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Constrained (L-)-*S*-adenosyl-L-homocysteine (SAH) analogues as DNA methyltransferase inhibitors

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ABSTRACT

Potent SAH analogues with constrained homocysteine units have been designed and synthesized as inhibitors of human DNMT enzymes. The five membered (2*S*,4*S*)-4-mercaptopyrrolidine-2-carboxylic acid, in **1a**, was a good replacement for homocysteine, while the corresponding six-member counterpart was less active. Further optimization of **1a**, changed the selectivity profile of these inhibitors. A Chloro substituent at the 2-position of **1a**, compound **1d**, retained potency against DNMT1, while N^6 alkylation, compound **7a**, conserved DNMT3b2 activity. The concomitant substitutions of **1a** at both 2- and N^6 positions reduced activity against both enzymes.

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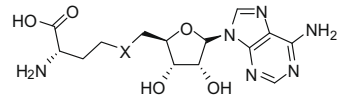
Abnormalities in human DNA methylation patterns are commonly found in human tumors and are implicated in development and maintenance of human cancers.[1a–c] DNA hypermethylation in CpG islands in cancer cells results in alteration of gene expression patterns and most notably in the loss of expression or silencing of tumor suppressor genes.[2a–c] Changes of DNA methylation could be used for the identification of specific and sensitive tests for the early detection of some cancers.[2d–f] DNA methyltransferase 1 (DNMT1) protein is a major contributor to DNMT activity in human cells and is required to maintain methylation patterns in differentiated cells[3a] while the de novo DNMTs, DNMT3a, DNMT3b1, and DNMT3b2 establish DNA methylation during early embryogenesis.[3b–e] A recent review of the enzymatic mechanism of DNMT1 and its implication in the design of novel mechanism-based inhibitors was published in 2008 by Svedružić.[3f] A second review by Yu et al., gave an account of the different DNMTs and their biological functions with the focus on the design of various inhibitors of DNA hypermethylation as anticancer drugs.[3g]

The nucleotide analogues 5-azacytidine[4a] (Vidaza®) and 5-aza-2′-deoxycytidine[4b] (Decitabine) are approved anti-cancer drugs targeting DNA methylation, while a number of other inhibitors are in different stages of development.[3g] Such inhibitors, however, act as mechanism-based (suicide or covalent) inhibitors that are incorporated into the DNA of the target cell leading to a depletion (Vidaza) or degradation (Decitabine) of the active enzymes. Other small molecule DNMT inhibitors such as procaine and procainamide (interrupt binding of DNMT1 to DNA), EGCG, hydralazine, RG108 and psammaplin A (block the active site of DNMT1 non-covalently) have been reported.[3g,4c] More recently Liu et al. have identified curcumin from virtual screening and docking utilizing a homology model of DNMT1.[4c] Curcumin is proposed to work by blocking the catalytic C1226 of DNMT1 inhibitor enzyme without incorporation into the DNA.

The cofactor (L-)-*S*-adenosyl-L-methionine (SAM) is the methyl group donor in the methylation reaction and is required for the DNA cleavage by most DNMTs. It covalently modifies the DNA at the carbon-5 of cytosine residues and in the process is converted to its demethylated metabolite (L-)-*S*-adenosyl-L-homocysteine (L-SAH).[3f,g] In tissue, the levels of SAM and of SAH are equivalent (Fig. 1).[5a,b] SAH can bind to DNMTs and inhibit their catalytic reaction and has an important role in the regulation of biological transmethylation.[6a–c]



SAM: X= $^{+}$SMe
SAH: X=S
Sinefungin: X= CH-NH2

Figure 1. Structure of SAM, SAH and Sinefungin.

