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DMI-2 AND DMI-3, DNA METHYLTRANSFERASE INHIBITORS PRODUCED BY *STREPTOMYCES* SP. STRAIN NO. 560

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Streptomyces sp. strain No. 560 produces several types of DNA methyltransferase inhibitors in the culture filtrate. Two of them, DMI-2 and DMI-3, were distinguished from the previously reported DMI-1 by their inhibitory spectrum and inhibition characteristics against DNA methyltransferase. The molecular weights of DMI-2 and DMI-3 were 854 and 435, respectively. The structure of DMI-2 was determined to be 4‴R,6aR,10S,10aS-8-acetyl-6a,10a-dihydroxy-2-methoxy-12-methyl-10-[4′-[3″-hydroxy-3″,5″-dimethyl-4″ (Z-2‴,4‴-dimethyl-2‴-heptenoyloxy) tetrahydropyran-1″-yloxy]-5′-methylcyclohexan-1′-yloxy] -1,4,6,7,9-pentaoxo-1,4,6,6a,7,8,9,10,10a,11-decahydronaphthacene. The chemical structure of DMI-2 was established as a tautomer of dutomycin which is an antitumor antibiotic produced by *Streptomyces* sp. 1725.

DMI-2 and DMI-3 showed strong inhibition against N^6-methyladenine-DNA methyltransferase (M. *Eco* RI). DMI-2 inhibited M. *Eco* RI in a competitive manner with respect to plasmid pUC19 used as DNA substrate and in an uncompetitive manner with respect to *S*-adenosylmethionine (SAM) used as methyl donor. DMI-3 inhibited M. *Eco* RI in a competitive manner with respect to plasmid pUC19 and SAM. The inhibitory activities of both inhibitors depended upon the pH and temperature in the assay media.

KEY WORDS: DNA methyltransferase inhibitor, DMI, M. *Eco* RI, DMI-2, DMI-3, *Streptomyces* sp.

INTRODUCTION

Methylation of DNA by DNA methyltransferase is an important factor in the regulation of gene expression, malignant alteration of cell and senescence.[1–3] However, the biological functions and the fate of the enzyme in the cells are not well known. The existence of a specific DNA methyltransferase inhibitor will facilitate studies concerning the role of DNA methyltransferases and the regulation of the enzyme activities in the cells. 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 which transfers the methyl group from SAM to the N^6-position of the second adenine in the double stranded DNA sequence 5′-GAATTC-3′.

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In the previous papers,[4] we reported a DNA methyltransferase inhibitor, designated as DMI-1 (8-methylpentadecanoic acid), produced in the culture filtrate of *Streptomyces* sp. strain No. 560, and described the purification procedure, the structure and some properties of the inhibitor. Recently, we found two additional inhibitors in the culture filtrate and designated these as DMI-2 and DMI-3. In this paper, we describe the purification procedure, the structure and some properties of both inhibitors.

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).

Measurement of DNA Methyltransferase Activity

The assay of M. *Eco* RI[5] activity was based on its ability to protect plasmid pUC19 from digestion by *Eco* RI[6] which cleaves the unmethylated 5′-GAATTC-3′ site. Plasmid pUC19 (0.4 μg) was methylated in 20 μl of the buffer for M. *Eco* RI reaction containing 100 mM Tris-HCl (pH 8.0), M. *Eco* RI (10 units), 80 mM *S*-adenosylmethionine (SAM), 4 mM EDTA and 2 mM dithiothreitol at 37°C for 60 min. After incubation, 30 μl of the buffer for the *Eco* RI reaction containing 10 mM Tris-HCl (pH 8.0), *Eco* RI (4 units), 10 mM $MgCl_2$ and 100 mM NaCl was added and then further incubated at 37°C for 60 min for digestion of unmethylated pUC19. After enzyme reaction, the incubation mixture was subjected to 1% agarose gel electrophoresis, and methylated pUC19 (undigested pUC19) on the gel was measured by a densitometer with transilluminator (Atto Co., AE-6900M) after electrophoresis at 200 V for 60 min. One unit of inhibitory activity was defined as the amount of inhibitor causing a decrease of methylated pUC19 concentration by 50%. M. *Alu* I[7] and M. *Bam* HI[8] were assayed by the same method as described above.

