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Activation and inhibition of DNA methyltransferases by *S*-adenosyl-L-homocysteine analogues

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Abstract—The inhibition of methyltransferases is currently of high interest, particularly in the areas of microbial infection and cell proliferation, as there have been serious attempts to develop novel anti-microbial agents. In the present investigation, a series of 11 *S*-adenosyl-L-homocysteine analogues have been synthesized and effect of these analogues on DNA methylation catalyzed by DNA methyltransferases was studied. It was found that, while 5′-*S*-(propionic acid)5′-deoxy-9-(1′-β-D-ribofuranosyl)1,3-dideazaadenine was an activator of EcoP15I and HhaI DNA methyltransferases, 5′-*S*-(propionic acid)5′-deoxy-9-(1′-β-dribofuranosyl)adenine inhibited the methyltransferases in a non-competitive manner. An understanding of the binding of analogues to DNA methyltransferases will greatly assist the design of novel anti-microbial compounds.

1. Introduction

S-Adenosyl-L-methionine (AdoMet) is a source of diverse chemical groups used in biosynthesis and modification of virtually every class of biomolecules and is a commonly used cofactor, second only to ATP in the variety of reactions in which it participates.[1] AdoMet is a conjugate of nucleoside adenosine and amino acid methionine and is the methyl donor in the majority of methyl transfer reactions, including methylation of DNA, RNA, proteins, and small molecules.[2] One of the most notable reactions requiring AdoMet, the transfer of methyl group, is performed by a large class of enzymes, AdoMet-dependent DNA methyltransferases (DNA MTases), which have been the focus of considerable structure–function studies.[3] A conserved AdoMet-dependent methyltransferase fold, in which AdoMet is bound in the same orientation, has been observed in structurally characterized DNA MTases.[4] DNA MTases catalyze the transfer of a methyl group from AdoMet to N^4 or C^5 atoms of cytosine or to N^6 atom of adenine bases in DNA. The enzymatic transfer of the methyl group of AdoMet yields *S*-adenosyl-L-homocysteine (AdoHcy) as one of the products of the reaction.[3]

In prokaryotes, all three types of DNA methylation described above are observed and DNA methylation has three major biological roles: (i) distinction of self and nonself DNA as in restriction modification systems,[5], (ii) direction of postreplicative mismatch repair[6], and (iii) control of DNA replication and cell cycle.[7,8] Since several bacteria are important human pathogens and adenine MTases are not known to exist in higher eukaryotes, design and synthesis of promising inhibitors that are specific for adenine MTases have generated a lot of interest in the understanding of the chemistry and biology of DNA MTases.[9]

DNA adenine methyltransferases (Dam) are highly conserved in many pathogenic bacteria,[10,11] that cause significant morbidity and mortality. *Salmonella typhimurium* strains lacking Dam were fully proficient in colonization of mucosal sites but showed severe defects in colonization of deeper tissue sites.[12] Studies have shown that hypermethylation of upstream regulatory sequences by human C^5 cytosine DNA MTases led to inactivation of tumor suppressor genes in numerous cancers. It was postulated that inhibitors of human

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DNA MTase could reverse the effects of DNA hypermethylation and therefore be important in the treatment of cancer.[13]

It is increasingly evident that epigenetic mechanisms play a significant role in the regulation of virulence-associated functions in pathogenic bacteria.[14] Thus, DNA methyltransferase inhibitors are likely to have broad anti-microbial action. Sinefungin (5′-deoxy-5′-1,4-diamino-4-carboxybutyl)adenosine, a competitive inhibitor of all DNA methyltransferases, has been shown to have therapeutic properties including antifungal, antiviral, antiparasitic, and antitumor activities.[15] DNA methyltransferases are inhibited by the demethylated product AdoHcy.[16] Other than AdoHcy analogues, single-stranded oligonucleotides have been shown to act as inhibitors of murine cytosine C^5 methyltransferase[17] and human DNA methyltransferase.[18] Single-stranded oligonucleotides specifically inhibit the DNA MTases, whereas AdoHcy analogues inhibit most of the AdoMet-dependent MTases leading to toxicity.[18] In this study, we report the modulating effects of different AdoHcy analogues on the activity of two N^6 adenine MTases, EcoP15I DNA MTase (M.EcoP15I) and DNA adenine MTase (Dam), and one C^5 cytosine MTase HhaI DNA MTase (M.HhaI).

2. Results and discussion

AdoHcy is structurally similar to AdoMet in that it binds to MTase in the same orientation and with almost similar affinities but lacks the reactive CH_3 group and acts as a competitive inhibitor with respect to AdoMet.[16] Earlier studies had indicated that terminal amino and terminal carboxyl groups present in the amino acid side chain are an absolute requirement for the AdoHcy molecule to bind with enzymes catechol *O*-methyltransferase, phenylethanolamine *N*-methyltransferase, histamine *N*-methyltransferase, and hydroxyindole *O*-methyltransferase. Modification in the sulphur atom on the homocysteine portion of AdoHcy results in the decrease in the inhibitory activity.[19] Furthermore, the adenine portion of AdoHcy, with an absolute requirement of the 6-amino group, was essential for the inhibitory activity of AdoHcy.[20] The 2′ hydroxyl group of AdoHcy was shown to be important for inhibition.[21] Inhibition studies on two cytosine C^5 DNA methyltransferases, M.HhaI and M.HaeIII, by AdoHcy analogues showed that different AdoHcy analogues have different inhibition ability, suggesting that multiple protein–ligand interactions are involved in binding and recognition.[22]

In order to further investigate the role of different functional groups of AdoHcy in the inhibition of adenine and cytosine methyltransferases, we synthesized 11 AdoHcy analogues by altering the purine ring (**2**, **4**, and **6**), thioester linkage (**5** and **6**), and/or amino acid portion (**2**–**12**) (Table 1).

Analogue (**2**) differs from AdoHcy (**1**) in that it lacks both nitrogen atoms of the adenine base and terminal amino group and one carbon atom of amino acid side chain. The deaminated AdoHcy analogue, 5′-*S*-(propionic acid)5′-deoxy-9-(1′-β-D-ribofuranosyl)adenine (**3**), has one carbon atom less in the side chain and with an aim to increase its effectiveness, it was converted to its amide (**7**) and ester (**8**) derivatives. These modifications are likely to make the compound more lipophilic in nature which would help in its better cellular uptake and thus might result in showing better activity. In analogues (**5**) and (**6**), the amino acid side chain was replaced by chloride and hydroxyl groups, respectively. Analogue (**6**) had a deaminated adenine ring in which N^6 amino group was replaced by a nitro group. Analogues (**9**) to (**12**) differ from AdoHcy by lacking carboxyl and amino group in the amino acid side chain and differ from each other in terms of the length of the hydrocarbon side chain. The catalytic activity of M.EcoP15I, Dam, and M.HhaI was measured by incorporation of radiolabeled methyl group from ^{3}H-methyl AdoMet in a synthetic DNA substrate.