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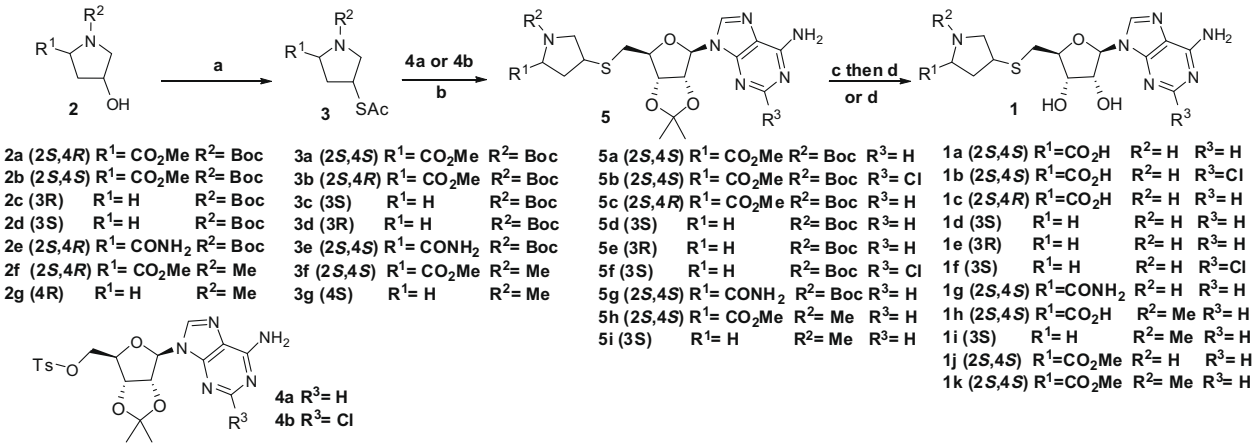
Borchardt et al.,[7a–f] Cohen et al.,[8a] and others[8b–e] prepared SAH analogues as inhibitors for specific non-human DNMTs. More recently, some N^6-substituted SAH analogues have been described as inhibitors of protein arginine methyltransferases.[9a,b] Nitrogen analogues of SAM and SAH have been tested against catechol O-methyltransferase and tRNA methylases.[10a–c] In addition, Sinefungin has been reported to inhibit human DNMT, however, it is a non-selective inhibitor with potential for toxicity (Fig. 1).[11] In spite of the differences in their sequence identity, the different classes of DNMTs have a conserved cofactor binding site. There are numerous published structures of DNMTs in complex with SAM or SAH, and the position of SAM is relatively similar in each structure, but the interactions of the methionine moiety are not conserved between the different classes of DNMTs.[8a,12a] All the functional groups of the homocysteine moiety of L-SAH, the terminal amino group, the carboxylic acid group and the sulfur atom, are involved in binding to the DNMT enzymes.[3f,7b,12a] SAH analogues with amino acid modifications tested against the bacterial DNMT enzymes M.HhaI and M.HaeIII provided further evidence for the important role of both the amino and carboxylic acid functional groups for binding to the enzyme.[8a]

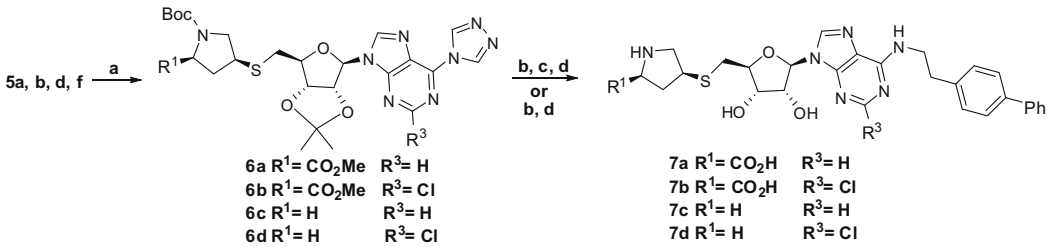
The conformation of the flexible homocysteine moiety in the active site of DNMT1 or DNMT3b2 has not been reported. The homocysteine unit could adopt either an extended or folded conformation, with the extended conformation possibly representing the catalytically competent conformation.[12b,8a] We prepared a large set of compounds focusing on replacements for the homocysteine unit to identify analogues with improved drug-like properties lacking the zwitter-ionic character of SAH. This Letter will describe replacement of the homocysteine moiety by constrained groups. In addition, the results of simultaneous modifications of SAH at the homocysteine, 2- and N^6 positions on binding and selectivity against DNMT1 and DNMT3b2 enzymes will be shown.