Measurement of Other Enzyme Activities

Activities of *Alu* I,[9,10] *Bam* HI,[11] *Eco* RI, *Pst* I,[12] *Sca* I,[13] DNase I[14] and DNase II[15] were determined by measuring the concentration of plasmid pUC19 after the enzymic reaction. The reaction mixture (50 μl) consisting of 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 100 mM NaCl, each enzyme (4 units) and plasmid pUC19 (0.4 μg) was incubated at 37°C for 60 min. After incubation, the concentration of remaining plasmid pUC19 (undigested

pUC19) in the reaction mixture was measured using a densitometer after agarose gel electrophoresis. Activity of topoisomerase I[16] was determined by measuring the relaxed plasmid pUC19. The reaction mixture (20 μl) consisting of 50 mM Tris-HCl, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, BSA (0.6 μg), plasmid pUC19 (0.4 μg) and topoisomerase I (1 unit) was incubated at 37°C for 60 min. The reaction was terminated by adding 10 μl of loading buffer consisting of 200 mM Tris, 200 mM boric acid, 5 mM EDTA, 50% glycerin and 10% bromphenol blue. The mixture was subjected to 1% agarose gel electrophoresis containing ethidium bromide (0.5 mg/ml), and relaxed plasmid pUC19 on the gel was measured by the densitometer after electrophoresis. Electrophoresis was performed by the usual method using Tris-borate buffer system.[17] One unit of inhibitory activity was defined as the amount of inhibitor which reduced each enzyme activity by 50%.

Production and Isolation of DMI-2 and DMI-3

Streptomyces sp. strain No. 560 was cultivated at 28°C 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 (11,000 × g, 10 min). The culture filtrate was adjusted to pH 7 and extracted with ethyl acetate to give DMI-2. The organic layer was concentrated *in vacuo* and applied to a Bondapak C_{18} column (1.9 × 40 cm, Waters). After washing with 30% methanol, DMI-2 was eluted with 90% methanol. The eluate was concentrated and subjected to Sephadex LH-20 column (1.9 × 40 cm, Pharmacia) chromatography with methanol. The active fractions containing DMI-2 were combined, concentrated and then subjected to Sephadex LH-20 column (1.2 × 35 cm) chromatography with chloroform. The active fraction obtained was applied to a Silica gel 60 column (1.3 × 35.5 cm, Merck Co.), and DMI-2 was eluted with the solvent of chloroform-methanol-acetic acid (95:5:1). The accumulated active fractions were concentrated and then also subjected to Silica gel 60 column (1.2 × 40 cm) chromatography with the solvent of chloroform-methanol-acetic acid (99:1:1). The active fractions (purified DMI-2) were combined and concentrated.

To obtain DMI-3, the culture filtrate was adjusted to pH 10 and extracted with ethyl acetate. The organic layer containing DMI-3 was concentrated *in vacuo* and applied to a Bondapak C_{18} column (1.6 × 25 cm). After washing with 30% ethanol, DMI-3 was eluted with 60% ethanol. The eluate was concentrated and subjected to Sephadex LH-20 column (1.0 × 25 cm) chromatography with chloroform. The accumulated active fractions were concentrated and subjected to preparative thin layer chromatography on silica gel with the solvent of chloroform-methanol (97:3). The purified DMI-3 on the silica gel plate was eluted with chloroform and concentrated.

Instrumental Analyses

^1H NMR, ^{13}C NMR, ^1H-^1H COSY, ^{13}C-^1H COSY and heteronuclear multiple bond correlation spectroscopy (HMBC) were measured by a JMX GX-400 spectrometer. NMR spectra with tetramethylsilane (TMS) as an internal standard were taken in $CDCl_3$ solution at 400 MHz. 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

TABLE 1
Summary of purification steps of DMI-2 and DMI-3 from the culture filtrate.