Of the 11 analogues studied in terms of their effect on the activity of M.EcoP15I (Fig. 1A) and M.HhaI (Fig. 1B), all compounds deficient in terminal carboxyl group such as 5′-allylthio-5′-deoxy-9-(1′-β-D-ribofuranosyl)6-nitrodideazaadenine (**4**), 5′-chloro-5′-deoxyadenosine (**5**), 9-(1′-β-D-ribofuranosyl)6-nitro-1,3-dideazaadenine (**6**), 5′-*S*-(propionamide)5′-deoxy-9-(1′-β-D-ribofuranosyl)-adenine (**7**), 5′-*S*-(ethylpropionate)5′-deoxy-9-(1′-β-D-ribofuranosyl)adenine (**8**), 5′-*S*-5′-methylthio deoxyadenosine (**9**), 5′-*S*-propargylthio adenosine (**10**), 5′-*S*-allenylthio adenosine (**11**), and 5′-*S*-propynylthio adenosine (**12**) had no effect on M.EcoP15I and M.HhaI activity (Fig. 1A and B). AdoHcy strongly inhibits M.EcoP15I and M.HhaI activities (Fig. 1A and B).

Analogue **3** was shown to inhibit both M.EcoP15I (Fig. 2A) and M.HhaI activities (Fig. 2B). In order to characterize the inhibition, we carried out detailed kinetic analysis with M.EcoP15I. Figure 3A shows the initial velocity as a function of increasing concentrations of AdoMet at the fixed concentration of inhibitor. As concentration of inhibitor increases, the slope of the reciprocal plot increases and $1/v$-axis, intercept pivots counterclockwise about the point of intersection with the control curve (at $-1/K_m$ on the 1/[S]-axis). The pattern of lines clearly indicates that it acts as a non-competitive inhibitor (Fig. 3B). Next, $1/v$-axis intercept of the above reciprocal plot was plotted against inhibitor concentration and K_i value determined as [I]-axis intercept which is equal to 5 μM (Fig. 3C). From the Dixon plot (Fig. 3D), IC_{50}^{23} value was estimated as 5 μM by plotting $1/v$ versus inhibitor concentration (Fig. 3D). Thus, K_i is equal to the IC_{50}, which is a characteristic of non-competitive inhibition.[23]

Sinefungin is one of the most potent inhibitors of MTases.[24] Compared to sinefungin, analogue **3** is a weak inhibitor. At the same concentration, sinefungin inhibits 20% more than analogue **3** (data not shown). Earlier, Cohen et al.[22] synthesized series of analogues of AdoHcy each containing a single modification and tested for the ability to inhibit methylation by M.HhaI and

Table 1.

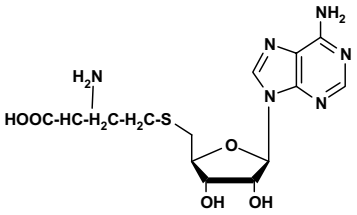
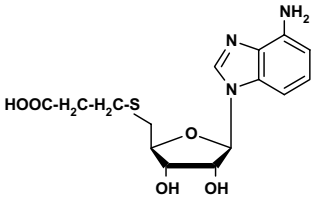
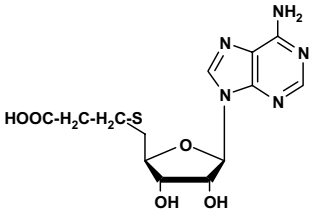
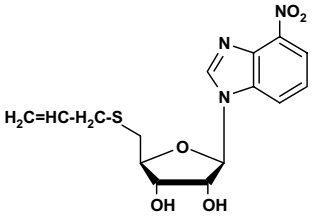
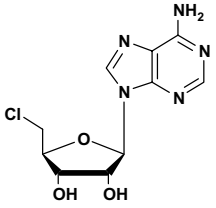
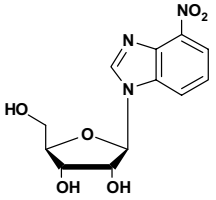
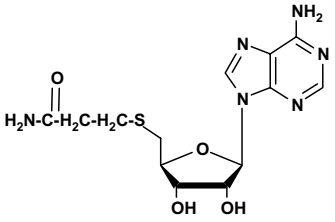
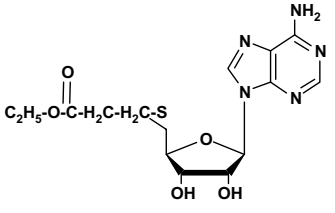
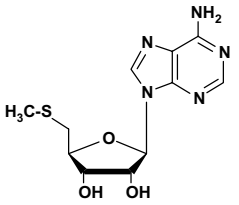
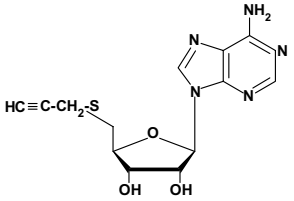
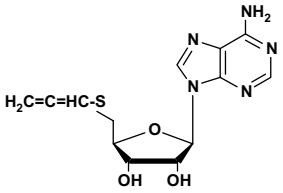
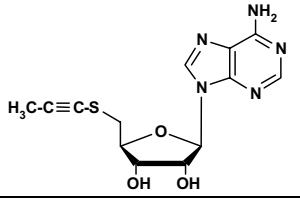
S. No.	Structure	Name
1		*S*-Adenosyl-homocysteine
2		5′-*S*-(Propionic acid)5′-deoxy-9-(1′-β-D-ribofuranosyl)1,3-dideaza adenine
3		5′-*S*-(Propionic acid)5′-deoxy-9-(1′-β-D-ribo furanosyl)adenine
4		5′-Allylthio-5′-deoxy-9-(1′-β-D-ribofuranosyl)6-nitrodideazaadenine
5		5′-Chloro-5′-deoxyadenosine
6		9-(1′-β-D-Ribofuranosyl)6-nitro-1,3-dideaza adenine
7		5′-*S*-(Propionamide)5′-deoxy-9-(1′-β-D-ribofuranosyl)adenine
8		5′-*S*-(Ethylpropionate)5′-deoxy-9-(1′-β-D-ribofuranosyl)adenine

Table 1 (*continued*)

S. No.	Structure	Name
9		5′-*S*-5′-Methylthio deoxyadenosine
10		5′-*S*-Propargylthio adenosine
11		5′-*S*-Allenylthio adenosine
12		5′-*S*-Propynylthio adenosine

