J. Enzyme Inhibition, 1996, Vol. 10, pp. 271–280
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INHIBITION OF DNA METHYLTRANSFERASE BY MICROBIAL INHIBITORS AND FATTY ACIDS

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(Received 5 November 1995)

Streptomyces sp. strain No. 560 produces four kinds of DNA methyltransferase inhibitors in the culture filtrate. One of them, DMI-4 was distinguished from DMI-1, -2 and -3 previously reported with respect to certain properties. DMI-4 is considered to be a triglyceride consisting of the fatty acids anteisopentadecanoic acid ($C_{15:0}$), isopalmitic acid ($C_{16:0}$) and isostearic acid ($C_{18:0}$) from the results of gas chromatography analysis. Since DMI-4 contains three molecules of fatty acid, and the previously reported DMI-1, 8-methylpentadecanoic acid, is analogous to a fatty acid, the inhibitory activity has been examined of various fatty acids and their methyl esters against *Eco* RI DNA methyltransferase (M. *Eco* RI).

Oleic acid ($C_{18:1}$) was found to be a potent inhibitor of M. *Eco* RI. The inhibitory activity of oleic acid was shown to be pH- and temperature-dependent and inhibited M. *Eco* RI in a noncompetitive manner with respect to DNA or *S*-adenosylmethionine (SAM).

The number of carbon atoms and double bonds in the fatty acid molecule affected the inhibitory activity, but their methyl esters were not inhibitors. Our results suggest that the length of the carbon chain, the number of double bonds and the presence of a carboxyl group and branched methyl group in the fatty acid molecule may play an important role in the inhibition of DNA methyltransferase.

KEY WORDS: *Streptomyces* sp., methyltransferase inhibitor, fatty acid, oleic acid, DMI-4, M. *Eco* RI

INTRODUCTION

DNA contains a few methylated nucleic acids such as C^2-methyladenine, C^5-methylcytosine, N^6-methyladenine, N^4-methylcytosine or O^6-methylguanine together with four normal nucleic acids, adenine, cytosine, guanine and thymine. Methyladenine and methylcytosine generally exist in prokaryote and eukaryote cells, respectively.[1] Methylation of DNA by DNA methyltransferase is an important step in the regulation of gene expression in the eukaryote.[2] Manipulation of the level of DNA methylation *in vivo* and *in vitro* changes the pattern of gene expression.[3] In bacteria, methylation prevents the digestion of gene by restriction endonucleases. However, little is known about the state of DNA methyltransferase activities in the cells. The existence of a specific DNA methyltransferase inhibitor will facilitate studies concerning the role of DNA methyltransferases in the cells and the regulation of their activities.

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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. This enzyme transfers the methyl group from *S*-adenosylmethionine (SAM) to the N^6 position of the second adenine in the double stranded DNA sequence 5′-GAATTC-3′.[4] We found three kinds of DNA methyltransferase inhibitors, designated as DMI-1, DMI-2 and DMI-3, 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.[5,6] Recently, we found an additional inhibitor in the culture filtrate which was designated as DMI-4. DMI-4 is considered to be a triglyceride consisting of the fatty acids anteisopentadecanoic acid ($C_{15:0}$), isopalmitic acid ($C_{16:0}$) and isostearic acid ($C_{18:0}$).

As reported previously,[5] DMI-1, 8-methylpentadecanoic acid ($C_{16:0}$) is analogous to a fatty acid. Also, it is known that long-chain fatty acids inhibit methylation of phosphatidylethanolamine.[7] Consequently, we have examined the inhibitory activities of various fatty acids and their methyl esters against *Eco* RI DNA methyltransferase. In the present paper, we report the purification procedure and the structure of DMI-4, and effects of various fatty acids on DNA methyltransferase activity.

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. *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. DNase I (EC 3.1.4.5) from bovine pancreas, DNase II from porcine spleen and RNase A from bovine pancreas were obtained from Sigma Chemicals. 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.

Fatty acids

Caproic acid ($C_{6:0}$), caprylic acid ($C_{8:0}$), capric acid ($C_{10:0}$), lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$) and behenic acid ($C_{22:0}$) were obtained from Nacalai Tesque, Inc. Linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$), methylmyristate ($C_{15:0}$), methylpalmitate ($C_{17:0}$) and methylstearate ($C_{19:0}$) were obtained from Wako Pure Chemical Industries, Ltd. 8-Methylpentadecanoic acid (DMI-1, $C_{16:0}$) and methyl-8-methylpentadecanoate ($C_{17:0}$) were prepared in our laboratory.

