 0.002% adenine sulfate. Yeast at 1×10^5 cells/ml in 0.1 ml of culture medium were incubated with compounds at 30°C for 48 hours. The yeast growth inhibition was measured by optical density (O.D. 620 nm).

Relaxation Assay of Topoisomerase I

The reaction mixture contained 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 0.25 μg pBR322 DNA, 1 μl sample solution, and 1 unit topoisomerase I in total volume of 20 μl. The mixture was incubated at 37°C for 30 minutes and reaction was terminated by the addition of 2 μl of 10% SDS and 2 μl of proteinase K (1 mg/ml). Following additional incubation at 37°C for 30 minutes, the reaction mixture was subjected to electrophoresis through a 1.0% agarose gel in TAE buffer.

Relaxation Assay of Topoisomerase II

The reaction mixture contained 30 mM Tris-HCl (pH 7.6), 3 mM ATP, 15 mM mercaptoethanol, 8 mM $MgCl_2$, 60 mM NaCl, 0.25 μg pBR322 DNA, 1 μl sample solution, and 2 units topoisomerase II in total volume of 20 μl. The mixture was incubated at 37°C for 30 minutes and reaction was terminated by the addition of 2 μl of 10% SDS and 2 μl of proteinase K (1 mg/ml). Following additional incubation at 37°C for 1 hour, it was subjected to electrophoresis through a 1.0% agarose gel containing 0.5 μg/ml ethidium bromide in TAE buffer.

DNA Unwinding Assay

DNA unwinding effects of drugs were assayed according to the method described by YAMASHITA *et al.*[11] and CAMILLONI *et al.*[12] with minor modification. pBR322 DNA

was linearized with *Hind*III restriction endonuclease and recovered by phenol extraction and ethanol precipitation. The reaction mixture (20 μl) containing 66 mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 mM ATP, 0.2 μg of linearized DNA and drugs was equilibrated at 16°C for 15 minutes and then incubated with excess amount of T4 DNA ligase at 16°C for 1 hour. DNA was analyzed by agarose gel electrophoresis after extraction with phenol and ether.

In Vitro Antitumor Activity

All cell lines were cultured with Eagle's minimum essential medium (Eagle's MEM) containing 10% fetal bovine serum and 1 mM L-glutamine. Cells in 0.1 ml of culture medium were incubated with various concentrations of the compound at 37°C for 48 hours in an atmosphere of 5% CO_2 in air. The growth of cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. IC_{50} value represents the concentration required for 50% reduction of cell viability.

Antiviral Activity

HEL11-21 cell lines were grown in Eagle's MEM supplemented with 10 % fetal bovine serum and 1 mM L-glutamine. Confluent cells were treated with different dose of drugs and infected with varicella zoster virus. After incubation at 37°C for 6 days in an atmosphere of 5% CO_2 in air, the cell viability was determined by the MTT reduction assay. EC_{50} value represents the concentration required for 50% protection of cell viability from viral-induced cytopathic effect.

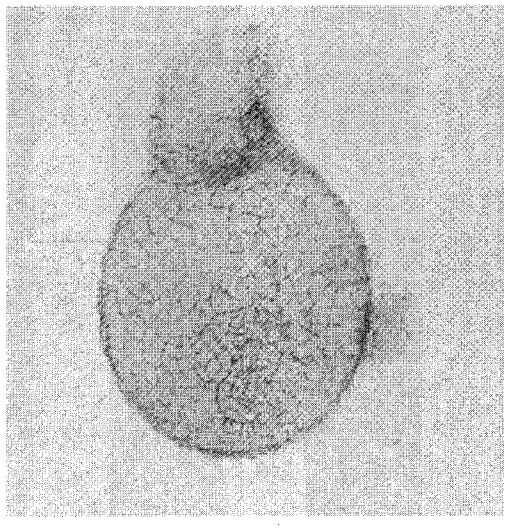
Antimicrobial Activity

Antifungal activity was determined by agar dilution method using Sabouraud dextrose agar. All fungi were grown at 35°C for 24 hours. MIC was defined as the lowest concentration in which 75% inhibition of growth was observed. Antibacterial activity was determined by agar dilution method using sensitivity test agar. All bacteria were grown at 37°C for 24 hours. MIC was defined as the lowest concentration in which 99.9% inhibition of growth was observed.

