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Determinants of cofactor binding to DNA methyltransferases: insights from a systematic series of structural variants of *S*-adenosylhomocysteine†

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S-Adenosylmethionine (AdoMet) is a commonly used cofactor, second only to ATP in the variety of reactions in which it participates. It is the methyl donor in the majority of methyl transfer reactions, including methylation of DNA, RNA, proteins and small molecules. Almost all structurally characterised methyltransferases share a conserved AdoMet-dependent methyltransferase fold, in which AdoMet is bound in the same orientation. Although potential interactions between the cofactor and methyltransferases have been inferred from crystal structures, there has not been a systematic study of the contributions of each functional group to binding. To explore the binding interaction we synthesised a series of seven analogues of the methyltransferase inhibitor *S*-adenosylhomocysteine (AdoHcy), each containing a single modification, and tested them for the ability to inhibit methylation by HhaI and HaeIII DNA methyltransferase. Comparison of the K_i values highlights the structural determinants for cofactor binding, and indicates which nucleoside and amino acid functional groups contribute significantly to AdoMet binding. An understanding of the binding of AdoHyc to methyltransferases will greatly assist the design of AdoMet inhibitors.

Introduction

Methyl transfer is a reaction central to cellular biochemistry, with 140 different classes of methyltransferase alkylating such diverse substrates as DNA, RNA, proteins, lipids, polysaccharides and small molecules.[1] *S*-Adenosylmethionine (AdoMet) contains a highly reactive methylthiol group and is the cofactor and methyl donor for the majority (over 90%) of these enzymes (www.expasy.ch/enzyme).[2] With enzymes catalysing the methylation of N, O or S the substrates contain a polarisable nucleophile, which directly attacks the activated methyl group, whereas enzymes catalysing methylation of carbon atoms usually proceed *via* addition and subsequent elimination from a thiol group in the enzyme active site. The structures of over 50 different AdoMet-dependent methyltransferases are known and include five distinct AdoMet-binding folds.[3] Despite the extensive chemical and structural diversity of methylation substrates, the great majority of known methyltransferases contain the Class I fold and share a common evolutionary origin.[3,4] Currently the structures of 31 methyltransferases have been solved in complex with AdoMet or the reaction product, *S*-adenosylhomocysteine (AdoHcy) and the relative position of AdoMet is almost identical in each structure.

Analogues of AdoMet that inhibit methyltransfer, such as the natural product sinefungin, have therapeutic properties including antifungal, antiviral, antiparasitic and antitumour activities.[5,6] However the high conservation of the AdoMet-binding domain means that these analogues often inhibit a wide range of methyltransferases, leading to *in vivo* toxicity. Previous studies have investigated the ability of AdoMet analogues to inhibit enzymes such as the essential bacterial adenine N^6-methyltransferases, involved in cell cycle regulation, and the erythromycin resistance methyltransferases, which methylate prokaryotic rRNA.[7,8] Analogues of the methylation target, or mechanism-based inhibitors, are more specific than inhibitors such as sinefungin. A multisubstrate adduct synthesised by Wahnon *et al*., for example, inhibits adenine N^6, but not cytosine C^5, DNA methyltransferases.[8]

Despite the structural conservation of the cofactor binding site there is little sequence identity between methyltransferases in different classes. Only three motifs are conserved amongst the AdoMet-dependent methyltransferase family, located in the P loop, G loop and part of strand β4.[9] Notably each of these positions is involved in cofactor binding.

There is often greater sequence conservation within methyltransferase classes and DNA and RNA methyltransferases contain particularly strongly conserved motifs. Cytosine C^5 DNA methyltransferases share ten well defined and highly conserved motifs.[10] Three structures of C^5 DNA methyltransferases are known: two bacterial type II restriction modification system methyltransferases, M.HhaI (recognition sequence GCGC, solved in complex with AdoHcy and DNA)[10] and M.HaeIII (recognition sequence GGCC, solved in complex with DNA),[11] and human DNMT2, which shows homology to other prokaryotic and eukaryotic cytosine C^5 DNA methyltransferases (solved in complex with AdoMet).[12] Seven residues are proposed to form hydrogen bonds between M.HhaI and AdoMet, with four of these identical (six conserved) in M.HaeIII and three identical (seven conserved) in DNMT2.

In order to characterise the functional groups that allow cofactor recognition by the active site of cytosine C^5 DNA methyltransferases we measured the inhibition of M.HhaI and M.HaeIII by AdoHcy analogues. AdoHcy is structurally similar to AdoMet, binding to methyltransferases in the same orientation, and with a similar K_d to AdoMet, but it lacks the reactive methyl group and acts as a competitive inhibitor with respect to AdoMet.[13] A series of analogues was prepared,

† Electronic supplementary information (ESI) available: Table S1: proposed hydrogen bonds between methyltransferase and cofactor based on the crystal structure of each methyltransferase with bound AdoHcy. See http://www.rsc.org/suppdata/ob/b4/b415446k/

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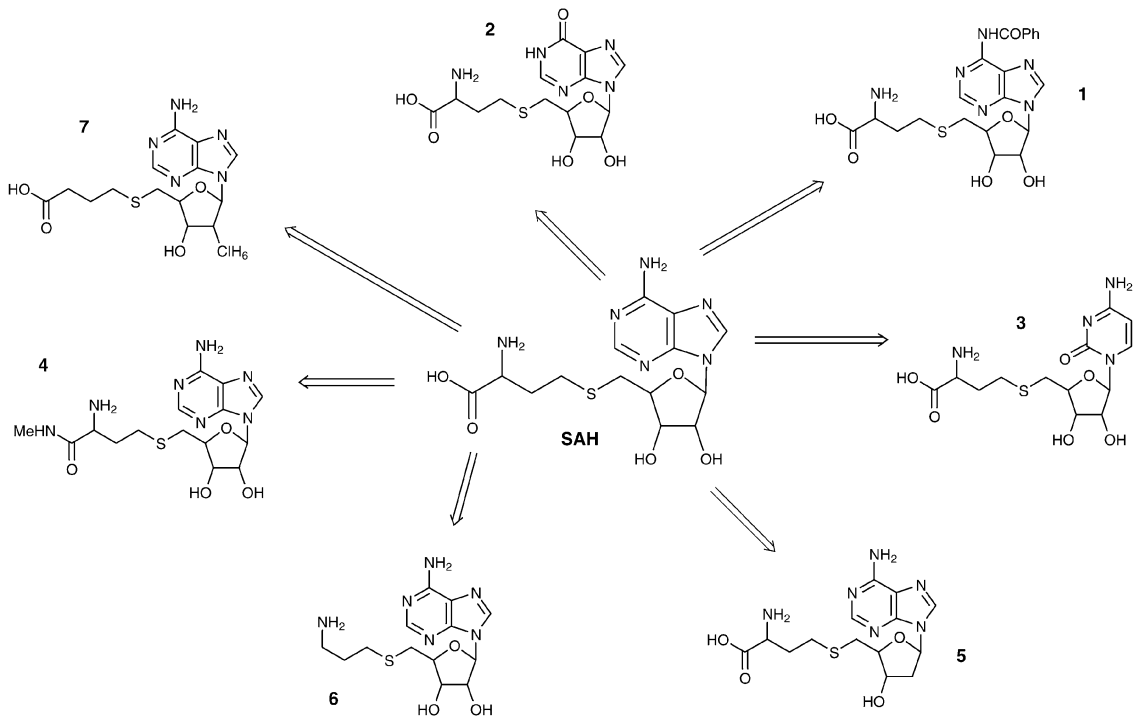


Fig. 1 AdoHcy analogues synthesized. Arrows show the functional groups that have been modified in each case.

with single modifications to the adenine, ribose and methionine moieties (Fig. 1).

