NEW NAPHTHACENECARBOXAMIDE ANTIBIOTICS, TAN-1518 A AND B, HAVE INHIBITORY ACTIVITY AGAINST MAMMALIAN DNA TOPOISOMERASE I

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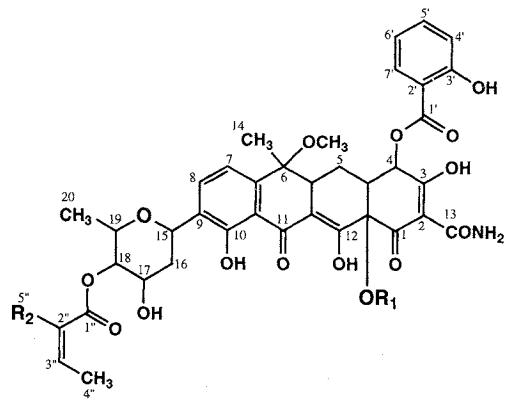
New naphthacenecarboxamide antibiotics, TAN-1518 A and B, were isolated from a culture broth of *Streptomyces* sp. AL-16012. Their structures were elucidated from their reactions and from spectroscopic analyses. The relaxation of supercoiled pBR322 DNA by calf thymus DNA topoisomerase I was inhibited by these metabolites as potently as by camptothecin. However, the decatenation of kinetoplast DNA by calf thymus DNA topoisomerase II was little affected by these agents. The major metabolite, TAN-1518 A, strongly suppressed the growth of various murine and human tumor cells, inducing apoptosis. Unlike camptothecin, TAN-1518 A did not stimulate cleavable complex formation in the nuclei of CHO-K1 cells and had weak activity in intercalating into DNA strands. This metabolite arrested the growth of human tumor cell lines in G1 phase of the cell cycle. These results suggest that TAN-1518 A and B are novel antitumor agents targeting topoisomerase I.

DNA topoisomerases are nuclear enzymes responsible for DNA topology, and they play an important role in DNA metabolism in mammalian cells, including 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 large number of antitumor agents that inhibit topoisomerase II (Topo II) are known, thus far, few agents that inhibit topoisomerase I (Topo I) have been identified.[4~7]

In the search for Topo I inhibitors of microbial origin, we found three metabolites in the culture broth of an actinomycete strain isolated from a soil sample. Two of them, TAN-1518 A and B, were proven to be new metabolites belonging to the tetracycline family (Fig. 1). The other one, TAN-1518 X, was identical to SF-2575.[8,9]

Fig. 1. Structure of TAN-1518 A, B and X.



TAN-1518	R_1	R_2
A (**1**)	H	Me
B (**2**)	Me	Et
X (**3**)	Me	Me

Materials and Methods

Taxonomic Studies

Culture characterization was carried out following the International Streptomyces Project procedure.[10] The color recorded for the mature culture was described according to the Color Harmony Manual.[11] Whole-cell analysis performed by the method of HASEGAWA *et al.*[12] Scanning electron microcopy was conducted by the method of TANIDA *et al.*[13]

Fermentation

A frozen seed stock (5 ml) of strain AL-16012 was inoculated into a 2-liter Sakaguchi flask containing 500 ml of a sterile seed medium consisting of glucose 2%, soluble starch 3%, soybean flour 1%, corn steep liquor 0.3%, polypeptone 0.5%, NaCl 0.3%, and $CaCO_3$ 0.5%. The flask was shaken on a reciprocal shaker at 28°C for 48 hours. One liter of the seed culture was transferred to a 200-liter fermenter containing 120 liters of a production medium consisting of soluble starch 3% and cotton seed flour 0.5%. The fermentation was carried out at 28°C for 72 hours with aeration of 120 liters/minute and agitation of 200 rpm. The production culture (100 liters) was transferred to a 6000-liter fermentor containing 3200 liters of the same medium, and the fermentation was carried out at 24°C with aeration of 3200 liters/minute and agitation of 120 rpm.

Analytical Measurement

The IR spectra were measured in KBr pellets and EI-MS spectra were measured on a JEOL JMS-DX303 instrument. The δ-values in the NMR spectra were recorded in ppm downfield from tetramethylsilane (TMS) using, a Bruker AC-300 spectrometer.

Isolation of TAN-1518 A (**1**) and B (**2**)

Culture broth (3,200 liters) was extracted with EtOAc (1,200 liters) at pH 2.0. The organic layer was washed with 2% $NaHCO_3$ and water successively, and was concentrated, giving an oily residue. To the residue, hexane was added, and a brown powder (250 g) was obtained. This was subjected to silica gel (8 liters) column chromatography, eluted with toluene - EtOAc - formic acid (92 : 8 : 2, 8 liters) and (88 : 12 :2, 10 liters) to give crude **1** and a mixture of **2** and TAN-1518 X (**3**). The former was purified by silica gel (800 ml) chromatography, eluted with $CHCl_3$ - MeOH - formic acid (99 : 1 : 2, 2 liters), giving **1** (18 g) as a yellow powder. The latter was evaporated to dryness and the residue was treated with MeOH to exclude precipitate **3** (67 g). The filtered liquid was concentrated, giving a brown powder (4.2 g) containing **2**. The powder was chromatographed on a silica gel (200 ml) column, and eluted with toluene - EtOAc - formic acid (95 : 5 : 2, 1 liter) and (90 : 10 : 2, 500 ml); the latter fraction was concentrated, giving crude **2** (715 mg). The crude **2** was applied to preparative HPLC (ODS, YMC-Pack S-363, I-15) with a mobile phase of 73% acetonitrile - 0.01 M phosphate buffer (pH 3.0). The fractions containing **2** were concentrated and extracted with EtOAc, giving **2** (70 mg) as a yellow powder.