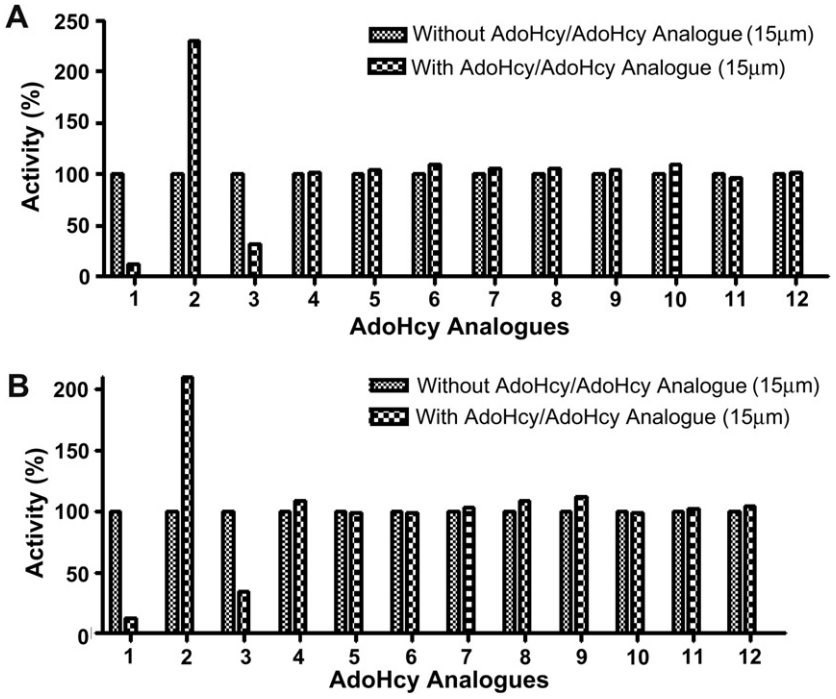


Figure 1. Effect of AdoHcy analogues on MTase activity. Effect was observed by incubating 100 nM of (A) M.EcoP15I or (B) M.HhaI with 15 μM of AdoHcy analogues and reactions were performed with 2 pmol of substrate DNA and 1 μM of [^{3}H]AdoMet (74.5 Ci/mmol) in the methylation buffer at 37 °C for 30 min. The enzyme activity in the absence of the inhibitor was normalized to 100.

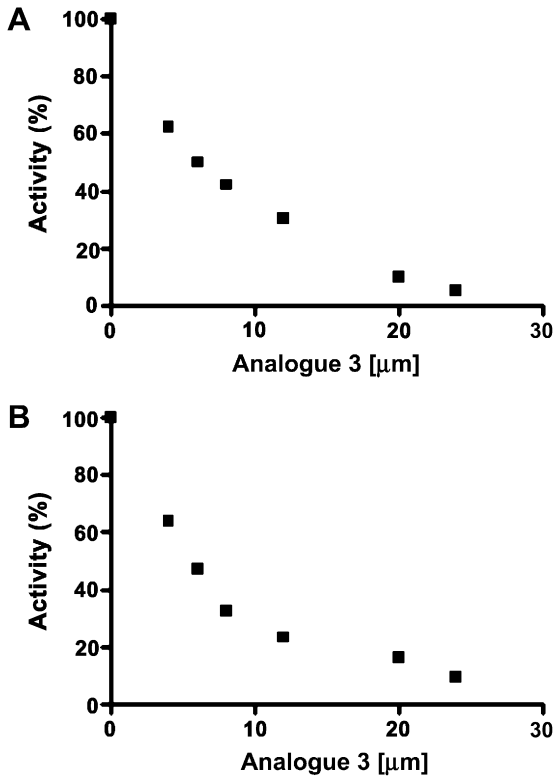


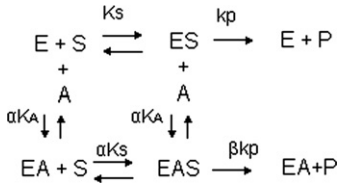
Figure 2. Effect of 5′-*S*-(propionic acid)5′-deoxy-9-(1′-β-D-ribofuranosyl)adenine on MTase activity. Inhibitory effect of 5′-*S*-(propionic acid)5′-deoxy-9-(1′-β-D-ribofuranosyl)adenine was studied by incubating 100 nM of (A) M.EcoP15I or (B) M.HhaI with increasing concentrations (0, 4, 6, 8, 12, 20, and 24 μM) of analogue and reactions were performed with 2 pmol of substrate DNA and 1 μM of [^{3}H]AdoMet (74.5 Ci/mmol) in the methylation buffer at 37 °C for 30 min.

M.HaeIII. In that study, all analogues inhibited methylation with K_i values ranging from 8 to 3000 μM.

Interestingly in the presence of analogue **2** there is twofold increase (Fig. 1) in the activity of both M.EcoP15I and M.HhaI. Analogue **2** activates in a complex manner. It acts as an activator at low concentration up to 20 μM for M.EcoP15I (Fig. 4A) and up to 16 μM for M.HhaI (Fig. 4B), but at higher concentrations it has less effect on the enzyme activity. Figure 4C shows the initial velocity as a function of increasing concentrations of AdoMet at the fixed concentration of inhibitor. The family of reciprocal plots for the activator is shown in Figure 4D. As the concentration of activator increases, the reciprocal plot pivots clockwise about the point of intersection with the control plot. We have found that the apparent change in the K_s of M.HhaI is by a factor α, which is equal to 0.66 and V_{max} is by a factor β, which is equal to 2.003 (Fig. 4D).

There are two major types of activation: (i) non-essential activation, in which the reaction can occur in the absence of the activator. Non-essential activation is similar to partial or mixed type of inhibition; however, the changes are in opposite direction. (ii) Essential activation in which the true substrate is an SA complex, where *A* is a metal ion.[25]

An activator enhances the enzymatic reaction by altering the rate of dissociation of ES complex. α is the factor by which dissociation of ES to E and S is changed and β is the factor by which the rate constant for the breakdown of ES to E and P is changed. For such activators, α will be less than unity and β will be greater than unity.



We were also interested in observing the effect of AdoHcy analogues on Dam methylase as it plays a vital role in regulating the expression of various bacterial genes related to virulence in diverse pathogens. We found that analogue **2** and analogue **3** affect the Dam methylase in the same way as they affect the M.EcoP5I and M.HhaI (Fig. 5).

It is worth mentioning that structurally analogue **2** differs from analogue **3** in terms of the absence of two nitrogen atoms in the purine ring (Table 1). To the best of our knowledge analogue **2** is the only known AdoHcy analogue which acts as an activator of DNA MTases. Thus, in assays measuring low MTase activity, as in cell lines and tissues, inclusion of analogue **2** will be useful.