Materials

Supercoiled pBR322 DNA and T4 DNA ligase were purchased from Takara Shuzo Co. (Kyoto, Japan). Camptothecin, VP-16, human Topoisomerase I and human Topoisomerase II were purchased from TopoGEN Inc. (Ohio, U.S.A.). *m*-AMSA, actinomycin D, acyclovir, amphotericin B and 5-fluorocytosin were purchased from Sigma Chemical Co. (Missouri, U.S.A.). Yeast Nitrogen Base w/o a.a. and Sabouraud dextrose agar were purchased from Difco Laboratories Co. (Michigan, U.S.A.). Jamarin S was purchased from Jamarin Laboratory (Osaka, Japan).

Fig. 2. Optical micrograph of strain BAUA2861.



Results

Characterization of the Producing Strain

Strain BAUA2861 exhibited good growth at 15~25°C on PDA. Colonies growing on CMA were floccose and gray, and the margin was velvety and brown. A reverse side of the culture was brown. Formation of pycnidium was observed and its shape was spherodial or subglobose (Fig. 2). Conidiospores were uncolored and elliptical. Strain BAUA2861 also generated chlamydospores. Soluble pigment was not observed around colonies.

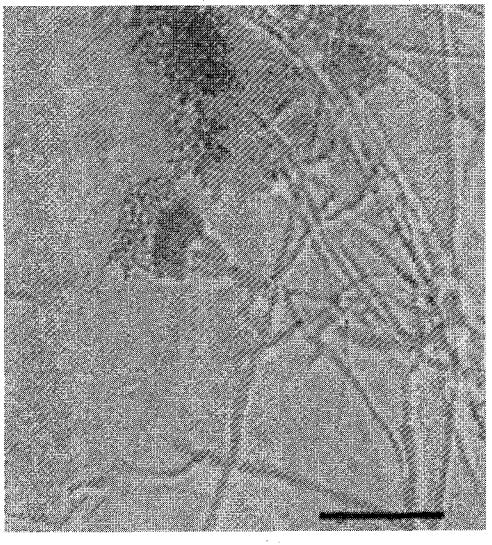
Based on cultural and microscopic characteristics described above, strain BAUA2861 is considered to belong to the genus *Phoma*. The strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as *Phoma* sp. BAUA2861 with the accession No. FERM P-17384.

Strain BAUA4206 grew well on PDA. Colonies growing on PDA were light whitish olive gray. The reverse side of colonies was pale yellowish brown. When the strain BAUA4206 was grown on LCA, the conidiophores were formed from the substrate hypae. Penicillia were

biverticillate as shown in Fig. 3. The conidia were globose, achroic and their surface was smooth.

From the above characteristics, strain BAUA4206 was identified as belonging to the genus *Penicillium*. The strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as *Penicillim* sp. BAUA4206 with the accession No. FERM P-17643.

Fig. 3. Optical micrograph of strain BAUA4206.



