J. Enzyme Inhibition, 1995, Vol. 9, pp. 243–252
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DMI-1, A NEW DNA METHYLTRANSFERASE INHIBITOR PRODUCED BY *STREPTOMYCES* SP. STRAIN NO. 560

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(Received 24 April 1995)

A new inhibitor of DNA methyltransferase named DMI-1 has been discovered in the culture filtrate of *Streptomyces* sp. strain No. 560. DMI-1 was purified by extraction with ethyl acetate followed by Diaion HP-20SS and silica gel column chromatography. The structure of DMI-1 was determined to be 8-methylpentadecanoic acid ($C_{16}H_{32}O_2$). DMI-1 is a novel inhibitor of methyltransferase isolated from microorganisms and is structurally different from sinefungin and A9145C which are structural analogs of S-adenosylmethionine (methyl donor). DMI-1 was a strong inhibitor of N^6-methyladenine-DNA methyltransferase (M. *Eco* RI, EC 2.1.1.72) in a noncompetitive manner and its inhibition depended on the pH and temperature in the assay media.

KEY WORDS: Methyltransferase inhibitor, DMI-1, *Streptomyces* sp., M. *Eco* RI, 8-methylpentadecanoic acid, fatty acid

INTRODUCTION

DNA methylation directly affects sequence-specific interactions of proteins with DNA, adding an additional level of genetic information to the DNA helix. In bacteria, methylation prevents the digestion of host DNA by the host restriction endonucleases, and foreign DNA lacking this modification is digested by these enzymes. In mammals, the biological functions of DNA methyltransferases are not well known, although they may be involved in important steps such as gene expression, malignant alteration of cell and senescence.[1–3] The existence of a specific DNA methyltransferase inhibitor will facilitate studies concerning the role of DNA methyltransferases in cells and the regulation of their activities. In this context, DNA methyltransferase inhibitors such as sinefungin[4] and A9145C[5] have been isolated from microorganisms. As they are structural analogs of S-adenosylmethionine (SAM) and are known as natural inhibitors against methylation of proteins,[6] phospholipids[7] and other molecules,[8] they are not specific for DNA.

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Little has been reported concerning DNA methyltransferase inhibitors except for structural analogs of SAM. In the search for specific inhibitors of DNA methyltransferases, we have screened various microorganisms isolated in our laboratory using *Eco* RI DNA methyltransferase (M. *Eco* RI, EC 2.1.1.72) as a 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′.

Recently, we have found some inhibitors of M. *Eco* RI in the culture filtrate of *Streptomyces* sp. strain No. 560 isolated from a soil sample. One of them, designated as DMI-1, was an inhibitor of three kinds of DNA methyltransferases (M. *Eco* RI, M. *Alu* I and M. *Bam* HI). In this paper, we describe the purification procedure, the structure and some properties of DMI-1.

MATERIALS AND METHODS

Enzymes and Substrates

M. *Eco* RI from *Escherichia coli* RY13, M. *Alu* I (EC 2.1.1.73) from *Arthrobacter luteus* and M. *Bam* HI (EC 2.1.1.73) from *Bacillus amyloliquefaciens* H 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[9] activity was based on its ability to protect plasmid pUC19 from digestion by *Eco* RI[10] 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 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. The reaction was terminated by adding 10 μl of the 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, and methylated pUC19 (undigested pUC19) on the gel was measured by a densitometer after electrophoresis. One unit of inhibitory activity was defined as the amount of inhibitor causing a decrease of methylated pUC19 concentration by 50%. M. *Alu* I[11] and M. *Bam* HI[12] were also assayed by the method described above.

Measurement of Other Enzyme Activities

Activities of *Alu* I,[13–14] *Bam* HI,[15] *Eco* RI, *Pst*, I[16] *Sca* I,[17] DNase I[18] and DNase II[19] 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 electorophoresis. One unit of inhibitory activity was defined as the amount of inhibitor which reduced the enzyme activity by 50%.

Cultural Conditions for DMI-1 Production

Streptomyces sp. strain No. 560 was isolated from a soil sample collected in Japan by us and this strain was used in this work. To obtain DMI-1, the strain was cultivated 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).