Enzyme reactions

Activities of DNA methyltransferases (M. *Eco* RI,[4] M. *Alu* I[8] and M. *Bam* HI[9]) were assayed for their ability to protect plasmid pUC19 from digestion by restriction enzymes

(*Eco* RI, *Alu* I and *Bam* HI), respectively. 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 incubation continued at 37°C for 60 min for digestion of unmethylated pUC19. After 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.

Activities of restriction enzymes (*Alu* I,[10,11] *Bam* HI,[12] *Eco* RI,[13] Pst I[14] and *Sca* I[15]) and nucleases (DNase I,[16] DNase II[17] and RNase A[18]) were determined by measuring the concentration of plasmid pUC19 or RNA after the enzyme reactions. 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 or RNA (0.4 μg) was incubated at 37°C for 60 min. After 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 after electrophoresis at 200 V for 60 min. The assay conditions for inhibitory activity and electrophoresis have been described previously.[5,6]

Production and isolation of DMI-4

Streptomyces sp. strain No. 560 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 (10,000 rpm, 10 min). The culture filtrate was adjusted to pH 10 and extracted with ethyl acetate. The organic layer was concentrated *in vacuo* and dissolved in a minimal volume of methanol. This methanol solution was stood at –20°C overnight to retain DMI-1, -2 and -3 in solution, whilst DMI-4 settled out. The precipitate containing DMI-4 was applied to a silica gel 60 column (1.6 × 15 cm, Merck Co.) and DMI-4 was eluted with *n*-hexane-diethyl ether (9:1). The eluate was concentrated and applied to a silica gel 60 column. DMI-4 was eluted with *n*-hexane-diethyl ether (92:8). The active fractions obtained were combined and concentrated to give purified DMI-4.

Instrumental analyses

Proton nuclear magnetic resonance (^{1}H NMR) was measured by 400 MHz JMX GX-400 spectrometer. ^{1}H NMR spectra was obtained in $CDCl_3$ with tetramethylsilane (TMS) as internal standard. Gas chromatography was carried out with on a Hitachi 163 type. The conditions for the chromatography were as follows; 25% diethyleneglycol succinate on Chromosorb W packed in a stainless steel column (0.3 × 300 cm), column temperature at 200°C, N_2 gas as carrier gas.

RESULTS AND DISCUSSION

Production and Purification of DMI-4

Cultivation of *Streptomyces* sp. strain No. 560 was carried out under the cultural conditions described in MATERIALS AND METHODS. This strain produced different kinds of M. *Eco* RI inhibitors, *Eco* RI inhibitors and DNases in the different phase of cultivation. M. *Eco* RI inhibitory activity in the culture filtrate 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 was maintained at this level for a further 9 days. However, DNase activity reached a maximum in 1–3 days, in the early phase of cultivation, and then decreased rapidly.

Overall, 56.7 mg of purified DMI-4 was obtained from 1000 ml of the culture filtrate with a yield of 0.15% by the procedure described in MATERIALS AND METHODS. 250 μg of DMI-4 per 20 μl of incubation mixture gave 50% inhibition (1 unit) against M. *Eco* RI, but *Eco* RI was not inhibited by the addition of as much as 1000 μg of DMI-4.

Physicochemical properties and structure of DMI-4

DMI-4 was obtained as white powder which was soluble in chloroform and *n*-hexane but insoluble in water, ethanol and 2-propanol. It showed a positive H_2SO_4 reaction, and negative Dittmer, ninhydrin, $FeCl_3$ and $SbCl_3$ reactions on thin layer chromatography (TLC). DMI-4 gave a single spot, *Rf* 0.49, with *n*-hexane-ether (4:1) on TLC of a Silica Gel 60 F_{254} plate (Merck), *Rf* 0.62 with 2-propanol-acetonitrile (1:1) on RP-8F_{254} plate (Merck) and *Rf* 0.89 with benzene-ether (4:1) on silica gel (type G) plate containing 20% $AgNO_3$. The UV spectrum showed end absorption and ^{1}H NMR signals showed a typical pattern for triglyceride. Moreover, gas chromatography of the methyl esters obtained by esterification of DMI-4 revealed that DMI-4 contained the fatty acids anteisopentadecanoic acid ($C_{15:0}$), isopalmitic acid ($C_{16:0}$) and isostearic acid ($C_{18:0}$). From these results, DMI-4 was determined to be methyltetradecanoly-methylpentadecanoyl-methylheptadecanoyl-glycerol (see Figure 1).