Alkaline Hydrolysis of **1**

A solution of **1** (2 g) in 1 N NaOH (13 ml) was stirred for 6 hours at room temperature. The solution was adjusted to pH 2.0 and extracted with EtOAc (2 × 50 ml). The combined organic layers were washed with water, dried over anhydrous sodium sulfate and evaporated, giving a yellow powder (1.9 g). The powder was chromatographed on silica gel (50 g), eluted with toluene - EtOAc - formic acid (78 : 20 : 2, 600 ml) and (58 : 40 : 2, 600 ml). The former fraction was concentrated, giving a mixture of salicylic acid (**4**) (190 mg) and 2-methyl-2-butenoic acid (**5**). The mixture of **4** and **5** was again chromatographed on slica gel (6 g), eluted with $CHCl_3$ - MeOH (80 : 20, 100 ml), affording **4** (50 mg) and **5** (100 mg) as colorless crystals; **4** was identified with an authentic sample by TLC and ^1H NMR.

5: EI-MS *m/z* 100 (M^+); ^{13}C NMR ($CDCl_3$, δ ppm) 174.0 (s), 141.4 (d), 127.2 (s), 20.3 (q), 14.5 (q);

Anal. Calcd for $C_5H_8O_2$: C 59.98, H 8.05
Found: C 60.12, H 8.07

Relaxation Assay of Topo I

Topo I activity was measured by detecting the conversion of supercoiled pBR322 DNA to its relaxed form. The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 30 μg/ml bovine serum albumin (BSA), 0.25 μg pBR322 DNA, 2 μl sample solution, and 1 unit Topo I, in a total volume of 20 μl. The mixture was incubated at 37°C for 40 minutes and reactions were stopped by the addition of 4 μl of loading buffer, consisting of 0.25% bromophenol blue, 40% glycerol, and 2.5% SDS. They were electrophoresed in agarose gel at 100 volts (V) for 35 minutes in TBE buffer consisting of 89 mM Tris-borate (pH 8.9) and 2 mM EDTA containing 0.1% SDS. Gels were stained with ethidium bromide and washed throughly with distilled water. The DNA band was visualized

over UV light and photographed with Polaroid type 667 positive/negative films. One unit of Topo I activity was defined as the amount of the enzyme that converted 0.25 μg of supercoiled pBR322 DNA to its relaxed form in 40 minutes at 37°C.

Decatenation Assay of Topo II

Topo II activity was measured by detecting the conversion of catenated kinetoplast DNA (kDNA) to minicircle monomers. The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, 0.5 mM DTT, 30 μg/ml BSA, 0.2 μg kDNA, 2 μl sample solution, and 1 unit Topo II, in a total volume of 20 μl. The mixture was incubated at 37°C for 20 minutes. DNA was analyzed by agarose gel electrophoresis, as described above. One unit of Topo II activity was defined as the amount of the enzyme that decatenated 0.2 μg of kDNA for 20 minutes at 37°C.

Cytotoxicity

EL4 murine lymphoma, P815 murine mastcytoma, and SW48 human colon adenocarcinoma were purchased from the American Type Culture Collection (ATCC). The CHO-K1 Chinese hamster ovary cell line was purchased from Flow Laboratories Inc. (Irvine, Scotland). B16 murine melanoma was kindly provided by the Japanese Research Resources Bank. The HeLa S3 human epitheloid carcinoma, WiDr human colon adenocarcinoma, A549 human lung carcinoma, and G361 human melanoma cell lines were obtained from the Institute for Fermentation, Osaka. All cell lines, except for EL4, P815, and CHO-K1, were cultured with EAGLE's minimum essential medium (EAGLE's MEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine, and 20 μg/ml gentamicin. The EL4, P815, and CHO-K1 cell lines were cultured with RPMI-1640 containing FBS, 2 mM glutamine, 20 μg/ml gentamicin, and 5×10^{-5} M 2-mercaptoethanol. Cells at various concentrations in 0.1 ml of culture medium were incubated with TAN-1518 A at 37°C for 72 hours in an atmosphere of 5% CO_2 in air. The growth of the cell lines was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay.[14]

Detection of DNA Fragmentation

Cells (10^6) in 3 ml of culture medium were seeded in a 6-well plate. After 18-hours incubation, 1 μM of TAN-1518 A was added, and the cells were incubated for another 18 hours. They were then harvested, and genomic DNA was isolated, essentially following the method of MILLER *et al.*[15] The harvested cells were resuspended in 300 μl of nuclear lysis buffer consisting of 10 mM Tris-HCl (pH 8.2), 400 mM NaCl, and 2 mM EDTA. The cell lysates were digested overnight at 37°C by the addition of 25 μl of 10% SDS and 50 μl of 10 mg/ml proteinase K in distilled water. Then 100 μl of saturated NaCl was added, and the suspension was shaken vigorously. After centrifugation, 350 μl of supernatant was transferred to another tube and 700 μl of ethanol was added. Precipitated DNA was dried, rehydrated in buffer consisting of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, and incubated for 1 hour at 37°C in the presence of 10 units of ribonuclease A (RNase A). The purified chromosomal DNA was analyzed by agarose gel electrophoresis, as described above.