DMI-2	Volume (ml)	Inhibitory activity (unit/ml)	Total activity (unit)	Yield (%)	DMI-3	Volume (ml)	Inhibitory activity (unit/ml)	Total activity (unit)	Yield (%)
Culture filtrate	520.0	794	412698	100.0	Culture filtrate	2000.0	210	420000	100.0
AcOEt layer	10.5	15152	159091	38.5	AcOEt layer	20.0	8700	174000	41.4
Active fraction[a]	7.8	12500	97826	23.6	Active fraction[b]	2.0	5130	10260	2.4
Purified DMI-2	12.6	5555	70000	17.0	Purified DMI-3	0.1	71	71	0.02

[a] After gel filtration on Sephadex LH-20. [b] After column chromatography on Bondapak C18.

a Jasco DIP-360 digital polarimeter, Hitachi F-4010 spectrofluorometer, Hitachi U-2000 spectrometer and Hitachi 270-30 spectrophotometer, respectively.

RESULTS AND DISCUSSION

Production and Purification of DMI-2 and DMI-3

Streptomyces sp. strain No. 560 produced several M. *Eco* RI inhibitors in the different phase of cultivation. To obtain DMI-2 and DMI-3, the strain was cultivated at 28°C for 4 and 7 days, respectively. Table 1 gives a summary of the purification of DMI-2 and DMI-3 from the culture filtrate. The yields of DMI-2 and DMI-3 obtained from 1000 ml of the culture filtrate were 24.2 mg and 10 mg, respectively. As shown in Figure 1, 180 ng of DMI-2 and 154 ng of DMI-3 gave 1 unit (50% inhibition) in the assay system as described in MATERIALS AND METHODS.

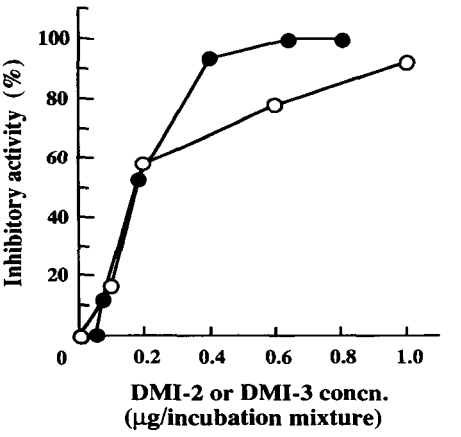


FIGURE 1 Inhibitory activities of DMI-2 (○) and DMI-3 (●) against M. *Eco* RI. The concentration of DMI-2 and DMI-3 were expressed as μg in the incubation mixture (20 μl).

TABLE 2
Physico-chemical properties of DMI-2 and DMI-3.

	DMI-2		DMI-3
Nature	Orange powder	Nature	Pale yellow liquid
MP (°C)	143~145	$[\alpha]_D^{25}$ (c=0.05, EtOH)	Inactive
Molecular weight	854	Molecular weight	435
Molecular formula	$C_{44}H_{54}O_{17}$	Fluorescence $\lambda_{ex\,max}^{EtOH}$ nm	270
		Fluorescence $\lambda_{em\,max}^{EtOH}$ nm	374
λ_{max} (MeOH) nm (ε)	241 (39540), 280 (29378)	λ_{max} (MeOH) nm (ε)	268 (269265)
FT-IR ν max [ATR] cm^{-1}	3504, 1700, 1633, 1602	FT-IR max (ZnSe) cm^{-1}	3348, 3332, 2922, 2852, 1734, 1711

