Targeted labeling of DNA by methyltransferase-directed Transfer of Activated Groups (mTAG)

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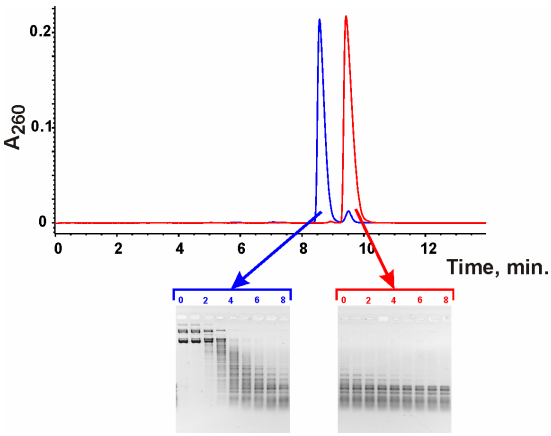
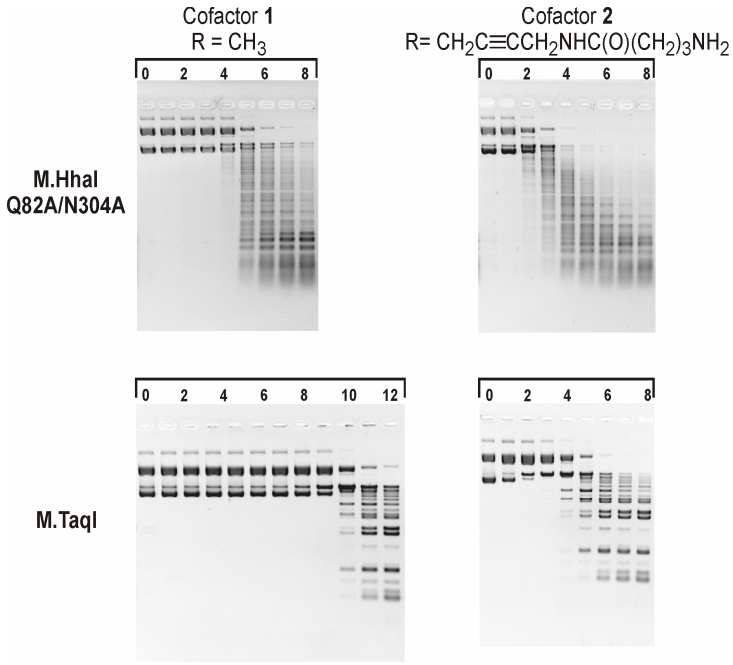
Supporting Figures and Table



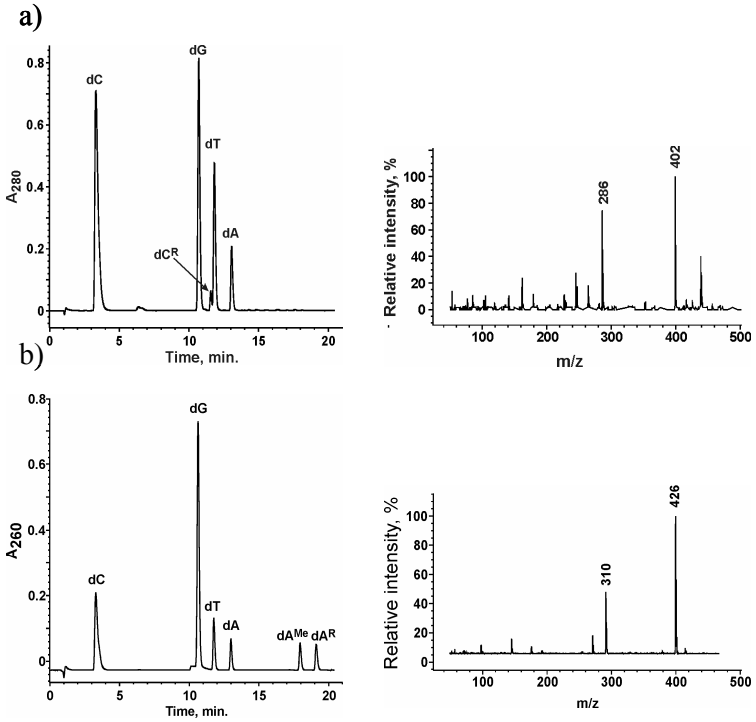
Supporting Figure 1. **Chromatographic and enzymatic analysis of sulfonium epimers of the synthetic AdoMet analog 2.**