Quantitative Analysis of Cleavable Complex in Nuclei

The amount of cleavable complex was measured by the method of CALDECOTT *et al.*[16] Five thousand CHO-K1 cells in 4 ml of culture medium were seeded in a 6-well plate. After 24-hours incubation, the cells were labeled with 2.5 μCi/ml of [6-^{3}H]thymidine and incubated for another 24 hours. One hour prior to drug treatment, the label was removed, the cells were washed once with phosphate buffered saline (PBS), and fresh medium was added. These log phase cells were then treated with each drug for 1 hour, after which they were washed with ice-cold PBS and lysed with 500 μl prewarmed (60°C) lysis solution consisting of 1.25% SDS, 5 mM EDTA, and 0.4 mg/ml salmon sperm DNA. The lysed cells were carefully passed 15 times through a 0.4-mm-diameter needle. Prewarmed (37°C) 325 mM KCl (250 μl) was then added, and the tubes were kept on ice for 10 minutes. The protein-DNA complex was pelleted by centrifugation for 10 minutes at 4°C. The supernatant was carefully removed and the precipitate was resuspended in 1 ml of prewarmed (37°C) wash solution consisting of 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM EDTA, and 0.1 mg/ml salmon sperm DNA by heating at 60°C for 10 minutes with periodic mixing. The tubes were then transferred to ice for 10 minutes and the cycle was repeated once. Finally, the protein-DNA

complex was dissolved in 100 μl of distilled water at 50°C and added to 4 ml of scintillant. Radioactivitity was measured with a liquid scintillation counter (Beckmann LS6000TA).

Competition of DNA Binding with Ethidium Bromide (EtBr)

DNA binding of TAN-1518 A was analyzed by an EtBr competition assay.[5] Sample solution (10 μl) was mixed with 100 μl of 5 μM EtBr and 40 μM (in base) salmon sperm DNA in a buffer consisting of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA. The fluorescence intensity of the solution was measured with a fluorescence concentration analyzer (Baxter Pnadex FCA-VIP); excitation wavelength was 545 nm for competition of EtBr versus TAN-1518 A, 4′-(9-acridinylamino)methanesulfon-*m*-anisidine (*m*-AMSA) or actinomycin D, and 300 nm for competition between EtBr and adriamycin; emission wavelength was 575 nm for all competitors.

Cell Cycle Analysis

HeLa S3 cells (5×10^5) in 10 ml of culture medium were seeded in a plate (9-cm diameter). After 24-hours incubation, each drug was added and the cells were incubated for another 24 hours. The following procedures were carried out at room temperature: The cells were harvested and resuspended in 180 μl of a lysis buffer consisting of 0.03 mg/ml trypsin, 0.5 mM Tris-HCl (pH 7.6), 3.4 mM trisodium citrate, 0.1% Nonidet P-40 (NP-40), and 1.5 mM spermine. After 10 minutes, 150 μl of a nuclease buffer, consisting of 0.1 mg/ml RNase A, 0.5 mg/ml trypsin inhibitor, 0.5 mM Tris-HCl (pH 7.6), 3.4 mM trisodium citrate, 0.1% NP-40, and 1.5 mM spermine was added, and the suspension was left to stand for 10 minutes. The resulting nuclei were stained with 150 μl of dye buffer consisting of 2 mg/ml propidium iodide (PI), 0.5 mM Tris-HCl (pH 7.6), 3.4 mM trisodium citrate, 0.1% NP-40, and 4.8 mM spermine for 10 minutes. After filtration through a nylon mesh, the PI-stained cells were subjected to flow cytometric analysis with a FACScan (Beckton-Dickinson) and the percentage of cells in each phase of the cell cycle was determined by analysis of DNA histograms of 1×10^4 cells, using the Cell-fit program (Beckton-Dickinson).

Inhibition of Macromolecular Syntheses

The effects of TAN-1518 A on DNA, RNA, and protein synthesis was determined by measuring the amounts of radiolabeled precursors incorporated into HeLa S3 cells. The cells (1×10^3/well) in 0.1 ml of culture medium were seeded in a 96-well plate. After 24-hours incubation, TAN-1518 A was added, and the cells were incubated for a further 18 hours after which they were treated with 0.1 μCi/ml of [6-^{3}H]thymidine, [6-^{3}H]uridine, or L-[^{35}S]methionine in the last 2 hours of incubation. The culture supernatant was then discarded, and monolayer cells were washed twice with PBS and treated with trypsin-EDTA solution, consisting of 0.25% trypsin and 0.02% EDTA, at 37°C for 10 minutes. The cells were harvested on glass fiber filter disks and washed twice with PBS; radioactivity associated with the filter disks was measured with a liquid scintillation counter (Beckman LS6000TA).

Antimicrobial Activity

The antimicrobial activity of TAN-1518 A was determined by the agar dilution method. Antibiotic medium 3 supplemented with yeast extract 0.5% was used for common bacteria; trypticase soy agar (TSA) supplemented with glycerol 3% was used for acid-fast bacteria; and TSA supplemented with glucose 1% was used for yeasts and fungi. Bacteria were grown at 37°C for 18 hours, and yeasts and fungi were grown at 28°C for 48 hours.

Materials

Supercoiled pBR322 DNA, RNase A, EtBr, and proteinase K were purchased from Wako Pure Chemical Co. (Osaka, Japan). Catenated kDNA was purchased from TopoGEN Inc. (Ohio, U.S.A.). Calf thymus Topo I was purchased from Takara Shuzo Co. (Kyoto, Japan). Topo II was purified to homogeneity from calf thymus gland, as described by SCHOMBURG *et al.*[17] EAGLE's MEM, RPMI-1640 medium, and FBS were purchased from Whittaker M. A. Bioproducts Inc. (Maryland, U.S.A.). FBS was inactivated by heating at 56°C for 30 minutes prior to use. Camptothecin (CPT), *m*-AMSA, PI, salmon sperm DNA, and NP-40 were purchased from Sigma Chemical Co. (Missouri, U.S.A.). Culture plates were purchased from Corning Laboratory Sciences Co. (New York, U.S.A.). Antibiotic medium 3 and

TSA were purchased from Difco Loboratories Co. (Michigan, U.S.A.) and Becton Dickinson and Co. (Maryland, U.S.A.), respectively.