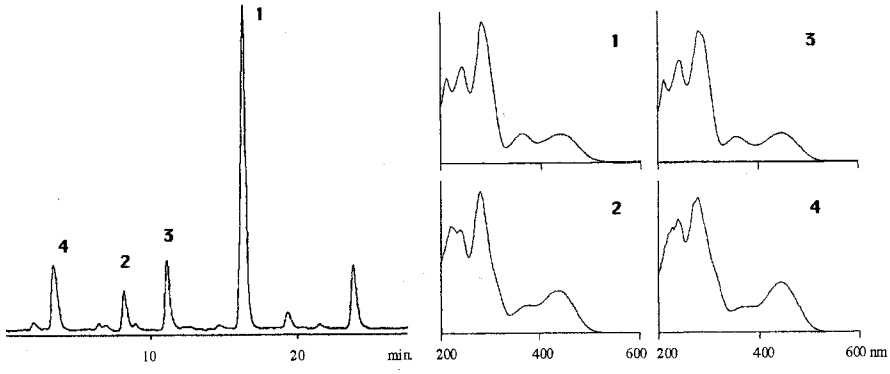
Bar represent 10μm

Preparation of Topopyrones A, B, C and D

A fermentation broth of *Phoma* sp. BAUA2861 was extracted with BuOH. After concentration, the residue was dissolved in DMSO and analyzed by HPLC. Four new active peaks were detected as shown in Fig. 4. UV-VIS spectrum of topopyrone A was similar to that of topopyrone C and the spectrum topopyrone B was also similar to that topopyrone D. Topopyrone A was converted to topopyrone B, and topopyrone C was converted to topopyrone D by alkaline treatment, respectively. We also found that addition of sodium bromide to the fermentation enhanced the production of topopyrone C. To prepare topopyrone A and B, a culture broth of *Phoma* sp. BAUA2861 was extracted with BuOH. After concentration, the extract was dissolved in DMSO. Topopyrone A and B were purified by preparative HPLC, using ODS column. To prepare topopyrone C more efficiently, strain BAUA2861 was inoculated with a medium containing 0.01% sodium bromide. Topopyrone C was purified by preparative HPLC, using ODS column. Since topopyrone D was very minor in the culture broth, purified topopyrone C was converted into topopyrone D with 1% NaOH/MeOH solution.

A fermentation broth of *Penicillium* sp. BAUA4206 was extracted with BuOH. After concentration, the residue was analyzed by HPLC. Two active peaks were detected and identified as topopyrones C and D from its UV-VIS spectrum and MS data.

Fig. 4. HPLC analysis of BAUA2861.



Broth of BAUA2861 was extracted with BuOH. After concentration , extract was dissolved in DMSO and analyzed.
HPLC condition
Column : Capcell pak UG120 4.6×100mm
Flowrate : 1ml/minute, Temp. : 50°C, Detect. : 450nm
Mobile phase : MeCN - water (0.05% TFA), linear gradient from 30% MeCN to 60% MeCN till 23 minutes

1 : topopyrone A, **2** : topopyrone B, **3** : topopyrone C, **4** : topopyrone D

Table 1. Physico-chemical properties of topopyrones A, B, C and D.

	topopyrone A	topopyrone B	topopyrone C	topopyrone D
Appearance	Orange powder	Yellow powder	Orange powder	Orange powder
Molecular formula	$C_{18}H_9O_7Cl$	$C_{18}H_9O_7Cl$	$C_{18}H_{10}O_7$	$C_{18}H_{10}O_7$
Melting point	> 280℃	> 280℃	> 280℃	> 280℃
ESI-MS (m/z)	371 $(M-H)^-$	371 $(M-H)^-$	337 $(M-H)^-$	337 $(M-H)^-$
HRFAB-MS				
found	370.9962 $(M-H)^-$	370.9964 $(M-H)^-$	337.0322 $(M-H)^-$	337.0353 $(M-H)^-$
calcd.	370.9959 $(M-H)^-$ for $C_{18}H_8O_7Cl$	370.9959 $(M-H)^-$ for $C_{18}H_8O_7Cl$	337.0348 $(M-H)^-$ for $C_{18}H_9O_7$	337.0348 $(M-H)^-$ for $C_{18}H_9O_7$
UV nm λmax (ε)	210 (27554), 240 (21901)	222 (10632), 237 (10521)	209 (30375), 240 (24450)	221 (12223), 237 (12487)
in MeOH	260 (21630), 284 (22487)	264 (8377), 281 (8409)	261 (23878), 279 (23813)	266 (10165), 276 (9742)
	297 (21918), 332 (16316)	341 (6807), 449 (3694)	294 (24231), 331 (13446)	344 (6180), 452 (3231)
	380 (6086), 436 (4540)	480 (3133)	380 (5711), 452 (4860)	515 (2193)
	520 (2502)		508 (3254)	

Table 2. Growth inhibition of recombinant yeast by topopyrones.

