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Novel Selective Inhibitors for Human Topoisomerase I, BM2419-1 and -2 Derived from Saintopin

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Compounds BM2419-1 and -2 were isolated from a culture broth of a fungus *Paecilomyces* sp. BM2419. It was shown that these novel compounds were artifacts derived from saintopin, a dual inhibitor of topoisomerase I and II by independent processes. In the human topoisomerase I inhibition assay using the recombinant *Saccharomyces cerevisiae*, BM2419-1 and -2 inhibited selectively the yeast growth dependent on human topoisomerase I induction with IC_{50} values of 0.3 ng/ml and 6.0 ng/ml, respectively.

DNA topoisomerases are nuclear enzymes essential for controlling DNA topology in mammalian cells, such as replication, transcription and recombination processes[1]. Many clinically important anticancer drugs are known to be lethal to cells by inhibiting topoisomerase II (Top2)[2]. Topoisomerase I (Top1) was shown to be a principal target for the anticancer drugs, such as camptothecin and its derivatives[3,4].

WANG *et al.*[5] reported a human Top1 (hTop1) inhibition assay using recombinant yeasts, in which the cleavable complex type hTop1 inhibition can be detected by yeast growth inhibition. In the course of a screening program for hTop1 selective inhibitors by this method, we have found new active compounds BM2419-1 and -2 which are considered to be derived from saintopin during the isolation process[6,7]. This paper reports the isolation, structure determination and biological activity of the two new compounds.

Results

Preparation of BM2419-1 (1) and -2 (2)

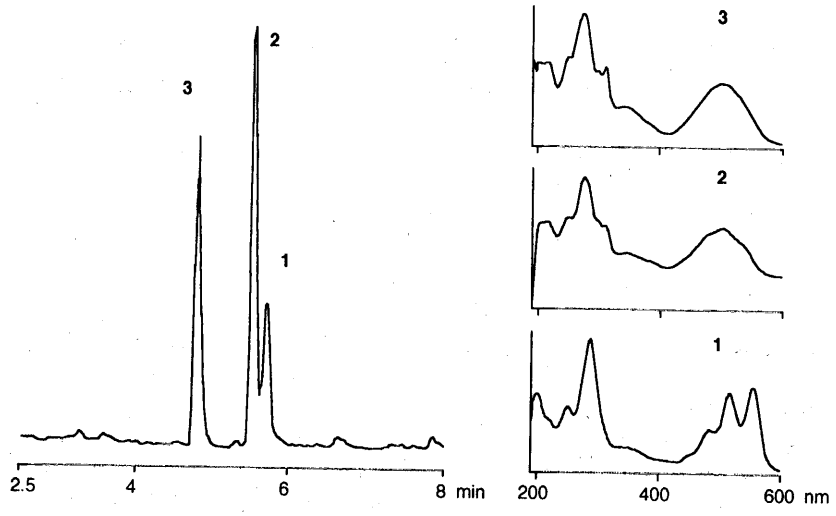
Mycelia of *Paecilomyces* sp. BM2419 was extracted with 70% aqueous acetone. After filtration and concentration, the extract was dissolved in DMSO and analyzed by HPLC. Two new active peaks, **1** and **2** were detected as shown in Fig. 1. Another peak **3** was identified as saintopin from its UV, MS and NMR data. However, **1** and **2** were not present in a fresh BM2419 culture broth. Finally it was found that **1** and **2** were each derived from saintopin by a different process. **1** required light for conversion from saintopin, such as sunlight, fluorescent or halogen lamp, while **2** required DMSO and acetone extracts for the conversion. The isolation procedures of these compounds **1** and **2** were summarized as shown in Fig. 2.

Structure Determination

The molecular formula of **1** was determined to be $C_{18}H_{10}O_8$ by HRFAB-MS ($[M-H]^-$ m/z 353.0291, calcd. 353.0297) (Table 1). Since the ^{1}H-NMR spectrum indicated only three broad protons and the ^{13}C-NMR spectrum showed only nine carbons, **1** was considered to have a symmetric structure. Acetylation of **1** was performed with pyridine and acetic anhydride, giving the 1,3,8,10-tetraacetate (**4**), 1,3,6,8,10-pentaacetate and 1,3,6,8,10,11-hexaacetate. In the ^{1}H-NMR spectrum, the pentaacetate and hexaacetate had four aromatic proton signals, however **1** and **4** had two. The spectrum of **4** showed a new phenolic proton at 14.99 ppm instead of H-6 singlet at 8.22 ppm in saintopin-1,3,8,10-tetraacetate (**6**). Therefore, the structure of **1** was determined as shown in Fig. 3. The symmetric structure of **1** results from the formation of the two hydrogen bonds, between 6-OH and 5-keto and between 11-OH and 12-keto, followed by keto-enol tautomerism as shown in Fig. 4.