It is possible that the analogues which had no effect on MTase activity failed to bind to the enzymes. To check this possibility, fluorescence emission spectra and fluorescence intensities were measured for M.EcoP15I in the presence of different AdoHcy analogues. M.EcoP15I showed significant quenching in the presence of AdoHcy, analogues **2** and **3**, and no quenching was observed in the presence of analogue **8** (Fig. 6A). K_{SV} values for analogues, **2** and **3** were calculated by using Stern Volmer plot as 5.66 and 21.99 μM, respectively (Fig. 6B and C). $1/K_{SV}$ is the quencher concentration at which 50% of the intensity is quenched.

DNA methylation and its role in lower eukaryotes especially in protozoans are of great interest because large groups of protozoan are human pathogens. N^6 adenine DNA methylation has been reported in *Tetrahymena* and its orthologous open-reading frames have been identified in three species of malarial parasite *Plasmodium*.[26] It has been shown that 5-azacytidine, a potent inhibitor of DNA methyltransferase, significantly reduced *Entamoeba histolytica* virulence in vitro and in vivo.[27] Sinefungin is a very potent antileishmanial agent in vitro and in vivo[28] but because of drug pressure it has been reported that *Leishmania donovani* promastigotes develop resistance.[29]

In the view of these observations it has become important to design more efficient inhibitors against MTases. This study analyzes the inhibition of two N^6 adenine DNA MTases, M.EcoP15I and Dam, and one cytosine C^5 DNA MTase, M.HhaI, by a series of 11 AdoHcy analogues to characterize quantitatively the contribution of different functional groups to affinity. These studies are helpful in understanding the role of different groups of

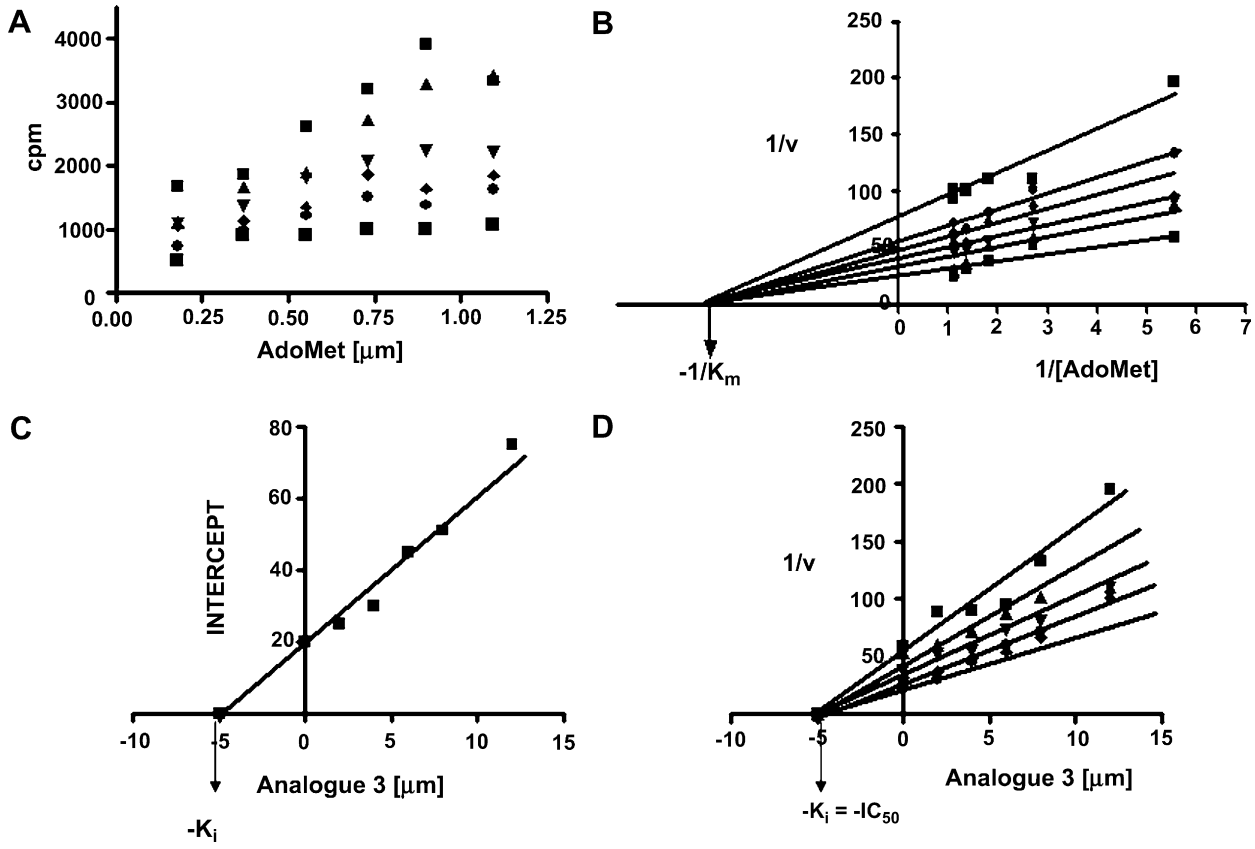


Figure 3. Kinetics of inhibition of M.EcoP15I by 5′-*S*-(propionic acid)5′-deoxy-9-(1′-β-D-ribofuranosyl)adenine. (A) Inhibition of M.EcoP15I at different concentrations of inhibitor (■0, ▲2, ▼4, ♦6, •8, and ■12 μM). (B) Lineweaver-Burk plot. (C) Secondary plot for the estimation of K_i value. (D) Dixon plot.

AdoHcy in inhibition which in turn will be helpful in the designing of more effective inhibitors for MTases, which play a vital role in pathogenesis. Further investigation is required to understand the uptake and in vivo mode of action of inhibitor used in this investigation.

3. Conclusions

We report the inhibiting and activating effects of a series of AdoHcy analogues on the activity of N^6 adenine and C^5 cytosine MTases. In this study, one analogue (analogue **3**) was found to be a strong inhibitor for MTases and interestingly analogue **2** acts as an activator. This study highlights the importance of two nitrogen atoms in the purine ring in the modulating effects of these AdoHcy analogues.