Results

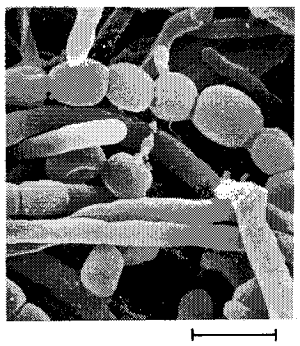
Taxonomy of the Producing Organism

Strain AL-16012 was isolated from a soil sample collected in Amino-cho, Takeno county, Kyoto prefecture, Japan. Whole-cell analysis of the strain showed the presence of LL-diaminopimelic acid. The aerial mycelia of the strain were gray. Each spore chain of this strain was bent and spiral, and consisted of about 10~50 spores, which were cylindrical and had a smooth surface (Plate 1). No soluble pigment production was observed. The culture and physiological characteristics of the strain are shown in Table 1.

These characteristics of strain AL-16012 indicate that it belongs to the genus *Streptomyces*. Therefore, the strain was named *Streptomyces* sp. AL-16012 and has been deposited in the Institute for Fermentation, Osaka under the accession No. IFO 15242. The strain was also deposited in the National Institute of Bioscience and Human-

Plate 1. Scanning electron micrographs of producing strain AL-16012.

Bar represents 1 μm.



The organism was cultured on ISP-2 agar for 2 weeks at 28°C.

Table 1. Culture and physiological characteristics of strain AL-16012.

Culture characteristics		
Yeast extract-malt extract agar (ISP-2)	G:	Moderate, Lt wheat (2ea)
	A:	Moderate, gray (3fe)
	R:	Lt mustard tan (2ie) to mustard (2ni)
	P:	None
Oatmeal agar (ISP-3)	G:	Moderate, Lt wheat (2ea)
	A:	Moderate, gray (3fe)
	R:	Mustard (2le) to mustard brown (2ni)
	P:	None
Inorganic salts-starch agar (ISP-4)	G:	Moderate, Lt wheat (2ea)
	A:	Moderate, gray (3fe)
	R:	Mustard (2le) to mustard brown (2ni)
	P:	None
Glycerol-asparagine agar (ISP-5)	G:	Moderate, Lt wheat (2ea)
	A:	Moderate, gray (3fe)
	R:	Mustard (2le) to mustard brown (2ni)
	P:	None
Peptone-yeast extract-iron agar (ISP-6)	G:	Moderate, Lt wheat (2ea)
	A:	Moderate, gray (3fe)
	R:	Mustard (2le) to mustard brown (2ni)
	P:	None
Tyrosine agar (ISP-7)	G:	Moderate, Lt wheat (2ea)
	A:	Moderate, gray (3fe)
	R:	Mustard (2le) to mustard brown (2ni)
	P:	None
Physiological characteristics		
Temperature range for growth	12 to 30°C	
Nitrate reduction	–	
Starch hydrolysis	+	
Milk peptonization	–	
Milk coagulation	–	
Gelatin liquefaction	–	
Utilization of carbon sources		
Positive	Glucose, rhamnose, raffinose[a]	
Negative	Arabinose, xylose, fructose, sucrose, inositol, mannitol	

[a] Weakly positive.

G: Growth, A: aerial mass color, R: reverse side color of colony, P: soluble pigment.

Technology, Agency of Industrial Science and Technology, Japan, with the accession No. FERM BP-3660.

Production and Isolation

TAN-1518 A and B were produced by culturing strain AL-16012. The production of the major metabolite, TAN-1518 A, reached a maximum (87 μg/ml) at 138 hours after inoculation.

TAN-1518 components consist of three compounds, A (**1**), B (**2**) and X (**3**). Since they are fat-soluble and weakly acidic, the acidified culture broth was extracted with EtOAc. The organic layer was then washed with aqeous $NaHCO_3$ and water and concentrated. The residue was chromatographed on a silica gel column, giving **1** and **3**. A minor constituent, **2**, was purified by preparative HPLC, using a column of octadecylsilane (ODS).

Chemical Characterization and Determination of Structure

Compounds **1** and **2** were soluble in MeOH, $CHCl_3$, EtOAc, and Me_2CO, but insoluble in water and hexane. They gave positive color reactions with potassium permanganate, phosphomolybdic acid, and sulfuric acid, and negative color reaction with ninhydrin and Dragendorff reagents. The physico-chemical properties of TAN-1518 A (**1**) and B (**2**) are summarized in Table 2. The molecular formulae were determined to be $C_{39}H_{41}NO_{15}$ for **1** and $C_{41}H_{45}NO_{15}$ for **2**, on the basis of elemental analyses, mass spectra, and ^{13}C NMR spectra. The IR spectra in KBr showed the absorption bands at 3410 cm^{-1} (NH and OH), 1720 (sh, ester), 1660 (amide). The UV spectra resembled those of tetracyclines. The ^1H and ^{13}C NMR data are summarized in Tables 3 and 4. The signals were assigned based on 2D NMR experiment, *i.e.*, ^1H-^1H correlation spectroscopy (COSY), ^1H-^{13}C COSY, and long range ^1H-^{13}C COSY. These spectra indicated the presence of *C*-glycoside, salicylic acid, and 2-methyl-2-butenoic acid moieties in **1** and **3**. In fact, alkaline hydrolysis of **1** with 1 N NaOH for 6 hours at room temperature gave salicylic acid (**4**) and 2-methyl-2-butenoic acid (**5**). The configuration of the double bond in **5** was determined to be *Z*, by comparing the chemical shift of β-methyl carbon (δ 20.3) in **5** (angelic acid and that in tiglic acid ([*E*]-2-methyl-2-butenoic acid, δ 11.5).