The molecular formula of **2** was determined to be $C_{19}H_{12}O_7$ (14 mass units over saintopin) by HRFAB-MS ($[M-H]^-$ m/z 351.0537, calcd. 351.0305). The UV spectrum was quite similar to saintopin, indicating that

Fig. 1. HPLC analysis of BM2419.



Mycelia of BM2419 were extracted with acetone and filtered, after concentration extract was dissolved in DMSO and analyzed.

HPLC conditions

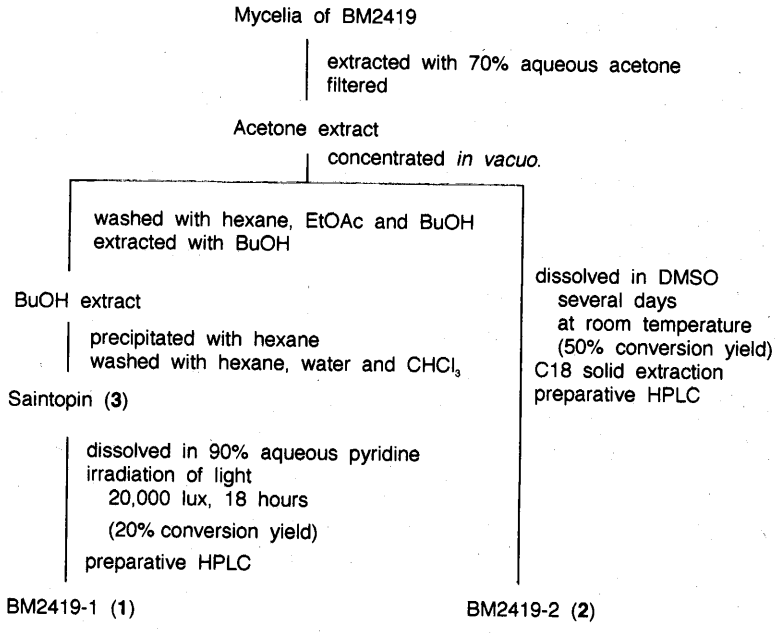
Column: Capcell pak C 18 UG 120 4.6 mm i.d. × 100 mm.

Flow: 1 ml/minute, temp.: 50°C, detect.: UV 503 nm.

Solvent: MeCN - water (0.05% TFA), linear gradient from 40% MeCN to 70% MeCN till 8 minutes.

1: BM2419-1, **2**: BM2419-2, **3**: Saintopin.

Fig. 2. Isolation of BM2419-1 and-2.



it is an analogue of saintopin. The 1,3,8,10-tetraacetate of **2** (**5**) was prepared by the same method as in the case of **1**. The ^{1}H-NMR spectrum of **5** was quite similar to **6**. However, **5** had a methyl proton at 2.96 (s) instead of H-6 in **6**, and a NOE was observed between the methyl proton and H-7 at 7.95 (d, J=2.36 Hz) in a difference NOE experiment. Therefore, the structure of **2** was elucidated as shown in Fig. 3.

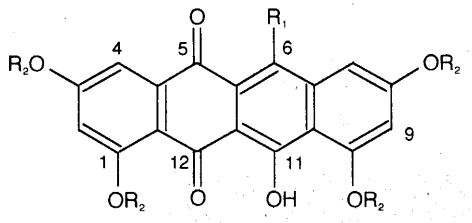
Table 1. Physico-chemical properties of BM2419-1 (**1**) and -2 (**2**).

	BM2419-1 (**1**)	BM2419-2 (**2**)
Appearance	purple powder	orange powder
UV λmax (MeOH) nm	243, 283, 533, 569	247, 276, 307, 345, 508
FAB-MS (m/z)	353 $(M-H)^-$	351 $(M-H)^-$
HRFAB-MS (m/z)		
Found	353.0291 $(M-H)^-$	351.0537 $(M-H)^-$
Calcd.	353.0297	351.0505
Molecular formula	$C_{18}H_{10}O_8$	$C_{19}H_{12}O_7$

Table 2. ^{1}H-NMR data of the 1, 3, 8, 10-tetraacetate derivatives.