Scheme 1 depicts the general procedure utilized to prepare analogues **1a–k**.[13a] Racemic **1a**, **1c**, **1d**, and **1e** have been reported previously as inhibitors of polyamine biosynthesis.[13b,c] Alcohol **2a,** (2*S*,4*R*)-methyl 1-(*tert*-butoxycarbonyl)-4-hydroxypyrrolidine-2-carboxylate, was converted to thioacetate **3a** via the mesylate resulting in inversion of configuration. In situ formation of the thiol with NaOMe followed by the reaction with either intermediate **4a**[13d] or **4b**[13e] gave the fully protected **5a** or **5b**, respectively. Ester hydrolysis of **5a** or **5b** with KOH (not shown in scheme) followed by the treatment with a mixture of TFA/H_2O resulted in removal of both the Boc and the acetonide protecting groups to yield the desired **1a** or **1b**. Similarly, compound **1c** was prepared from alcohol **2b**, (2*S*,4*S*)-methyl 1-(*tert*-butoxycarbonyl)-4-hydroxypyrrolidine-2-carboxylate. Compounds **1d** and **1f** were prepared from alcohol **2c**, (*R*)-*tert*-butyl 3-hydroxypyrrolidine-1-carboxylate, via the thioacetate **3c.** The liberated thiol (not shown in scheme) was then reacted with **4a** or **4b** to give acetonides **5d** and **5f**, respectively, and the desired **1d** and **1f** were obtained by deprotection of the protecting groups. Likewise, compounds **1e** and **1g** were prepared starting from alcohols **2d**, (*S*)-*tert*-butyl 3-hydroxypyrrolidine-1-carboxylate, and **2e**, (2*S*,4*R*)-1-(*tert*-butoxycarbonyl)-4-hydroxypyrrolidine-2-carboxamide, correspondingly. The N-methyl-pyrrolidine analogues **1h** and **1i** were accessible from alcohol **2f**, (2*S*,4*R*)-methyl 4-hydroxy-1-methylpyrrolidine-2-carboxylate, and **2g**, (*R*)-1-methylpyrrolidin-3-ol in the order mentioned. Esters **1j** and **1k** were available from intermediates **5a** and **5h**, respectively, by treatment with TFA/H_2O (Scheme 1).

The synthesis of analogues **7a–d** (Scheme 2) with N-6-modifications began from intermediates **5a**, **5b**, **5d**, and **5f** which were



Scheme 1. Reagents and conditions: (a) (i) MsCl, pyridine; (ii) KSAc, DMF, 80 °C, 2 h; (b) (i) NaOMe, MeOH, 15 min rt; (ii) intermediate from **3a** and **4a** to yield **5a**; **3a** and **4b** to yield **5b**; **3b** and **4a** to yield **5c**; **3c** and **4a** to yield **5d**; **3d** and **4a** to yield **5e**; **3c** and **4b** to yield **5f**; **3e** and **4a** to yield **5g**; **3f** and **4b** to yield **5h**; and **3g** and **4a** to yield **5i**; (c) KOH, H_2O, THF; (d) TFA, H_2O, rt.

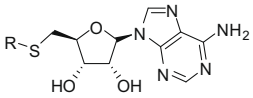


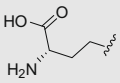
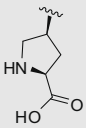
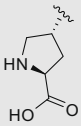
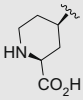
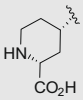
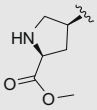
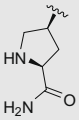
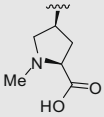
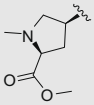
Scheme 2. Reagents and conditions: (a) Intermediates **5a**, **5b**, **5d**, or **5f** and azine dihydrochloride, pyridine, reflux, 16 h to give **6a**, **6b**, **6c** and **6d**, respectively; (b) 2-(biphenyl-4-yl)ethanamine, DMF, rt, 48 h; (c) (i) KOH, H_2O, THF; (d) TFA, H_2O, rt.

converted to triazoles **6a**, **6b**, **6c**, and **6d** utilizing azine dihydrochloride. Displacement of the triazole group with 2-(biphenyl-4-yl)ethanamine followed by removal of the protecting groups gave the desired products **7a–d**.[13f]

