J. Enzyme Inhibition, 1998, Vol. 13, pp. 135–146
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1513-DMIa AND 1513-DMIb, DNA METHYLTRANSFERASE INHIBITORS PRODUCED BY *STREPTOMYCES* SP. STRAIN NO. 1513

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(Received 16 May 1997)

Two new methyltransferase inhibitors were isolated from the culture filtrate of *Streptomyces* sp. strain No. 1513 and named 1513-DMIa and 1513-DMIb. 1513-DMIa and 1513-DMIb were distinguished in certain properties from DMI-1, DMI-2, DMI-3 and DMI-4 previously reported. The molecular weight of 1513-DMIa and 1513-DMIb were estimated to be 576 and 8400 from the results of FAB-MS and gel filtration, respectively. The inhibitory activities of 1513-DMIa and 1513-DMIb were shown to be pH- and temperature-dependent and both inhibited M. *Eco*RI in an uncompetitive manner with respect to DNA or *S*-adenosylmethionine (SAM).

Keywords: *Streptomyces*; Methyltransferase inhibitor; DMI; M. *Eco*RI

INTRODUCTION

S-Adenosylmethionine (SAM) is a methyl donor in a wide range of critical biological reactions and organisms expend considerable energy in maintaining intracellular levels of this cofactor. SAM-dependent methyltransferases methylate nucleic acids, proteins or lipids in cells. In these, DNA methylation is a key element in the control mechanisms that govern gene function and

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differentiation.[1] The existence of a specific DNA methyltransferase inhibitor would facilitate studies concerning the role of DNA methyltransferases in the cell and the regulation of their activities. In the search for specific inhibitors of DNA methyltransferases, we have screened various microorganisms isolated in our laboratory using the *Eco*RI DNA methyltransferase (M. *Eco*RI, EC 2.1.1.72) as marker enzyme. We found four kinds of DNA methyltransferase inhibitors (designated as DMI-l, DMI-2, DMI-3 and DMI-4) in the culture filtrate of *Streptomyces* sp. strain No. 560, and described the purification procedures, the structures and some properties of these inhibitors in previous papers.[2-4] Recently, we found new inhibitors in the culture filtrate of *Streptomyces* sp. strain No. 1513, designated as 1513-DMIa and 1513-DMIb, and here, we report their purification procedure and inhibitory activities.

MATERIALS AND METHODS

Enzymes and Substrates

M. *Eco*RI from *Escherichia coli* RY13, M. *Alu*I (EC 2.1.1.73) from *Arthrobacter luteus*, M. *Bam*HI (EC 2.1.1.73) from *Bacillus amyloliquefaciens* H and topoisomerase I from calf thymus gland were obtained from Takara Shuzo Co. Ltd. (Kyoto). *Alu*I (EC 3.1.23.1) from *Arthrobacter luteus*, *Sca*I (EC 3.1.21.4) from *Streptomyces caespitosus*, *Bam*HI (EC 3.1.23.6) from *Bacillus amyloliquefaciens* H, *Eco*RI (EC 3.1.23.13) from *Escherichia coli* RY13 and *Pst*I (EC 3.1.23.31) from *Providencia stuartii* were obtained from Gibco BRL (New York). DNase I (EC 3.1.4.5) from bovine pancreas, DNase II (EC 3.1.4.6) from porcine spleen and RNase A (EC 3.1.27.5) from bovine pancreas were obtained from Sigma Chemicals (Saint Louis). Plasmid pUC19 used as DNA substrate was obtained from Gibco BRL, and maintained in our laboratory. RNA from yeast was obtained from Kohjin Co. Ltd. (Tokyo).

Enzyme Reactions

Activities of DNA methyltransferases (M. *Eco*RI[5], M. *Alu*I[6] and M. *Bam*HI[7]) were assayed as the ability to protect plasmid pUC19 from digestion by restriction enzymes (*Eco*RI, *Alu*I and *Bam*HI), respectively. Activities of restriction enzymes (*Alu*I[8,9], *Bam*HI[10], *Eco*RI[11], *Pst*I[12] and *Sca*I[13]) and nucleases (DNase I[14], DNase II[15] and RNase A[16]) were determined by measuring the concentration of plasmid pUC19 or RNA after enzyme reactions. Activity of topoisomerase I[17] was determined by measuring the relaxed

plasmid pUC19. After enzyme reaction the incubation mixture was subjected to 1% agarose gel electrophoresis and the plasmid or RNA on the gel was measured by a densitometer with transilluminator (Atto Co., AE-6900M) after electrophoresis at 200 V for 60 min. The assay conditions for inhibitory activity and electrophoresis have been described in a previous paper.[2] One unit of inhibitory activity was defined as the amount of inhibitor which reduced each enzyme activity by 50%.