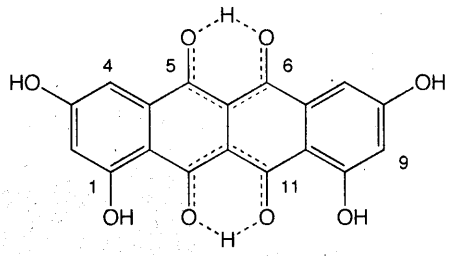
	^{1}H-NMR ppm (multiplicity, J in Hz, $CDCl_3$)		
Pos.	**4**	**5**	**6**
2	7.28 (d, 2.45)	7.24 (d, 2.36)	7.30 (d, 2.14)
4	8.18 (d, 2.45)	7.97 (d, 2.36)	8.06 (d, 2.14)
6	-	-	8.22 (s)
7	8.18 (d, 2.45)	7.95 (d, 2.36)	7.67 (d, 2.14)
9	7.28 (d, 2.45)	7.16 (d, 2.36)	7.13 (d, 2.14)
6-Me	-	2.96 (s) (3H)	-
6-OH	14.99 (s)	-	-
11-OH	16.09 (s)	15.96 (s)	15.28 (s)
Ac	2.46 (s)	2.47 (s)	2.49 (s)
	2.46 (s)	2.44 (s)	2.44 (s)
	2.38 (s)	2.38 (s)	2.37 (s)
	2.38 (s)	2.37 (s)	2.37 (s)

Fig. 3. Structure of BM2419-1 and -2.



Compounds	R_1	R_2
BM2419-1 (**1**)	OH	H
BM2419-2 (**2**)	Me	H
Saintopin (**3**)	H	H
4	OH	Ac
5	Me	Ac
6	H	Ac

Fig. 4. The symmetric structure of BM2419-1 (**1**).



Biological Properties

The hTopl inhibition activity of **1** and **2** using recombinant yeasts is shown in Table 3. Saintopin, a Topl, 2 dual inhibitor exhibited the growth inhibition independent of hTopl induction. However **1** and **2** inhibited yeast growth dependent on hTopl induction with IC_{50} values 0.3 ng/ml and 6.0 ng/ml, respectively. The selectivity of **1** and **2** were superior to that of saintopin, and were comparable to that of camptothecin, a Topl specific inhibitor. These compounds did not inhibit the growth of *S. cerevisiae* W303-1A and *Candida albicans* ATCC 38247 at 50,000 ng/ml (50 μg/ml) as did saintopin and camptothecin (data not shown).

The effect of **1** and **2** on the relaxation of supercoiled DNA by hTopl is shown in Fig. 5. **1** and **2** inhibited the Topl conversion of supercoiled DNA to relaxed DNA in dose-dependent manner. Partial inhibition was observed at 3.2 μg/ml and 6.3 μg/ml, respectively. This result indicated the hTopl inhibition by **1** and **2** was

Table 3. Growth inhibition of recombinant yeasts by BM2419-1 (**1**) and -2 (**2**).

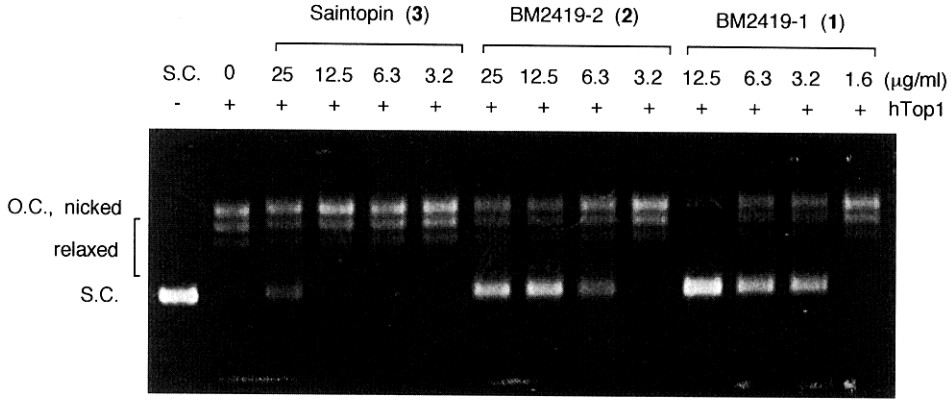
	IC_{50} μg/ml			selectivity*
Organism	*Saccharomyces cervisiae*			
Strains	JN2-134 hTOP1		JN2-134	
Remarks	Top1 induction	Top1 non-induction	Top1 deficient	
BM2419-1 (**1**)	0.3	>50,000	50,000	>160,000
BM2419-2 (**2**)	6.0	>50,000	>50,000	>8,000
Saintopin (**3**)	1.0	20	12	20
Camptothecin	0.1	>50,000	>50,000	>500,000

* $\frac{IC_{50} \text{ value of Top1 non-induction}}{IC_{50} \text{ value of Top1 induction}}$

Table 4. Cytotoxicity of BM2419-1 (**1**) and -2 (**2**).