Top: Reversed-phase HPLC analysis of the two sulfonium epimer fractions, blue – *S*-epimer and red – *R*-epimer (assignments are based on enzymatic activities), under isocratic conditions (20 mM ammonium formate, pH 3.5). Bottom: DNA protection analysis of isolated fractions. pBR322 plasmid DNA and AdoMet analogs were incubated with different amounts (twofold serial dilutions from lanes 0 to 8 starting with equimolar amounts of DNA MTase and target sites in lane 0) of the HhaI MTase (variant Q82A/N304A) for 1 h, treated with a methylation sensitive restriction endonuclease, R.Hin6I, and the degree of protection was analyzed by agarose gel electrophoresis.



Supporting Figure 2. **Analysis of enzymatic transalkylation reactions.**

DNA protection assay for DNA MTase-directed transfer of different chemical groups R from the natural cofactor **1** or its analog **2** to pBR322 plasmid DNA. DNA and cofactors **1** or **2** were incubated with different amounts of DNA MTases (twofold serial dilutions starting with equimolar amounts of DNA MTase and target sites in lane 0) for 1 h, treated with a methylation sensitive restriction endonuclease (R.Hin6I for M.HhaI or R.TaqI for M.TaqI) and the degree of protection was analyzed by agarose gel electrophoresis. Enzymatic turnover numbers can be estimated based on the minimal molar ratio of DNA MTase to its target sites that is required for complete protection of DNA in 1 h (defined as lanes N). The molecular ratio of DNA MTase to target sites in lane N is $1/2^N$ and at this molecular ratio the DNA MTase went at least through 2^N enzymatic turnovers: 16 h^{-1} for M.HhaI (variant Q82A/N304A) with AdoMet **1** and 2 h^{-1} with AdoMet analog **2**, 512 h^{-1} for M.TaqI with AdoMet **1** and 8 h^{-1} with AdoMet analog **2**.



Supporting Figure 3. **Analysis of the transalkylation product formed in DNA.**

(a) Left: RP-HPLC analysis of enzymatically fragmented duplex oligodeoxynucleotide III-IV (see Supporting Methods below) obtained after modification with the cytosine-specific MTase HhaI in the presence of cofactor analog **2** (R = $CH_2C{\equiv}CCH_2NHC(O)(CH_2)_3NH_2$). Right: ESI-MS analysis of the modified nucleoside dC^R. The observed molecular masses match the expected masses for the modified 2'-deoxycytidine (402 $[M + Na]^+$) and modified cytosine (286 $[M + Na]^+$). (b) Left: RP-HPLC analysis of enzymatically fragmented duplex oligodeoxynucleotide I-II obtained after modification with the adenine-specific MTase TaqI in the presence of analog **2**. Right: ESI-MS analysis of the modified nucleoside dA^R. The observed molecular masses match the expected masses for the modified 2'-deoxyadenosine (426 $[M + Na]^+$) and modified adenine (310 $[M + Na]^+$).