From its physico-chemical properties, we determined that compound **3** was identical to SF2575,[9] a tetracycline antibiotic with *C*-glycoside, salicylic acid, and angelic moieties. Compound **1** was structurally similar to **3**; we compared its physico-chemical properties with those of **3**, and found that one of the two methoxy groups observed in **3** was not observed in **1**. A long-range coupling was found between the methoxy protons (6-OCH_3, δ 3.20) and C-6 (δ 77.9). Further, the chemical shift of C-12a (δ 75.1) in **1** showed an

Table 2. Physico-chemical properties of TAN-1518 A (**1**) and B (**2**).

	1	**2**
Appearance	Yellow powder	Yellow powder
$[\alpha]_D^{25}$	+190° (*c* 0.6, $CHCl_3$)	+206° (*c* 0.5, $CHCl_3$)
MS (*m/z*)	763 $(M)^+$ (FD-MS)	792 $(M+H)^+$ (SI-MS)
Molecular formula	$C_{39}H_{41}NO_{15}$	$C_{41}H_{45}NO_{15}$ ($\frac{1}{2}H_2O$)
Anal Calcd:	C 61.33, H 5.41, N 1.83	C 61.24, H 5.75, N 1.73
Found:	C 60.72, H 5.51, N 1.76	C 61.42, H 5.71, N 1.81
UV λ_{max}^{MeOH} nm (ε)	206 (49,600), 227 (sh, 28,600), 243 (sh, 24,400), 270 (sh, 17,500), 315 (10,700), 368 (15,600)	205 (56,800), 226 (sh, 32,800), 241 (sh, 29,200), 270 (19,600), 310 (12,800), 375 (17,600)
IR (KBr) cm^{-1}	3410, 2980, 2940, 1660, 1610, 1430, 1290, 1160, 1080, 840, 760	3410, 2970, 1660, 1610, 1560, 1430, 1290, 1160, 840, 760

Table 3. ^{1}H NMR spectral data of TAN-1518 A (**1**) and B (**2**) (δ ppm, in DMSO-d_6, J=Hz).

	1	2
4	6.32 (d, 5.1)	6.12 (d, 5.1)
4a	2.86 (ddd, 13.0, 5.1, 2.5)	3.24 (m)
5	1.60 (m), 2.18 (m)	1.64 (q, 12.1), 2.21 (m)
5a	3.49 (dd, 11.0, 5.5)	3.48 (m)
6-OMe	3.20 (s)	3.19 (s)
7	6.98 (d, 7.9)	6.98 (d, 7.8)
8	7.72 (d, 7.9)	7.72 (d, 7.8)
12-OMe	—	3.52 (s)
14	1.05 (s)	1.05 (s)
15	4.82 (d, 11.2)	4.82 (d, 11.2)
16	1.50 (m), 2.21 (m)	1.49 (q, 11.6), 2.21 (m)
17	3.80 (m)	3.80 (m)
18	4.61 (t, 9.5)	4.68 (t, 9.3)
19	3.62 (dq, 9.5, 6.1)	3.62 (dq, 9.3, 6.1)
20	1.13 (d, 6.1)	1.14 (d, 6.1)
4′	7.05 (d, 7.9)	7.05 (d, 8.0)
5′	7.57 (t, 7.9)	7.57 (t, 8.0)
6′	7.02 (t, 7.9)	7.02 (t, 8.0)
7′	7.86 (d, 7.9)	7.86 (d, 8.0)
3″	6.12 (q, 7.2)	6.02 (q, 7.2)
4″	1.94 (d, 7.2)	1.92 (d, 7.2)
5″	1.88 (s)	2.24 (m)
6″	—	1.01 (t, 7.4)
3-OH	17.3 (br)	17.1 (br)
10-OH	12.4 (s)	12.1 (s)
12-OH	14.9 (s)	14.9 (s)
12a-OH	7.26 (s)	—
13-NH_2	9.48 (br)	9.40 (br)
17-OH	5.13 (d, 5.6)	5.10 (d, 5.9)
3′-OH	10.7 (s)	10.3 (s)

Table 4. ^{13}C NMR spectral data of TAN-1518 A (**1**) and B (**2**). (δ ppm, in DMSO-d_6).

	1	2		1	2
1	192.0[a]	189.4[a]	14	24.3	24.4
2	95.4	95.8	15	70.3	70.3
3	189.0[a]	189.2[a]	16	40.3	40.4
4	70.3	69.4	17	69.1	69.1
4a	40.9	36.5	18	77.5	77.4
5	19.0	19.2	19	73.6	73.6
5a	34.9	34.9	20	18.0	18.0
6	77.9	77.7	1′	167.2	167.0
6a	147.3	147.2	2′	113.2	113.2
6-OMe	50.2	50.2	3′	160.0	159.9
7	114.8	114.8	4′	117.6	117.6
8	133.7	133.8	5′	135.8	135.8
9	126.8	126.8	6′	119.4	119.4
10	158.1	158.0	7′	130.2	130.2
10a	114.5	114.4	1″	166.9	167.0
11	192.2	192.3	2″	126.7	128.6
11a	106.0	108.2	3″	136.8	134.0
12	175.9	173.9	4″	15.4	15.2
12a	75.1	80.9	5″	20.2	27.0
12-OMe	—	54.2	6″	—	13.5
13	173.1	173.9			

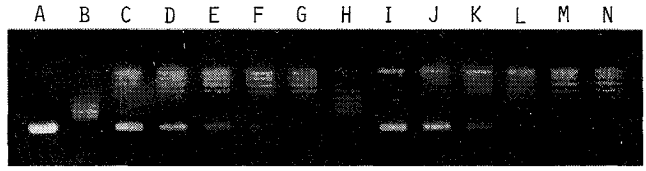
[a] May be interchangeable.