	IC_{50} (ng/ml)			selectivity *
Organism	*Saccharomyces cerevisiae*			
Strains	JN2-134 hTOP1		JN2-134	
Remarks	Topl induction	Topl non-induction	Topl deficient	
topopyrone A	1.22	>10000	5000	>8000
topopyrone B	0.15	>10000	>10000	>66000
topopyrone C	4.88	156.25	39.06	32
topopyrone D	19.63	>10000	>10000	>500
camptothecin	0.10	>10000	>10000	>100000

* IC50 value of Top1 non-induction / IC50 value of Top1 induction

Physico-chemical Properties

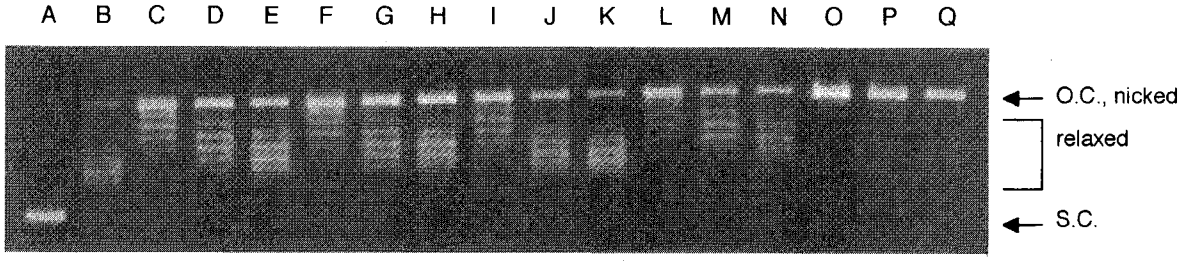
The physico-chemical properties of topopyrones are summarized in Table 1. The UV-VIS spectra indicated that topopyrones are anthraquinone compounds. In the electrospray ionization MS (ESI-MS) negative mode measurement, topopyrones A and B showed the peaks of *m/z* 371 and 373, with a peak ratio of 3 : 1. Thus, the molecular weight of 372 containing one chlorine atom was suggested. The molecular formulae of topopyrones A and B were determined to be $C_{18}H_9O_7Cl$ by HRFAB-MS. The UV-VIS spectrum of topopyrone C was similar to that of topopyrone A and the spectrum topopyrone D was also similar to that topopyrone B. The molecular formulae of topopyrone C and D were determined to be $C_{18}H_{10}O_7$ by HRFAB-MS (see the accompanying paper).

Biological Activity

Inhibition of human topoisomerase I by topopyrones A, B, C and D using recombinant yeast is shown in Table 2. Topopyrones A, B, C and D inhibited yeast growth in human topoisomerase I inductive conditions with IC_{50} value of 1.22, 0.15, 4.88 and 19.63 ng/ml, respectively. Although topopyrones A, B and D did not inhibit yeast growth in non-inductive conditions at 10,000 ng/ml, topopyrone C inhibited at a concentration of 156 ng/ml. The activity and selectivity of topopyrone B were comparable to those of camptothecin, a specific inhibitor of topoisomerase I.