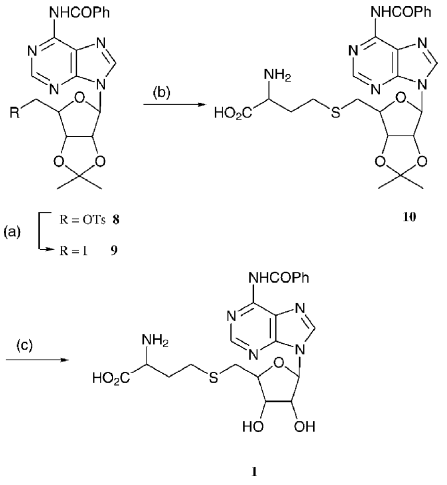
Results

Synthesis of AdoHyc analogues

A series of seven AdoHcy analogues were synthesised in order to probe the structural determinants of cofactor binding (Fig. 1). Initially we chose the N^6-acetyl derivative. This analogue has the advantage of weakening the hydrogen bonding ability of the amino group, but with minimal steric effects. However, it was found that the acetyl group was both acid and base labile and it was never possible to prepare the material free from AdoHcy. We therefore chose to make the N^6-benzoyl derivative (**1**) which has greater stability, although the benzoyl group adds steric bulk, but still weakens the hydrogen bonding capability of the amino group. In addition, the inosine derivative (SIH, **2**) was prepared, which has an altered hydrogen bonding pattern. The effect of altering the nucleobase was examined by the use of SIH and also the cytosine derivative (**3**). Modification of the amino acid carboxyl group to alter the hydrogen bonding behaviour and probe for steric effects was carried out by conversion to the methylamide derivative **4**. The remaining modifications arise from the removal of functional groups, namely the 2′-hydroxyl group of the ribose to give **5** (S2′dAH), and removal of either of the amino acid functionalities to give 5′-(3-aminopropylthio)-5′-deoxyadenosine, **6**, and 5′-(3-carboxypropylthio)-5′-deoxyadenosine, **7**, as described.[14]

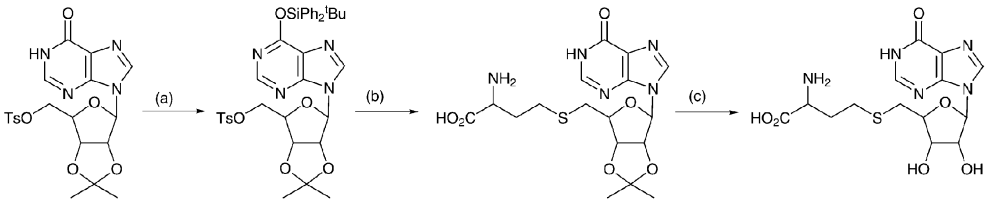
Synthesis of AdoHcy and derivatives has been previously described *via* coupling of 5′-*O*-tosyl[15] or 5′-deoxy-5′-halo derivatives of adenosine[16] with the di-sodium salt of L-homocysteine. Coupling of 5′-*O*-tosyl derivatives is generally carried out in liquid ammonia after reduction of homocysteine, whereas 5′-halo derivatives are coupled with the di-sodium salt of L-homocysteine in water.[17]

The first modification was the addition of a benzoyl group to the adenine N^6-amino group (analogue **1**, Fig. 1). The N^6-benzoyl-isopropylidene adenosine derivative was converted to its 5′-*O*-tosylate **8**, and then reaction with sodium iodide in acetone yielded the 5′-deoxy-5′-iodo derivative **9** in moderate yield (Scheme 1). This was reacted with the di-sodium salt of L-homocysteine in aqueous DMF solution to give **10**. This compound partially lost the benzoyl group, but in a slower reaction than was observed with the acetyl group. Finally, removal of the isopropylidene protection was carried out in 5% aqueous TFA to give **1**. N^6-benzoyl-AdoHcy was stable under these conditions, and no AdoHcy was observed, as was found with the N^6-acetyl route.



Scheme 1 Synthesis of N^6-benzoyl-AdoHyc. (a) NaI–acetone, 69%. (b) Homocysteine, Na–NH_3, 57%. (c) 5% aqueous TFA, 70%.

S-Inosinyl-L-homocysteine (SIH, **2**) has been previously described,[18] prepared by coupling 5′-*O*-tosyl-isopropylidine inosine and the di-sodium salt of L-homocysteine in liquid ammonia. However, no characterisation of the product was provided. As we had found that coupling of 5′-iodo derivatives was more efficient, 5′-*O*-tosyl-isopropylidine inosine was converted to 5′-deoxy-5′-iodo-isopropylidine inosine, and then reacted with the di-sodium salt of L-homocysteine (Scheme 2). However, the product, which was not identified, did not contain homocysteine, and was possibly the cyclonucleoside. Repeating the previously described synthesis,[18] it became apparent from the NMR spectra that the product was not the SIH derivative,



Scheme 2 Synthesis of *S*-inosyl-AdoHcy. (a) DMAP, TEA, *tert*-butyldiphenylchlorosilane. (b) Homocysteine, Na–NH_3, 53%. (c) 5% aqueous TFA, 69%.

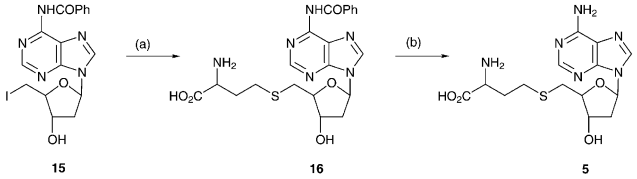
and again may have been the cyclonucleoside; evidently the inosine-6-oxo group required protection. Using a modification of a procedure described by Grøtli *et al*.,[19] isopropylidene inosine was converted to its 5′-*O*-tosylate and then reacted with *tert*-butyldiphenylchlorosilane. The authors stated that the silyl protecting group is unstable, in particular to column chromatography[19] so the silylated inosine derivative was used without purification.

The silylated derivative was reacted with the di-sodium salt of L-homocysteine in liquid ammonia. At the end of the reaction the product was dissolved in aqueous ethanol and acidified with acetic acid to remove the silyl protection. The product, after reverse phase purification, was shown to be the desired isopropylidene protected **11**. Treatment with 5% aqueous TFA removed the isopropylidene protection to give SIH, **2**.

S-Cytidinyl-L-homocysteine (SCH, **3**) has also been previously described,[18] but the product was not characterised. In this work SCH was prepared by an alternative route. Conversion of cytidine to 5′-deoxy-5′-chlorocytidine was carried out by reaction with thionyl chloride in hexamethylphosphoramide.[16,20] This was then reacted with the di-sodium salt of L-homocysteine in water to give, after purification by reverse phase chromatography, SCH.

S-(2′-Deoxy-β-D-ribofuranosyladenosin-5′-yl)-L-homocysteine (SdAH, **5**) was prepared by an alternative route to that previously described (Scheme 3).[21] N^6-benzoyl-5′-*O*-tosyl-2′deoxyadenosine was converted to its 5′-iodo derivative **15**, which was then treated with the di-sodium salt of L-homocysteine in aqueous DMF to give **16**. Removal of the benzoyl group with ammonia solution yielded **5**.

The final modification we required was to the carboxylic acid function, which was modified to the methylamide **4** (Scheme 4). This required the protection of most of the functional groups

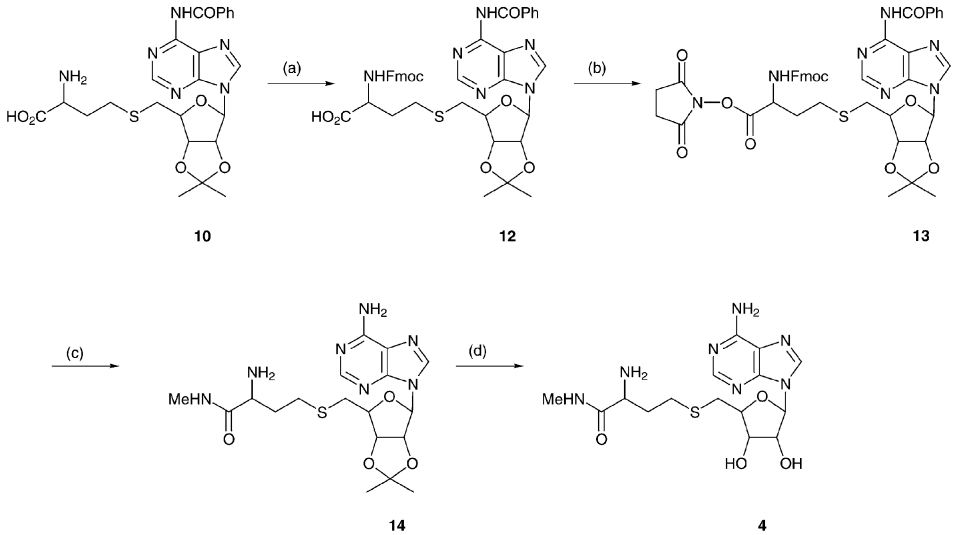


Scheme 3 Synthesis of 2′-deoxy-AdoHcy. (a) Homocysteine, Na–NH_3, 57%. (b) 5% aqueous TFA, 89%.

of AdoHcy in order to activate the carboxylic acid group. A number of different protecting group strategies were employed, primarily to avoid using acid labile protecting groups. However, it was found that acid labile protecting groups could not be avoided. The synthesis was carried out using the intermediate **10**. Attempts to protect the homocysteine amino function with fluorenylmethoxycarbonyloxy-succinimide in pyridine failed, but using aqueous dioxane with sodium carbonate the desired Fmoc derivative, **12**, was prepared. This was then reacted with *N*-hydroxysuccinimide with DCC to yield the active ester **13**, which was reacted with aqueous methylamine in DMF. This yielded the desired methylamide derivative, **14**, while removing the Fmoc and benzoyl protecting groups in one step. Final removal of the isopropylidene protecting group was carried out using 5% aqueous TFA as before to give **4**.