4. Experimental

4.1. Synthesis of AdoHcy analogues

Chemical synthesis of AdoHcy analogues used (**2**–**12**) (Table 1) in the present study was carried out. The analogue 5′-*S*-(propionic acid)5′-deoxy-9-(1′-β-D-ribofuranosyl)adenine (**3**) was prepared according to the method of Heildesheim et al.[30] and then converted to its amide (**7**) and ester (**8**).[31] The analogues 5′-*S*-(propionic acid)5′-deoxy-9-(1′-β-D-ribofuranosyl)1,3-dideazaadenine (**2**) and 5′-allylthio-5′-deoxy-9-(1′-β-D-ribofuranosyl)-nitrodideazaadenine (**4**) were synthesized.[31] 9-(1′-β-D-Ribofuranosyl)6-nitro-1,3-dideazaadenine (**6**) was prepared according to the method of Sinha et al.[32] 5′-Chloro-5′-deoxyadenosine (**5**) was prepared in 75% yield from adenosine and thionyl chloride in hexamethylphosphoramide by a modification mentioned by Borchardt et al.[33] of the procedure of Kikugawa and Ichino.[34] The synthesis of 5′-*S*-5′-methylthio deoxyadenosine (**9**) was carried out using methyl disulfide and tributylphosphine by the procedure described by Nakagawa and Hata[35] in 60% yield. 5′-*S*-Propargylthio adenosine (**10**), 5′-*S*-allenylthio adenosine (**11**), and 5′-*S*-propynylthio adenosine (**12**) were synthesized using tosylated N^6-benzoyl-2′,3′-isopropylidene adenosine according to Guillerm et al.[36] in 65%, 50% and 38% yield, respectively. All the analogues were characterized by their ^{1}H NMR, UV spectral properties, and elemental analysis. UV measurement was carried out on Hitachi 220S spectrophotometer. ^{1}H NMR was recorded using DRX 300 instrument with d6-DMSO as solvent. AdoHcy was obtained from Sigma.

Characterization details of the synthesized analogues. 5′-*S*-(Propionic acid)5′-deoxy-9-(1′-β-D-ribofuranosyl)1,3-dideaza adenine (**2**): UV (MeOH) λ_{max} 235, 266, 314 nm. ^{1}H NMR (DMSO-d_6) δ 4.58–4.60 (s, 2H, $NH_{2'}$) δ 7.26–7.70 (m, 3H, aro); δ 8.48–8.70 (s, 1H, C-8); δ 5.65–6.03 (d, 1H, H1′); δ 3.50–3.70 (m, 1H, H2′); δ

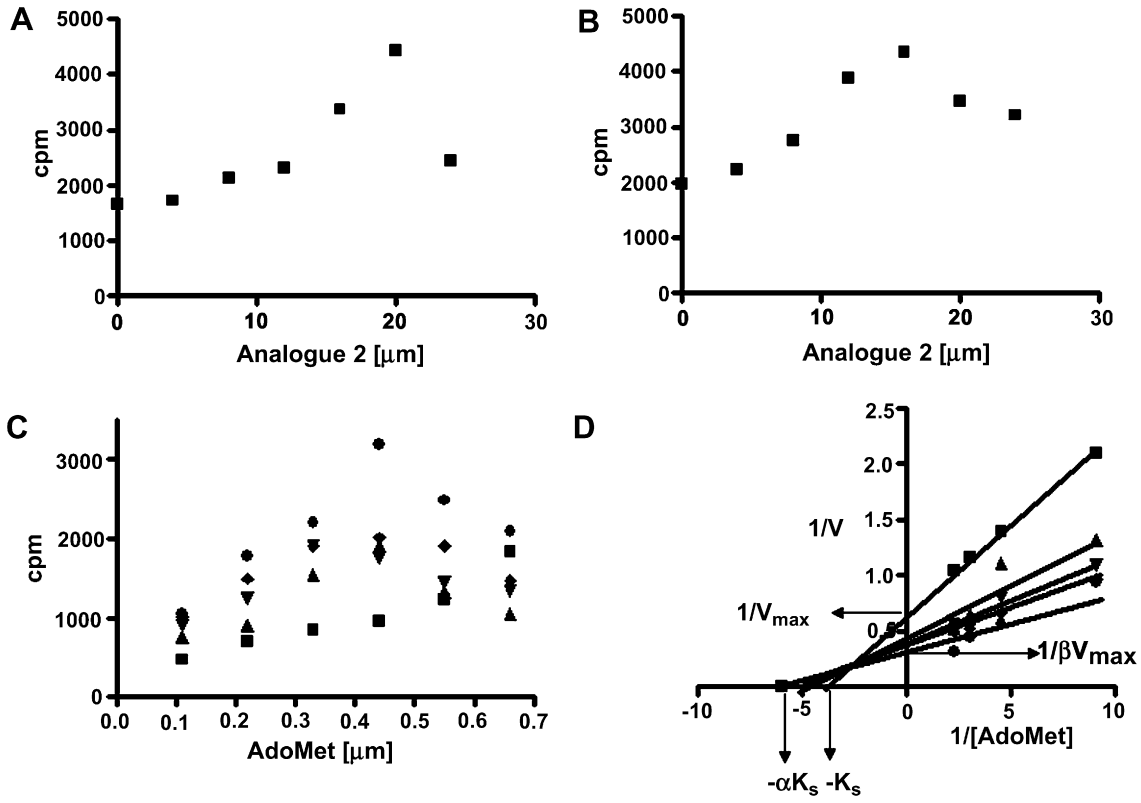


Figure 4. Kinetics of activation of (A) M.EcoP15I and (B) M.HhaI at different concentrations of 5′-*S*-(propionic acid)5′-deoxy-9-(1′-β-D-ribofuranosyl)1,3-dideazaadenine (0, 4, 8, 12, 16, 20, and 24 μM). (C) Activation of M.HhaI at fixed concentration of activator (■0, ▲4, ▼8, ♦12, and ●16 μM) as a function of increasing AdoMet concentration. (D) Line weaver-Burk plot.

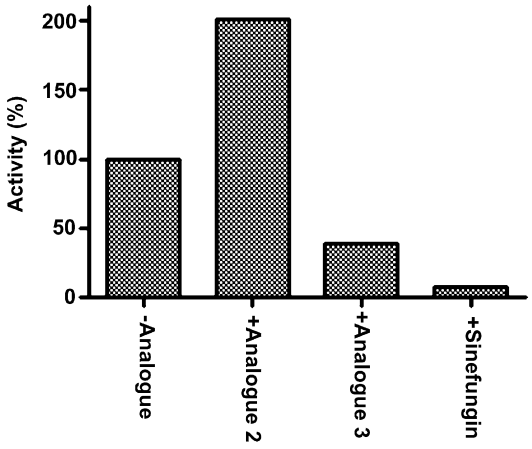


Figure 5. Effect of 5′-*S*-(propionic acid)5′-deoxy-9-(1′-β-D-ribofuranosyl)1,3-dideazaadenine (analogue **2**) and 5′-*S*-(propionic acid)5′-deoxy-9-(1′-β-D-ribofuranosyl)adenine (analogue **3**) on Dam MTase. Dam (1 U) was incubated with 15 μM of AdoHcy analogue (analogue **2**, analogue **3**, and sinefungin) and reactions were performed with 2 pmol of substrate DNA and 1 μM of [^{3}H]AdoMet (74.5 Ci/mmol) in the methylation buffer at 37 °C for 30 min. The enzyme activity in the absence of the inhibitor was normalized to 100.