up field shift by 5.8 ppm, compared with the corresponding carbon (δ 80.9) in **3**. Thus, **1** was determined to be 12a-*O*-demethyl compound **3**. Compound **2** was also structurally similar to **1**. The differences between **2** and **1** were found in the angelic acid moiety and the methoxy group. Comparing the ^{13}C NMR spectra, a methylene carbon signal at 27.0 ppm was observed in **2**. The proton signal at 1.88 ppm (3H, s, 5″-CH_3) of **1** was missing, and signals at 2.24 ppm (2H, m, $-CH_2-$) and 1.01 ppm (3H, t, J=7.4 Hz, $-CH_3$) were observed in **2**. Furthermore, in the NOESY experiment of **2**, a cross peak was observed between the methyl signal (δ 1.01) and the olefin signal at 6.02 ppm (1H, q, J=7.2 Hz). From these results, we determined that **2** had a (*Z*)-2-ethyl-2-butenoic acid moiety instead of angelic acid. Further, methoxy protons (12a-OCH_3, δ 3.52) correlated to C-12a (δ 80.9) in the COLOC spectrum were observed in **2**. Thus, the structure of **2** was determined, as shown in Fig. 1.

Biological Activity

The inhibitory activity of TAN-1518 A on the relaxation of plasmid DNA by Topo I is shown in Fig. 2. TAN-1518 A inhibited the Topo I activity in a dose-dependent manner; partial inhibition was observed even at a concentration as low as 2.5 μM. The inhibitory activity was virtually comparable to that of CPT, a specific inhibitor of Topo I. The inhibitory activity of TAN-1518 B was slightly weaker than that of TAN-1518 A (Table 5). These findings demonstrate that both metabolites are potent inhibitors of Topo I.

Fig. 2. Effects of TAN-1518 A on the relaxation activity of calf thymus Topo I.



Electrophoresis was carried out in a 0.7% agarose gel with 0.1% SDS at 100 volts for 35 minutes. Lane A, ccc DNA control; lane B, relaxed DNA control; lanes C to H, TAN-1518 A; lanes I to N, CPT. Drug concentrations were: lanes C and I, 100 μM; lanes D and J, 50 μM; lanes E and K, 25 μM; lanes F and L, 10 μM; lanes G and L, 5 μM; lanes H and N, 2.5 μM.

Table 5. Inhibitory activity of TAN-1518 A and B against calf thymus Topo I.

Conc. (μM)	TAN-1518 A	TAN-1518 B	CPT
100	+	+	+
50	+	+	+
25	+	+	+
10	w	w	+
5	w	w	w
2.5	w	−	w

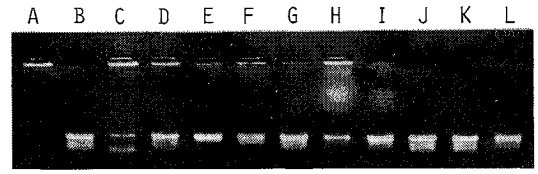
+, Inhibition; w, weak inhibition; −, no inhibition.

Table 6. Growth inhibitory activity of TAN-1518 A and B against murine and human tumor cell lines.

Cell	IC_{50} (ng/ml)	
	TAN-1518 A	TAN-1518 B
P815 murine mastcytoma	16.5	7.2
EL4 murine lymphoma	10.3	11.3
B16 murine melanoma	8.1	1.3
HeLaS3 human epitheloid carcinoma	0.7	2.0
WiDr human colon adenocarcinoma	233.5	11.4
A549 human lung carcinoma	167.7	4.2
G361 human melanoma	38.5	7.8
SW48 human colon adenocarcinoma	103.5	12.2

Each cell line was cultured with each metabolite in a 96-well plate for 72 hours. Proliferation was evaluated by MTT assay. Initial cell concentrations were: EL4, P815 and A31, 2×10^4/ml; HeLa S3, WiDr, A549, and G361, 4×10^4/ml; B16, 5×10^4/ml; SW48, 1×10^5/ml.

Fig. 3. Effects of TAN-1518 A on the decatenation activity of calf thymus Topo II.



Electrophoresis was carried out in a 1.0% agarose gel with 0.1% SDS at 100 volts for 20 minutes. Lane A, catenated kDNA control; lane B, decatenated minicircle control; lanes C to G, *m*-AMSA; lanes H to L, TAN-1518 A. Drug concentrations were: lanes C and H, 125 μM; lanes D and I, 62.5 μM; lanes E and J, 31.3 μM; lanes F and K, 15.7 μM; lanes G and L, 7.9 μM.

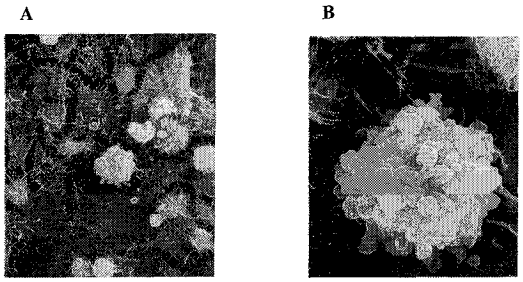
To determine whether or not these metabolites were specific inhibitors of Topo I, we examined the effect of TAN-1518A on the decatenation of kDNA by Topo II. TAN-1518 A slightly inhibited the enzyme activity at a concentration of 62.5 μM, whereas *m*-AMSA, a specific inhibitor o Topo II, inhibited the activity even at the concentration of 7.9 μM (Fig. 3). This indicates that the activity of TAN-1518 A is relatively specific to Topo I. Similar results were also obtained using TAN-1518 B.

The mode of action of these metabolites was investigated by the following studies; we used the major metabolite TAN-1518 A because of its stronger inhibitory activity against Topo I.

The cytotoxicity of TAN-1518 A is shown in Table 6. This metabolite strongly suppressed the growth of all tumor cell lines tested.