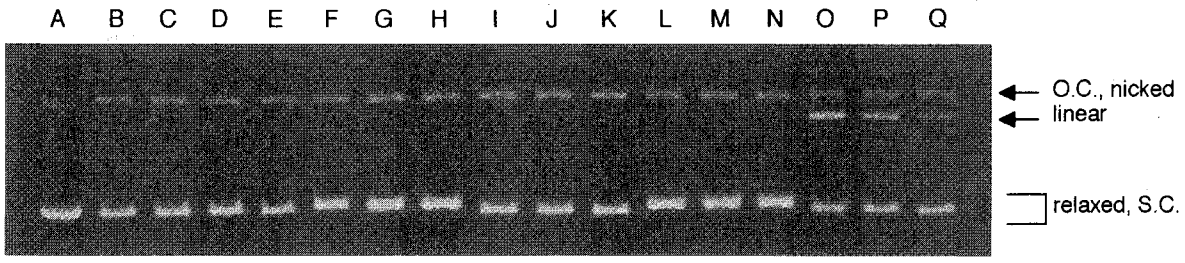
The inhibitory activity of topopyrones on relaxation of plasmid DNA by topoisomerase I is shown in Fig. 5. As the concentration of topopyrones was increased, the amount of nicked DNA was increased. The topoisomerase I mediated

Fig. 5. Effect of topopyrones on the relaxation activity of human topoisomerase I.



Electrophoresis was carried out in a 1.0% agarose gel at 50 volts for 90 minutes. Lane A, substrate supercoiled DNA; lane B, relaxed DNA; lanes C to E, topopyrone A; lane F to H, topopyrone B; lane I to K, topopyrone C; lane L to N, topopyrone D; lane O to Q, camptothecin. Drug concentrations were: lane C, F, I, L and O, 100μM; lane D, G, J, M and P, 33.3μM; lane E, H, K, N and Q, 11.1μM.
O.C. : open circular DNA, S.C. : supercoiled DNA

Fig. 6. Effect of topopyrones on the relaxation activity of human topoisomerase II.



Electrophoresis was carried out in a 1.0% agarose gel containing 0.5μg/ml ethidium bromide at 50 volts for 90 minutes. Lane A, substrate supercoiled DNA; lane B, relaxed DNA; lanes C to E, topopyrone A; lane F to H, topopyrone B; lane I to K, topopyrone C; lane L to N, topopyrone D; lane O to Q, VP-16. Drug concentrations were: lane C, F, I, L and O, 100μM; lane D, G, J, M and P, 33.3μM; lane E, H, K, N and Q, 11.1μM.
O.C. : open circular DNA, S.C. : supercoiled DNA

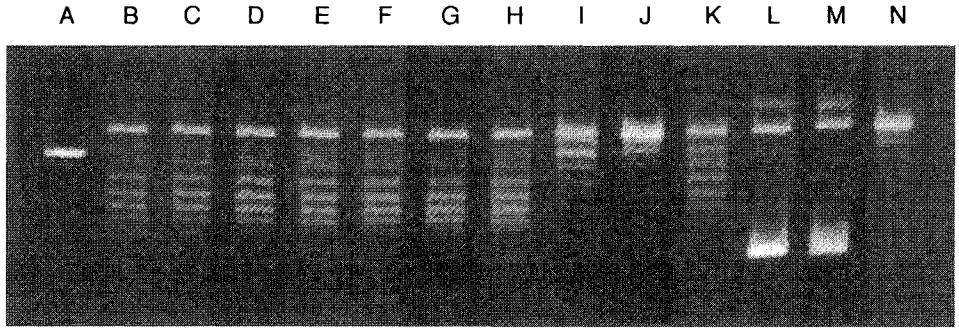
DNA cleavage activity of topopyrones A and B was dose-dependent and slightly weaker than that of camptothecin. The inhibitory activity of topopyrones C and D was less effective than that of topopyrones A and B.

To determine if topopyrones were selective inhibitors of topoisomerase I, we examined the effect of these compounds on the relaxation assay of topoisomeraseII (Fig. 6). None of the compounds inhibited the enzyme activity at a concentration of 100 μM, whereas VP-16, a specific inhibitor of topoisomerase II, inhibited at the concentration of 11.1 μM. This indicates that the activity of topopyrones A, B, C and D are selective to topoisomerase I.