***Supporting Table*.** **Fluorescence intensity distribution in GsuI fragments of MTase-labeled pBR322 DNA**

Fragment (size, bp)	HhaI methyltransferase			TaqI methyltransferase		
	Calculated		Observed	Calculated		Observed
	Number of target sites	% of total	Peak area, % of total	Number of target sites	% of total	Peak area, % of total
F1 (1757)	14	45.2	42.7	4	57.1	59.7
F2 (1434)	10	32.3	31.4	1	14.3	12.6
F3 (616)	4	12.9	14.4	0	0.0	-
F4 (554)	3	9.7	11.5	2	28.6	27.7

Supporting Methods

General

Recombinant DNA MTases M.TaqI [1] and M.HhaI [2] were produced as previously described. M.BseCI was kindly provided by Prof. M. Kokkinidis, University of Crete, Greece. The Q82A/N304A variant of M.HhaI was constructed by subcloning the Eco91I-HindIII fragment carrying the N304A mutation into the pHH5.3 plasmid carrying the Q82A mutation [3,4]; the mutations were verified by complete sequencing of the gene and ESI-MS analysis of the purified protein. Short DNA duplexes were produced by annealing commercial HPLC-purified oligodeoxyribonucleotides (5′…3′) GCCGCTCGATGCCG (I), CGGCATCGA^{Me}GCGGC (II, A^{Me} = N6-methyl-2′-deoxyadenosine) CATTACGCGCCGGG TCCTGGCTAT (III), ATAGCCAGGACCCGGCGCGTAATG (IV) as described [4]. S-Adenosyl-L-homocysteine (**6**), anion exchanger Dowex-1 and Nuclease P1 were purchased from Sigma-Aldrich, and biotinamidohexanoic acid N-hydroxysuccinimide ester (biotin-NHS), 2-butyne-1,4-diol, hydrazine hydrate, triethylamine, carbonyldiimidazole (CDI), 4-nitrobenzenesulfonyl chloride, trifluoroacetic acid, chloroform, was from Fluka. Acetic acid, formic acid, sodium hydroxide, methanol, dichloromethane, Roti®-Phenol (pH 8.0), Roti®-Phenol/C/I were from ROTH, and cyanine-5 N-hydroxysuccinimide ester (Cy5-NHS) from Amersham. Dynabeads® M-280 Streptavidin-coated magnetic beads were obtained from Invitrogen, G-25 columns from GE Healthcare and Microcon YM-3 spin columns from Amicon. All other enzymes were obtained from Fermentas Life Sciences. ESI-MS spectra were acquired on a LC/MS spectrometer HP 1100 series MSD single quadruple detector in positive ion mode. ^{1}H NMR spectra were recorded on a Varian Innova 300 nuclear magnetic resonance spectrometer using the HDO signal (δ = 4.78) or TMS (δ = 0.00) as internal reference.

Synthesis of AdoMet analog 2

4-Aminobut-2-yn-1-ol hydrochloride (3)

A methanolic solution (150 mL) of 4-phthalimidobut-2-yn-1-ol (7.66 g, 35.6 mmol, prepared from 2-butyn-1.4-diol according to reference 5) was treated with hydrazine hydrate (3.46 mL, 69.8 mmol). The reaction mixture was heated at reflux for 2 h and after cooling to room temperature the solvent was removed

under reduced pressure. Water and ethanol (100 mL, 1:1 mixture) and conc. hydrochloric acid (100 mL) were added to the residue. The mixture was heated at reflux for 20 min and the precipitate removed by filtration. The filtrate was concentrated under reduced pressure and the resulting residue crystallized from methanol to yield compound **3** (3.57 g. 82%) as a white solid. ^{1}H-NMR (300 MHz, D_2O): δ = 3.77 (t, ^{5}J = 2.0 Hz, 2H, CH_2), 4.18 (t, ^{5}J = 2.0, 2H, CH_2); ^{13}C-NMR (75 MHz, D_2O): δ = 32.09, 52.23, 79.15, 87.96.