	IC_{50} (μg/ml)			
Cell lines	BM2419-1 (**1**)	BM2419-2 (**2**)	Saintopin (**3**)	Camptothecin
MKN28	0.7	3.1	2.1	0.12
KB	0.1	0.8	0.3	0.003
HeLaS3	0.2	1.5	0.4	0.005
HeLa	0.6	1.5	2.1	0.005
MOLT4	0.2	2.0	1.0	0.016
K562	0.2	1.5	0.8	0.016
NIH/3T3	1.6	3.1	4.1	0.26
EL-4	0.7	1.5	1.5	0.012
B-16	1.2	2.1	3.8	0.13

Fig. 5. Effect of BM2419-1 and -2 on human Top1-induced DNA conversion from supercoiled DNA to relaxed DNA.



O.C.: open circular DNA, S. C.: supercoiled DNA.

stronger than that of saintopin.

The cytotoxicity of **1** and **2** is shown in Table 4. These compounds inhibited the growth of all the tumor cell lines tested equally as saintopin, but more weakly than camptothecin.

Discussion

Electrospray ionization MS (ESI-MS) of **2** prepared from saintopin by treatment with DMSO-d_6 indicated a molecular weight of 355. Therefore, the 6-Me of **2** originates from DMSO. However **2** was not formed by

treatment of pure saintopin with DMSO. The conversion requires the acetone extracts of the culture broth. A methylation reaction with DMSO was reported, which requires strong base as a catalyst[8,9]. Thus the conversion from saintopin to **2** was considered to be a novel reaction. The possible reaction mechanism from saintopin to **1** may be a Diels-Alder type addition reaction of molecular oxygen, followed by cleavage of the peroxide.

The results of two hTop1 inhibition assays suggested that substitution at position 6 of saintopin is important for Top1, 2 selectivity. Addition of functional groups to saintopin can affect the biological activity as reported for saintopin analogs UCE6[10], UCE1022[11] and saintopin E[12].

In the hTop1 inhibition assay with recombinant yeasts, compounds **1**, **2** and saintopin showed considerable decrease of activity upon addition of fetal calf serum (FCS) (data not shown), but no decrease was observed for camptothecin.

Experimental

General Methods

NMR data were collected on a JEOL JNM-A500 spectrometer. FAB-MS data were obtained on a JEOL JMS-SX102 spectrometer. Ultraviolet spectra were obtained on a Shimadzu UV-240. HPLC was carried out using a Hewlett Packard M1090 system. Preparative HPLC was carried out on a Shimadzu LC-6AD system. Growth Inhibition of yeast was measured with a TOSOH micro plate reader MPR-A4i.

Fermentation

The fungal strain BM2419 was isolated from a soil sample collected at Ooita prefecture, and was classified as *Paecilomyces* sp. BM2419. The strain was inoculated into 500 ml flasks containing 40 ml of a sterile seed medium consisting of glucose 3.5%, soluble starch 1.0%, soybean flour 2.0%, polypeptone 0.5%, meat extract 0.5%, yeast extract 0.3%, NaCl 0.2%, KH_2PO_4 0.05% and $MgSO_4$ 0.05%. The flasks were shaken on a reciprocal shaker at 25°C for 48 hours. One ml of the first seed culture was transferred into 100 ml of the same medium, and incubated on a reciprocal shaker at 25°C for 44 hours. 200 ml of the second seed culture was transferred to a 30-liter jar fermentor containing 20 liters of the production medium consisting of glucose 1.0%, soluble starch 15.0%, soybean flour 2.0%, meat extract 0.3%, NaCl 0.2%, KH_2PO_4 0.05% and $MgSO_4$ 0.05%. The fermentation was carried out at 25°C for 144 hours with aeration of 30 liters/minute and agitation at 500 rpm.