Kinetics and inhibition of methylation

The catalytic activities of M.HhaI and M.HaeIII were measured by incorporation of radiolabelled methyl groups into synthetic double stranded DNA substrates, Hhasub and Haesub, each containing a single unmethylated recognition site, GCGC and GGCC respectively. Steady state parameters, derived by fitting



Scheme 4 Synthesis of AdoHcy-methylamide. (a) Dioxane–H_2O–Na_2CO_3–*N*-(9-Fmoc)succinimide, 94%. (b) *N*-Hydroxysuccinimide–DCCD, 72%. (c) Aqueous $MeNH_2$, 62%. (d) 5% aqueous TFA, 61%.

the data to the Michaelis–Menten model (eqn. 1) are given in Fig. 2 and Table 1.

$$v_0 = V_{max}[S]_0/([S]_0 + K_m) \quad (1)$$

Our measurement of K_m^{AdoMet} for M.HhaI, 110 nM, is comparable to the published value of 161 nM, measured using a similar unmethylated 30 base pair DNA substrate.[22] K_m^{AdoMet} and K_i^{AdoHcy} of M.HhaI have also been determined using the substrate poly(dG–dC), with values of 15 nM and 2.1 nM respectively.[13] We have previously measured kinetic constants for M.HaeIII with an N-terminal FLAG tag:[23] these values of K_m^{AdoMet}, 620 nM, and k_{cat}, 0.0033 s^{-1}, are comparable with those measured in this study, but the higher K_m^{DNA} of 130 nM may have been caused by the presence of the tag.

To determine the relative effect of each modification to AdoHcy the analogues were tested for inhibition of M.HhaI (Fig. 3) and M.HaeIII by fitting data to eqn. 2. All analogues inhibit methylation by both enzymes, with K_i values ranging from 8–3800 μM (Table 2), but none are as efficient inhibitors as AdoHcy, which was determined to have a K_i of 15 ± 2 nM

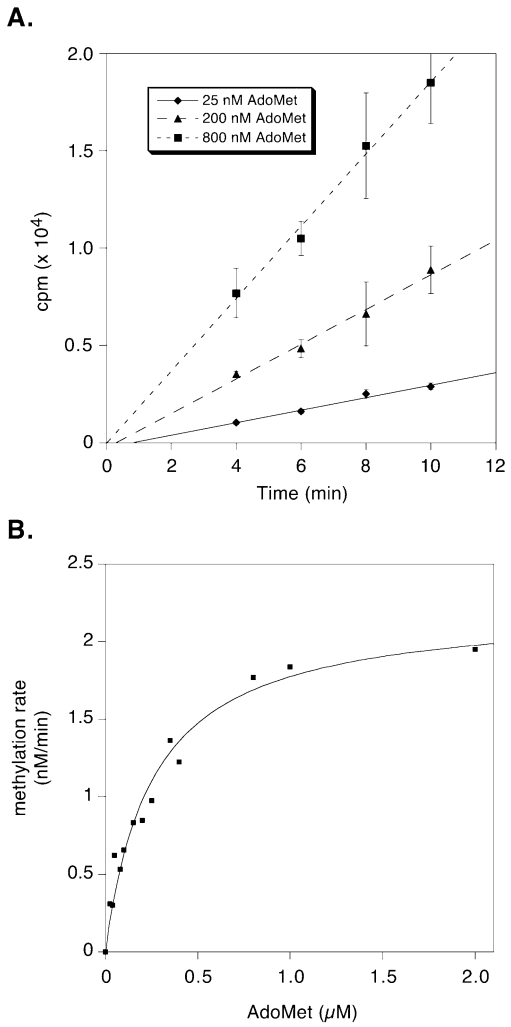


Fig. 2 Steady state kinetics of M.HaeIII. A. Plots of methyl group incorporation by M.HaeIII *versus* time. Methyltransferase activity of M.HaeIII was measured at 13 concentrations of AdoMet (three of which are shown), in the range 25–2000 nM. Error bars are standard error of the mean. B. Determination of the K_m^{AdoMet} of M.HaeIII. The K_m^{AdoMet} was determined by fitting the data to the Michaelis–Menten model (eqn. 1).

Table 1 Steady state parameters of M.HhaI and M.HaeIII with unmethylated 30-mer DNA. Rate constants have been previously measured for M.HhaI using a similar DNA substrate.[22] Here we verify the K_m^{AdoMet} for this enzyme (previously measured as 161 nM) and establish the steady state parameters for M.HaeIII

	M.HhaI	M.HaeIII
K_m^{AdoMet}/nM	[a]110 ± 20	[b]280 ± 40
K_m^{DNA}/nM	[d]4.0 ± 0.7	[c]3.7 ± 1.1
k_{cat}/s^{-1}	[d]0.085 ± 0.005	[c]0.0068 ± 0.0006

[a] Measurements were taken at 0.5 μM DNA. [b] 1.25 μM DNA. [c] or 1 μM AdoMet. [d] Measured previously.[22]

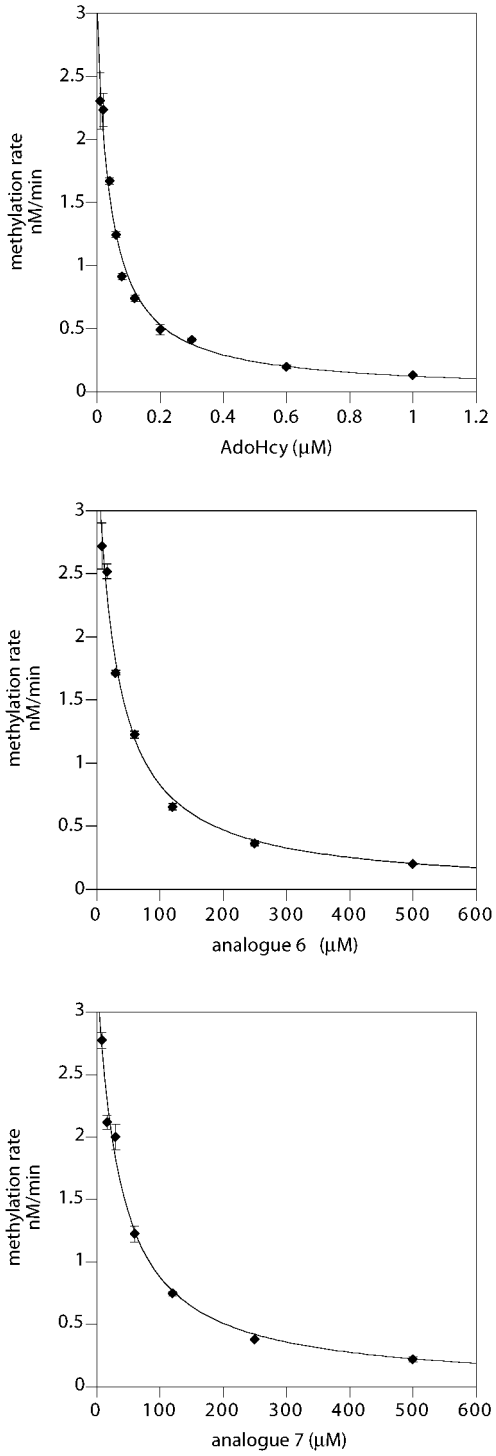


Fig. 3 Plots of M.HhaI methyltransferase activity *versus* inhibitor concentration for AdoHcy and two analogues with modifications to the methionine moiety, 5′-(3-aminopropylthio)-5′-deoxyadenosine, **6**, and 5′-(3-carboxypropylthio)-5′-deoxyadenosine, **7**. Inhibition of M.HhaI and M.HaeIII by all AdoHcy analogues were measured in a similar manner. K_i values listed in Table 2 were derived by fitting the data to eqn. 2.