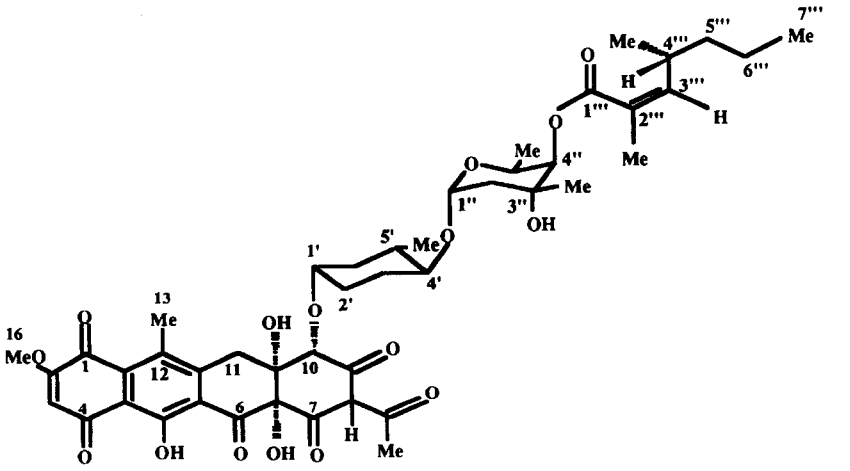
Physicochemical Properties of DMI-2 and DMI-3

The physicochemical properties of DMI-2 and DMI-3 are summarized in Table 2. DMI-2 was obtained as orange powder with melting point at 143–145°C. It was soluble in methanol, ethanol and chloroform, but insoluble in water. The UV spectrum in methanol showed peaks at 241 nm (ε, 39,540) and 280 nm (ε, 29,378). The molecular weight of DMI-2 was 854, and the molecular formula was calculated as $C_{44}H_{54}O_{17}$ from the results of FAB-MS, ^1H NMR and ^{13}C NMR. The structure of DMI-2 was elucidated from IR, ^1H NMR, ^{13}C NMR, ^1H-^1H COSY, ^{13}C-^1H COSY and HMBC spectra. The assignments for the carbon signals in the ^{13}C NMR spectrum and the structure of DMI-2 are shown in Table 3. The structure of DMI-2 was determined to be 4‴R,6aR,10S,10aS-8-acetyl-6a,10a-dihydroxy-2-methoxy-12-methyl-10-[4′-[3″-hydroxy-3″,5″-dimethyl-4″(Z-2‴,4‴-dimethyl-2‴-heptenoyloxy)tetrahydropyran-1″-yloxy]-5′-methylcyclohexan-1′-yloxy]-1,4,6,7,9-pentaoxo-1,4,6,6a,7,8,9,10,10a,11-decahydronaphthacene. The chemical structure of DMI-2 is similar to that of dutomycin,[18] which is an antitumor antibiotic produced by *Streptomyces* sp. 1725. DMI-2 has a carbonyl group at the 9th carbon in the structure, while dutomycin has a hydroxyl group, therefore, DMI-2 is considered to be a tautomer of dutomycin.

DMI-3 was obtained as a pale yellow liquid, soluble in ethanol and chloroform but insoluble in water. The UV spectrum in ethanol showed a maximum at 268 nm (ε, 269,265), and the IR spectrum showed peaks at 3348, 3332, 2922, 2852, 1734 and 1711 cm^{-1}. The molecular weight of DMI-3 was determined to be 435 from the results of EI-MS. DMI-3 was a fluorescent substance. The fluorescence emission maximum in ethanol was 374 nm when DMI-3 was excited at 270 nm as shown in Figure 2. Further investigation will be necessary to elucidate the structure of the inhibitor.

TABLE 3
^{13}C NMR data for DMI-2 (in $CDCl_3$).

Position	δ (ppm)	Position	δ (ppm)	Position	δ (ppm)
1	190.2	10a	75.5	3″	68.8
2	161.2	11	34.9	4″	74.0
3	108.7	11a	150.7	5″	62.7
4	181.3	12	132.7	3″-Me	25.7
4a	113.7	12a	132.3	5″-Me	16.6
5	161.9	13	16.6	1‴	167.3
5a	123.3	16	57.0	2‴	125.0
6	192.4	1′	102.1	2‴-Me	20.9
6a	81.0	2′	30.1	3‴	149.9
7	190.5	3′	29.3	4‴	33.3
8	100.2	4′	79.9	4‴-Me	20.6
8-CH_3CO	201.1	5′	74.3	5‴	39.6
8-CH_3CO	26.7	5′-Me	17.3	6‴	20.7
9	195.7	1″	100.2	7‴	14.2
10	73.4	2″	36.9		