All other analogues were prepared in a manner similar to the above compounds starting form the suitable alcohol.[14a] The synthesized compounds, **1a–k**, **7a–d**, **8a–c**, **9a–b**, **10a–b**, **11** and **12,** have the same configuration at the ribose moiety as that of SAH.[14b] These analogues were tested against recombinant human enzymes DNMT1 and DNMT3b2 and their activity is presented in Table 1.[15] For constrained SAH analogues incorporating cyclic amines bearing a carboxylic acid group in place of the homocysteine unit, the stereochemistry at the point of attachment to the sulfur atom and that at the alpha-amino acid carbon played a role in the observed activity. Of the five and six membered amino acid analogues (**1a**, **1c**, **8a**, **8b**, and **8c**) both **1a** and **8a** had reasonable potency against both enzymes. Remarkably, **1a** was almost equipotent to SAH itself retaining the same level of activity against both enzymes (Table 1). Compound **1c**, the epimer of **1a**, was devoid of activity against DNMT1 and was 270-fold less active against DNMT3b2 indicating that the inversion of stereochemistry at this center places either the amine or the carboxylic acid groups in unfavorable position for interaction with the enzyme. The difference in activity between

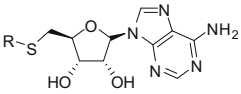
Table 1
DNMT1 and DNMT3b2 enzymatic activity of constrained SAH analogues[a]

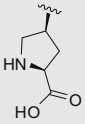
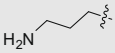


Compound	R	DNMT1 IC_{50} (μM)	DNMT3b2 IC_{50} (μM)
L-SAH		0.8	0.2
1a		1.1	0.3
1c		>100	54.0
8a		8.0	1.5
8b		>100	39
8c		>100	>100
1j[b]		4.0	50%
1g[b]		1%	42%
1h		>100	11.0
1k[b]		4.5	19%

[a] Values are means of at least two experiments, ±20%.
[b] Percent inhibition at 45 μM inhibitor concentration.

Table 2
DNMT1 and DNMT3b2 enzymatic activity of cyclic amines[a]



Compound	R	DNMT1 IC_{50} (μM)	DNMT3b2 IC_{50} (μM)
1a		1.1	0.3
1d		4.8	12.0
1e		44.0	26.0
1i[b]		>10	32%
9a[b]		18%	51%
9b[b]		0%	16%
10a		19.0	100
10b		31.0	21.0
11		94.0	66.0
12		44	>100
13		>45	17.0
14		18.0	41.0

[a] Values are means of at least two experiments, ±20%.
[b] Percent inhibition at 45 μM inhibitor concentration.

1a and **8a** implies that the distance as well as the configuration plays a role in the observed activity of these inhibitors.[16a] Compound **8c**, the epimer of **8a** at the α-carbon was not active against both enzymes. This is in agreement with the structure of SAH where only L-SAH is an inhibitor of DNMT enzymes.[16b]

The methyl ester of our lead **1a**, compound **1j**, maintained moderate activity against DNMT1, while amide **1g** lost activity completely against DNMT1 and retained slight activity towards DNMT3b2 (Table 1). Methylation of the amines of **1a** and **1j**, compounds **1h** and **1k**, respectively, abrogated DNMT1 activity indicating that the NH of **1a** is engaged in important hydrogen-bonding interactions in the active site of both enzymes.

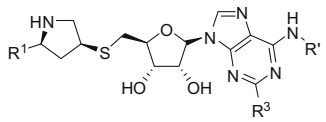
Deletion of the carboxylic acid group of **1a**, compound **1d**, resulted in a fivefold and 60-fold loss of enzymatic activity against DNMT1 and DNMT3b2, respectively (Table 2). The activity of the constrained amine **1d** against DNMT1 is superior to that of the linear amines **13** and **14**.[17] Encouraged by such observation we prepared other cyclic amines, **1e**, **1i**, **9a** and **9b**, **10a** and **10b**, **11** and **12**, and tested them in our assays (Table 2). Unfortunately, all were less active than **1d** against both DNMT enzymes. The inferior enzymatic activity of **1e** versus **1d** parallels that of **1c** versus. **1a** implicating that the (*S*)-configuration is superior to the (*R*). Generally, analogues with a pyrrolidine ring, compounds **1d**, **1e**, **10a**, **10b** and **9**, were more active than analogues with other rings such as azetidine, compound **12**, piperidine, compounds **9a** and **9b**, or 2-amino-cyclopropyl, compound **11** (Table 2).