Table 2 Inhibition constants of AdoHcy analogues for M.HhaI and M.HaeIII

[a]Inhibitor	Modification	M.HhaI			M.HaeIII		
		K_i/μM	K_i/K_i^{AdoHcy}	$\Delta\Delta G$/kJ mol^{-1}	K_i/μM	K_i/K_i^{AdoHcy}	$\Delta\Delta G$/kJ mol^{-1}
AdoHcy	—	0.015 ± 0.002	1.0	—	0.069 ± 0.011	1.00	—
1	N^6-benzoyl	8.1 ± 2.1	540	16	32 ± 4	460	16
2	Inosine	[b]3900 ± 800	26 0000	32	[b]450 ± 80	6600	23
3	Cytidine	27 ± 3	1800	19	[b]280 ± 60	4200	21
4	Methylamide	59 ± 9	3900	21	290 ± 46	4300	22
5	2′-Deoxy	44 ± 6	2900	21	69 ± 10	1000	18
6	Decarboxy	16 ± 4	1100	18	37 ± 5	540	16
7	Deamino	13 ± 2	850	17	230 ± 50	3400	21

[a] See Fig. 1. [b] These values are estimates only, as methylation proceeded at 35–41% of the uninhibited rate at the highest inhibitor concentrations tested.

for M.HhaI and 68 ± 11 nM for M.HaeIII.

$$v_0 = V_{max}[S]_0/(K_m(1 + [I]/K_i) + [S]_0) \quad (2)$$

Effect of base modifications (analogues 1, 2 and 3)

Analogue **1** is modified at the N^6 position of the adenine ring by the addition of a benzoyl group. Hydrogen bonding between N^6 on the adenine ring and an acidic side chain in the loop following strand β3 is a conserved feature of Class I AdoMet-dependent methyltransferases (Asp 60 in M.HhaI, Asp 50 in M.HaeIII, Fig. 4), although in some cases serine or asparagine replace the acidic side chain. Only three of the structurally characterised type I AdoMet-dependent methyltransferases lack any side-chain mediated interaction with N^6 (Table S1, ESI†). The addition of a benzoyl group at N^6 disrupts hydrogen bonds and acts as a steric probe. N^6-benzoyl AdoHcy, **1**, inhibits M.HhaI and M.HaeIII with K_i of 8.1 μM and 32 μM respectively (Table 1). These inhibition constants are 500 fold higher than those of AdoHcy, representing changes in ΔG of 16 kJ mol^{-1}. This is equivalent to the strength of a single hydrogen bond (typical hydrogen bond energies lie in the range of 12–38 kJ mol^{-1}).[24]

Despite the addition of a large hydrophobic group, analogue **1** was the best inhibitor of both methyltransferases, indicating that it is still able to compete with AdoMet for its binding site. In the structure of M.HhaI, and other methyltransferases, the edge of the AdoMet adenine ring is situated at the entrance to the AdoMet binding site where it is solvent accessible, explaining how the additional benzoyl group might be accommodated.

Analogues **2** and **3**, *S*-inosinyl-L-homocysteine and *S*-cytidinyl-L-homocysteine, contain alternative nucleobases. There is no conserved interaction between methyltransferases and the N^1, N^3, and N^7 atoms of the adenine ring. Hydrogen bonds to N^1 and N^3 are suggested in about half the structures, with hydrogen bonds predicted more frequently to N^1 than N^3 (Table S1†) and hydrogen bonds to N^7 only predicted in two methyltransferase structures. Where such hydrogen bonds are suggested they always involve main chain atoms, often using the same residues that form hydrophobic interactions with the adenine and ribose rings. The interaction with N^1 is often provided by the main chain amino group of a hydrophobic residue immediately following the conserved Asp or Glu that contacts N^6. Similarly N^3 is most frequently contacted by a main chain amino group of the residue immediately following or immediately preceding the residue that contacts the sugar hydroxyl groups. In the structure of M.HhaI these residues are Ile 61 and Trp 41 respectively. Ile 61 is conserved in M.HaeIII whereas Trp 41 aligns with Tyr 30.

Analogues **2** and **3** have substantial changes to the base. The modifications to analogue **2** would disrupt the hydrogen bonding interactions normally formed by the methyltransferase to N^6 and N^1. In the case of the cytidine derivative, analogue **3**, the positions of all the potential hydrogen bond-forming groups are altered. Despite the large structural differences from AdoHcy, the cytidine derivative inhibits both M.HhaI and M.HaeIII, albeit with higher K_i values than AdoHcy. The K_i of analogue **2** for M.HhaI, 3.9 mM, is 260 000 fold higher than that of AdoHcy, indicating poor competition with AdoMet for the cofactor binding site. The K_i for analogue **2** for M.HaeIII is lower (0.45 mM), perhaps indicating some difference between M.HhaI and M.HaeIII in the hydrophobic pocket that binds the adenosine moiety.

Effect of sugar modification (analogue 5)

Compound **5**, 2′-deoxy-AdoHcy, lacks one of the hydroxyl groups on the sugar ring. With only one exception (the mycolic acid synthetase family) methyltransferases with the type I fold have a conserved glutamate or aspartate residue (Glu 40 in M.HhaI, Glu 29 in M.HaeIII, Fig. 3) at the end of strand β2 that forms a pair of hydrogen bonds to the 2′- and 3′-ribose hydroxyl groups (Table S1†). This conserved pair of hydrogen bonds cannot be formed with **5**. The K_i of **5** for M.HhaI is 2900 fold higher than that of AdoHcy, allowing an estimate of the strength of the interaction between M.HhaI and the 2′-OH of AdoHcy of 21 kJ mol^{-1}. Similarly, the interaction between M.HaeIII and the 2′-OH of AdoHcy is estimated as 18 kJ mol^{-1}.

Effects of amino acid modifications (analogues 4, 6 and 7)

Interactions with the methionine moiety are not conserved between methyltransferases in different sub-classes within E.C. 2.1.1.- and there is some variation even within the DNA cytosine C^5 methyltransferases. M.HhaI is thought to make a number of side chain and main chain contacts with the cofactor carboxyl group but only water-mediated contacts to the cofactor amino group. Main chain contacts may be conserved in M.HaeIII, but Ser 305, which makes a hydrogen bond to the AdoHcy carboxyl group in the crystal structure of M.HhaI, is not conserved (Table S1†).

Three of the analogues were modified at the amino acid moiety; analogue **6** lacks the carboxyl group, analogue **7** lacks the amino group, and analogue **4** is modified at the carboxyl group by conversion to the methylamide. Each of these alterations caused a substantial increase in K_i compared to that of AdoHcy, (values of K_i were 540 to 4300 fold higher), evidence of the important role of these functional groups in cofactor binding and recognition by both enzymes.

Discussion

This study analyses the inhibition of two cytosine C^5 DNA methyltransferases, M.HhaI and M.HaeIII, by a series of AdoHcy analogues (Fig. 1) to characterise quantitatively the contribution of individual functional groups to affinity. All

modifications to AdoHcy increase K_i, suggesting that multiple protein–ligand interactions are involved in binding and recognition. We found evidence of significant interactions made by both methyltransferases to the ribose 2′-hydroxyl group, the adenine N^6 amino group and the methionine amino and carboxyl groups of AdoHcy. Additionally we found that a bulky substituent on the adenine N^6 amino group, or even replacement of the adenine with hypoxanthine or cytosine, do not preclude binding of AdoHcy analogue **1**, **2** and **3** to these enzymes.

The dissociation constant (K_d) of four analogues of AdoHcy for M.HhaI has previously been determined[14] with a view to understanding the role of the methionine moiety in protein–cofactor interaction in DNA methyltransferases and other methyltransferases. Two of these analogues were also tested in this study (compounds **6** and **7**).

In the previous study of M.HhaI compounds **6** and **7** were found to have K_d values 78 and 17 fold higher than that of AdoHcy respectively,[14] whereas we observed that K_i values 1100 and 850 fold higher than that of AdoHcy. The conclusion of the previous study is that the adenosyl moiety acts as a "molecular anchor" for cofactor binding, whereas our results emphasise the involvement of multiple groups on both the adenosyl and methionine moieties in cofactor binding.

K_d values were determined in the absence of DNA, by measuring the quenching of tryptophan fluorescence upon AdoHcy binding.[14] It has been previously reported that AdoHcy binds with high affinity to the M.HhaI–DNA complex but not to the free enzyme or the binary product complex (enzyme–methylated DNA),[13] in fact AdoMet is bound in a different orientation in the binary M.HhaI–AdoMet complex compared to the ternary complex of M.HhaI, AdoMet and DNA.[25,26] Our values of K_i are measurements of the competition of each analogue with AdoMet for occupation of the cofactor binding site in the catalytically competent enzyme–DNA complex. It is likely that our choice to measure K_i instead of K_d accounts for the differences in our estimated $\Delta\Delta G$ values.