Inhibitory activities of saturated fatty acids against M. Eco RI

Since DMI-1 and DMI-4 are a fatty acid and a triglyceride, respectively, the inhibitory activities of various fatty acids were examined against M. *Eco* RI. Inhibitory activities of some saturated fatty acids ($C_{6:0}$ to $C_{22:0}$) against M. *Eco* RI were shown in Table 1. $C_{6:0}$ to $C_{14:0}$ fatty acids did not inhibit M. *Eco* RI, but, with an increase in the length of the carbon chain above $C_{16:0}$ inhibitory activity appeared. Both palmitic acid and 8-methylpentadecanoic acid (DMI-1) are fatty acids of $C_{16:0}$, but the inhibitory activity of the latter having a branched methyl group was about 48-fold more than that of the former straight carbon chain fatty acid. From these results, it was suggested that the inhibition by saturated fatty acid depended on the number of carbon atoms present in the chain and the existence of a branched methyl group.

TABLE 1
Inhibitory activities of fatty acids against M. *Eco* RI.

Fatty acid	No. of carbon	pmole/μl of incubation mixture[a]
Caproic acid	6:0	>2500
Caprylic acid	8:0	>2500
Capric acid	10:0	>2500
Lauric acid	12:0	>2500
Myristic acid	14:0	>2500
Palmitic acid	16:0	1250
Stearic acid	18:0	133
Behenic acid	22:0	48
8-methylpentadecanoic acid (DMI-1)	16:0	26

[a]Amount required to cause 50% inhibition. The inhibitory activity was assayed under the standard assay conditions at 37°C and pH 8.0.

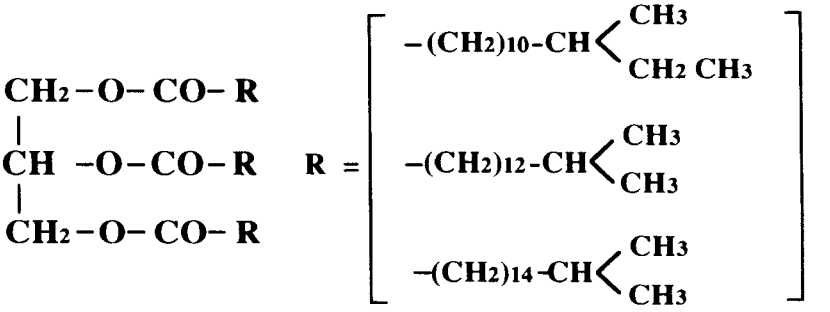

FIGURE 1 Proposed structure of DMI-4.

Inhibitory activities of unsaturated fatty acids against M. Eco RI

Inhibitory activities of unsaturated fatty acids (C_{18}) against M. *Eco* RI are shown in Table 2. Although stearic acid ($C_{18:0}$) required a concentration of 133 pmole to cause 50% inhibition oleic acid ($C_{18:1}$), containing a double bond in the molecule, required 16 pmole (about 8-fold inhibitory activity) compared with stearic acid. However, the activities of linolenic acid ($C_{18:2}$) and linoleic acid ($C_{18:3}$), containing two and three double bonds, respectively, were only 3-fold more potent than that of stearic acid. From these results, it was suggested that the existence of a double bond in a fatty acid molecule affected its inhibitory activity, but the intensity of the activity did not depend on the number of double bond.

TABLE 2
Inhibitory activities of saturated fatty acids against M. *Eco* RI.

Fatty acid	No. of carbon	pmole/μl of incubation mixture[a]
Stearic acid	18:0	133
Oleic acid	18:1	16
Linoleic acid	18:2	50
Linolenic acid	18.3	50
8-methylpentadecanoic acid (DMI-1)	16:0	26

[a]Amount required to cause 50% inhibition. The inhibitory activity was assayed under the standard assay conditions at 37°C and pH 8.0.

TABLE 3
Inhibitory activities of methyl esters of fatty acids against M. *Eco* RI.