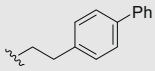
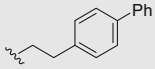
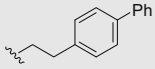
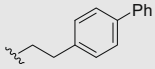
In a previous Letter, we have reported that analogues of SAH bearing a chlorine atom in the 2-position of the purine ring are more selective towards DNMT1.[18] To test whether the same applies to the constrained analogues of SAH, we prepared compounds **1b** and **1f** bearing a chlorine atom at the 2-position of the purine ring. From Table 3, it could be seen that compound **1b** retained all activity against DNMT1, but was sevenfold less active against DNMT3b2 enzyme as compared to **1a**. However, the 2-chloro substitution of **1d**, compound **1f**, resulted in a pronounced reduction of activity against both DNMT1 and DNMT3b2 enzymes.

In addition, we reported that lipophilic groups at the N^6 position of the purine ring of SAH are tolerated.[18] N^6 substitution of the constrained analogue **1a**, compound **7a**, produced an equipotent inhibitor against DNMT3b2 and a threefold reduction of activity against DNMT1 (Table 3). On the other hand, N^6 substitution of **1d,** compound **7c**, reduced the activity against both enzymes. Concomitant substitution of a 2-chloro and N^6 2-(biphenyl-4-yl)ethanamine groups on **1a** (compound **7b**) and **1d** (compound **7d**) was not beneficial for enzymatic activity. Compound **7b** was slightly more potent against DNMT1, and 3.5-fold less active against DNMT3b2, as compared to **1a**, while **7d** retained activity against DNMT1 and caused almost twofold reduction of activity against DNMT3b2 enzyme as compared to **1d**.

In conclusion, we have prepared inhibitors of DNMT1 and DNMT3b2 with constrained homocysteine units that are equipotent to SAH. The five membered (2*S*,4*S*)-4-mercaptopyrrolidine-2-carboxylic acid, in **1a**, was a good replacement for homocysteine, while the corresponding six-membered counterpart was less active. Deletion of the carboxylic group, compound **1d**, eliminating the Zwitter-ionic character was less active against both enzymes. Further optimization of **1a** failed to give more potent inhibitors. 2-Chloro substitution of **1a**, compound **1b**, retained potency against DNMT1, while N^6-substitution, compound **7a**, retained DNMT3b2 activity. The concomitant substitutions of **1a** at both 2- and N^6 positions lead to a threefold decrease of activity against both enzymes.

Table 3
DNMT1 and DNMT3b2 enzymatic activity of 2- and N-6-substituted analogues of **1a** and **1d**[a]



Compound	R′	R^1	R^3	DNMT1 IC_{50} (μM)	DNMT3b2 IC_{50} (μM)
1a	H	CO_2H	H	1.1	0.27
1b	H	CO_2H	Cl	0.82	1.9
1d	H	H	H	4.8	12.0
1f	H	H	Cl	36.0	>45
7a[b]		CO_2H	H	3.7	0.26
7b		CO_2H	Cl	2.5	0.92
7c		H	H	15.0	33.0
7d		H	Cl	5.0	27.0

[a] Values are means of at least two experiments, ±20%.
[b] N-6-Substituted SAH (2-amino-4-(((2*S*,3*S*,4*R*,5*R*)-5-(6-(2-(biphenyl-4-yl)ethylamino)-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methylthio)butanoic acid) IC_{50} DNMT1 = 5.4 μM; DNMT3b2 = 0.6 μM.[17]