Purification Procedure of DMI-1

After cultivation, the mycelium and other residues were removed by centrifugation (10,000 rpm, 10 min). The strain produced several different kinds of inhibitors in the culture filtrate. The culture filtrate was adjusted to pH 10 and extracted with ethyl acetate to give crude DMI-1. The organic layer was concentrated *in vacuo* and applied to a Diaion HP-20SS column (1.8×10 cm, Mitsubishi Chemical Industries Inc.). After washing with 50% ethanol, DMI-1 was eluted with 90% ethanol. The eluate (active fraction I) was concentrated and subjected to silica gel 60 column (1.6×10 cm, Merck Co.) chromatography with chloroform and ethanol (95:5). The active fractions containing DMI-1 (active fraction II) were combined, concentrated and then applied to a silica gel column (1.1×21 cm) with chloroform and ethanol (9:1). The active fractions obtained (purified DMI-1) were combined and concentrated.

Electrophoresis of Plasmid pUC19

Electrophoresis of plasmid pUC19 on 1% agarose slab gel containing ethidium bromide (0.5 μg/ml) was performed by the usual method using Tris-borate buffer system.[20] After electrophoresis at 200 V for 60 min, plasmid pUC19 concentration on the gel was measured by a densitometer with transilluminator (Atto Co., AE-6900M).

Analytical Methods

The methyl ester of DMI-1, obtained as follows, was analyzed by electron impact mass (EI-MS) and proton nuclear magnetic resonance (^{1}H NMR). DMI-1 (less than 50 mg) was dissolved in 1 ml of benzene and 2 ml of MeOH containing 5% HCl added and the mixture sealed *in vacuo* and then heated for 2 h in a water bath (90–100°C). After cooling, the solution was extracted three times with 5 ml of *n*-hexane, and the extract

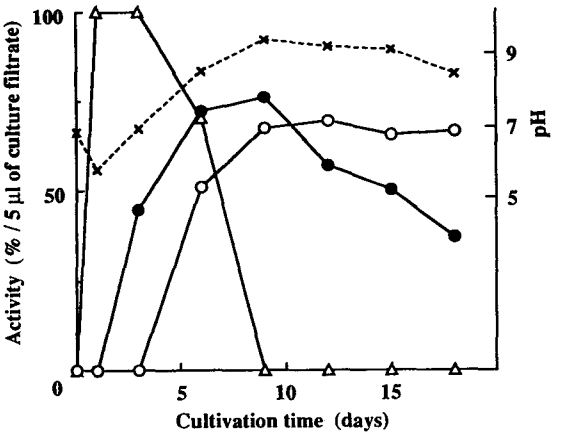

FIGURE 1 Time course of *Streptomyces* sp. strain No. 560 culture. •, M. *Eco* RI inhibitory activity; ○, *Eco* RI inhibitory activity; △, DNase activity; ×, pH.

dried *in vacuo*. A ^{1}H NMR spectrum was obtained with a JMX GX-400 spectrometer (400 MHz) in $CDCl_3$ solution with tetramethylsilane (TMS) as internal standard. The EI-MS spectrum was recorded with a JMS-DX 303 HF spectrometer.

RESULTS AND DISCUSSION

Time Course of DMI-1 Production by Strain No. 560

Cultivation of *Streptomyces* sp. strain No. 560 was carried out under the cultural conditions described in MATERIALS AND METHODS. Changes in M. *Eco* RI inhibitory activity, *Eco* RI inhibitory activity, DNase activity and pH of the broth are shown in Figure 1. For the measurement of M. *Eco* RI and *Eco* RI inhibitory activities, the culture filtrate was boiled at 100°C for 20 min to eliminate the effect of DNase in the filtrate (the inhibitors in culture filtrate are c. 90% stable to boiling). M. *Eco* RI inhibitory activity increased with pH and reached a maximum after 9 days of cultivation, thereafter decreasing gradually. *Eco* RI inhibitory activity also increased with M. *Eco* RI inhibitory activity and reached a maximum after 9 days of cultivation, and then remained almost stable for a further 9 days. DNase activity reached a maximum in 1 to 3 days, during the early phase of cultivation, and then rapidly decreased. It became clear that this strain produced M. *Eco* RI inhibitors, *Eco* RI inhibitors and DNases during the different phases of cultivation.