Isolation of 1

The fermentation broth (35 liters) was filtrated and mycelia (10 liters) were extracted with 70% acetone (60 liters), and after filtration acetone was removed *in vacuo*. The resulting solution was prepared at pH 3.0 and was washed with hexane: EtOAc = 4 : 1 (40 liters) and BuOH (40 liters), then was added BuOH (5 liters) (most saintopins was suspended at the bottom of BuOH layer because of its low solubility and high productivity of BM2419). After BuOH layer was washed with water (40 liters), the suspension (500 ml) was concentrated to small volume (75 ml), and was added hexane (300 ml) to precipitate saintopin. Precipitation was washed hexane (200 ml), water (200 ml) and $CHCl_3$ (200 ml), affording 1.8 g of saintopin. Saintopin (65 mg) dissolved in 90% aqueous pyridine (65 ml), and the solution was irradiated (18 hours) with halogen lamp (20,000 lux). The reaction mixture (20% conversion yield) was concentrated and subjected to preparative HPLC affording 3.5 mg of **1**.

BM2419-1 (**1**); ^{1}H-NMR (500 MHz, DMSO-d_6): 6.62 (1H, s), 7.20 (1H, s), 11.06 (1H, s), ^{13}C-NMR (125 MHz, DMSO-d_6): 101.8, 104.7, 107.6, 110.1, 133.9, 162.5, 163.7, 171.7, 173.3.

Isolation of 2

The flask cultured broth (120 ml) was diluted with acetone (240 ml), which was filtered and acetone was removed *in vacuo* . The residue was dissolved in DMSO (12 ml). The mixture was kept for several days at room temperature (50% conversion yield), and was purified with C18 solid extraction followed by preparative HPLC, affording 1.2 mg of **2**.

BM2419-2 (**2**); ^{1}H-NMR (500 MHz, DMSO-d_6): 2.69 (3H, s), 6.52 (1H, s), 6.55 (1H, s), 7.02 (1H, s), 7.05 (1H, s), 10.25 (1H, s), 10.67 (1H, s), 11.18 (1H, s), 11.84, (1H, br s), ^{13}C-NMR (125 MHz, DMSO-d_6): 16.4, 103.6, 104.7, 105.2, 107.3, 108.0, 108.4, 108.9, 125.6, 133.0, 137.5, 140.3, 160.3, 162.4, 163.3, 165.1, 183.7, 186.8

Top1 Inhibition Assay with Recombinant Yeasts

The yeast Top1 deficient strain JN2-134 (*top1-1 rad52 ise1 ura3-52*) and hTop1 vector YCpGAL1-hTOP1 were provided by Dr. WANG. Standard procedure followed the method of WANG *et al.*[5]. The human Top1 inducible strain JN2-134hTOP1 was transformed with YCpGAL1-hTOP1 by lithium acetate. JN2-134hTOP1 was cultured

with a base medium (Yeast Nitrogen Base w/o a.a. (Difco) 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 with a base medium, galactose 2.0% and adenine sulfate 0.002%. Yeasts at 1×10^5 cell/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 (UV 595 nm).

Cell Free Top1 Assay

This assay was performed using Topoisomerase I Drug Screening Kit and human type I Topoisomerase (TopoGEN, Inc.). Proteinase K was purchased from BIOTEX LABORATORIES, INC. Standard procedure followed as recommended by manufacturer. The reaction mixture contained 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.25 μg plasmid DNA, 2 μl sample solution, and 1 unit Top1, in a total volume of 20 μl. The mixture was incubated at 37°C for 30 minutes and 2 μl 10% SDS and 2 μl proteinase K were added. The mixture was incubated again at 37°C for 30 minutes and the reaction was stopped by adding 2 μl loading buffer, consisting of 25% bromophenol blue and 50% glycerol, and was extracted with 30 μl chloroform : isoamylalcohol = 24 : 1. After centrifugation at 14,000 rpm for 2 minutes, the supernatant was subjected to electrophoresis in 1% agarose gel at 50 volts for 90 minutes in TAE buffer pH 8.0. The gel was stained with ethidium bromide and washed with distilled water. The DNA band was visualized under UV light.

Cytotoxicity

All cell lines were cultured with Eagle's minimum essential medium (Eagle's MEM) containing 10% FCS. Cells in 0.1 ml of culture medium were incubated with at various concentrations of the compounds at 37°C for 72 hours in an atmosphere of 5% CO_2 in air. The cell growth inhibition was measured with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT).

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