Effects of pH and Temperature on Inhibitory Activity

The inhibitory activities of DMI-2 and DMI-3 against M. *Eco* RI were measured in the pH range from 4.0 to 9.5 and incubation temperatures from 0°C to 70°C, respectively. As shown in Figure 3, the activity of DMI-2 was shown to be pH- and temperature-dependent with a maximum at pH 7.0 and 37°C. The activity of DMI-3 was also shown to be pH- and temperature-dependent and increased with rising pH and temperature over the range pH 5–10 and 0–70°C respectively.

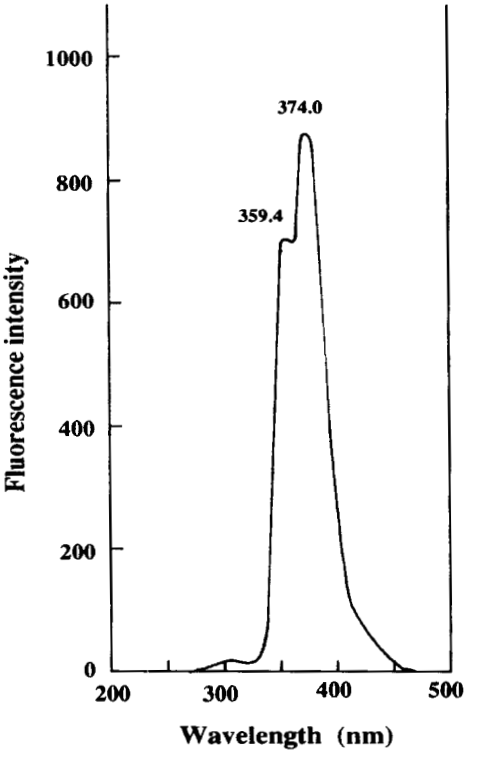


FIGURE 2 Fluorescence emission spectrum of DMI-3 in methanol, excited at 270 nm.

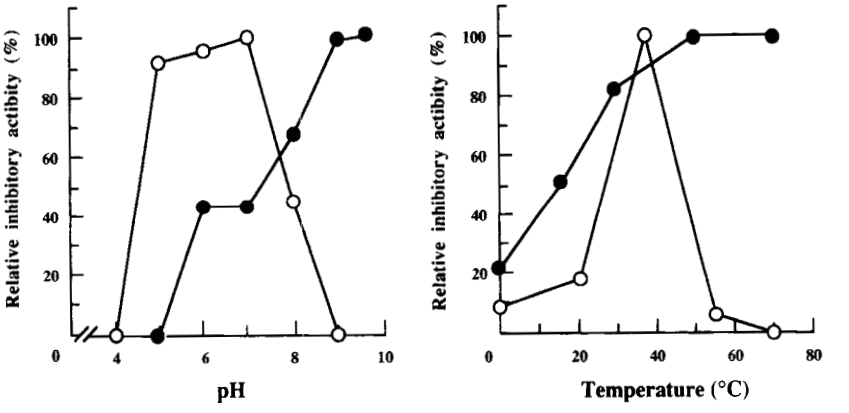


FIGURE 3 Effects of pH and temperature on the inhibitory activities of DMI-2 (○) and DMI-3 (●). DMI-2 and DMI-3 were used at a concentration of 2.3×10^{-5} M and 3.7×10^{-5} M respectively in the incubation mixture.

TABLE 4
Inhibitory spectra of DMI-2 and DMI-3.