Production and Isolation of 1513-DMIa and 1513-DMIb

Streptomyces sp. strain No. 1513 was grown at 28°C for 7 days on a rotary shaker (180 rpm, 5 cm radius) in S medium consisting of 2% glucose, 3% starch, 1% corn steep liquor, 1% soybean flour, 0.5% peptone, 0.3% NaCl and 0.5% $CaCO_3$ (pH 7.0). After cultivation, the mycelium and other residues were removed by centrifugation (5000 × *g*, 10 min). The culture filtrate was adjusted to pH 7 and extracted with ethyl acetate to give crude 1513-DMIa. The organic layer was concentrated *in vacuo*, dissolved in methanol and washed with *n*-hexane to remove hydrophobic substances. The methanol solution was concentrated *in vacuo* and subjected to a Sephadex LH-20 column (2.1 × 50 cm) chromatography with methanol. The eluate (Active fraction I) was concentrated and applied to a Bondapak C_{18} column (1.8 × 49 cm). After washing with water, 1513-DMIa was eluted with methanol. The eluate (Active fraction II) was applied to a Silica gel 60 column (1.0 × 25 cm), and 1513-DMIa was eluted with the chloroform–methanol (9 : 1). The active fractions containing 1513-DMIa (Active fraction III) were concentrated and then also subjected to a Silica gel 60 column (1.0 × 25 cm) chromatography with the chloroform–methanol (95 : 5). The active fractions obtained (Purified 1513-DMIa) were combined and concentrated.

To obtain 1513-DMIb, the culture filtrate was brought to 60% saturation by adding solid ammonium sulfate. The precipitate containing 1513-DMIb was dissolved in 0.01 M acetate buffer (pH 6) and dialyzed against the same buffer for 2 days. The dialysate was applied to a CM-cellulose column (2.4 × 20 cm) which had been equilibrated with 0.01 M acetate buffer (pH 6) and 1513-DMIb was eluted with the same buffer containing 0.5 M NaCl. The active fractions were combined and dialyzed against 0.01 M Tris-HCl buffer (pH 8) for 2 days. The active fraction was applied to a Sephadex G-100 column (1.2 × 90 cm) previously equilibrated with the above dialyzing buffer, and eluted with the same buffer. The active fractions obtained (Purified 1513-DMIb) were combined and dialyzed against distilled water and then lyophilized.

Instrumental Analyses

^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HMQC and heteronuclear multiple bond correlation (HMBC) spectra were obtained with a JNM α-500 spectrometer. NMR spectra with tetramethylsilane (TMS) as an internal standard were taken in $CDCl_3$ solution at 500 MHz (^{1}H) and 125 MHz (^{13}C). EI-MS and FAB-MS spectra were recorded with a JMS-DX 303 HF spectrometer. Optical rotation, fluorescence emission, UV spectrum and IR spectrum were measured by a Jasco DIP-1000KUY digital polarimeter, Hitachi F-4010 spectrofluorometer, Hitachi U-2000 spectrometer and Hitachi 270-30 spectrophotometer, respectively.

RESULTS AND DISCUSSION

Production and Purification of 1513-DMIa and 1513-DMIb

The cultivation of *Streptomyces* sp. strain No. 1513 was carried out under the cultural conditions described in MATERIALS AND METHODS. Changes of M. *Eco*RI inhibitory activity, growth of mycelium and pH of the broth are shown in Figure 1. This strain produced two kinds of M. *Eco*RI inhibitors,