Fatty acid	No. of carbon	pmole/μl of incubation mixture[a]
Myristic acid	14:0	>2500
Methylmyristate	15:0	>2500
Palmitic acid	16:0	1250
Methylpalmitate	17:0	>2500
Stearic acid	18:0	133
Methylstearate	19:0	>2500
8-methylpentadecanoic acid	16:0	26
Methyl 8-methylpentadecanoate	17:0	543

[a]Amount required to cause 50% inhibition. The inhibitory activity was assayed under the standard assay conditions at 37°C and pH 8.0.

Inhibitory activities of methyl esters of fatty acids against M. Eco RI

To examine the requirement for the presence of a carboxyl group in a fatty acid molecule for inhibition potency, the inhibitory activities of some fatty acids and their methyl esters were measured and compared (see Table 3). Methyl myristate, methyl palmitate and methyl stearate did not show inhibitory activity, and the inhibitory activity of the methyl ester of DMI-1 (methyl 8-methylpentadecanoate) was 21-fold weaker than that of DMI-1. It was considered that the presence of a carboxyl group in the molecule was important for inhibitory activity.

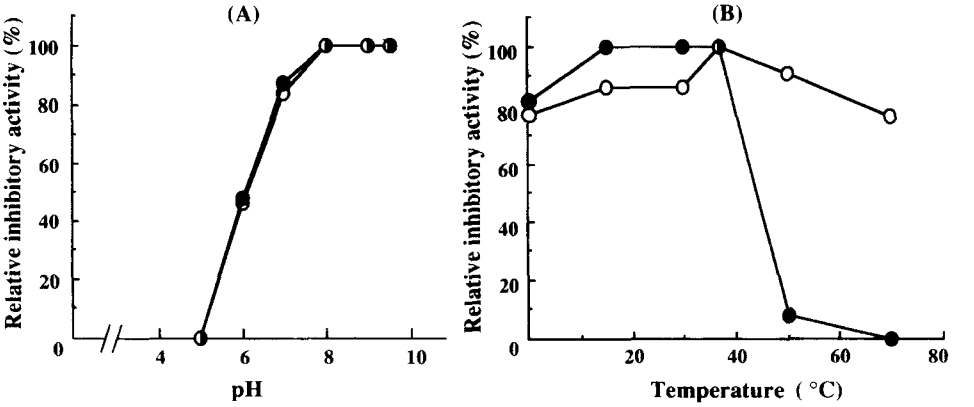

FIGURE 2 Effects of pH and temperature on the inhibitory activities of oleic acid (•) and DMI-4 (○). (A): oleic acid and DMI-4 were used at a concentration of 110 ng (2.0×10^{-5} M) and 370 μg (2.2×10^{-2} M) in the incubation mixture, respectively. The inhibitory activity was determined under the standard assay conditions at 37°C and various pHs, and the relative activity was expressed as 100% at pH 8.0. (B): oleic acid and DMI-4 were used at concentration of 92 ng (1.6×10^{-5} M) and 250 μg (1.5×10^{-2} M) in the incubation mixture, respectively. The inhibitory activity was determined under the standard assay conditions at pH 8.0 and various temperatures and the relative activity was expressed as 100% at 37°C.

Effects of pH and temperature inhibitory activity

Since oleic acid was the most potent inhibitor among the fatty acids examined here, further studies on the inhibitory properties of oleic acid and DMI-4 were conducted. First, the effects of pH and temperature on the inhibitory activities of both inhibitors were measured over the pH range pH 5.0–9.5 and at incubation temperatures from 0°C–70°C, respectively. As shown in Figure 2(A), the inhibitory activities of oleic acid and DMI-4 were found to be pH-dependent and increased with increasing pH. The activities measured at various temperatures are shown in Figure 2(B). The inhibitory activity of DMI-4 was independent on temperature whereas the activity of oleic acid was shown to be temperature-dependent and decreased with increasing temperature. Although DMI-1 is analogous to a fatty acid the inhibitory activity of DMI-1 did not show the remarkable decreases with incubation temperature over the range 50°C–70°C shown by oleic acid but behaved in a similar manner to DMI-4.

