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SAR around (L)-*S*-adenosyl-L-homocysteine, an inhibitor of human DNA methyltransferase (DNMT) enzymes

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ABSTRACT

The inhibitory activity of base-modified SAH analogues and the specificity of inhibiting human DNMT1 and DNMT3b2 enzymes was explored. The 6-amino group was essential while the 7-N of the adenine ring of SAH could be replaced by CH– without loss of activity against both enzymes. The introduction of small groups at the 2-position of the adenine moiety favors DNMT1 over DNMT3b2 inhibition whereas alkylation of the N^6-amino moiety favors the inhibition of DNMT3b2 enzyme.

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DNA methylation or the addition of a methyl group at the 5′ positions of cytosine residues within a CpG sequence is the main epigenetic modification in mammals and is responsible for gene expression in cancer and in normal cells.[1] DNA methyltransferase (DNMT) enzymes catalyze the transfer of a methyl group from (L)-*S*-adenosyl-L-methionine (SAM), onto the 5′ position of the cytosine ring of the DNA.[2] The demethylated metabolite, (L)-*S*-adenosyl-L-homocysteine (L-SAH) and methylated DNA are the products of this reaction.[3] L-SAH binds to DNMTs and inhibits their catalytic reaction and is important for the regulation of biological transmethylations.[4] DNMT1 is required for preservation of methylation patterns in differentiated cells, while the de novo DNMTs like DNMT3A and DNMT3B play a role in establishing methylation during early embryogenesis.[5,6] Changes in human DNA methylation patterns are commonly found in human tumors and are implicated in development and maintenance of human cancer.[7] The aberrant hypermethylation of the CpG islands in cancer cells with concomitant transcriptional inactivation of important tumor suppressor genes[8] is one of the hallmarks of cancer. DNMT inhibition is correlated with reactivation of tumor suppressor genes in cancer cells with consequent cancer remission[9] making it a valuable therapeutic target. Different types of DNMT1 inhibitors are under investigation or are currently in use for the treatment of cancer. Nucleoside analogs Vidaza® (5-aza-C), and Decitabine® (5-aza-CdR),[10] have recently been approved for myelodysplastic syndrome while DNMT1 antisense molecules such as MG98, have demonstrated evidence of clinical activity.[11] To exert their biological effects, 5-aza-C and 5-aza-CdR must be incorporated into the DNA where they covalently trap the bulky 190-kDa DNMT enzyme onto the DNA while agents such are MG98 interfere with the biosynthesis of DNMT1 at the transcriptional level.[12a] Other small molecule DNMT inhibitors such as procainamide, EGCG, RG108, and curcumin[12c] have been reported.[12b]

Borchardt et al.[13a–f] and others[14] described the synthesis of a number of L-SAH analogues having structural modifications of the amino acid, sugar, and base portions as inhibitors of non-human DNMTs. More recently, some N^6-substituted SAH analogues have been described as inhibitors of protein arginine methyltransferases.[14f] In addition, a number of simplified and less rigid analogues of SAH have been reported, however these compounds, were devoid of activity against human DNMT1.[15] The 2′- and 3′-hydroxy groups of the sugar portion and the 6-amino group of the adenine moiety of SAH were found to be important structural requirements for inhibition of DNMTs.[16] Some nitrogen analogues of SAM and SAH have been reported and tested against catechol *O*-methyltransferase and tRNA methylases.[17] However to the best of our knowledge, there are no reports on the activity of SAH or

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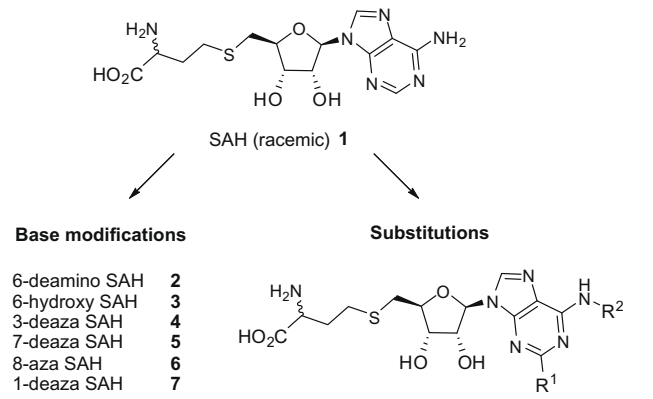


Figure 1. Derivatives of SAH synthesized to probe the binding sites of human DNMT1 and DNMT3b2.

its analogues against human DNMT1 or DNMT3b2 isosymes. In addition, this is the first account of any inhibitors of the de novo DNMT3b2 enzyme.