Purification of DMI-1

Table 1 summarizes the purification of DMI-1 from the culture filtrate. The inhibitor was purified 952 times on the basis of the optical density reading at 210 nm (DMI-1

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

Step	Volume (ml)	Inhibitory activity (unit/μl)	Total activity (unit$\times 10^{-3}$)	Yield (%)	Specific activity (unit/OD_{210})	Purification ratio
Culture filtrate	770	0.5	385	100.0	3	1
Crude	5	50.0	250	64.9	99	33
Active fraction I	5	5.0	25	6.5	200	67
Active fraction II	5	5.0	25	6.5	1563	521
Purified DMI-1	1.6 mg	7.5 unit/μg	12	3.1	2857	952

has an end absorbance). Overall, 1.6 mg of DMI-1 was obtained from 770 ml of the culture filtrate with a yield of 3.1%. As shown in Figure 2, 134 ng of the inhibitor gave 1 unit in the assay system as defined in MATERIALS AND METHODS.

Physiochemical Properties and Structure of DMI-1

DMI-1 was obtained as an oily liquid, which was soluble in acetone, ethyl alcohol and isopropyl alcohol but insoluble in water. The results of EI-MS and ^{1}H NMR on esterified DMI-1 are shown in Figures 3 and 4. A molecular ion peak at m/z 270 was detected and the characteristic fragment peaks of fatty acid, m/z 74, 87, M-43 (227) and M-31 (239), were observed. Moreover, the two fragments, m/z 143 and M-15 (255)

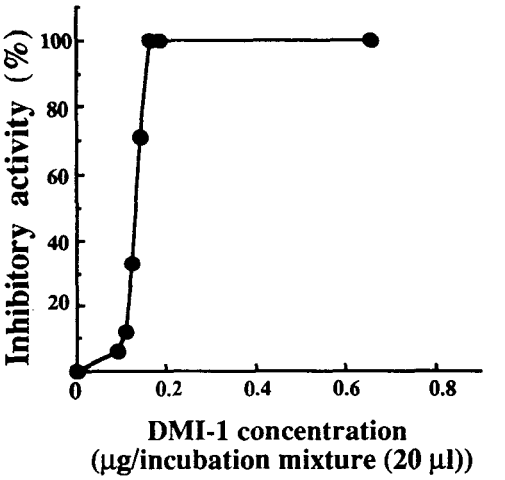

FIGURE 2 Inhibitory activity of DMI-1 against M. *Eco* RI. The concentration of DMI-1 was expressed as in the incubation mixture (20 μl).

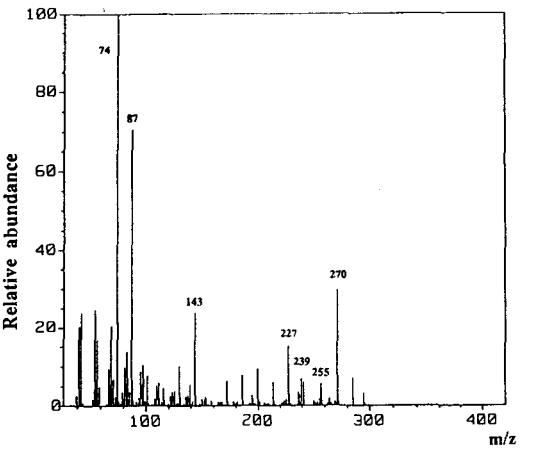

FIGURE 3 EI-MS spectrum of DMI-1.

indicated that DMI-1 is a branched chain fatty acid which had a methyl group at the 8th carbon. From these results, the molecular weight of DMI-1 was 256 ($C_{16}H_{32}O_2$) and the structure was determined to be 8-methylpentadecanoic acid as shown in Figure 4. The assignments for the hydrogen signals in the ^1H NMR spectrum of DMI-1 are also shown in the figure.

Effects of pH and Temperature on Inhibitory Activity

The inhibitory activity of DMI-1 against M. *Eco* RI was measured in the pH range from 6.0 to 9.5. As shown in Figure 5(A), the activity of DMI-1 was shown to be pH-dependent with a maximum at pH 8.0. The inhibitory activity determined at various temperatures from 0°C to 70°C at pH 8.0 are shown in Figure 5(B). The inhibitory activity of DMI-1 was also shown to be temperature-dependent with a maximum at 37°C.