3.49–3.65 (m, 1H, H3′); δ 4.36–4.66 (m, 1H, H4′); δ 3.34–3.46 (m, 2H, H5′); δ 1.90–2.0 (s, 2H, OH); δ 2.50–2.71 (t, 2H); δ 2.55–2.60 (t, 2H); MS *m/z* 352.4 (M^+). Anal. calcd for $C_{15}H_{19}N_3O_5S$: C, 50.99; H, 5.38; N, 11.89. Found: C, 50.81; H, 5.31; N, 11.78.

5′-*S*-(Propionic acid)5′-deoxy-9-(1′-β-D-ribo furanosyl)adenine (**3**): UV (MeOH) λ_{max} 240, 281, 317 nm. ^{1}H NMR (DMSO-d_6) δ 4.50–4.52 (s, 2H, $NH_{2'}$); δ 8.12 (s, 1H, C-2); δ 8.48–8.70 (s, 1H, C-8); δ 5.65–6.03 (d, J = 6.2, 1H, H1′); δ 3.50–3.70 (m, 1H, H2′); δ 3.49–3.65 (m, 1H, H′3); δ 4.36–4.66 (m, 1H, H4′); δ 3.34–3.46 (m, 2H, H5′); δ 1.90–2.0 (s, 2H, OH); δ 2.50–2.71 (t, 2H); δ 2.55–2.60 (t, 2H); MS *m/z* 354.5 (M^+). Anal. calcd for $C_{13}H_{17}N_5O_5S$: C, 43.94; H, 4.79; N, 19.72. Found: C, 43.76; H, 4.68; N, 19.57.

5′-Allylthio-5′-deoxy-9-(1′-β-D-ribofuranosyl)6-nitro-dideazaadenine (**4**): UV (MeOH) λ_{max} 290 nm λ_{min} 238 nm. ^{1}H NMR (DMSO-d_6) δ 7.52–8.19 (m, 4H, aro), 2.3–2.5 (2H, q), 1.3–1.57 (3H, t), 1.8–2.0 (2H, d), 4.0–4.2 (1H, m), 4.8–5.02 (2H, d). MS *m/z* 351.6 (M^+). Anal. calcd for $C_{15}H_{17}O_5N_3S$: C, 51.28; H, 4.84; N, 11.97. Found: C, 51.21; H, 4.80; N, 11.91.

5′-Chloro-5′-deoxyadenosine (**5**): mp 190 °C, UV (MeOH) λ_{max} 257, λ_{min} 229, R_f = 0.48 (DCM/MeOH: 8.5:1.5). Anal. calcd for $C_{10}H_{12}O_3N_5Cl$: C, 42.04; H, 4.23; N, 24.51; Cl, 12.41. Found: C, 42.40; H, 4.38; N, 24.41; Cl, 12.80.

9-(1′-β-D-Ribofuranosyl)6-nitro-1,3-dideaza adenine (**6**): UV (MeOH) λ_{max} 235, 292, 305 (sh) nm. ^{1}H NMR (300 MHz, DMSO/water) δ 1.85–2.5 (m, 1H, H2′); 3.34–3.46 (m, 1H, H4′); 4.36–4.46 (m, 2H, H5′); 4.67–4.81 (m, 1H, H3′); 5.68 (d, *J* = 6.2, 1H, H1′); 7.79–

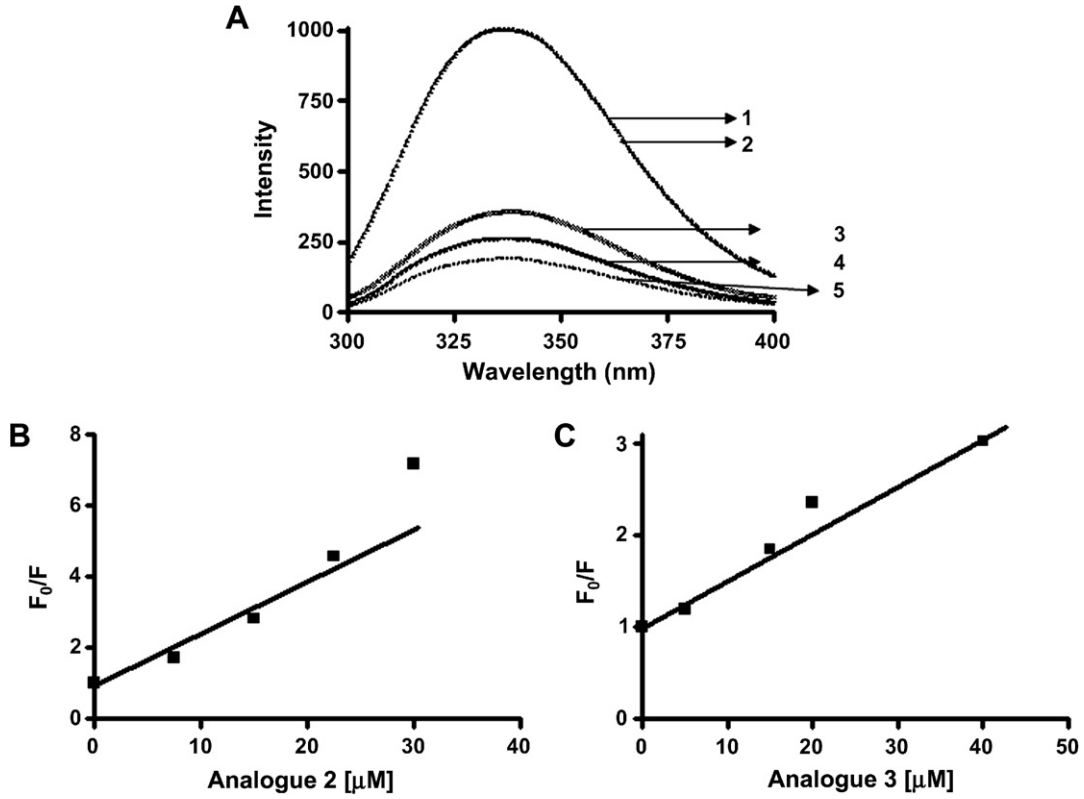


Figure 6. Fluorescence measurements. (A) 1 μM of M.EcoP15I was preincubated with 25 μM of AdoHcy analogues (**2**, **3**, and **8**) and AdoHcy for 1 min and then emission spectra were recorded over a wavelength of 300–400 nm with an excitation wavelength of 280 nm. [1—M.Ecop15I without analogue, 2—M.Ecop15I with analogue **8**, 3—M.Ecop15I with analogue **3**, 4—M.Ecop15I with AdoHcy, 5—M.Ecop15I with analogue **2**]. (B) 1 μM of M.EcoP15I was preincubated with increasing concentrations (7.5, 15, 22.5, and 30 μM) of 5′-*S*-(propionic acid)5′-deoxy-9-(1′-β-D-ribofuranosyl)1,3-dideazaadenine for 1 min and then emission spectra were recorded. The ratio of fluorescence intensities (F_0/F) was plotted against the total AdoHcy analogue concentration, and the data were analyzed according to Stern–Volmer plot. (C) 1 μM of M.EcoP15I was preincubated with increasing concentrations (5, 15, 20, 40, and 60 μM) of 5′-*S*-(propionic acid) 5′-deoxy-9-(1′-β-D-ribofuranosyl)adenine for 1 min and then emission spectra were recorded. The ratio of fluorescence intensities (F_0/F) was plotted against the total AdoHcy analogues concentration, and the data were analyzed according to Stern–Volmer plot.