The present paper summarizes the results of our investigation of modifications of the base, and 2- and N^6-substitutions (R^1 and R^2, respectively) of SAH (Fig. 1) and the effect on the binding affinity to DNMT1 and DNMT3b2 enzymes.

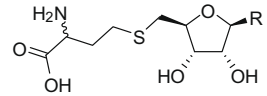
The synthesis of several aza or deaza SAH derivatives was described previously in the literature. *S*-nebularinehomocysteine **2**,[13f] *S*-inosinylhomocysteine **3**,[13b] 3-deaza-SAH **4**,[13b] *S*-tubercidinylhomocysteine **5**,[14d] and 8-aza-SAH **6**[13f] are known compounds (Table 1). The synthesis of 1-deaza-SAH **7** appeared in the literature[18] however, we prepared it as described in Scheme 1. Commercially available **8** reacted readily with a solution of **9** in methanol containing 1 equiv of sodium methoxide to give **10**. In situ exchange of the protecting groups afforded the glycoside donor **12**. Pre-activation of 7-nitro-3*H*-imidazo[4,5-*b*]pyridine[18a] and reaction with **12** and TMSOTf yielded **13**. Reduction of the nitro group of **13** and removal of the protecting groups gave 1-deaza-SAH **7**.

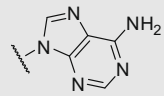
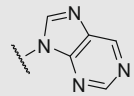
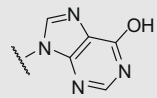
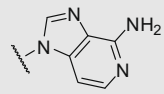
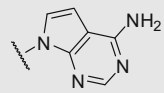
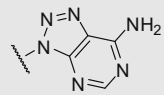
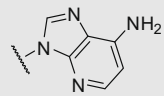
The 2-substituted analogs listed in Table 2 were prepared as shown in Schemes 2 and 3.

Reaction of **14**[19] with $SnBu_3Cl$ using the conditions described by Kato et al.[20a,b] gave **15** which upon electrophilic fluorination using XeF_2/AgTf, nucleophilic substitution employing NH_3/isopropanol, removal of the protecting group and tosylation of the liberated alcohol afforded **16**. Nucleophilic displacement of tosylate **16** with the in situ produced thiol from **9**, followed by removal of the protecting groups gave **18**.[14c]

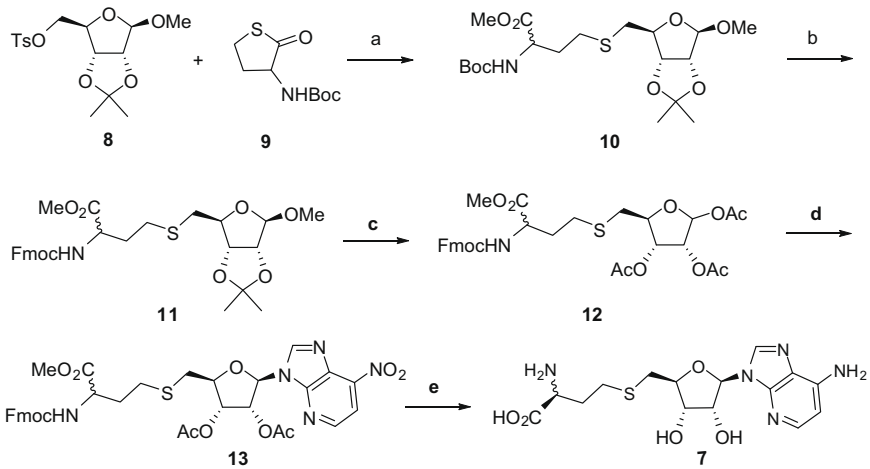
Reaction of intermediate **15** with I_2 and nucleophilic substitution using NH_3/isopropanol afforded **19**. Intermediate **19** was converted to **20** following the same reaction sequence as the one

Table 1
DNMT1 and DNMT3b2 *enzymatic activity*: base modifications, compounds **1-7**[a]



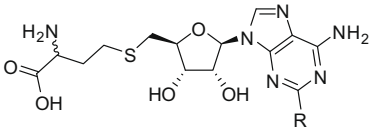
Compd	R	DNMT 1 IC_{50} (μM)	DNMT 3b2 IC_{50} (μM)
1[b]		2	0.3
2[14e]		300	28
3[13b]		>1 mM	>1 mM
4[13b,14i]		45	6.8
5[14e,i]		1.5	0.3
6[13f]		8	3
7		45	45

[a] Values are means of at least two experiments, ±20%.
[b] Racemic SAH.