References and notes

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14. (a) The piperidine type analogues, compounds **8a**, **8b** and **8c** (Table 1) were prepared in a manner similar to that for **1a**, starting from alcohols (2*S*,4*R*)-methyl 1-(*tert*-butoxycarbonyl)-4-hydroxypiperidine-2-carboxylate, (2*S*,4*S*)-methyl 1-(*tert*-butoxycarbonyl)-4-hydroxypiperidine-2-carboxylate, and (2*R*,4*R*)-methyl 1-(*tert*-butoxycarbonyl)-4-hydroxypiperidine-2-carboxylate, respectively, while compounds **9a** and **9b** were easily reached from alcohols (*R*)-*tert*-butyl 3-hydroxypiperidine-1-carboxylate and *tert*-butyl 4-hydroxypiperidine-1-carboxylate, respectively. The 2-methylpyrrolidine analogues **10a** and **10b** were made starting from (*R*)-*tert*-butyl 2-(hydroxymethyl)pyrrolidine-1-carboxylate and (*S*)-*tert*-butyl 2-(hydroxymethyl)pyrrolidine-1-carboxylate in the order mentioned. The 1,2-cyclopentyl analogue **11** was prepared from *tert*-butyl (1*S*,2*S*)-2-hydroxycyclopentylcarbamate and the azetidine analogue **12**, was prepared from *tert*-butyl 3-hydroxyazetidine-1-carboxylate following the same chemistry described for **1a**.; (b) All compounds were >95% pure and were routinely characterized by ^{1}H NMR and in some instances by ^{13}C NMR and NOE. Characterization of **1b**: ^{1}H NMR (D_2O) δ (ppm) 7.88 (s, 1H), 5.61 (d, 1H, *J* = 4.5 Hz, 1H), 4.46 (dd, *J* = 4.5 Hz, *J* = 5 Hz, 1H), 4.14 (dd, *J* = 5 Hz, *J* = 5.3 Hz, 1H), 4.05 (m, 2H), 3.45 (m, 2H), 3.16 (m, 1H), 2.88 (dd, *J* = 14 Hz, *J* = 5.1 Hz, 1H), 2.78 (dd, *J* = 14 Hz, *J* = 6.1 Hz, 1H), 2.57 (m, 1H). MS: calcd 430.08 (100%), 432.08 (37%); found 431.3 (100%), 433.3 (17%) (MH^+); **1f**: ^{1}H NMR (D_2O) δ (ppm) 8.26 (br, 1H), 8.08 (s, 1H), 5.8 (d, 1H, *J* = 4.7 Hz, 1H), 4.7 (dd, *J* = 4.7 Hz, *J* = 5.1 Hz, 1H), 4.27 (dd, *J* = 5.1Hz, *J* = 5.1 Hz, 1H), 4.16 (m, 1H), 3.48 (m, 1H), 3.33 (m, 1H), 3.33 (m, 2H), 3.21 (m, 1H), 3.02 (dd, *J* = 4.9 Hz, *J* = 12.1 Hz, 1H), 2.26 (dd, *J* = 4.5 Hz, *J* = 14.1 Hz, 1H), 2.89 (dd, *J* = 7 Hz, *J* = 14.1 Hz, 1H), 2.20 (m, 1H), 1.82 (m, 1H). MS: calcd 386.09 (100%), 388.09 (37%); found 387.2 (100%), 389.1 (40%) (MH^+); **7a**: ^{1}H NMR (DMSO-d_6) δ 8.26 (s, 1H), 8.18 (s, 2H), 7.86 (br s, 1H), 7.54 (m, 4H), 7.35 (m, 2H), 7.24 (m, 3H), 5.82 (d, 1H, *J* = 5.5 Hz), 4.64 (m, 1H), 4.06 (m, 1H), 3.94 (m, 1H), 3.65 (m, 3H), (3H assumed under H_2O at 4.87), 2.8–3.0 (m, 5H), 1.68 (m, 1H). MS: calcd 576; found 577 (MH^+); **7b:** ^{1}H NMR (DMSO-d_6) δ 8.49 (s, 1H), 7.6–7.7 (m, 4H), 7.1–7.5 (5H), 5.85 (d, 1H, 5.9 Hz), 4.66 (m, 1H), 4.13 (m, 1H), 4.04 (m, 1H), 3.3–3.8 (m, 7H), 2.98 (m, 4H), 1.76 (m, 1H). MS: calcd 610; found 611 (MH^+).
15. *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 10X 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.
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