Inhibitory Spectrum

The effects of DMI-1 on various enzymes were examined. As shown in Table 2, DMI-1 strongly inhibited three kinds of DNA methyltransferase and *Sca* I, and weakly *Eco* RI, *Bam* HI and *Pst* I, but did not inhibit *Alu* I, DNase I, DNase II and RNase A. Among three DNA methyltransferases, DMI-1 strongly inhibited M. *Eco* RI (N^6-methyladenine-DNA methyltransferase), and the inhibitions against M. *Alu* I (C^5-methylcytosine-DNA methyltransferase) and M. *Bam* HI (N^4-methylcytosine-DNA methyltransferase) were 3.3 and 3.7 times weaker than that against M. *Eco* RI. Therefore, it is considered that DMI-1 has high selectivity against N^6-methyladenine-DNA methyltransferase.

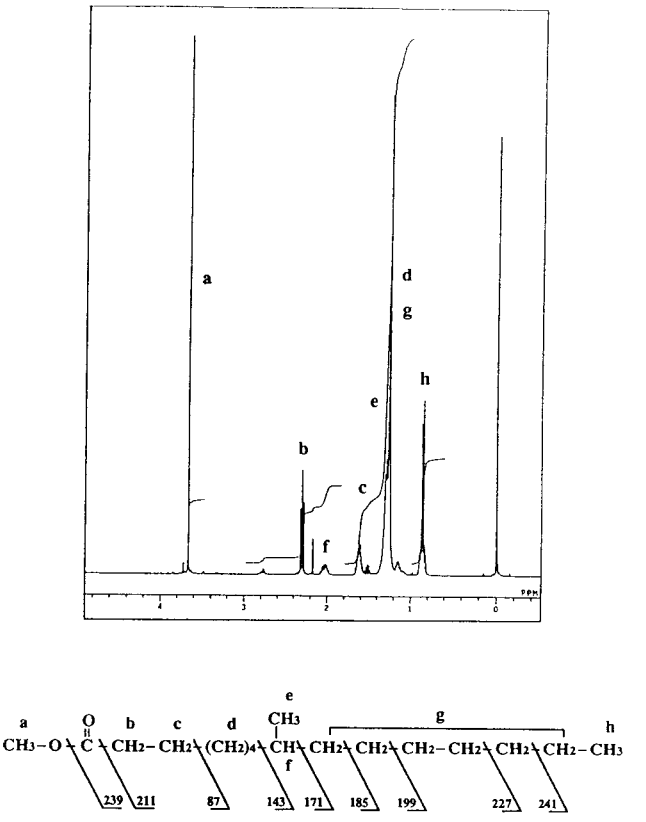

FIGURE 4 ^{1}H NMR spectrum and structure of DMI-1.

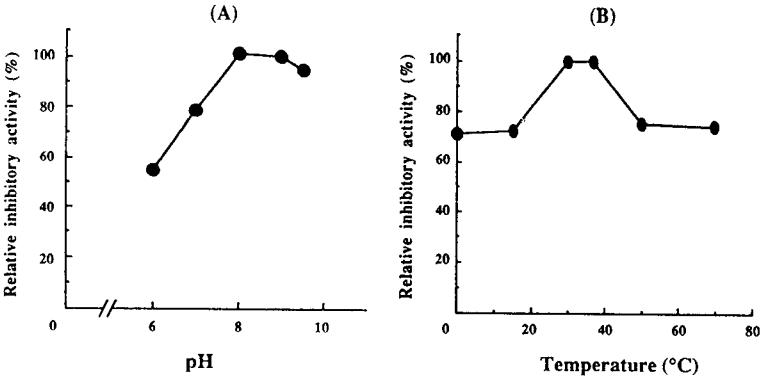

FIGURE 5 Effects of pH and temperature on the inhibitory activity of DMI-1. DMI-1 was used at a concentration of 4.2×10^{-5} M (1.6 unit) in the incubation mixture.

TABLE 2
Inhibitory spectrum of DMI-1.