Scheme 1. Reagents and conditions: (a) **9**/NaOMe/MeOH, rt, 30 min then **8**, reflux, 3 h; (b) TMSOTf/DCE, rt, 1 h then Fmoc-Cl, rt, 5 h; (c) (i) TFA/water, rt, 30 min; (ii) AcCl/pyrinine/DCM, rt, 16 h; (d) (i) 7-nitro-3*H*-imidazo[4,5-*b*]pyridine/*N*,O-bistrimethylsilylacetomide/MeCN, rt, 1 h then 2,6-dichloro-9*H*-purine/TMSOTf, reflux, 30 min; (e) (i) Fe/HCl/MeOH, reflux, 15 min; (ii) KOH/water/THF, 0 °C, 3 h.

Table 2
DNMT1 and DNMT3b2 *enzymatic activity*: 2-substituted analogues, compounds **1**, **19**, and **22**–**26**[a]



Compd	R	DNMT 1 IC_{50} (μM)	DNMT 32b IC_{50} (μM)
1	H	2.0	0.3
18[14c]	F	2.5	6.8
24	Cl	2.9	57.2
20	I	21.0	256
22	Me	6.0	1.0
26	OMe	75	NI
27	SMe	216	500

NI: no inhibition at 45 μM inhibitor concentration.
[a] Values are means of at least two experiments, ±20%.

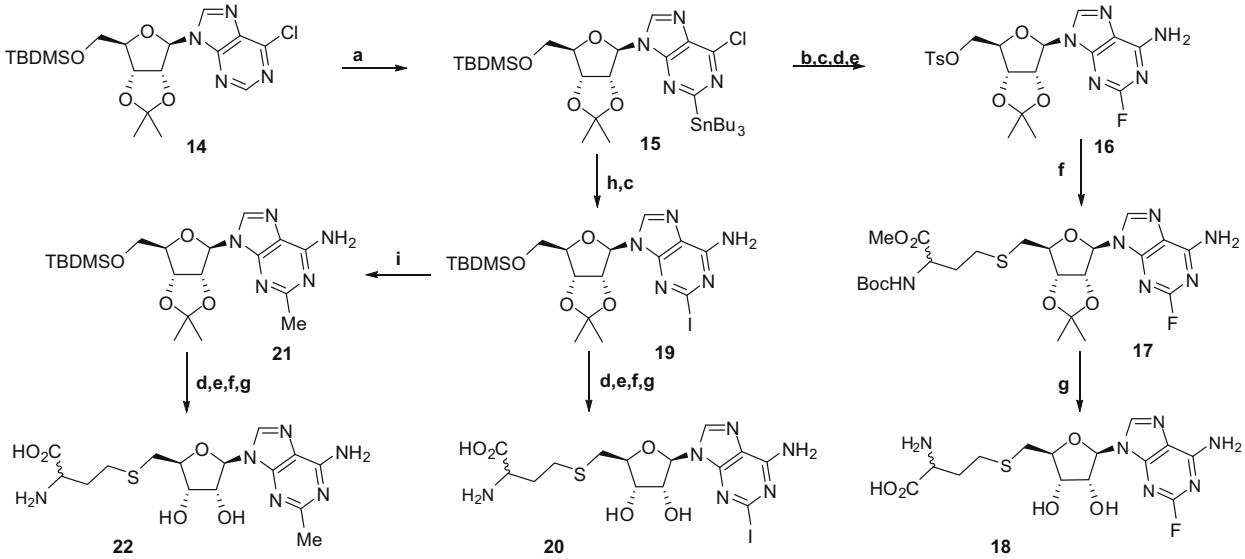
described for the transformation of **16** to **18**. The palladium catalyzed reaction of **19** with $AlMe_3$ gave **21**, which was converted to **22** as described above (Scheme 2). Compound **23**[21] was used for the preparation of compound **24** by transferring the free alcohol to the tosylate followed by the same reaction sequence as the one described for the transformation of **16** to **18**. Compound **23** was converted either to the 2-methoxy- or to the 2-methylthio-intermediates, **25a** and **25b**, respectively, employing sodium methoxide or sodium methanethiolate, respectively.

Compounds **25a** and **25b** were converted to **26** and **27**, respectively, in a manner similar to the synthesis of **18** from **16**.