4-[(*tert*.-Butoxycarbonylamino)butanamido]but-2-yn-1-ol (4)

4-[(*tert*.-butoxycarbonyl)amino]butanoic acid (5.20 g, 25.6 mmol, prepared in analogy to reference 6) was dissolved in anhydrous tetrahydrofuran (20 mL), carbonyldiimidazole (CDI) (4.56 g, 28.1 mmol) was added, and the resulting clear solution was stirred at room temperature for 2 h. Then, the primary amine **3** (3.11 g 25.6 mmol) and trietylamine (7.10 mL, 50.9 mmol) were added and stirring was continued at room temperature for 2 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (silica gel, 40 g, chloroform/ethylacetate 1:1) to give compound **4** (3.59 g, 52%) as a light yellow oil (R_f 0.3, chloroform/ethyl acetate 1:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.41 (s, 9H, CH_3), 1.78 (quint, ^{3}J = 6.8 Hz, 2H, CH_2), 2.22 (t, ^{3}J = 7.1 Hz, 2H, CH_2), 3.13 (q, ^{3}J = 6.4 Hz, 2H, CH_2), 4.03-4.09 (m, 2H, CH_2), 4.09-4.14 (m, 2H, CH_2), 4.84 (br. s, 1H, NH), 6.67 (br. s, 1H, NH); ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 26.38, 28.64, 29.62, 33.52, 40.05, 50.74, 79.63, 81.22, 81.86, 153.87, 171.53.

4-[4-(*tert*.-Butoxycarbonylamino)butanamido]but-2-ynyl-4-nitrobenzenesulfonate (5)

4-Nitrobenzenesulfonyl chloride (0.90 g, 4.07 mmol) and sodium hydroxide (0.74 g, 18.5 mmol) were added to a solution of alcohol **5** (1.0 g, 3.7 mmol) in methylene chloride (15 mL) at 0°C following a general literature procedure [5]. After stirring the reaction mixture for 3 h at room temperature the reaction was quenched with 20 mL of cold water, extracted with methylene chloride (3 × 10 mL) and the combined organic layers dried over sodium sulfate. The sample was passed through a glass filter and concentrated under reduced pressure to yield compound **5** (0.93 g, 55%) as a slightly yellow solid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.43 (s, 9H, CH_3), 1.76 (quint, ^{3}J = 6.6 Hz, 2H, CH_2), 2.19 (t, ^{3}J = 7.1 Hz, 2H, CH_2), 3.13 (q, ^{3}J = 6.4 Hz, 2H, CH_2), 3.92 (dt, ^{5}J = 1.8 Hz, ^{3}J = 5.3 Hz, 2H, CH_2), 4.74 (br. s, 1H, NH), 4.83 (t, ^{5}J = 1.8 Hz, 2H, CH_2), 6.61 (br. s, 1H, NH), 8.09-8.17 (m, 2H, arom. H), 8.38-8.45 (m, 2H, arom. H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 26.96, 28.64, 29.26, 33.37, 39.66, 59.33, 74.19, 79.89, 87.08, 124.69, 129.74, 142.36, 151.20, 162.76, 172.64.

5'-[(*S*)-[(*3S*)-3-Amino-3-carboxypropyl]-4-[4-aminobutanamido]but-2-ynylsulfonio]-5'-deoxyadenosine (2)