Crystal structures of HhaI and HaeIII in complex with AdoHcy indicate hydrogen bonds with each of the nucleobase, sugar and amino acid moieties, as shown in Fig. 4. However, we compared the data from 31 crystal structures of methyltransferases in complex with AdoHcy or analogues and found that the only conserved hydrogen bonds are between acidic side chains and the adenine N^6 amino group and the ribose hydroxyls (Table S1†). Hydrogen bonds to the nitrogens N^1 and N^3 in the adenine ring were made by the main chain amino groups of hydrophobic residues, and were only predicted in about half the methyltransferases studied. Hydrogen bonds to N^7 were predicted in two structures. In contrast to the interactions with the adenosine moiety, the residues making hydrogen bonds to the methionine moiety are not conserved. Interactions with both the amino and carboxyl functional groups are predicted in 27 of the structures, yet these hydrogen bonds are a mixture of main chain, side chain and water mediated contacts made by a wide variety of residues from two regions of the consensus fold or from outside the consensus fold. Examination of the phylogenetic data alone might suggest that the interactions with the methionine moiety are less significant than those with other parts of the molecule. Our results indicate that, despite the lack of conservation of hydrogen bond-forming residues, the methionine functional groups are an important determinant of cofactor binding by both M.HhaI and M.HaeIII. We observe that modification of either the amino or the carboxyl group of AdoHcy leads to an increase in K_i comparable to the change caused by modification of the 2′-hydroxyl or N^6 amino groups.

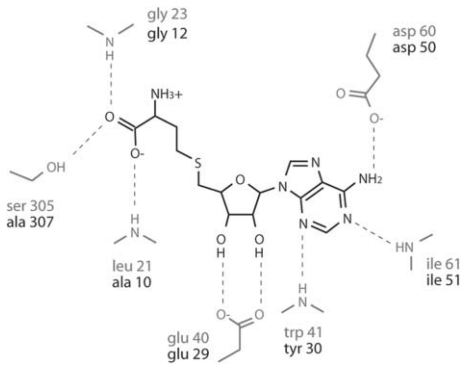


Fig. 4 Schematic diagram of the proposed hydrogen bonds between M.HhaI and AdoHcy. M.HhaI amino acids are written in grey, with the equivalent residues in M.HaeIII below. Water-mediated hydrogen bonds are omitted for clarity.

Our findings suggest that recognition of AdoMet by M.HhaI and M.HaeIII relies on multiple interactions with the adenine, ribose and methionine moieties. This pattern of methyltransferase-cofactor recognition is likely to be conserved amongst other cytosine C^5 DNA methyltransferases and may also be conserved in other proteins with the type I AdoMet-dependent methyltransferase fold. However the residues involved in cofactor binding are highly variable, particularly residues predicted to contact the methionine moiety, therefore the strength of interactions with this moiety may differ significantly. These AdoHcy analogues should prove useful tools for further study of methyltransferase function.

Experimental

General methods

^{1}H-NMR spectra were obtained on a Bruker DRX-300 spectrometer in d_6-DMSO unless otherwise stated. Mass spectra were recorded on a Bruker FTICR Bioapex II instrument. Ultraviolet spectra were recorded on a Perkin Elmer Lambda 40 spectrophotometer in 10% aqueous methanol unless otherwise stated. TLC was carried out on pre-coated F_{254} or Merck RP-18 F_{254s} silica plates, and column chromatography with Merck Kieselgel 60, or Merck RP-18 silica. Reactions were worked up as follows, unless otherwise stated. Reaction mixtures were evaporated to dryness and the products dissolved in chloroform and washed with saturated aqueous sodium bicarbonate solution. The organic fractions were combined and dried over anhydrous sodium sulfate, filtered and then evaporated to dryness. HPLC was carried out using a Phenomenex Luna 10 μm C-18 reverse phase column. Unless otherwise stated, HPLC conditions are buffer A, 0.1% aqueous TFA; buffer B, 0.1% aqueous TFA, 25% MeCN. 0% to 100% buffer B over 20 minutes, 100% buffer B 10 minutes.

***N*^{6}-Benzoyl-(*O*-2′,*O*-3′-isopropylidene-5′-*O*-toluenesulfonyl)-adenosine 8.** To a solution of N^6-benzoyl-*O*-2′,*O*-3′-isopropylidene-adenosine (11.5 g, 28 mmol) in pyridine (150 cm^3) was added toluene sulfonyl chloride (10.7 g, 56 mmol) and the solution stirred at room temperature overnight. The reaction was quenched with methanol, evaporated and worked up to give a brown gum. This was chromatographed on silica (CH_2Cl_2–(0–5%) MeOH) to give an off-white foam. Yield 14.89 g, 94%. Spectra were as described.[27]

***N*^{6}-Benzoyl-(*O*-2′,*O*-3′-isopropylidene-5′-deoxy-5′-iodo)-adenosine 9.** A solution of **8** (14.5 g, 25.6 mmol) in acetone (200 cm^3) containing sodium iodide (9.6 g, 64 mmol) was heated at reflux overnight. The solution was evaporated, dissolved in ethyl acetate and washed (2 M $Na_2S_2O_3$) and evaporated to give a pale brown foam. This was chromatographed on silica (CH_2Cl_2–(0–5%) MeOH) to give an off-white foam. Yield 9.23 g, 69%. δ_H 1.34, 1.55 (6H, 2 × s, isopropylidene CH_3), 3.33–3.49 (2H, m, H5′, H5″), 4.35–4.40 (1H, m, H4′), 5.04–5.07 (1H, m, H3′), 5.57–5.60 (1H, m, H2′), 6.37 (1H, d, *J* 2, H1′), 7.52–7.67 (3H, m, Ph–CH), 7.99–8.09 (2H, m, Ph–CH), 8.68 (1H, s, H2), 8.79 (1H, s, H8), 11.25 (1H, s, NH). UV λ_{max}/nm 281

(18 400), λ_{min}/nm 245; pH 1, λ_{max}/nm 290 (21 800), λ_{min}/nm 240; pH 12, λ_{max}/nm 302 (12300), λ_{min}/nm 258. *m/z* 544 (70%, M + Na)$^+$, 522 (15%, M + H)$^+$, 394 (100%, M − I)$^+$. Accurate mass measurement on $C_{20}H_{20}N_5O_4INa$ gives 544.04600, deviation 0.36 ppm.

***S*-(*O*-2′,*O*-3′-Isopropylidene-N^6-benzoyladenosin-5′-yl)-L-homocysteine 10.** Homocystine (2 g, 7.4 mmol) was dissolved in liquid ammonia (*ca*. 100 cm^3) and freshly cut sodium was added until the deep blue colour remained for 20 minutes. The blue colour was quenched by the addition of small portions of ammonium chloride, and the reaction allowed to warm to room temperature. Under a nitrogen atmosphere the residue was dissolved in dry ethanol (15 cm^3) and filtered through Celite, and washed with further portions of dry ethanol (3 × 5 cm^3). To the solution was then added dry ether and the white solid was filtered and dried *in vacuo* over phosphorus pentoxide. The product is highly hygroscopic and was used immediately, assuming 100% conversion to L-homocysteine di-sodium salt.

The di-sodium salt of L-homocysteine from above was dissolved in DMF (20 cm^3) and water (10 cm^3), and to this was added potassium iodide (10 mg) and (**9**)[27] (6.50 g, 12.5 mmol), and the solution heated at 100 °C for 45 minutes. The solution was neutralized (acetic acid) and evaporated to dryness to give a yellow–brown gum, which, by reverse phase TLC, showed two products. The mixture was chromatographed (Merck RP-18 silica, water to 25% acetonitrile in water gradient). The first product eluted in 10–15% MeCN, was shown to be the de-benzoylated product, ***S*-(*O*-2′,*O*-3′-isopropylidene-adenosin-5′-yl)-L-homocysteine**. Yield 1.25 g, 24%. δ_H 1.30, 1.51 (6H, 2 × s, isopropylidene CH_3), 1.78–1.99 (2H, m, CH_2CH_2S), 2.57–2.61 (2H, m, CH_2S), 2.63–2.82 (2H, m, H5′, H5″), 3.30–3.34 (1H, m, CO*CH*NH_2), 4.22–4.26 (1H, m, H4′), 4.96–4.99 (1H, m, H3′), 5.46–5.49 (1H, m, H2′), 6.14–6.15 (1H, m, H1′), 7.39 (2H, br. s, NH_2), 8.17 (1H, s, H2), 8.35 (1H, s, H8). UV λ_{max}/nm 259 (13 000), λ_{min}/nm 227; pH 1 λ_{max}/nm 257 (13 700), λ_{min}/nm 228. *m/z* 425 (100%, M + H)$^+$, 381 (10%, M − CO_2)$^+$. Accurate mass measurement on $C_{17}H_{24}N_6SO_5$ gives 425.1603, deviation −0.98 ppm.