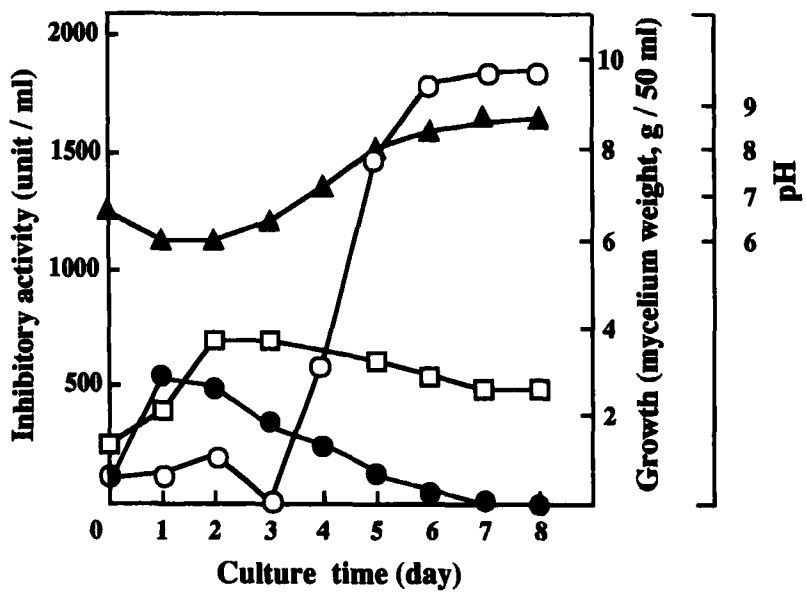

FIGURE 1 Time course of *Streptomyces* sp. strain No. 1513 culture. ●, M. *Eco*RI inhibitory activity of 1513-DMIa; ○, M. *Eco*RI inhibitory activity of 1513-DMIb; □, growth; ▲, pH.

1513-DMIa and 1513-DMIb, in the different phase of cultivation. The inhibitory activity of 1513-DMIa in the culture filtrate reached a maximum after 1 or 2 days of cultivation and thereafter gradually decreased with increasing pH. The inhibitory activity of 1513-DMIb reached a maximum after 7 or 8 days of cultivation. Purification procedures for 1513-DMIa and 1513-DMIb are shown in Figure 2 and described in MATERIALS AND METHODS. Table I shows a summary of the purification of 1513-DMIa and 1513-DMIb from each culture filtrate. Yields (%) were calculated from total activity (unit). Purified 1513-DMIa and 1513-DMIb obtained from 1000 ml of each culture filtrate were 0.19 mg and 13.7 mg, respectively. Fourteen ng of 1513-DMIa and 294 ng of 1513-DMIb gave 1 unit (50% inhibition per 20 μl of incubation mixture) in the assay system for M. *Eco*RI as described in MATERIALS AND METHODS, respectively.

Some Properties of 1513-DMIa and 1513-DMIb

1513-DMIa was obtained as a white powder, $[\alpha]^{20} - 36.5°$(c = 0.12, $CH_3OH : CHCl_3 = 1 : 1$). The molecular weight of 1513-DMIa was 576, and the molecular formula was calculated as $C_{35}H_{48}O_6$ from the results of FAB-MS, ^1H NMR and ^{13}C NMR. The structure of 1513-DMIa was elucidated from IR, ^1H NMR, ^{13}C NMR, ^1H-^1H COSY, HMQC and HMBC spectra. The assignments for the proton and carbon signals, and the structure of 1513-DMIa are shown in Table II and Figure 3, respectively. The structure of 1513-DMIa was determined to be β-sitosterol-3-*O*-β-D-glucopyranoside.

TABLE I Summary of purification steps of 1513-DMIa and 1513-DMIb from the culture filtrate

	Volume (ml)	*Total activity* ($\times 10^{-3}$ *unit*)	*Yield* (%)	*Specific activity* (*unit*/OD_{280})	*Purification rate*
1513-DMIa					
Culture filtrate	1800	180	100.0	3	1
Crude solution	100	44	24.4	14	5
Active fraction I	10	37	20.5	106	35
Active fraction II	7	37	20.5	1736	579
Active fraction III	7	30	16.7	13514	4505
Purified 1513-DMIa	6	24	13.3	20000	6667
	(0.34 mg)				
1513-DMIb					
Culture filtrate	1030	4120	100.0	90	1
Dialysate	66	3208	77.9	480	5
Active fraction	20	250	6.1	10580	118
Purified 1513-DMIb	10	48	1.2	106670	1185
	(14.1 mg)				