S-Adenosyl-L-homocysteine (**6**) (20 mg, 52 µmol) was dissolved in a 1:1 mixture of formic acid and acetic acid (0.4 mL) and nosylate **5** (16 equivalents) was added at 0°C. The solution was allowed to warm up at room temperature and incubated at room temperature with shaking for 8 h. The reaction was quenched by adding water (4 mL) and the aqueous phase was extracted with diethyl ether (3 × 4 mL). The aqueous phase was concentrated under reduced pressure and redissolved in ammonium formate buffer (10 mL, 20 mM, pH 3.5). The sample was passed through a Dowex 1 column (pre-equilibrated with 20 mM ammonium formate, pH 3.5) to remove 4-nitrobenzensulfonic acid formed during the reaction. Boc-deprotection was achieved by treatment with trifluoroacetic acid (final concentration 66%) at room temperature for 1 h [6]. The sample was concentrated under reduced pressure and redissolved in ammonium formate (20 mM, pH 3.5). Separation of epimers was performed by preparative reversed-phase HPLC (Discovery C18, 150 x 25 mm, 5 µm, Supelco). Compounds were eluted with ammonium formate buffer (20 mM, pH 3.5) at a flow rate of 4.5 mL/min at 30ºC under isocratic conditions. The purified product **2** (1.74 µmol, 3%) was quantified by UV-spectroscopy using an extinction coefficient of 15400 L mol^{-1} cm^{-1} at 260 nm for the adenine chromophore. ^{1}H-NMR (300 MHz, D_2O): δ = 1.93 (quint, ^{3}J = 7.6 Hz, H7''), 2.29-2.44 (m, 4H, H8'', Hβ), 3.01 (t, ^{3}J = 7.7 Hz, 2H, H6''), 3.45-3.75 (m, 2H, Hγ), 3.83 (t, ^{3}J = 6.3 Hz, 1H, Hα), 4.00 (d, ^{3}J = 5.9 Hz, 2H, H5'), 4.05 (br. s, 2H, H4''), 4.41 (br. s, 2H, H1''), 4.53 (q, ^{3}J = 5.9 Hz, 1H, H4'), 4.65 (t, ^{3}J = 5.7 Hz, 1H, H3'), 4.93 (dd, ^{3}J = 3.8, 5.3 Hz, 1H, H2'), 5.09 (t, ^{3}J = 1.7 Hz, 1H, NH), 6.11 (d, ^{3}J = 4.2 Hz, 1H, H1'), 8.30-8.35 (m, 2H, arom. H); ESI-MS m/z (relative intensity): 537.3 (25) $[M]^+$, 458.1 (33) [5'-(4-(4-aminobutanamido)but-2-ynyl)thio-5'-deoxyadenosine + Na]$^+$, 436.2 (100) [5'-(4-(4-aminobutanamido)but-2-ynyl)thio-5'-deoxyadenosine + H]$^+$.

Enzymatic procedures

MTase-assisted modification of plasmid DNA, DNA protection assays and composition analysis of transalkylated DNA were performed as previously described [4,7]. M.BseCI-methylated pBR322 was obtained by incubating two identical samples of pBR322 (40 µg) in buffer (2 mL, 10 mM Tris-HCl pH 7.4, 50 mM sodium chloride, 20 mM magnesium chloride, 50 µM EDTA, 2 mM β-mercaptoethanol) containing AdoMet (80 µM) and M.BseCI (78 ng) at 55°C for 2 h followed by heat inactivation at 70°C for 10 min. Completeness of methylation was verified by the absence of fragmentation with R.ClaI. Both samples were concentrated to approximately 200 µL by lyophilization, combined and filled up with water to 500 µL. The sample was applied on a NAP 5 column (equilibrated with water), and eluted with water (1 mL) yielding 60 µg of M.BseCI-methylated pBR322.