Although camptothecin and VP-16 are classified as a non-intercalative drugs, most of the antitumor drugs which can induce a cleavable complex with topoisomerase II are intercalators, such as *m*-AMSA, adriamycine and saintopin. To investigate if topopyrones A and B intercalate with DNA strands, an unwinding assay was performed using linearized pBR322 DNA and T4 DNA ligase. Fig. 7 shows agarose gel electrophoresis of the products of the DNA unwinding assay with topopyrones A, B, and the known intercalators *m*-AMSA and actinomycin D. Upon ligation in the presence of increasing concentration of the intercalators, the resulting population of molecules became more negatively supercoiled as compared with the control (*m*-AMSA and actinomycin D). However topopyrones A and B did not affect DNA conformation. These results suggested that topopyones A and B are classified as a non-intercalative topoisomerase I inhibitor. Similar results were also obtained using topopyrones C and D (data not shown).

In vitro antitumor activity of topopyrones A, B, C and D is shown in Table 3. These compounds inhibited the growth of all the tumor cell lines tested, but the activities were weaker than camptothecin.

Fig. 7. Effects of topopyrones A and B on the DNA unwinding assay with T4 ligase.



Electrophoresis was carried out in a 1.0% agarose gel at 50 volts for 90 minutes. Lane A, substrate linearized DNA; lane B, No drug; lanes C to E, topopyrone A; lane F to H, topopyrone B; lane I to K, amsacrine; lane L to N, actinomycin D. Drug concentrations were: lane C, F, I and L, 50μM; lane D, G, J and M, 10μM; lane E, H, K and N, 2μM.

Table 3. *In vitro* antitumor activity of topopyrones A, B, C and D.

	IC_{50} (μg/ml)					
cell lines	topopyrone A	topopyrone B	topopyrone C	topopyrone D	camptothecin	Adriamycin
HeLa	0.56	3.5	6.9	>5	0.01	0.96
B16	1.5	4.1	7.5	>5	0.18	0.16
Colon 26	0.57	2.4	6.1	>5	0.031	0.031
3LL	0.29	2.2	5	2.5	0.031	0.14
P388	0.52	2.5	4.4	0.5	0.013	1.96
L1210	0.5	2	4	0.47	0.016	1.25
Vero	0.56	3.7	7.8	>5	0.05	>2.5
HEL11-21	0.57	4.6	8.2	>5	0.073	0.59

Several researchers have shown that camptothecin inhibits replication of equine infectious anemia virus (EIAV)[13] and herpes simplex virus (HSV)[14], and topoisomerase I plays a role in virus replication. Therefore, we investigated effect of topopyrones on the replication of herpesvirus. Of the four compounds tested, only topopyrone B exhibited a potent inhibitory activity against herpesvirus, especially varicella zoster virus (VZV). It inhibited VZV growth with EC_{50} value of 0.038 μg/ml, which is 24-fold stronger than that of acyclovir (Table 4). Since non-cytotoxic dose of topopyrone B inhibited VZV replication, virus-inhibitory effect of topopyrone B is not due to its cytotoxic activity.

The antimicrobial activity of topopyrones A, B, C and D is shown in Table 5. Although topopyrones A, B and C were strongly active against Gram-positive bacteria including quinolone-resistant MRSA, topopyrone D was only weakly active against Gram-positive bacteria.

Table 4. Antiviral activity of topopyrones.

	EC_{50} (μg/ml) VZV	IC_{50} (μg/ml) HEl11-21	S.I.
topopyorne B	0.038	0.81	21.3
acyclovir	0.9	>100	>111

EC_{50} : The concentration required for protecting 50% of cell viability from viral-induced cytopthic effect.
IC_{50} : The concentration required for reducing to 50% of cell viability.
S.I. : selective index.

However, topopyrones showed no antimicrobial activity against Gram-negative bacteria, yeast or fungi.

Discussion

We have described here the novel topoisomerase I

Table 5. Antimicrobial activity of topopyrones.