Enzyme	IC_{50} (ng/μl of incubation mixture)
M. *Eco* RI	6.7
M. *Alu* I	22.2
M. *Bam* HI	24.8
Eco RI	40.0
Alu I	>40.0
Bam HI	28.4
Pst I	38.0
Sca I	2.9
DNase I	>40.0
DNase II	>40.0
RNase A	>40.0

K_i *Value of DMI-1 against M. Eco RI*

The type of inhibition was determined by a Lineweaver-Burk plot[21] of substrates (pUC19 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 DMI-1. As shown in Figures 6 and 7, DMI-1 inhibited M. *Eco* RI in a noncompetitive manner against both substrates. The K_m value of the enzyme against pUC19 was 1.85×10^{-8} M and the K_i value of DMI-1 was 4.93×10^{-5} M (Figure 6). In the case of SAM (Figure 7), the K_m value of the enzyme was 4.76×10^{-7} M and the K_i value of DMI-1 was 1.02×10^{-5} M. From these results, it is suggested that DMI-1 binds at a different site from the binding sites of pUC19 and SAM on the enzyme.

DMI-1 is a different type of DNA methyltransferase inhibitor in structure, from a structural analog of the natural cofactor (SAM) such as sinefungin, since it is a fatty acid derivative (8-methylpentadecanoic acid), and is considered to have a different type of inhibition mechanism. Recently, we found that esterification of the carboxyl group in DMI-1 induced a remarkable decrease in the inhibitory activity suggesting that the carboxyl group may play an important role in the enzyme inhibition. More detailed research concerning the inhibition of DNA methyltransferase by DMI-1 and fatty acids will allow us to understand the physiological functions and regulation of their enzyme activity in the living cell.

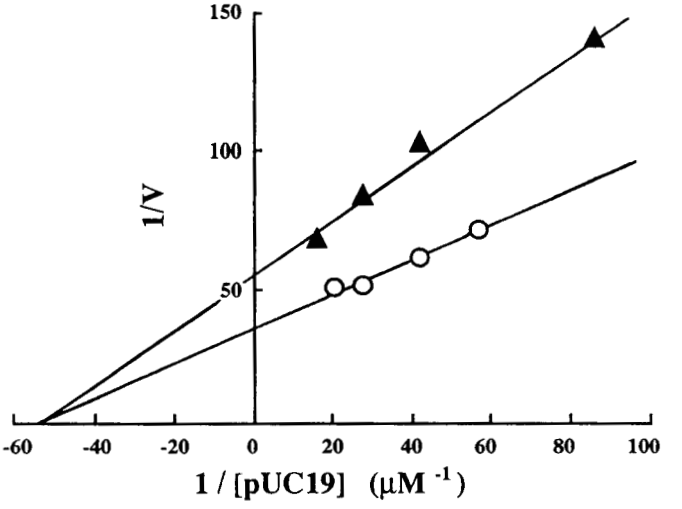

FIGURE 6 A Lineweaver-Burk reciprocal plot of plasmid pUC19 concentration against rate of methylation by M. *Eco* RI with DMI-1 (▲) and without DMI-1 (○). The inhibitor concentration used was 2.6×10^{-5} M (1 unit) in the incubation mixture.

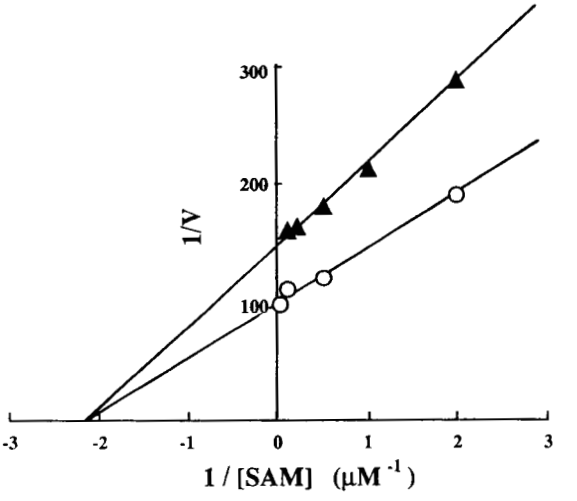

FIGURE 7 A Lineweaver-Burk reciprocal plot of S-adenosylmethionine (SAM) concentration against rate of methylation by M. *Eco* RI with DMI-1 (▲) and without DMI-1 (○). The inhibitor concentration used was 5.1×10^{-6} M (0.2 unit) in the incubation mixture.

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