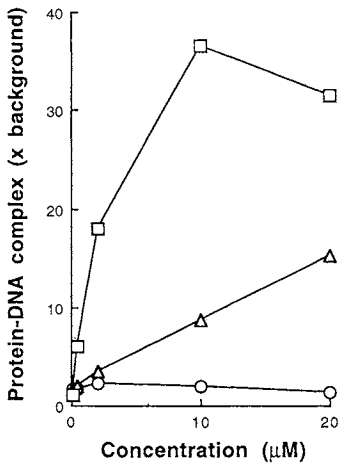
The morphology of HeLa S3 cells treated with TAN-1518 A is shown in plate 2. The treated cells formed typical apoptotic bodies in which surface microvilli were lost. Similar results were also obtained with SW48 cells (data not shown). To confirm the induction of apoptosis, we extracted chromosomal

Plate 2. Scanning electron micrographs of HeLa S3 cells treated with TAN-1518 A.



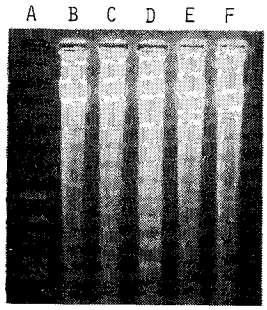
Cells were treated with 1 μM of TAN-1518 A for 18 hours. A, $\times$1,000; B, $\times$4,000.

Fig. 5. Effects of TAN-1518 A on the stimulation of cleavable complex formation.



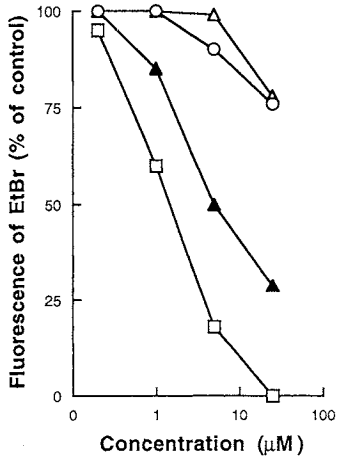
CHO-K1 cells labeled with [6-^{3}H] thymidine were incubated with each drug for 1 hour. Protein-DNA complex in cell nuclei was measured by the procedure described in "Materials and Methods". Circle, TAN-1518 A; triangles, CPT; squares, *m*-AMSA.

Fig. 4. Effects of TAN-1518 A on the induction of apoptosis in various human tumor cell lines.



Each cell line was treated with 1 μM of TAN-1518 A for 18 hours. Electrophoresis was carried out in a 1.5% agarose gel with 0.1% SDS at 100 volts for 40 minutes. Lane A, $\phi\times$-174/*Hinc*II marker; lane B, SW48 human colon adenocarcinoma; lane C, G361 human melanoma; lane D, WiDr human colon adenocarcinoma; lane E, HeLa S3 human epitheloid carcinoma; lane F, human lung adenocarcinoma.

Fig. 6. Effects of TAN-1518 A on DNA binding competition with ethidium bromide.

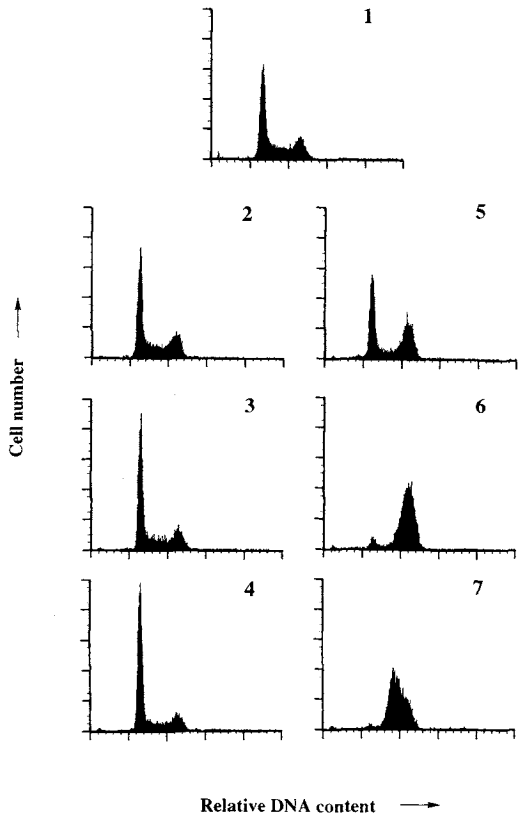


Each drug was mixed with 5 μM EtBr and 40 μM calf thymus DNA. The percent fluorescence intensity was calculated by setting the intensity of 5 μM EtBr alone as 0% and that of 5 μM EtBr in the presence of 40 μM of calf thymus DNA as 100%. Circles, TAN-1518 A; triangles, *m*-AMSA; closed triangles, actinomycin D; squares, adriamycin.

DNA from the nuclei of tumor cells treated with TAN-1518 A and analyzed it by agarose gel electrophoresis. DNA fragmentations corresponding to the nucleosomal ladders were detected in all cell lines tested (Fig. 4). These findings indicate that TAN-1518 A strongly induces apoptosis in tumor cells.

Most topoisomerase inhibitors inhibit the enzyme by stabilizing the enzyme-DNA reaction intermediate cleavable complex.[2] In our experiments, TAN-1518 A did not increase the protein-DNA complex in the nuclei of CHO-K1 cells at the concentrations tested, whereas CPT and *m*-AMSA, both of which stabilize the cleavable complex, increased the complex in a dose-dependent manner (Fig. 5). These findings indicate

Fig. 7. Effects of TAN-1518 A on the cell cycle of HeLa S3 cells.