8.01 (m, 4H, aro). MS *m*/*z* 294.7 (M^+). Anal. calcd for $C_{12}H_{13}N_3O_6$: C, 48.81; H, 4.41; N, 14.24. Found: C, 48.80; H, 4.35; N, 14.00.

5′-*S*-(Propionamide)5′-deoxy-9-(1′-β-D-ribofuranosyl)-adenine (**7**): UV (MeOH) λ_{max} 238, 281, 317 nm. ^{1}H NMR (DMSO-d_6) δ 4.48–4.50 (s, 2H, $NH_{2'}$); δ 8.09 (s, 1H, C-2); δ 8.45–8.68 (s, 1H, C-8); δ 5.68–6.00 (d, 1H, H1′); δ 3.49–3.69 (m, 1H, H2′); δ 3.46–3.63 (m, 1H, H3′); δ 4.33–4.62 (m, 1H, H4′); δ 3.32–3.40 (m, 2H, H5′); δ 1.88–2.0 (s, 2H, OH); δ 2.70 (t, 2H); δ 2.49 (t, 2H); δ 5.49–6.0 (s, 2H, NH_2); MS *m*/*z* 353 (M^+). Anal. calcd for $C_{13}H_{18}N_6O_4S$: C, 44.07; H, 5.08; N, 23.66. Found: C, 43.93; H, 4.98; N, 23.62.

5′-*S*-(Ethylpropionate)5′-deoxy-9-(1′-β-D-ribofuranosyl)adenine (**8**): UV (MeOH) λ_{max} 257, 281, 317 nm. ^{1}H NMR (DMSO-d_6) δ 4.52–4.54 (s, 2H, $NH_{2'}$); δ 8.10 (s, 1H, C-2); δ 8.45–8.67 (s, 1H, C-8); δ 5.69–6.06 (d, J = 6.1, 1H, H1′); δ 3.54–3.75 (m, 1H, H2′); δ 3.44–3.56 (m, 1H, H3′); δ 4.32–4.59 (m, 1H, H4′); δ 3.33–3.44 (m, 2H, H5′); δ 1.90–2.0 (s, 2H, OH); δ 2.79–2.83 (t, 2H); δ 2.55–2.62 (t, 2H); δ 4.12 (q, 2H); δ 1.30 (t, 3H); MS *m*/*z* 383.7 (M^+). Anal. calcd for $C_{15}H_{21}N_5O_5S$: C, 47; H, 5.47; N, 18.23. Found: C, 46.86; H, 5.42; N, 18.19.

5′-*S*-5′-Methylthio deoxyadenosine (**9**): UV (MeOH) λ_{max} 262, 257 nm. ^{1}H NMR (DMSO-d_6) δ 4.52–4.56 (s, 2H, $NH_{2'}$); δ 8.11 (s, 1H, C-2); δ 8.52–8.68 (s, 1H, C-8); δ 5.52–5.78 (d, 1H, H1′); δ 3.56–3.62 (m, 1H, H2′); δ 3.53–3.58 (m, 1H, H3′); δ 4.28–4.46 (m, 1H, H4′); δ 3.32–3.40 (m, 2H, H5′); δ 1.88–2.0 (s, 2H, OH); δ 2.53–2.65 (s, 3H). MS *m*/*z* 297.8 (M^+). Anal. calcd for $C_{11}H_{15}N_5O_3S$: C, 42.70; H, 4.56; N, 22.66. Found: C, 42.73; H, 4.98; N, 22.62.

5′-*S*-Propargylthio adenosine (**10**): UV (MeOH) λ_{max} 267, 257 nm. ^{1}H NMR (DMSO-d_6) δ 4.48–4.56 (s, 2H, $NH_{2'}$); δ 8.11 (s, 1H, C-2); δ 8.60–8.68 (s, 1H, C-8); δ 5.66–5.72 (d, 1H, H1′); δ 3.60–3.68 (m, 1H, H2′); δ 3.53–3.58 (m, 1H, H3′); δ 4.32–4.48 (m, 1H, H4′); δ 3.26–3.38 (m, 2H, H5′); δ 1.88–2.0 (s, 2H, OH); δ 2.23–2.28 (s, 2H); δ 2.53–2.65 (s, 1H). MS *m*/*z* 321.2 (M^+). Anal. calcd for $C_{13}H_{15}N_5O_3S$: C, 42.70; H, 4.56; N, 22.66. Found: C, 42.73; H, 4.98; N, 22.62.

5′-*S*-Allenylthio adenosine (**11**): UV (MeOH) λ_{max} 272, 260 nm. ^{1}H NMR (DMSO-d_6) δ 4.52–4.62 (s, 2H, $NH_{2'}$); δ 8.23 (s, 1H, C-2); δ 8.68–8.72 (s, 1H, C-8); δ 5.52–5.58 (d, 1H, H1′); δ 3.53–3.58 (m, 1H, H2′); δ 3.55–3.58 (m, 1H, H3′); δ 4.12–4.18 (m, 1H, H4′); δ

3.22–3.34 (m, 2H, H5′); δ 2.02–2.12 (s, 2H, OH); δ 2.17–2.22 (s, 1H); δ 3.53–3.65 (s, 2H). MS *m*/*z* 321.8 (M^+). Anal. calcd for $C_{13}H_{15}N_5O_3S$: C, 43.94; H, 4.79; N, 19.72. Found: C, 43.76; H, 4.68; N, 19.57.