Enzyme	Substrate (concn., ng/μl of incubation mixture)	IC_{50} (ng/μl of incubation mixture) DMI-2	DMI-3
M. *Eco* RI	pUC19 (20)	9.0	7.7
M. *Alu* I	pUC19 (20)	>40.0	>40.0
M. *Bam* HI	pUC19 (20)	>40.0	>40.0
Sca I	pUC19 (8)	2.9	>40.0
Pst I	pUC19 (8)	5.2	>40.0
Eco RI	pUC19 (8)	8.0	>40.0
Bam HI	pUC19 (8)	20.4	>40.0
Alu I	pUC19 (8)	>40.0	>40.0
DNase I	pUC19 (8)	>40.0	>40.0
DNase II	pUC19 (8)	5.5	>40.0
RNase A	RNA (600)	>40.0	>40.0
Topoisomerase I	pUC19 (20)	>40.0	ND

ND; not determined.

TABLE 5
Comparison of types of inhibition and inhibitory constants against M. *Eco* RI between DMI-2 and DMI-3.

	pUC19		SAM	
	Type	K_i (μM)	Type	K_i (μM)
DMI-2	competitive inhibition	11.70	uncompetitive inhibition	46.70
DMI-3	competitive inhibition	53.16	competitive inhibition	1.19

K_m values of M. *Eco* RI with respect to pUC19 and SAM were 0.02 μM and 0.48 μM, respectively.

Inhibitory Spectrum

The effects of DMI-2 and DMI-3 on various enzymes were examined and are summarized in Table 4. DMI-2 and DMI-3 strongly inhibited M. *Eco* RI (N^6-methyladenine-DNA methyltransferase), and did not inhibit M. *Alu* I (C^5-methylcytosine-DNA methyltransferase) and M. *Bam* HI (N^4-methylcytosine-DNA methyltransferase). Therefore, it is considered that DMI-2 and DMI-3 have selectivity against N^6-methyladenine-DNA methyltransferase. However, DMI-2 was distinguished from DMI-3 in the inhibitions against endonucleases such as *Sca* I, *Pst* I, *Eco* RI, DNase II and *Bam* HI. DMI-3 inhibited only M. *Eco* RI, and did not inhibit the other enzymes tested. Therefore, DMI-3 was considered to be a specific inhibitor for M. *Eco* RI.

Type of Inhibition and K_i Value against M. Eco RI

The types of inhibition were determined by a Lineweaver-Burk plot[19] of the reciprocal of the substrates (pUC19 as an acceptor and SAM as a donor of methyl group) concentration against the reciprocal of the rate of methylation of M. *Eco* RI in the presence and absence of DMI-2 and DMI-3, respectively. As shown in Table 5, DMI-2 inhibited M. *Eco* RI in a competitive manner with respect to pUC19 and in an uncompetitive manner with respect to SAM. The K_m value of the enzyme with respect to pUC19 was 2.00×10^{-8} M and the K_i value of DMI-2 was 1.17×10^{-5} M. In the case of SAM, the K_m value of the enzyme was 0.48×10^{-6} M and the K_i value of DMI-2 was 4.67×10^{-5} M. From these results, it was considered that the inhibitor bound to the binding site of pUC19 and prevented pUC19 from binding to the enzyme molecule.

DMI-3 inhibited M. *Eco* RI in a competitive manner with respect to both substrates. The K_i values of DMI-3 with respect to pUC19 and SAM were 5.32×10^{-5} M and 1.19×10^{-6} M, respectively. This inhibitor was considered to bind strongly to the binding site of pUC19 on the enzyme molecule.

DMI-2 and DMI-3 were different DNA methyltransferase inhibitors from a structural analog of the natural cofactor (SAM) such as sinefungin[20] and A9145C,[21] and showed a different inhibitory spectrum and inhibition mechanism. DMI-2 and DMI-3 strongly inhibited M. *Eco* RI (N^6-methyldenine-DNA methyltransferase), and did not inhibit M. *Alu* I (C^5-methylcytosine-DNA methyltransferase) and M. *Bam* HI (N^4-methylcytosine-DNA methyltransferase). Therefore, it is thought that both inhibitors were specific inhibitors for N^6-methyladenine-DNA methyltransferase. More detailed work concerning inhibition of DNA methyltransferase by both inhibitors will allow us to understand the physiological functions and regulation of the enzyme activity in living cells.

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