The second product, eluting in 20–25% MeCN, was shown to be the title product as a white foam. Yield 3.75 g, 57%. δ_H (D_2O) 1.34, 1.55 (6H, 2 × s, isopropylidene CH_3), 1.73–1.99 (2H, m, CH_2CH_2S), 2.58–2.63 (2H, m, CH_2S), 2.70–2.83 (2H, m, H5′, H5″), 3.25–3.29 (1H, m, CO*CH*NH_2), 4.28–4.30 (1H, m, H4′), 5.02–5.04 (1H, m, H3′), 5.56–5.58 (1H, m, H2′), 6.29 (1H, d, *J* 1.9, H1′), 7.44–7.65 (3H, m, Ph–CH), 7.92–8.08 (2H, m, Ph–CH) 8.69 (1H, s, H2), 8.79 (1H, s, H8). UV λ_{max}/nm 281 (22 000), λ_{min}/nm 246; pH 1, λ_{max}/nm 288 (23 300), λ_{min}/nm 263; pH 12, 303 (13 100), λ_{min}/nm 260. *m/z* 529.19 (70%, M + H)$^+$, 439 (10%, M − COPh)$^+$, 412 (10%, M − COPh − CO)$^+$. Accurate mass measurement on $C_{24}H_{29}N_6SO_6$ gives 529.18570, deviation −2.330 ppm.

***S*-(N^6-Benzoyladenosin-5′-yl)-L-homocysteine 1.** A solution of **10** (150 mg, 0.28 mmol) in 5% aqueous TFA (10 cm^3) was stirred at room temperature for 6 hours. The reaction was lyophilised to give a white solid, which was chromatographed (RP-C18) eluting with H_2O then 0–15% MeCN in H_2O. Yield 97 mg, 70%. δ_H (D_2O) 1.75–2.04 (2H, m, CH_2CH_2S), 2.59 (2H, t, *J* 7.7, CH_2S), 2.79–2.94 (2H, m, H5′, H5″), 3.27–3.33 (1H, m, NH_2CHCO_2H), 4.03–4.08 (1H, m, H4′), 4.15–4.21 (1H, m, H3′), 4.78–4.81 (1H, m, H2′), 5.78 (1H, br, OH), 6.01 (1H, d, *J* 5.9, H1′), 7.51–7.66 (3H, m, Ph–H), 8.00–8.03 (2H, m, Ph–H), 8.68 (1H, s, H2), 8.75 (1H, s, H8), 11.20 (1H, br, NH). UV (H_2O) λ_{max}/nm 281 (19 100), λ_{min}/nm 245; pH 1, λ_{max}/nm 290 (23 600), λ_{min}/nm 242; pH 12, λ_{max}/nm 301 (12 800), λ_{min}/nm 258. ε260 (μM) 11.2, ε280 (μM) 19. *m/z* 511.14 (50%, M + Na)$^+$, 489.17 (40%, M + H)$^+$, 425 (5%, M − Ph)$^+$, 399 (5%, M − CoPh)$^+$. Accurate mass measurement on $C_{21}H_{24}N_6SO_6Na$ gives 511.13990, deviation 4.49 ppm.

***S*-(*O*-2′,*O*-3′-Isopropylidene-inosin-5′-yl)-L-homocysteine 11.** Prepared by a modified procedure described by Grøtli *et al*.[19] To a solution of (*O*-2′,*O*-3′-isopropylidene-5′-*O*-toluenesulfonyl)-inosine[28] (0.6 g, 1.1 mmol) in dichloromethane (25 cm^3) was added DMAP (66 mg, 0.54 mmol) and triethylamine (1.5 cm^3, 11 mmol) followed by *tert*-butyldiphenylchlorosilane (0.71 cm^3, 2.77 mmol) and the solution stirred at room temperature for 48 hours. TLC showed complete conversion. The reaction was quenched with methanol, evaporated, and the product coevaporated twice with toluene to give 6-*O*-(*tert*-butyldiphenylsilyl)-(*O*-2′,*O*-3′-isopropylidene-5′-*O*-toluenesulfonyl)-inosine as a yellow foam which was used without purification.

Homocystine (0.4 g, 1.5 mmol) in liquid ammonia (approximately 50 cm^3) was converted to its di-sodium salt as described above. Once the blue colour had dissipated the reaction was cooled to −78 °C and the above silyl protected inosine derivative (0.4 g, 0.5 mmol) added, and the solution stirred at −78 °C for 6 hours. The reaction was allowed to warm to room temperature. Under a nitrogen atmosphere ethanol (20 cm^3) was added followed by water (20 cm^3) and the solution acidified (HOAc) and stirred at room temperature overnight to remove the silyl protection. The solution was evaporated, dissolved in water and purified on RP-C18 silica eluting with water and then a gradient of 0–10% acetonitrile in water to elute the product as an off-white foam. Yield 128 mg, 53%. δ_H (D_2O) 1.24, 1.45 (6H, 2 × s, isopropylidene CH_3), 1.85–2.00 (2H, m, CH_2CH_2S), 2.49 (2H, t, *J* 7.2, CH_2S), 2.64–2.67 (2H, m, H5′, H5″), 3.68 (1H, t, *J* 5.7, NH_2CHCO_2H), 4.25 (1H, br. s, H4′), 4.90 (1H, br. s, H3′), 5.24–5.27 (1H, m, H2′), 5.98 (1H, s, H1′), 7.99 (1H, s, H2), 8.05 (1H, s, H8). UV λ_{max}/nm 249 (11 600), λ_{min}/nm 222; pH 12 λ_{max}/nm 254 (12 100), λ_{min}/nm 232. *m/z* 448 (70%, M + Na)$^+$, 399 (10%, M − CO)$^+$. Accurate mass measurement on $C_{17}H_{23}N_5SO_6Na$ gives 448.12670, deviation −0.01 ppm.

***S*-Inosin-5′-yl-L-homocysteine (SIH) 2.** A solution of **11** (100 mg, 0.24 mmol) in 5% aqueous TFA (5 cm^3) was stirred at room temperature for 5 hours. The solution was applied to an RP-C18 column and washed with water to neutrality and the product eluted with 10% aqueous acetonitrile to give a white foam. Yield 63 mg. 69%. δ_H (D_2O) 1.89–2.06 (2H, m, CH_2CH_2S), 2.55 (2H, t, *J* 7.2, CH_2S), 2.79–2.93 (2H, m, H5′, H5″), 3.69 (1H, t, *J* 5.8, NH_2CHCO_2H), 4.15–4.17 (1H, m, H4′), 4.25–4.27 (1H, m, H3′), 4.58–4.68 (under H_2O peak, H2′), 5.90 (1H, d, *J* 4, H1′), 8.02 (1H, s, H2), 8.14 (1H, s, H8). UV (H_2O) λ_{max}/nm 249 (12 000), λ_{min}/nm 223; pH 12 λ_{max}/nm 254 (12 500), λ_{min}/nm 231. *m/z* 408.09 (100%, M + Na)$^+$, 386.09 (20%, M + H)$^+$. Accurate mass measurement on $C_{14}H_{19}N_5SO_6Na$ gives 408.0957, deviation 0.72 ppm.

***S*-Cytid-5′-yl-L-homocysteine (SCH) 3.** The title compound was prepared from 5′-chloro-5′-deoxycytidine according to Ramalingam and Woodward.[20] The title compound has been reported,[16,18,20] but no characterisation provided. δ_H (D_2O) 1.95–2.13 (2H, m, CH_2CH_2S), 2.61 (2H, t, *J* 7.6, CH_2S), 2.76–2.95 (2H, m, H5′, H5″), 3.71 (1H, t, *J* 6.5, NH_2CHCO_2H), 4.00–4.09 (2H, m, H4′, H3′), 4.19–4.23 (1H, m, H2′), 5.72 (1H, d, *J* 3.9, H1′), 5.92 (1H, d, *J* 7.6, H6), 7.58 (1H, d, *J* 7.6, H5). UV (H_2O) λ_{max}/nm 270 (7900), λ_{min}/nm 250; pH 1 λ_{max}/nm 279 (11 250), λ_{min}/nm 240; pH 12 λ_{max}/nm 272 (7800), λ_{min}/nm 251. *m/z* 383 (100%, M + Na)$^+$. Accurate mass measurement on $C_{13}H_{20}N_4SO_6Na$ 383.09980, deviation −0.93 ppm.