5′-*S*-Propynylthio adenosine (**12**): UV (MeOH) λ_{max} 267, 257 nm. ^{1}H NMR (DMSO-d_6) δ 4.42–4.46 (s, 2H, $NH_{2'}$); δ 8.12 (s, 1H, C-2); δ 8.62–8.66 (s, 1H, C-8); δ 5.60–5.68 (d, 1H, H1′); δ 3.60–3.68 (m, 1H, H2′); δ 3.53–3.58 (m, 1H, H3′); δ 4.32–4.48 (m, 1H, H4′); δ 3.26–3.38 (m, 2H, H5′); δ 1.88–2.0 (s, 2H, OH); δ3.23–3.28 (s, 3H); MS *m*/*z* 321.2 (M^+). Anal. calcd for $C_{13}H_{15}N_5O_3S$: C, 43.00; H, 4.78; N, 22.78. Found: C, 42.93; H, 4.60; N, 22.72.

4.2. Purification of EcoP15I DNA MTase, HhaI MTase and *Serratia marcescens* nuclease

Wild-type M.EcoP15I was purified according to the method described by Rao et al.[37] to near homogeneity. Peak fractions from the heparin–Sepharose column containing the enzyme were pooled and concentrated by using Amicon ultrafiltration unit with 30-kDa cutoff membrane.

ER1727 cells containing the plasmid pUHE25 expressing M.HhaI were grown to an absorbance (600 nm) of 0.8–1.0 at 37 °C and subsequently induced with 0.15 mM IPTG. The cells were allowed to grow for 2 h after induction. The cells were harvested and either stored at −20 °C or used immediately. Cells were sonicated and the M.HhaI protein extracted from the cell pellet using buffer containing high salt, 10 mM potassium phosphate, pH 7.4, 5 mM EDTA, 10% glycerol, 0.1% β-mercaptoethanol, and 400 mM NaCl. This suspension was centrifuged at 12,000 rpm for 15 min and the supernatant collected. The supernatant was then dialyzed against a low salt buffer, 10 mM potassium phosphate, pH 7.4, 5 mM EDTA, 10% glycerol, 0.1% β-mercaptoethanol, and 100 mM NaCl. The dialyzed protein was further purified on Q-Sepharose and SP-Sepharose ion exchange columns as described.[38] *Escherichia coli* Dam MTase was obtained from NEB, USA.

JM109 cells containing the plasmids pCI875 (Kan) and pHisNUC (Amp) coding for *Serratia marcescens* nuclease were grown in 500 ml LB having 100 μg/ml ampicillin and 50 μg/ml kanamycin at 28 °C, untill OD_{600} = 0.5–0.6. The temperature was then raised to 42 °C for 2 h for induction. After harvesting cells were sonicated and the endonuclease protein extracted from cell pellet using 10 mM Tris–HCl buffer, pH 8.2, containing 10 mM imidazole and 6 M urea. The extracted protein was purified on Ni–NTA agarose which was previously equilibrated with the above buffer. The enzyme was eluted by using 2 ml of 10 mM Tris–HCl, pH 8.2, buffer containing 200 mM imidazole and 6 M urea. The enzyme eluted was dialyzed overnight at 4 °C against 10 mM Tris–HCl, pH 8.2, and 30% glycerol. *Serratia* endonuclease activity was checked by incubating 1 μg pUC19 DNA with 500 ng of protein in 10 μl of reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $MgCl_2$. The DNA was completely digested by the endonuclease at 37 °C in 10 min.

The purity of the enzymes was judged as being greater than 99% on SDS–PAGE with Coomassie brilliant blue staining.[39] Protein concentration was estimated by the method of Bradford using bovine serum albumin as standard.[40]

4.3. Biotin–avidin microplate assay for the quantitative analysis of enzymatic methylation of DNA

Biotin–avidin microplate assay[41] was used for the quantitative analysis of enzymatic methylation of DNA. MTase activity of M.EcoP15I, Dam or M.HhaI was monitored by incorporation of [^{3}H]methyl groups in a 35 bp biotinylated ds DNA (5′-Bt-TGG AGA GCG CGG TAC CGG TCT GCT GGA TCA CAA AC-3′) containing recognition sites of M.EcoP15I (5′-CAG CAG-3′), M.HhaI (5′-GCGC-3′), and Dam (5′-GATC-3′). The reaction was carried out in 20 μl of 10 mM Hepes buffer, pH 8.0, containing 6.4 mM $MgCl_2$, 0.25 mM EDTA, and 7 mM β-mercaptoethanol (methylation buffer). Typically, the reactions were performed with 2 pmol of substrate DNA, 100 nM of purified M.EcoP15I or M.HhaI or 1 U of Dam (NEB), and 1 μM of [^{3}H]AdoMet (74.5 Ci/mmol) from NEN, Life Science Products, USA, at 37 °C for 30 min.

Microplates were coated with 1 μg avidin (Sigma) dissolved in 100 μl of 100 mM $NaHCO_3$ (pH 9.6) and incubated overnight at 4 °C. The wells were washed five times with 200 μl PBST (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM K_2HPO_4, 0.05% v/v Tween 20, pH 7.2). To measure the activity of the enzyme, the methylation reaction mixture was pipetted into the wells of a microplate. PBST supplemented with 500 mM NaCl and 1 mM EDTA was added to a total volume of 50 μl and the reaction mixture incubated for 30 min to allow binding of the biotinylated DNA to the microplate. The wells were washed five times with 200 μl PBST supplemented with 500 mM NaCl to remove the unreacted AdoMet and the enzyme. High salt in the washing buffer was used to prevent binding of the MTase to the DNA. Complete removal of the MTase was important, because unreacted AdoMet could bind to the protein and thereby be retained. Subsequently, the DNA was degraded using 500 ng *Serratia marcescens* nuclease in 100 μl of 50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$ for 30 min at 37 °C. The released radioactivity was estimated by liquid scintillation counting of the reaction mixture after adding 3 ml of scintillation fluid. Each experiment was done in duplicate, repeated three times, and average values reported.

4.4. Fluorescence quenching

Fluorescence emission spectra and fluorescence intensities were measured for EcoP15I MTase on a Perkin-Elmer spectrofluorimeter LS 55 using a 1-cm quartz cuvette at 25 °C. The emission spectra were recorded over a wavelength of 300–400 nm with an excitation wavelength of 280 nm. EcoP15I MTase was allowed to equilibrate for 1 min in methylation buffer before measurements were recorded. Small aliquots of AdoHcy analogues (final concentration 5–60 μM) or AdoHcy

(25 μM) were added to EcoP15I MTase (1 μM), and spectra recorded. Each spectrum recorded was an average of three scans. The fluorescence intensities were plotted against the total AdoHcy analogue concentration, and the data were analyzed according to Stern–Volmer relationship.[42]

$$F_0/F = 1 + K_{SV}[Q]$$

where F_0 and F are fluorescent intensities in the absence and presence of cofactor, respectively, K_{SV} is the collision Stern–Volmer constant, and Q is the quencher (AdoHcy analogues) concentration.

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