Strain		MIC (μg/ml)			
		topopyrone A	topopyrone B	topopyrone C	topopyrone D
Aspergillus fumigatus	TIMM0069	>8	>8	>16	>16
A. fumigatus	TIMM0070	>8	>8	>16	>16
A. fumigatus	MF-13	>8	>8	>16	>16
A. flavus	ATCC9643	>8	>8	>16	>16
A. flavus	TIMM0057	>8	>8	>16	>16
A. niger	TIMM2814	>8	>8	>16	>16
A. terreus	NUD3265	>8	>8	>16	>16
Candida albicans	ATCC10259	>8	>8	>16	>16
C. glabrata	ATCC90030	>8	>8	>16	>16
C. parapsilosis	ATCC90018	>8	>8	>16	>16
Cryptococcus neoformans	ATCC90112	>8	>8	>16	>16
Staphylococcus aureus	FDA209P JC-1	0.5	1	0.5	≧2
S. aureus	Smith	0.125	0.25	0.25	0.5
S. aureus	JS-1	0.25	0.5	0.5	≧2
S. aureus	KP90-3	0.125	0.25	0.25	≧2
Streptococcus pyogenes	Cook	0.5	4	2	≧2
Micrococcus luteus	ATCC9431	0.5	0.5	1	≧2
Bacillus subtilis	ATCC6633	0.125	0.25	0.125	≧2
Escherichia coli	NIHJ JC-2	>8	>8	≧4	≧2
E. coli	ML4707	>8	>8	≧4	≧2
Klebsiella pneumoniae	KC-1	>8	>8	≧4	≧2
Serratia marcescens	No 16-2	>8	>8	≧4	≧2
Pseudomonas aeruginosa	PAO-1	>8	>8	≧4	≧2
P. aeruginosa	KP-254	>8	>8	≧4	≧2

inhibitors, topopyrones A to D. Among these compounds, topopyrones A and B were chlorinated anthraquinone compounds, whereas topopyrones C and D were dechloro-derivatives of topopyrones A and B, respectively. We found that fermentation of strain BAUA2861 with sodium bromide resulted in accumulation of topopyrone C. The observation indicates that sodium bromide inhibits the chlorination of topopyrone A. It is assumed that fungal chloroperoxidase was inhibited by bromide ion[15]. We also found that the irreversible conversion from topopyrone A into topopyrone B and from topopyrone C into topopyrone D. Detailed studies on the mechanism of conversion will be described in an accompanying paper.

The results of two topoisomerase I inhibition assays suggested that the chlorinated compounds have higher activity than the dechlorinated compounds. This indicates that a chlorine atom at position 7 contributes for enhancement of topoisomerase I inhibition activity.

ISHIYAMA *et al.*[16] reported that fetal calf serum (FCS) influenced the topoisomerase I inhibition activity of BM2419-1 and -2, saintopin derivatives, but no influence was observed for camptothecin. In the human topoisomerase I inhibition assay using recombinant yeasts, topopyrones showed the remarkable decrease of the inhibitory activity upon addition of FCS. Moreover, the influence of FCS on topopyrone B was higher than that of topopyrone A (data not shown).

In the antiviral assay, only topopyrone B exhibited a potent inhibitory activity, however, topopyrone D slightly inhibited viral replication (data not shown). These results suggest that the inhibitory effect of topopyrones B and D on viral replication is likely due to topoisomerase I inhibition. In contrast, topopyrones A and C did not exhibit inhibitory activity completely, because these compounds showed a potent cytotoxicity.

Although topopyrone C inhibited yeast growth in non-inductive conditions, it did not exhibit antimicrobial activity against both fungi and yeast. NITISS and WANG[17] reported that intact cells of ordinary laboratory strain of the yeast are refractory to the drugs, however, yeast strain JN-134 carrying a drug permeability mutation *ise1* are sensitive to crystal violet, cycloheximide and camptothecin. Probably for this reason, topopyrone C did not exhibit antifungal activity. In the topoisomerase I deficient

condition, despite topopyrones did not inhibit topoisomerase II, not only topopyrone C but topopyrone A inhibited yeast growth at concentration of 5 μg/ml. Clearly the isomerization of 1,4-pyrone ring resulted in the increased inhibitory activity.

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