Comparison of inhibitory spectra

The effects of oleic acid on various enzymes were examined, and the results are compared with those of DMI-1, DMI-2 and DMI-3 produced by the strain No. 560 (see Table 4). Oleic acid as well as DMI-1 inhibited three kinds of DNA methyltransferases, but oleic acid was strongly distinguished from DMI-1 in that oleic acid inhibited *Eco* RI. DMI-2 was distinguished from other inhibitors in the inhibitions against *Pst* I and DNase II. DMI-1 showed high selectivity against three DNA methyltransferases and DMI-3 was a

TABLE 4
Comparison of inhibitory spectra.

Enzyme	Inhibition (ng/μl of incubation mixture)[a]				
	oleic acid	DMI-1	DMI-2	DMI-3	DMI-4
M. *Eco* RI	4.6	6.7	9.0	7.7	12.5 μg
M. *Alu* I	12.0	22.2	>40.0	>40.0	–
M. *Bam* HI	19.0	24.8	>40.0	>40.0	–
Eco RI	3.7	40.0	8.0	>40.0	>50.0 μg
Alu I	>40.0	>40.0	>40.0	>40.0	–
Bam HI	15.0	28.4	20.4	>40.0	–
Pst I	>40.0	38.0	5.2	>40.0	–
Sca I	4.0	2.9	2.9	>40.0	–
DNase I	>40.0	>40.0	>40.0	>40.0	–
DNase II	>40.0	>40.0	5.5	>40.0	–
RNase A	>40.0	>40.0	>40.0	>40.0	–

[a] Amount required to cause 50% inhibition.

TABLE 5
Comparison of types of inhibition and inhibitory constants against M. *Eco* RI among DMIs, oleic acid and sinefungin.

Inhibitor	pUC19		SAM	
	Type	K_i (μM)	Type	K_i (μM)
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
Oleic acid	N	12.6	N	57.4
Sinefungin	U	–	C	–

K_m value of M. *Eco* RI against pUC19 and SAM were 0.02 μM and 0.48 μM, respectively. C: competitive inhibition; N: non-competitive inhibition; U: uncompetitive inhibition.

specific inhibitor of M. *Eco* RI. These inhibitors showed different inhibitory spectra, but none of the inhibitors examined could inhibit *Alu* I, DNase I and RNase A.

Comparison of K_i values and type of inhibition against M. Eco RI

The types of inhibition exhibited by DMI-4 and oleic acid were determined by a Lineweaver-Burk plot[19] of substrates (pUC19 and SAM) concentration against rate of methylation of M. *Eco* RI in the presence and absence of the inhibitor.

The results are compared with those for other inhibitors in Table 5. Sinefungin is a natural analog of SAM isolated from *Streptomyces griseolus*; the methyl sulfonium of SAM is replaced with an amine-methine substituent.[20,21] Sinefungin binds to the SAM binding site of M. *Eco* RI molecule in the presence of DNA to form the DNA-M. *Eco* RI-Sinefungin complex.[22,23] Consequently it is a competitive inhibitor of M. *Eco* RI with respect to SAM and an uncompetitive inhibitor with respect to DNA.

The five inhibitors tested showed different types of inhibition from that of Sinefungin. DMI-1, DMI-4 and oleic acid noncompetitively inhibited with respect to SAM and DNA but DMI-1 differed from DMI-4 and oleic acid with regard to the comparative K_i values for SAM and DNA. The K_i value of DMI-1 for SAM was 4.8-fold smaller than that for DNA whereas the values of DMI-4 and oleic acid for SAM were, contrary to DMI-1, 4 to 5-fold larger than those for DNA. DMI-2, reported previously,[6] was a competitive inhibitor with respect to DNA, therefore, this inhibitor was thought to bind the DNA binding site of the enzyme. DMI-3 was a highly potent inhibitor ($K_i = 1.2$ μM) and bound to the SAM binding site of the enzyme. Although both DMI-1 and oleic acid are fatty acids, DMI-1 acted strongly on the SAM binding site and oleic acid acted on the DNA binding site. This difference may be caused by the existence of a branched methyl group or double bond in the fatty acid molecules. It would seem that each fatty acid may show a different inhibitory spectrum and inhibition manner.

It would be interesting to establish the correlation between structure and inhibitory activity of fatty acids since various fatty acids exist in living cells and these structures ceaselessly alter by oxidation, reduction, esterification or epoxidation and so on and exhibit various physiological effects. Our data suggests that a double bond, carboxyl group and branched methyl group in the fatty acid molecule plays an important role in the enzyme inhibition process.

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