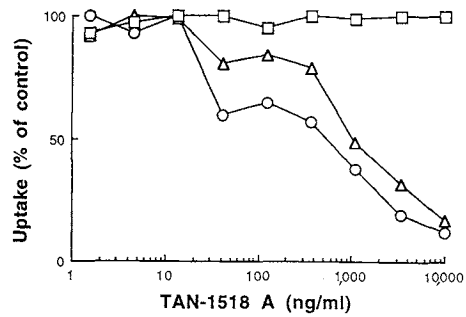
Exponentially growing cells were treated with TAN-1518 A for 24 hours. 1, no drug control; 2, TAN-1518 A, 50 nM; 3, TAN-1518 A, 200 nM; 4, TAN-1518 A, 1 μM; 5, CPT, 20 nM; 6, CPT, 50 nM; 7, CPT, 100 nM.

that TAN-1518 A inhibits Topo I in a different manner from CPT.

To determine whether TAN-1518 A had the ability to intercalate into DNA strands, we employed an EtBr-competition assay with salmon sperm DNA. This metabolite weakly reduced the fluoresence intensity of EtBr (Fig. 6). The inhibitory effect of TAN-1518A was weaker than that of actinomycin D or adriamycin, but stronger than that of *m*-AMSA, these results suggesting that TAN-1518 A is weakly intercalated into double-stranded DNAs.

The effects of TAN-1518 A on the cell cycle of HeLa S3 cells in logarithmic growth are shown in

Fig. 8. Effects of TAN-1518 A on the incorporation of labeled precursors into DNA, RNA, and protein.



Cells (1×10^3/well) were precultured for 24 hours and then treated with TAN-1518 A for 18 hours. During the last 2 hours of culture, the cells were treated with each radiolabeled precursor. Circles, [6-^{3}H]thymidine; triangles, [6-^{3}H]uridine; squares, L-[^{35}S]-methionine.

Table 7. Antimicrobial spectra of TAN-1518 A.

Test organism	MIC (μg/ml)
Escherichia coli K2	>100
E. coli NIHJ JC-2	>100
Proteus mirabillis ATCC 21100	>100
P. vulgaris IFO3045	>100
P. morganii IFO 3168	>100
Klebsiela pneumonia IFO 3168	>100
Serratia marcescens IFO 3046	>100
Salmonella typhimurium IFO 12529	>100
S. enteritidis IFO 3313	>100
Citrobacter freundii IFO 12681	>100
Pseudomonas aeruginosa IFO 3080	>100
Alcaligenes faecalis IFO 13111	>100
Bacillus subtilis PCI219	12.5
B. cereus IFO 3514	50
B. pumilus IFO 3813	6.25
B. megaterium IFO 12108	12.5
Staphylococcus aureus FDA 209P	25
Micrococcus luteus IFO 12708	25
M. flavus IFO 3242	25
Mycobacterium avium IFO 3154[a]	>100
M. phlei IFO 3158[a]	>100
M. segmatis ATCC 607	>100
Candida albicans IFO 0583[b]	>100
Cryptococcus neoformans IFO 0410[b]	>100
Saccharomyces serevisiae IFO 0209[b]	>100
Candida parakrusei IFO 0640[b]	>100
Penicillium chrysogenum IFO 4626[b]	>100
Aspergillus niger IFO 4066[b]	>100

Minimum inhibitory concentration (MIC) was determined by the agar dilution method with antibiotic medium 3+0.5% yeast extract, [a]TSA+3% glycerin, [b]TSA+1% glucose.

Fig. 7. The cells in G1 phase increased, while the cells in S and G2/M phase decreased in a dose-dependent manner. In contrast, CPT, at concentrations of 20 to 50 nM, increased the cells in G2 phase, while at a concentration of 100 nM, most of the cells were arrested in S phase. Similar results were also obtained with WiDr cells (data not shown).

The effects of TAN-1518 A on macromolecular synthesis in HeLa S3 cells are shown in Fig. 8. TAN-1518 A predominantly inhibited the uptake of [6-^{3}H]thymidine in the cells and this also impaired the uptake of [6-^{3}H]uridine. The uptake of L-[^{35}S]methionine was not affected by the addition of the metabolite, even at a concentration of 10 μg/ml. These results indicate that TAN-1518 A impairs the synthesis of both DNA and RNA in HeLa cells.

The antimicrobial activity of TAN-1518 A is shown in Table 7. This metabolite was weakly active against some Gram-positive bacteria but showed no antimicrobial activity against Gram-negative bacteria, acid-fast bacteria, yeasts, or fungi.

In vivo antitumor activity was tested in an experimental murine tumor model. Meth A fibrosarcoma cells (1×10^6 per mouse) were implanted subcutaneously in CDF1 mice and tumor weight was measured on day 10. TAN-1518 A (10 mg/kg) given intraperitoneally on day 1 and day 2 significantly suppressed the growth of Meth A tumor (T/C=57%).

Discussion

We have described here the novel Topo I inhibitors, TAN-1518 A and B, which belong to the tetracycline family.[18] TAN-1518 A was shown to predominantly impair the uptake of [6-^{3}H]thymidine in HeLa cells, to strongly suppress the growth of various tumor cells, and to induce apoptosis in these cells. These findings imply that the inhibitory activity of these metabolites against Topo I may be involved in the apoptotic cell death of tumor cells.[19,20] These metabolites also showed weak inhibitory activity against Topo II; this phenomenon may be due to their weak activity in intercalating into DNA strands.

Unlike CPT, TAN-1518 A did not stabilize the cleavable complex in the nuclei of HeLa cells.[21~25] Further, this metabolite prevented the death of yeast cells caused by CPT (data to be published elsewhere). These findings suggest that TAN-1518 A may inhibit some earlier steps that occur before cleavable complex formation in the catalytic cycle of Topo I.[24,25] These metabolites would therefore be expected to be useful for clarifying the function of Topo I. TAN-1518 A arrested HeLa cells in G1 phase of the cell cycle, and this indicates that Topo I may play a crucial role in this phase of the mammalian cell cycle.

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