***N^6*-Benzoyl-(2-deoxy-β-D-ribofuranosyl-5-deoxy-5-iodo)-adenosine 15.** A solution of N^6-benzoyl-(2-deoxy-β-D-ribofuranosyl-5-toluenesulfonyl)-adenosine[29] (0.49 g, 0.96 mmol) in acetone (50 cm^3) containing sodium iodide (0.72 g, 4.8 mmol) was heated at reflux overnight. The solution was evaporated, dissolved in ethyl acetate and washed (2 M $Na_2S_2O_3$) and evaporated to a pale brown foam. This was chromatographed on silica (CH_2Cl_2–(0–5%) MeOH) to give an off-white foam. Yield 0.32 g, 71%. δ_H 2.82–2.90 (2H, m, H2′, H2″), 4.00–4.02

(1H, m, H4′), 4.20–4.33 (2H, m, H5′, H5″), 4.47 (1H, br. s, H3′), 5.56 (1H, d, *J* 3.7, OH), 6.43 (1H, t, *J* 6.5, H1′), 7.53–7.93 (5H, m, Ph–H), 8.53 (1H, s, H2), 8.66 (1H, s, H), 811.23 (1H, s, NH). *m/z* 466 $(M + H)^+$.

***S*-(*N*^{6}-Benzoyl-2′-deoxy-β-D-ribofuranosyladenosin-5′-yl)-L-homocysteine 16.** Homocystine (0.25 g, 0.09 mmol) was converted to its disodium salt as described above. The salt was added to a solution of **15** (0.4 g, 0.086 mmol) in DMF (10 cm^3) and water (10 cm^3) and the solution stirred at 100 °C for 1 hour. The solution was cooled, neutralised (HOAc) and evaporated, and the residue chromatographed (RP-C18, H_2O) and the product eluted in a gradient of 0–10% MeCN in H_2O to give a white powder. Yield 0.23 g, 57%. δ_H (D_2O) 1.85–2.04 (2H, m, H2′, H2″), 2.30–2.59 (4H, m, SCH_2CH_2), 2.61–2.79 (2H, m, H5′, H5″), 3.62–3.66 (1H, m, NCH), 4.02 (1H, br. s, H4′), 4.29 (1H, br. s, OH), 4.40 (1H, br. s, H3′), 6.22 (1H, t, *J* 5.7, H1′), 7.11–7.31 (3H, m, Ph–H), 7.53–7.56 (2H, m, Ph–H), 8.26 (1H, s, H2), 8.34 (1H, s, H8). UV λ_{max}/nm 281 (21 000), λ_{min}/nm 245; pH 1, λ_{max}/nm 290 (25 400), 253 (10 800), λ_{min}/nm 261, 240; pH 12, λ_{max}/nm 301 (13 900), λ_{min}/nm 258. *m/z* 473.2 (100%, $M + H)^+$, 408 (10%, $M - Ph)^+$. Accurate mass measurement on $C_{21}H_{25}N_6SO_5$ gives 473.16010, deviation −1.30 ppm.

***S*-(2′-Deoxy-β-D-ribofuranosyladenosin-5′-yl)-L-homocysteine 5.** A solution of **16** (130 mg, 0.28 mmol) in concentrated ammonia solution (10 cm^3) was stirred at room temperature overnight. The solution was evaporated and the product chromatographed (RP-C18, H_2O) and the product eluted in a gradient of 0–10% MeCN in H_2O to give a white powder. Yield 90 mg, 89%. The ^{1}H-NMR spectrum was as previously described.[21] UV (H_2O) λ_{max}/nm 260 (15 200), λ_{min}/nm 227; pH 1 λ_{max}/nm 258 (14 900), λ_{min}/nm 229; pH 12 λ_{max}/nm 260 (14 300), λ_{min}/nm 234. *m/z* 369.1 (40%, $M + H)^+$, 391.1 (100%, $M + Na)^+$. Accurate mass measurement on $C_{14}H_{20}N_6SO_4Na$ gives 391.11620, deviation −0.71 ppm.

***N*-α-Fmoc-*S*-(*O*-2′,*O*-3′-isopropylidene-*N*^{6}-benzoyladenosin-5′-yl)-L-homocysteine 12.** To a solution of **10** (2.50 g, 4.7 mmol) in an ice-cold solution of dioxane (25 cm^3) and water (25 cm^3) containing sodium carbonate (4.3 g, 15 mmol) was added a solution of *N*-(9-fluorenylmethoxycarbonyloxy)-succinimide (3.35 g, 9.9 mmol) in dioxane (20 cm^3) dropwise over 30 minutes. The solution was then stirred at room temperature for 2 hours. The reaction was then neutralised (acetic acid) and concentrated and then worked up as described to give a yellow foam. This was chromatographed on silica (CH_2Cl_2–(0–5%) MeOH) to give an off-white foam. Yield 3.33 g, 94%. δ_H 1.31, 1.51 (6H, 2 × s, isopropylidene CH_3), 1.80–1.90 (2H, m, CH_2CH_2S), 2.31–2.34 (2H, m, CH_2S), 2.56–2.81 (2H, m, H5′, H5″), 3.84–3.87 (1H, m, $NHCHCO_2H$), 4.19–4.29 (4H, m, $CO.OCH_2$, $CO.OCH_2CH$, H4′), 5.00–5.02 (1H, m, H3′), 5.54–5.56 (1H, m, H2′), 6.28 (1H, s, H1′), 7.26–8.06 (13H, m, Ph–CH, Fmoc-CH), 8.66 (1H, s, H2), 8.77 (1H, s, H8), 12.2 (1H, br. s, NH). UV λ_{max}/nm 266 (30 200), λ_{min}/nm 234; pH 12, λ_{max}/nm 255 (28 100), λ_{min}/nm 240. *m/z* 773.23 (80%, $M + Na)^+$. Accurate mass measurement on $C_{39}H_{38}N_6SO_8Na$ gives 773.23720, deviation 0.28 ppm.

***N*-α-Fmoc-*S*-(*O*-2′,*O*-3′-isopropylidene-*N*^{6}-benzoyladenosin-5′-yl)-L-homocysteine *N*-hydroxysuccinimide ester 13.** To a solution of the above acid (**12**) (2.80 g, 3.73 mmol) in dichloromethane (50 cm^3) was added *N*-hydroxysuccinimide (0.86 g, 7.47 mmol), followed by dicyclohexylcarbodiimide (2.31 g, 11.2 mmol) and the solution stirred at room temperature overnight. The solid was filtered through Celite and the liquors worked up as described to give a white foam, which was chromatographed on silica (CH_2Cl_2–(0–2%) MeOH) to give a white foam. Yield 2.29 g, 72%. δ_H 1.33, 1.54 (6H, 2 × s, isopropylidene CH_3), 1.84 (2H, br. s, CH_2CH_2S), 2.49–2.58 (2H, m, CH_2S), 2.76–2.79 (2H, m, H5′, H5″), 3.60 (4H, s, 2 × CH_2CO), 4.11–4.31 (5H, m, $NHCHCO_2H$, $CO{\cdot}OCH_2$, $CO{\cdot}OCH_2CH$, H4′), 5.01–5.03 (1H, m, H3′), 5.54–5.56 (1H, m, H2′), 6.30 (1H, s, H1′), 7.29–8.05 (13H, m, Ph–CH, Fmoc-CH), 8.67 (1H, s, H2), 8.78 (1H, s, H8), 11.2 (1H, s, NH). UV λ_{max}/nm 270 (36 200), 209 (69 000), λ_{min}/nm 245; pH 1, λ_{max}/nm 281 (36 600), λ_{min}/nm 245; pH 12, λ_{max}/nm 300 (21 100), 255 (31 300), λ_{min}/nm 285, 240. *m/z* 870 (100%, $M + Na)^+$, 848 30%, $M + H)^+$. Accurate mass measurement on $C_{43}H_{41}N_7SO_{10}Na$ gives 870.2533, deviation −2.60 ppm.

***S*-(*O*-2′,*O*-3′-Isopropylideneadenosin-5′-yl)-L-homocysteine methylamide 14.** To a solution of **13** (0.5 g, 0.59 mmol) in DMF (20 cm^3) was added methylamine (40% solution in H_2O, 10 cm^3) and the solution stirred at room temperature overnight. The solution was evaporated and the product chromatographed on silica (CH_2Cl_2–(0–20%) MeOH) to give a white foam. Yield 0.16 g, 62%. δ_H 1.32, 1.52 (6H, 2 × s, isopropylidene CH_3), 1.45–1.89 (2H, m, CH_2CH_2S), 2.13–2.29 (2H, m, CH_2S), 2.55 (3H, br. d, *J* 3.5, CH_3NH), 2.65–2.81 (2H, m, H5′, H5″), 3.10–3.14 (1H, m, $COCHNH2$), 4.20–4.25 (1H, m, H4′), 4.97–5.00 (1H, m, H3′), 5.47–5.50 (1H, m, H2′), 6.14–6.16 (1H, m, H1′), 7.35 (2H, s, NH_2), 7.77 (2H, br, NH_2), 8.16 (1H, s, H2), 8.32 (1H, s, H8). UV λ_{max}/nm 260 (10 100), λ_{min}/nm 227; pH 1, λ_{max}/nm 258 (10 000), λ_{min}/nm 227. *m/z* 460.2 (100%, $M + Na)^+$, 438 (80%, $M + H)^+$. Accurate mass measurement on $C_{18}H_{27}N_7SO_4Na$ gives 460.17280, deviation −3.31 ppm.