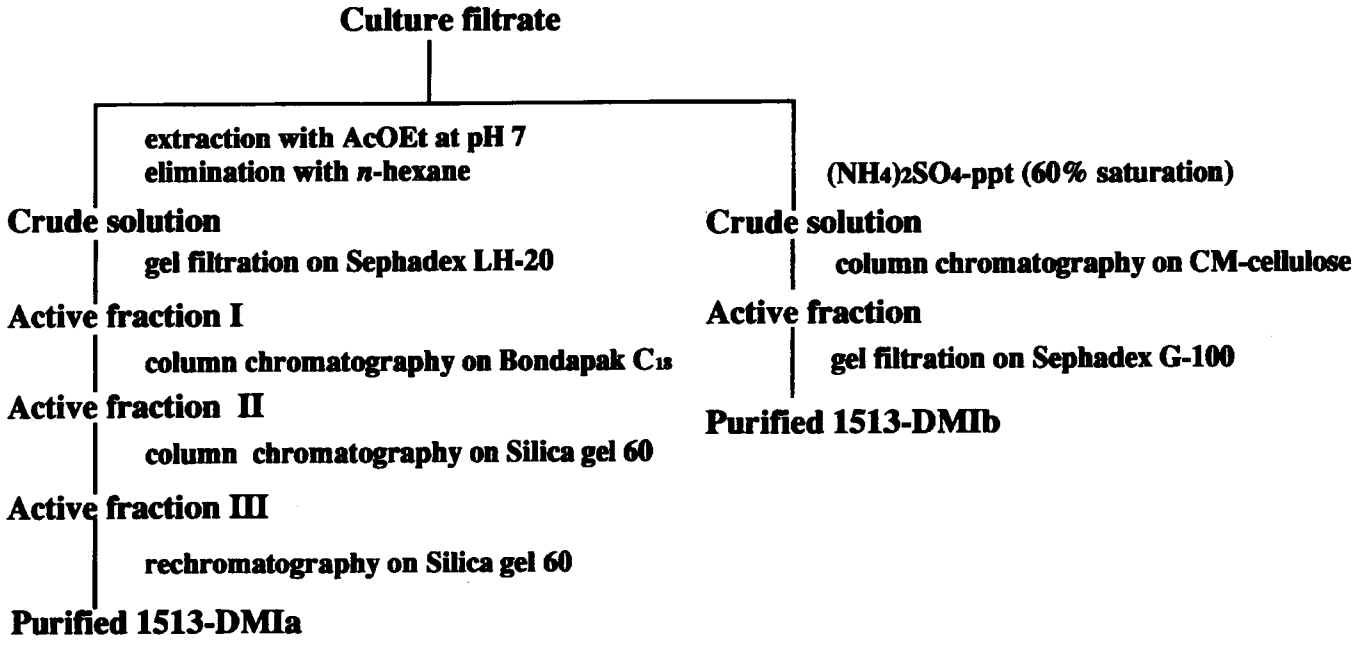

FIGURE 2 Purification procedure for 1513-DMIa and 1513-DMIb.

TABLE II ^{13}C- and ^{1}H-NMR assignments, and ^{1}H, ^{13}C-long-range correlations of 1513-DMIa by ^{1}H-^{1}H-COSY, FG-HMBC and FG-HMQC in $CDCl_3$–CD_3OD (1 : 1) on JNM α-500 spectrometer