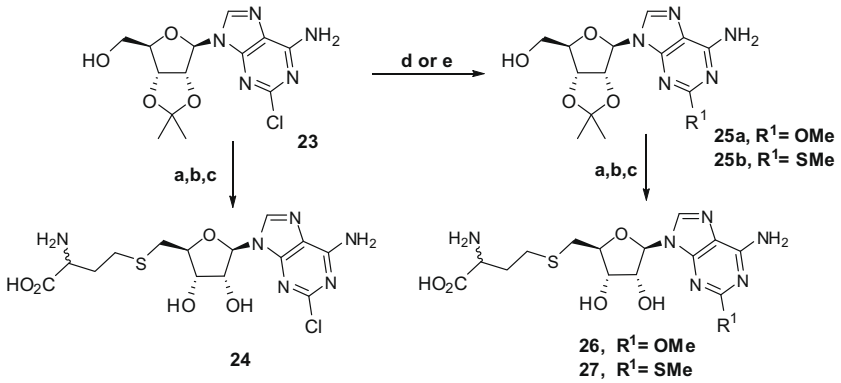
The N^6 substituted analogues, compounds **28**–**34**, were prepared according to the procedure of Lin et al.[14f] For the concomitant substitution of the 2- and N-6 positions, we utilized intermediate **12** and reacted it with activated 2,6-dichloro-9*H*-purine to afford **35**. Displacement of the chloro group of **35** with 2-(biphenyl-4-yl)ethanamine and removal of the protecting groups gave **36** (Scheme 4).

All compounds[22a–c] were tested against recombinant human DNMT1 and DNMT3b2 enzymes[23] and the results are presented in Tables 1–3.

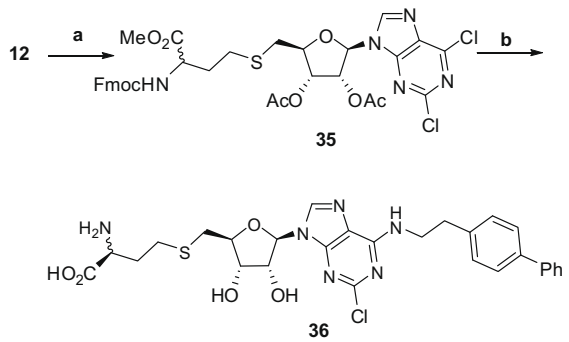
We attempted first to explore the importance of the 6-amino group for inhibition of human DNMT1 and DNMT3b2 enzymes. As it can be seen for compounds **2** and **3** the replacement of the amino group by a hydrogen or by a hydroxyl group resulted in a marked reduction in inhibitory activity against both enzymes. This



Scheme 2. Reagents and conditions: (a) LTMP/THF, −78 °C, 30 min then $SnBu_3Cl$, −78 °C, 1 h; (b) 2,6-di-tert-butyl-4-methylpyridine/XeF_2/AgTf/DCM, rt, 15 min; (c) NH_3/isopropanol, 2 days, 100 °C, sealed tube; (d) TBAF/THF, 0 °C to rt, 1 h; (e) NaH/THF, 0 °C, 30 min then TsCl, 1 h; (f) **9**/NaOMe/MeOH, rt, 30 min then **16**, reflux, 1 h; (g) (i) KOH/THF/water, rt, 1 h, (ii) TFA, rt, 30 min, (iii) TFA/H_2O, rt 2 h; (h) I_2, THF, 1 h; (i) $AlMe_3$, $PdCl_2$, PPh_3, THF, reflux, 2 h.

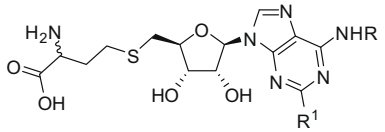


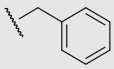
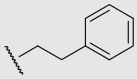
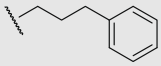
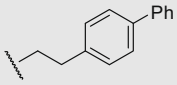
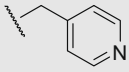
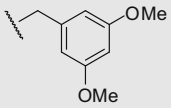
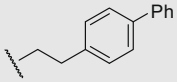
Scheme 3. Reagents and conditions: (a) NaH/THF, 0 °C, 30 min then TsCl, 1 h; (b) **9**/NaOMe/MeOH, rt, 30 min then **16**, reflux, 1 h; (c) (i) KOH/THF/water, rt, 1 h, (ii) TFA, rt, 30 min, (iii) TFA/H_2O, rt 2 h; (d) $NaOCH_3$, DMF, 90 °C, 1 h; (e) $NaSCH_3$, DMF, 80 °C, 1 h.