***S*-Adenosin-5′-yl-L-homocysteine methylamide 4.** A solution of **14** (140 mg, 0.32 mmol) in 5% aqueous TFA (5 cm^3) was stirred at room temperature overnight. The solution was evaporated, and the product chromatographed (RP-C18) eluting with H_2O then 0–5% MeCN in H_2O to give a white powder. Yield 77 mg, 61%. δ_H (D_2O) 1.88–1.95 (2H, m, CH_2CH_2S), 2.39–2.54 (2H, m, CH_2S), 2.55 (3H, s, CH_3N), 2.75–2.90 (2H, m, H5′, H5″), 3.84 (1H, t, *J* 6.8, $COCHNH_2$), 4.13–4.18 (1H, m, H4′), 4.23–4.26 (1H, m, H3′), 4.63–4.68 (1H, m, H2′), 5.86 (1H, d, *J* 4.6, H1′), 7.98 (1H, s, H2), 8.13 (1H, s, H8). UV (H_2O) λ_{max}/nm 260 (11 200), λ_{min}/nm 228. *m/z* 398.16 (80%, $M + H)^+$. Accurate mass measurement on $C_{15}H_{23}N_7SO_4$ gives 398.1610, deviation −0.13 ppm.

DNA substrates. Haesub, a synthetic 30 base pair DNA substrate for M.HaeIII which contains a single unmethylated recognition site (GGCC), was prepared by annealing complementary oligonucleotides Haesub1, 5′-TTCGAGAAGCTGAGGCCGGCGTACCTGGAG-3′, and Haesub2, 5′-CTCCAGGTACGCCGGCCTCAGCTTCTCGAA-3′, as described.[30] Hhasub, a similar 30 base pair substrate containing a single M.HhaI recognition site (GCGC), was prepared by annealing Hhasub1, 5′-TTCGAGAAGCTGAGCGCGGCGTACCTGGAG-3′, and Hhasub2, 5′-CTCCAGGTACGCCGCGCTCAGCTTCTCGAA-3′, as above.

For electrophoretic mobility shift assays a substrate with a single hemimethylated HaeIII site was prepared by annealing complementary oligonucleotides Gsub (5′-TTCGAGTGACTGAGGCCGGTGTACCTGGAG-3′) and Gsub-meth (5′-CTCCAGGTACACCGGMCTCAGTCACTCGAA-3′), where M represents C^5 methylcytosine. The substrate was ^{32}P end labeled.

Methyltransferase assays. Filter binding assays monitored the incorporation of tritium labelled methyl groups into DNA. DNA and *S*-adenosyl-L-[methyl-^{3}H]methionine, (20 Ci $mmol^{-1}$; Amersham-Pharmacia) were added to M.HhaI reaction buffer (50 mM Tris–HCl (pH 7.5), 10 mM EDTA, 5 mM β-mercaptoethanol) or M.HaeIII reaction buffer (50 mM NaCl, 50 mM Tris–HCl (pH 8.5), 10 mM dithiothreitol). Assays were performed at 37 °C, started by the addition of methyltransferase, and quenched by the addition of unlabelled AdoMet to a final concentration of 80 μM. Quenched reactions were either spotted onto DE81 filters (Whatman) and processed as described,[30] or

spotted onto Multiscreen DE plates, and washed three times with 200 μl ammonium carbonate (0.2 M), and once with 100 μl ethanol, before punching out individual filters for counting. The background level of radioactivity was determined by performing blank reactions (without enzyme) and was subtracted from all readings. Enzymes were obtained from New England Biolabs at 10 units μl^{-1} (one unit is the amount of enzyme required to protect 1 μg λ DNA in one hour at 37 °C in a 10 μl reaction mixture) and diluted in the appropriate methylation reaction buffer, supplemented with 1 mg ml^{-1} bovine serum albumin.

The concentration of M.HaeIII active sites was estimated by electrophoretic mobility shift assay as described previously.[31] Reactions contained 1–150 nM DNA, 2–5 nM enzyme, 200 μM AdoHcy and 10 mM EDTA in M.HaeIII reaction buffer. Reactions were incubated at 37 °C for 30 minutes before addition of glycerol to 5% of the final volume and electrophoresis using 10% TBE gels (Novex, Invitrogen). Gels were fixed and dried according to the manufacturer's instructions, exposed for 18 hours and scanned using a phosphorimager (Typhoon 8600, Molecular Dynamics). Results were analysed using Imagequant software (Molecular Dynamics).

Determination of K_m^{AdoMet} for M.HaeIII. Time course assays were started by the addition of M.HaeIII to a final concentration of 5.9 nM. The concentration of Haesub, 1.25 μM, was more than a hundred fold higher than K_m^{DNA}, and the concentration of AdoMet ranged from 25 to 2000 nM. Aliquots of the reaction mixture were removed at two minute intervals and quenched as described. Under these conditions the incorporation of methyl-^{3}H into DNA was linear for at least ten minutes (Fig. 2A). A plot of reaction velocity *versus* AdoMet concentration was fit to the Michaelis–Menten model (eqn. 1), using the Levenberg–Marquardt algorithm, as implemented in Kaleidagraph (Synergy Software, Reading, PA, USA). $[S]_0$ is the initial concentration of AdoMet and K_m is the apparent Michaelis–Menten constant of M.HaeIII for AdoMet.

Determination of K_m^{DNA} and K_{cat} for M.HaeIII. Time course assays were started by the addition of M.HaeIII to a final concentration of 0.4 nM. The AdoMet concentration of 1 μM was over three fold greater than K_m, and the final DNA concentration ranged from 1 to 30 nM. Aliquots of the reaction mixture were removed at one minute intervals for six minutes and quenched as described. Under these conditions the incorporation of methyl-^{3}H into DNA was linear. A plot of reaction velocity *versus* AdoMet concentration was fit to the Michaelis–Menten model (eqn. 1), as above, where $[S]_0$ is the initial concentration of DNA and K_m is the apparent Michaelis–Menten constant of M.HaeIII for the DNA substrate Haesub.

Determination of K_m^{AdoMet} for M.HhaI. Time course assays were started by the addition of M.HhaI to a final concentration of 0.067 units μl^{-1}. The concentration of Hhasub was 0.5 μM, a hundred fold higher than K_m^{DNA}, and the concentration of AdoMet ranged from 40 to 1000 nM. Four aliquots of the reaction mixture were removed at two minute intervals and quenched as described. Under these conditions the incorporation of methyl-^{3}H into DNA was linear. A plot of reaction velocity *versus* AdoMet concentration was fit to the Michaelis–Menten model (eqn. 1), as above. $[S]_0$ is the initial concentration of AdoMet and K_m is the apparent Michaelis–Menten constant of M.HhaI for AdoMet.

Determination of K_i for AdoHcy and analogues. The initial rate of methyltransfer by M.HaeIII was measured at a range of concentrations of the eight inhibitors using single time point assays. Final concentrations of 5.9 nM M.HaeIII, 1.25 μM Haesub, 200 nM AdoMet and 10 nM–15 μM AdoHcy analogue were used. Reactions were started with the addition of enzyme, quenched after ten minutes and processed as above. The inhibition of M.HhaI by AdoHcy and analogues was measured in the same way, using final concentrations of 0.067 units μl^{-1} M.HhaI, 1.25 μM Hhasub, 200 nM AdoMet and 10 nM–15 μM AdoHcy analogue. Reactions were quenched after eight minutes.

Plots of reaction velocity *versus* inhibitor concentration were fit to the curve in eqn. 2 using the Levenberg–Marquardt algorithm, where $[I]$ is the concentration of AdoHcy or analogue. Inhibition is assumed to be competitive with AdoMet, as observed for inhibition of M.HhaI by AdoHcy (13), $\Delta\Delta G$ was calculated from the inhibition constants for AdoHcy and analogues using eqn. 3.

$$\Delta\Delta G = -\mathrm{RTln}(K_i^{\text{analogue}} / K_i^{\text{AdoHcy}}) \quad (3)$$

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