Position	$^{13}C\delta$		$^{1}H\delta$	*HMBC cross peaks* ($^{13}C\delta$)
C-1	36.8	CH_2	1.10 (m)	
			1.88 (m)	
C-2	25.5	CH_2		
C-3	78.4	CH	3.63 (m)	
C-4	38.1	CH_2	2.28 (ddd, 13.5, 11.1, 2.4)	
			2.42 (ddd, 13.5, 4.9, 2.5)	
C-5	139.9	C		
C-6	121.4	CH	5.39 (ddd, 7.3, 1.8, 1.8)	
C-7	31.4	CH_2		
C-8	31.4	CH		
C-9	49.7	CH		
C-10	36.1	C		
C-11	20.5	CH_2		
C-12	29.0	CH_2		
C-13	41.8	C		
C-14	56.3	CH		
C-15	23.7	CH_2		
C-16	39.3	CH_2	1.21 (m)	
			2.04 (m)	
C-17	55.5	CH	1.13 (m)	
C-18	11.1	CH_3	0.72 (s)	39.3 (16), 41.8 (13), 55.5 (17), 56.3 (14)
C-19	18.5	CH_3	1.04 (s)	36.1(10), 36.8 (1), 49.7 (9), 139.9 (5)
C-20	35.6	CH	1.40 (m)	
C-21	18.0	CH_3	0.95 (d, 6.7)	33.4 (22), 35.6 (20), 55.5 (17)
C-22	33.4	CH_2		
C-23	27.6	CH_2		
C-24	45.4	CH		
C-25	28.6	CH		
C-26	18.9[a]	CH_3	0.86[a] (d, 6.7)	18.2 (27), 28.6 (25), 45.4 (24)
C-27	18.2[b]	CH_3	0.84[b] (d, 6.7)	18.9 (26), 28.6 (25), 45.4 (24)
C-28	22.5	CH_2	1.26 (m), 1.29 (m)	
C-29	11.1	CH_3	0.87 (t, 7.3)	22.5 (28)
glucose				
C-1′	100.7	CH	4.41 (d, 7.9)	78.4 (3)
C-2′	73.1	CH	3.23 (dd, 9.2, 7.9)	
C-3′	76.1	CH	3.42 (dd, 9.2, 9.2)	69.8 (4′)
C-4′	69.8	CH	3.38 (m)	
C-5′	75.7	CH	3.30 (m)	
C-6′	61.2	CH_2	3.73 (dd, 12.2,5.5)	
			3.87 (dd, 12.2, 2.4)	

[a,b] These assignments may be interchanged in each column. Glucose; β-D-glucopyranosyl.

1513-DMIb was soluble in water but insoluble in organic solvent and the molecular weight was estimated to be about 8,400 by gel filtration on Sephadex G-100 column as shown in Figure 4. 1513-DMIb was passed through a column of anion exchange resin such as DEAE-cellulose and adsorbed on CM-cellulose (cation exchange resin). From these results,

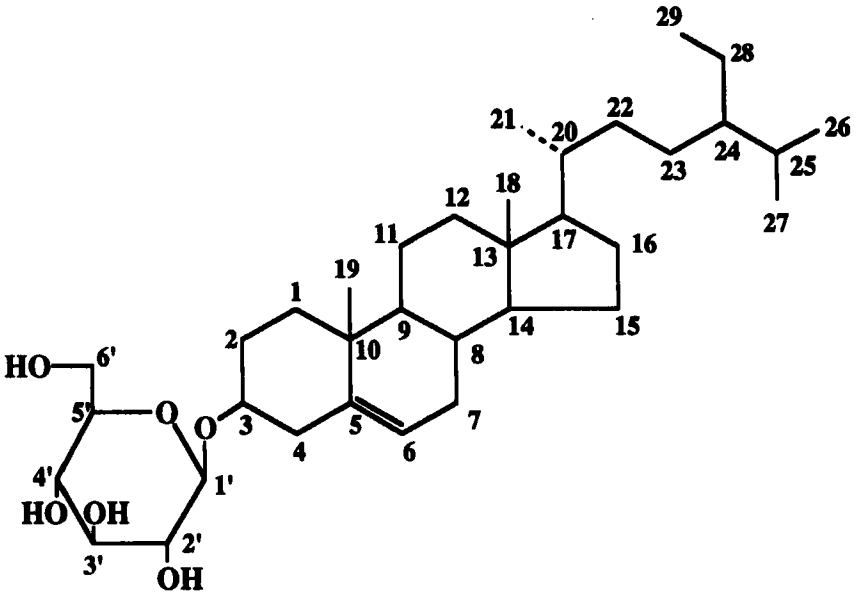

FIGURE 3 Structure of 1513-DMIa.

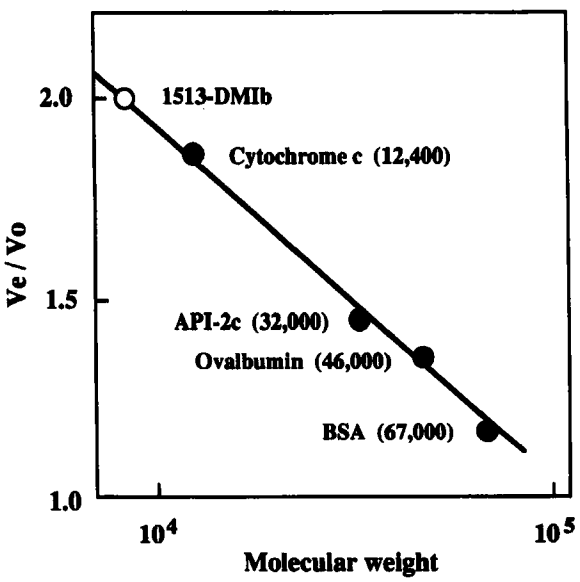

FIGURE 4 Determination of the molecular weight of 1513-DMIb. ○, 1513-DMIb; ●, marker proteins (molecular weight). API-2c is an alkaline protease inhibitor.[18,19]