Scheme 4. Reagents and conditions: (a) **12**/*N*,*O*-bistrimethylsilylacetomide/MeCN, rt, 1 h then 2,6-dichloro-9*H*-purine/TMSOTf, reflux, 30 min; (b) (i) 3-phenylpropan-1-amine/DCE, rt, 16 h; (ii) KOH/water/THF, rt, 16 h.

Table 3
DNMT1 and DNMT3b2 enzymatic activity: N^6 alkylation compounds **28**–**36**[a]



Compd	R	R^1	DNMT 1 IC_{50} (μM)	DNMT 3b2 IC_{50} (μM)
1	H	H	2.0	0.3
28[14f]		H	154	2
29[14f]		H	61	1
30		H	27	2
31		H	7.4	0.6
32		H	5.4	0.6
33		H	22	3.6
34		H	18	0.9
36		Cl	6.7	29

[a] Values are means of at least two experiments, ±20%.

result is in line with the reported lack of activity of **2** and **3** against other methyltransferases[13b] and implicates the requirement of the 6-amino group for activity.

Next we investigated the importance of the different adenine ring nitrogen atoms for inhibition of DNMT1 and DNMT3b2 enzymes. As can be seen from Table 1, compounds **4**, **6**, and **7** resulted in a marked loss of activity while *S*-tubercidinylhomocysteine (7-deaza SAH) **5** retained all activity against both enzymes and was as active as racemic SAH **1** which indicates that the nitrogen in the 7 position of adenine is not required for binding to DNMT1 and DNMT3b2 enzymes. This is in agreement with the reported observation that the N-7 of the adenine moiety of SAH is not important for binding to other O and N methyltransferases such as indoleethylamine *N*-methyltransferase (INMT),[14d] tRNA methylases (tRNA MT),[14d] catechol *O*-methyltransferase (COMT), phenylethanolamine *N*-methyltransferase (PNMT), histamine N methyltransferase (HMT), and hydroxyindole *O*-methyltransferase (HIOMT).[13b]

We explored a variety of substituents at the 2-position of the adenine ring of SAH, some of which are listed in Table 2. Generally, DNMT3b2 is less tolerant for substitution at this position than DNMT1, and none of the analogues prepared was more active than SAH itself. A 2-methyl group, compound **22**, produced a threefold loss of activity against both enzymes, while halogens led to partial selectivity for DNMT1 enzyme. Both the 2-fluoro- and 2-chloro analogues, compounds **18** and **24**, respectively, are almost equipotent to SAH against DNMT1 whereas the same compounds caused 22- and 190-fold decrease of inhibitory activity against DNMT3b2. However, a 2-iodo group lead to loss of activity against both enzymes. It is noteworthy that a 2-fluoro or 2-chloro-group conferred selectivity amongst human DNMT enzymes and this could be a beneficial tool for the preparation of more selective inhibitors. All other substituents such as a methoxy or a thiomethyl group, compounds **26**, and **27**, respectively, produced inactive compounds (Table 2).

We also investigated the tolerance of substitution at the N^6 position of the adenine ring. A phenyl and a benzyl group resulted in a marked decrease of activity against DNMT1, while the more flexible lipophilic moeities such as 3-phenylpropanyl and *p*-phenyl-phenethyl groups produced compounds, **31** and **32**, respectively, with moderate activity. The DNMT3b2 enzyme seems to be more tolerant for substitution at this position however the best compounds, **31** and **32**, are twofold less active than SAH itself. The concomitant 2- and N^6 substitution, compound **36**, resulted in slight decrease of activity against DNMT1 and a substantial loss of activity against DNMT3b2, as compared to **32**.

In conclusion, some differences in binding requirements of base modified SAH analogues have been observed between human DNMT1 and DNMT3b2 enzymes. The 6-amino group is essential while the 7-N of the adenine ring of SAH can be replaced without loss of activity against both enzymes. The introduction of small groups at the 2-position of the adenine moiety favors DNMT1 over DNMT3b2 whereas alkylation of the N^6-amino moiety favors the inhibition of DNMT3b2 enzyme. The study, however, failed to identify modifications capable of increasing inhibitory activity of SAH against either enzyme.