MTase-directed amino-modification of short duplex DNA with cofactor 2

Analyses were performed essentially as described previously [4,7]. For the reaction catalyzed by M.TaqI, duplex oligodeoxynucleotide I-II (10 µM), M.TaqI (12.5 µM) and AdoMet analog **2** (300 µM) in M.TaqI buffer were incubated at 37°C for 7 h. Enzymatic modification with M.HhaI (Q82A/N304A variant) was performed by incubation of the duplex oligodeoxynucleotide III-IV (10 µM) with AdoMet analog **2** (300 µM) and M.HhaI-Q82A/N304A (12.5 µM) in M.HhaI buffer at 37°C for 7 h. Samples were then mixed with Proteinase K (0.1% SDS and 0.18 mg/mL final concentration) and the solutions incubated at 55°C for 1 h followed by 80°C for 10 min. The modified duplexes were desalted by gel filtration using G-25 columns. Nuclease PI buffer (10 mM Tris-HCl, 10 mM magnesium chloride, 1 mM zinc acetate, pH 7.5) containing Nuclease PI (1.5 u) and calf intestine alkaline phosphatase (30 u) was added and the samples were incubated at 42°C for 4 h. For nucleoside analysis by reversed-phase HPLC-coupled ESI-MS samples were passed through a Microcon YM-3 spin column, loaded onto a reversed-phase HPLC column (Discovery HS C18, 3 µm, 75 × 2.1 mm, Supelco) and eluted with methanol (0% for 3 min, followed by linear gradients to 20% in 15 min and to 80% in 2 min) in ammonium formate buffer (20 mM, pH 3.5) at a flow of 0.3 mL/min and at 30°C. Post-column equal co-flow of 96% methanol, 4% formic acid and 1 mM sodium formate was used for the MS detection of modified nucleosides and its derivatives in the 50–500 m/z range (see Supporting Figure 3).

MTase-directed amino-modification of plasmid DNA with cofactor 2

Reaction solutions containing equimolar amounts of DNA MTases and plasmid pBR322 DNA (M.TaqI: 0.5 µM; M.HhaI variant Q82A/N304A: 2.5 µM) in M.TaqI or M.HhaI buffer and cofactor **2** (300 µM) were incubated at 60°C (M.TaqI) or 37°C (M.HhaI variant Q82A/N304A) for 1 h. Afterwards samples were diluted twice with water and extracted once with Roti®-

Phenol (pH 8.0), two times with Roti®-Phenol/C/I and three times with chloroform. Aqueous phases were separated and DNA was precipitated by adding isopropanol (0.9 volume) and sodium acetate (0.1 volume, 3 M, pH 7.0). Pellets were washed once with ice cold ethanol (75%) and dried. Modified DNA was dissolved in water to final concentration 0.4–0.5 μg/μL as determined from ethidium stained agarose gels (0.8%).

Labeling and analysis of MTase-modified plasmid DNA

pBR322 DNA (7 μg) amino-modified with M.HhaI (variant Q82A/N304A) or with M.TaqI in the presence of AdoMet analog **2** were treated with a Cy5-NHS (125 μM) in buffer (30 μL, 0.15 M sodium bicarbonate, pH 9.0) at room temperature for 1 h. Approximately 1 μg of labeled DNA was fragmented with R.GsuI (according to manufacturer's recommendations) and analyzed by agarose gel (1.5%) electrophoresis. Gels were scanned with a Fuji FLA-5100 imaging system using a 635 nm laser and LPR filter set for Cy5 emission. Densitometry of the label distribution in pBR322-GsuI fragments was performed using OptiQuant (v. 3) software.

Biotin attachment reactions were carried out with M.TaqI amino-modified pBR322 or M.BseCI premethylated pBR322 under the same conditions except that biotin-NHS was used at a final concentration of 7 mM. Approximately 0.75 μg of labeled pBR322 DNA was then fragmented with the R.FspAI and R.MbiI (according to manufacturer's recommendations, reaction mix volume 30 μL). One half of a sample (15 μL) was treated with Dynabeads® M-280 Streptavidin-coated magnetic beads (200 μg, suspended in 5 μL restriction enzyme buffer 66 mM Tris-acetate, pH 7.9, 20 mM magnesium acetate, 132 mM potassium acetate and 0.2 mg/mL BSA) and incubated with shaking at 43°C for 2 h. Beads were removed with a magnet, washed twice with restriction enzyme buffer (30 μL) and resuspended in restriction enzyme buffer (15 μL). DNA fragments were recovered by extracting with an equal volume of Roti®-Phenol/C/I solution. Finally, samples obtained after REase fragmentation, after treatment with streptavidin-coated beads and after elution from the beads were analyzed by agarose gel (1.2%) electrophoresis.

Supporting References

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