1513-DMIb was considered to be an alkaline substance. Further investigation will be necessary to elucidate the structure of 1513-DMIb.

Effects of pH and Temperature on Inhibitory Activity

The inhibitory activities of 1513-DMIa and 1513-DMIb against M. *Eco*RI were measured in the range, pH 5.2–8.8 and incubation temperatures from 0° to 50°C, respectively. As shown in Figure 5, the activity of 1513-DMIa was shown to be pH- and temperature-dependent with a maximum activity at pH 8.0 and 15°C.

The activity of 1513-DMIb was also pH-dependent and increased with rising pH over the range pH 5.2–8.8. The activity was also temperature-dependent with a maximum at 15°C.

Type of Inhibition and K_i Value against M. *Eco*RI

The type of inhibition was determined by a Lineweaver–Burk plot[20] of substrates (pUC19 DNA as an acceptor and SAM as a donor of methyl group) concentration against rate of methylation of M. *Eco*RI in the presence and absence of inhibitor, and the results are compared with those for other inhibitors in Table III. The K_m values of M. *Eco*RI for DNA and SAM were 0.02 μM and 0.48 μM, respectively. The seven inhibitors tested showed a different type of inhibition from Sinefungin[21,22] which is a natural analog of SAM isolated from *Streptomyces griseolus*. Sinefungin and its analogues are known as potent inhibitors of the enzyme 1513-DMIa and 1513-DMIb uncompetitively inhibited the enzyme with respect to DNA and SAM, but 1513-DMIa differed from 1513-DMIb in having a lower K_i value for DNA.

The K_i value of 1513-DMIa for SAM was similar that for DNA, whereas the value of 1513-DMIb for SAM was 14.4-fold lower than that for DNA. These results suggested that 1513-DMIa prevented DNA and SAM from binding to the enzyme molecule whereas 1513-DMIb mainly prevented SAM from binding to the enzyme molecule. Among the inhibitors obtained in our laboratories, 1513-DMIa is a highly potent inhibitor ($K_i = 1.8$ μM) with respect to DNA, and DMI-3 is a highly potent inhibitor ($K_i = 1.2$ μM) with respect to SAM.

Comparison of Inhibitory Spectra

The effects of 1513-DMIa and 1513-DMIb on various enzymes were examined and the results are compared with those for DMI-l, DMI-2, DMI-3