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22. (a) Experimental details are in MethylGene patent, Amal Wahhab, Jeffrey Besterman, Daniel Delorme, Ljubomir Isakovic, David Llewellyn, Jubrail Rahil, Oscar Saavedra, Robert Deziel, International Patent Application WO 2006/078752, 2006; (b) All compounds were >95% pure and were routinly characterized by ^{1}H NMR and MS, and in some instances by ^{13}C NMR and NOE. To further confirm the structural assignments, we prepared some analogues by different routs and compared their ^{1}H NMR spectra. A manuscript describing the efficient synthesis of some of these 2- and N^{6}-modified analogues is in preparation and will be submitted soon for publication; (c) Compound **22** ^{1}H NMR (CD_3OD): ppm: 8.32 (s, 1H), 6.06 (d, 1H, *J* = 4.8, 1-H), 4.80 (m, 1H), 4.68 (s, 1H), 4.40 (m, 1H), 4.30 (m, 1H), 3.74 (m, 1H), 3.08 (m, 2H), 2.80(m, 2H), 2.58(s, 3H, Me), 2.40(m, 1H), 2.10(m, 1H). MS: calcd 398.44; found 399.4 $(M+H)^{+}$;compound **32** ^{1}H NMR (DMSO-d_6) δ8.38 (s, 1H), 8.27 (s, 1H), 7.93 (br s, 1H), 7.63 (d, 2H, *J* = 8.4 Hz), 7.58 (d, 2H, *J* = 8.0 Hz), 7.44 (t, 2H, *J* = 7.4 Hz), 7.35 (m, 3H), 5.91 (d, 1H, *J* = 5.7 Hz), 4.75 (t, 1H, *J* = 5.3 Hz), 4.20 (m, 1H), 4.05 (m, 1H), 2.9–3.0 (m, 4H), 2.83 (m, 2H), 2.64 (t, 2H, *J* = 7.6 Hz), 2.02 (m, 1H), 1.84 (m, 1H). MS (ESI): calc 564; found 565 (MH^{+}).
23. *Enzymes and biological assays*: The full length cDNAs of DNMT1 (Swissprot accession number P26358) and of DNMT3b2 (Swissprot accession number Q9UBC3-2) were cloned in the pBlueBac4.5 vector (Invitrogen) These constructs were used to generate recombinant baculoviruses using the Bac-N-Blue™ DNA according to the manufacturer's instructions (Invitrogen) Nuclear extracts were prepared from High Five insect cells infected with the recombinant baculoviruses. The DNMT1 enzyme was purified on sequential Q-sepharose FF and Hitrap Heparin columns (Amersham Biosciences. The DNMT3b2 enzyme was purified on a Hitrap SP-sepharose column and underwent buffer exchange, using PD-10 column (Amersham Biosciences). Typically purities of DNMT1 and DNMT3b2 enzyme preparations were above 95% and 70%, respectively. Purified enzyme stocks were frozen at −80 °C in aliquots prior to use in enzymatic assays. Assay, DNMT1: the enzyme/Oligo mixture (10 μl) is added to the inhibitor (3 μl) in a round bottom 96-well plate and the mixture is pre-incubated for 10 min at 37 °C. Final enzyme concentration is 25 nM and the hemi-methylated oligonucleotide (MYG167: ATC GCA TCG ATC GCG ATT CGC GCA TCG GCGATC; MYG166: GAT XGC XGA TGX GXG AAT XGX GAT XGA TGX GAT (X: 5-methylcytosine, the two oligos are hybridized to form a duplex) at a final concentration of 2.6 μM. The SAM [methyl-^{3}H] mixture (10 μl, 20% hot and the remaining is cold SAM. SAM was 0.55 mCi/ml, with a specific activity of 55–85 Ci/mmol) was then added for a final concentration of 3 μM. All solutions are diluted with water and a 10× assay buffer (50 mM Tris–HCl pH 7.6, 5% Glycerol, 1 mM EDTA, 100 μg/ml BSA, 1 mM DTT). The final reaction was incubated for 15 min at 37 °C and the reaction was stopped with 150 μl of a solution of SAH, and harvested onto a DEAE filtermat (Wallac Cat# 1450-522) using a Tomtec Cell harvester. The Filtermat was washed using a cold 20 mM solution of NH_4HCO_3. The filtermat was dried on a hot plate (set at a low temperature) and MeltiLex™ scintillant (Wallac Cat#1450-441) was melted over the filtermat. The filtermat was read using a Wallac top-count beta-counter. The assay procedure for DNMT3b2 is exactly as DNMT except final enzyme concentration was 188 nM.