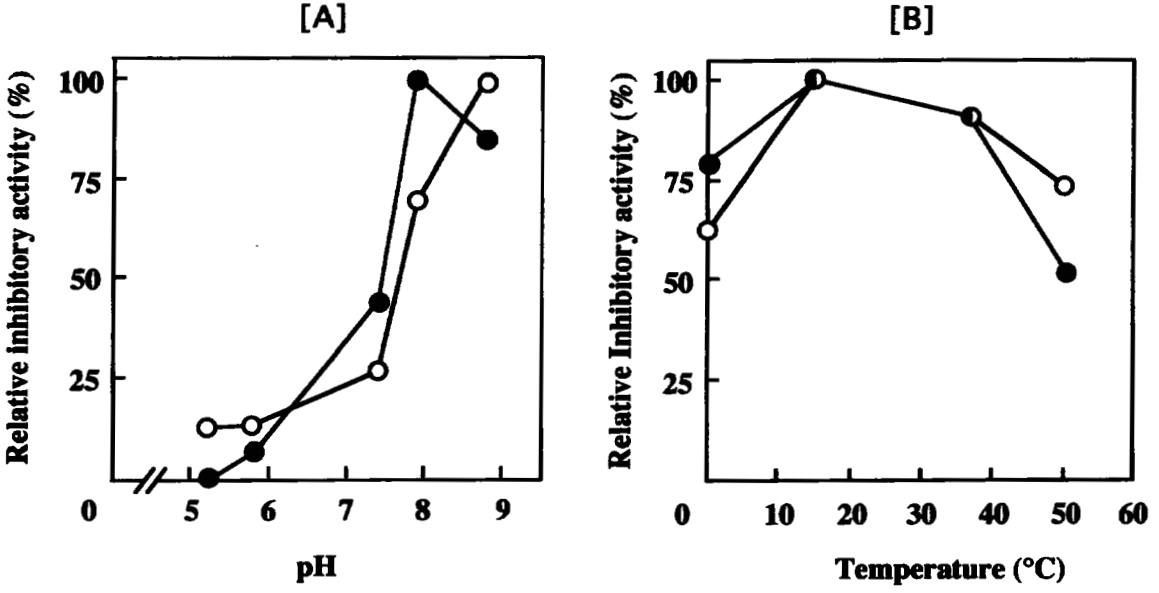

FIGURE 5 Effects of pH [A] and temperature [B] on the inhibitory activities of 1513-DMIa and 1513-DMIb. 1513-DMIa (●) and 1513 DMIb (○) were used at concentrations of 35 ng (1.5×10^{-10} M) and 735 ng (8.8×10^{-11} M) respectively in the incubation mixture.

TABLE III Comparison of types of inhibition and inhibitory constants against M. *Eco*RI for DNA methyltransferase inhibitors

Inhibitor	*pUC19 DNA*		*SAM*	
	Type	K_i (μM)	*Type*	K_i (μM)
1513-DMIa	U	1.8	U	2.4
1513-DMIb	U	38.9	U	2.7
DMI-1	N	49.3	N	10.2
DMI-2	C	11.7	U	48.3
DMI-3	C	53.2	C	1.2
DMI-4	N	8800.0	N	35830.0
Sinefungin	U	43.0	C	0.01

C: competitive inhibition, N: noncompetitive inhibition, U: uncompetitive inhibition.
K_m values for pUC19 DNA and SAM of M. *Eco*RI were 0.02 μM and 0.48 μM, respectively.

TABLE IV Comparison of inhibitory spectra

Enzyme	*Inhibition* (ng/μl *of incubation mixture*)[a]					
	1513-*DMIa*	1513-*DMIb*	*DMI*-1	*DMI*-2	*DMI*-3	*DMI*-4
M. *Eco*RI	0.7	14.7	6.7	9.0	7.7	12.5×10^3
M. *Alu*I	7.0	> 40.0	22.2	> 40.0	> 40.0	—
M. *Bam*HI	> 40.0	> 40.0	24.8	> 40.0	> 40.0	—
*Eco*RI	14.4	> 40.0	40.0	8.0	> 40.0	$> 50.0 \times 10^3$
*Alu*I	> 40.0	> 40.0	> 40.0	> 40.0	> 40.0	—
*Bam*HI	> 40.0	29.1	28.4	20.4	> 40.0	—
*Pst*I	> 40.0	> 40.0	38.0	5.2	> 40.0	—
*Sca*I	5.6	> 40.0	2.9	2.9	> 40.0	—
DNase I	> 40.0	> 40.0	> 40.0	> 40.0	> 40.0	—
DNase II	> 40.0	> 40.0	> 40.0	5.5	> 40.0	—
RNase A	> 40.0	> 40.0	> 40.0	> 40.0	> 40.0	—
Topoisomerase I	9.0	> 40.0	—	> 40.0	—	—

[a] amount required to give 50% inhibition per 1 μl of incubation mixture.

and DMI-4 produced by the strain No. 560. As shown in Table IV, 1513-DMIa was the most potent inhibitor against M. *Eco*RI. 1513-DMIa was distinguished from other inhibitors in inhibiting M. *Alu*I, *Sca*I and Topoisomerase I. 1513-DMIb as well as DMI-3 showed specific inhibition against M. *Eco*RI, but 1513-DMIb was distinguished from DMI-3 in its inhibition of *Bam*HI.

These inhibitors showed different inhibitory spectra but none of the inhibitors tested could inhibit DNase I and RNase A. More detailed research concerning the inhibition of DNA methyltransferase by these inhibitors will allow an understanding of the physiological functions and regulation of the enzyme activity in living cells.

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