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Design of a New Fluorescent Cofactor for DNA Methyltransferases and Sequence-Specific Labeling of DNA

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Abstract: Sequence-specific labeling of DNA is of immense interest for analytical and functional studies of DNA. We present a novel approach for sequence-specific labeling of DNA using a newly designed fluorescent cofactor for the DNA methyltransferase from *Thermus aquaticus* (M·*Taq*I). Naturally, M·*Taq*I catalyzes the nucleophilic attack of the exocyclic amino group of adenine within the double-stranded 5′-TCG**A**-3′ DNA sequence onto the methyl group of the cofactor *S*-adenosyl-L-methionine (AdoMet) leading to methyl group transfer. The design of a new fluorescent cofactor for covalent labeling of DNA was based on three criteria: (1) Replacement of the methionine side chain of the natural cofactor AdoMet by an aziridinyl residue leads to M·*Taq*I-catalyzed nucleophilic ring opening and coupling of the whole nucleoside to DNA. (2) The adenosyl moiety is the molecular anchor for cofactor binding. (3) Attachment of a fluorophore via a flexible linker to the 8-position of the adenosyl moiety does not block cofactor binding. According to these criteria the new fluorescent cofactor 8-amino[1′′-(*N*′′-dansyl)-4′′-aminobutyl]-5′-(1-aziridinyl)-5′-deoxyadenosine (**3**) was synthesized. **3** binds about 4-fold better than the natural cofactor AdoMet to M·*Taq*I and is coupled with a short duplex oligodeoxynucleotide by M·*Taq*I. The identity of the expected modified nucleoside was verified by electrospray ionization mass spectrometry after enzymatic fragmentation of the product duplex. In addition, the new cofactor **3** was used to sequence-specifically label plasmid DNA in a M·*Taq*I-catalyzed reaction.

Introduction

Sequence-specific labeling of native DNA still represents a more or less unsolved problem. Difficulties mainly arise from the necessity to combine two different functions: sequence-specific recognition of DNA and covalent bond formation between the label and DNA. Several systems, like triple helix-forming oligodeoxynucleotides,[2] polyamide nucleic acids,[3] pyrrole−imidazole−hydroxypyrrole polyamides[4] or zinc finger proteins,[5] are well suited to target many DNA sequences. However, these systems generally lack a catalytic function for covalent labeling. Labeling of DNA is often carried out using enzymatic or chemical methods.[6] Labeled deoxynucleoside triphosphates can be incorporated into DNA by DNA polymerases (nick translation, random-primed labeling, PCR[1]) or terminal deoxynucleotidyl transferases. Alternatively, chemical techniques can be used, e.g. photochemical coupling of aromatic azides with nucleobases (e.g., photobiotin) or bisulfite-catalyzed exchange of the cytosine amino group against primary amines. In addition, labeled oligodeoxynucleotides can be produced by solid-phase DNA synthesis and incorporated at the ends of DNA fragments by PCR. However, these enzymatic and chemical methods are usually not suitable for sequence-specific internal labeling of large DNA.

In contrast to these systems and methods mentioned above, DNA methyltransferases (MTases) are capable of recognizing specific sequences within DNA and catalyzing covalent bond formations. They naturally transfer the activated methyl group from the cofactor *S*-adenosyl-L-methionine (**1**, AdoMet) to the exocyclic amino group of adenine and cytosine or the 5-position of cytosine within their individual recognition sequences (Scheme 1, left).[7] Unfortunately, the methyl group itself is a very limited reporter group and only suitable for radioactive labeling with tritium or carbon-14. We recently designed the new cofactor *N*-adenosylaziridine (**2**) which is quantitatively, base- and sequence-specifically coupled with the double-

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(1) The following abbreviations are used: PCR, polymerase chain reaction; MTase, methyltransferase; AdoMet, *S*-adenosyl-L-methionine; M·*Taq*I, DNA methyltransferase from *Thermus aquaticus*; EDIA, *N*-ethyldiisopropylamine; M·*Hha*I, DNA methyltransferase from *Haemophilus haemolyticus*; M·*Pvu*II, DNA methyltransferase from *Proteus vulgaris*; DpnM, DNA methyltransferase from *Streptococcus pneumoniae*; M·*Rsr*I, DNA methyltransferase from *Rhodopseudomonas sphaeroides*; M·*Mbo*II, DNA methyltransferase from *Moraxella bovis*; ESI-MS, electrospray ionization mass spectrum/spectrometry; TLC, thin-layer chromatography.

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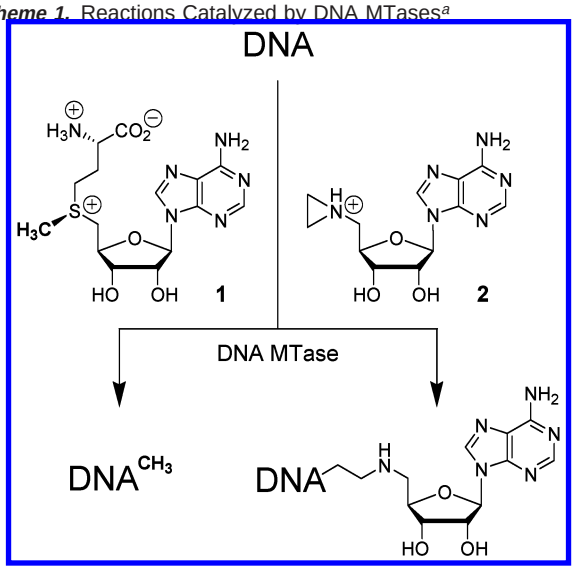
Scheme 1. Reactions Catalyzed by DNA MTases[a]



[a] DNA MTases activate nitrogen 6 of adenine, nitrogen 4 or carbon 5 of cytosine within their recognition sequences for nucleophilic attack onto the methyl group of AdoMet (**1**) resulting in methyl-group transfer (left) or for nucleophilic ring opening of the protonated aziridine group of *N*-adenosylaziridine (**2**) leading to sequence-specific coupling of the whole cofactor with DNA (right).

stranded 5′-TCG**A**-3′ DNA sequence by the adenine-specific DNA MTase from *Thermus aquaticus* (M·*Taq*I) (Scheme 1, right).[8] We have extended this work and now report on the design and synthesis of a fluorescent derivative of *N*-adenosylaziridine (**2**) which is used in combination with M·*Taq*I to sequence-specifically deliver a fluorophore to DNA.

Results and Discussion

Design and Synthesis of a New Fluorescent Cofactor for M·*Taq*I. We planned to use the cofactor **2**, which already contains the reactive aziridine group for DNA MTase-catalyzed coupling and the adenosyl moiety for cofactor binding,[9] as a delivery system for a fluorescent reporter group. The most critical part for the design of a new fluorescent cofactor appeared to be the choice of a suitable attachment position where the fluorophore attached via a flexible linker would not sterically interfere with binding to the enzyme. The three-dimensional structures of M·*Taq*I in complex with the natural cofactor AdoMet (**1**),[10,11] as well as with DNA and a cofactor analogue,[12] were determined by X-ray analysis. Figure 1 shows the cofactor binding site of M·*Taq*I with the extrahelical target adenine residue and *N*-adenosylaziridine (**2**) which was modeled in by replacing the cofactor analogue from the ternary complex structure. From this model it appears that the adenine 8-position of the bound cofactor is not recognized by M·*Taq*I and is accessible from the solvent. Thus, it is expected that attaching a reporter group at the 8-position of *N*-adenosylaziridine (**2**) should not interfere with cofactor binding.

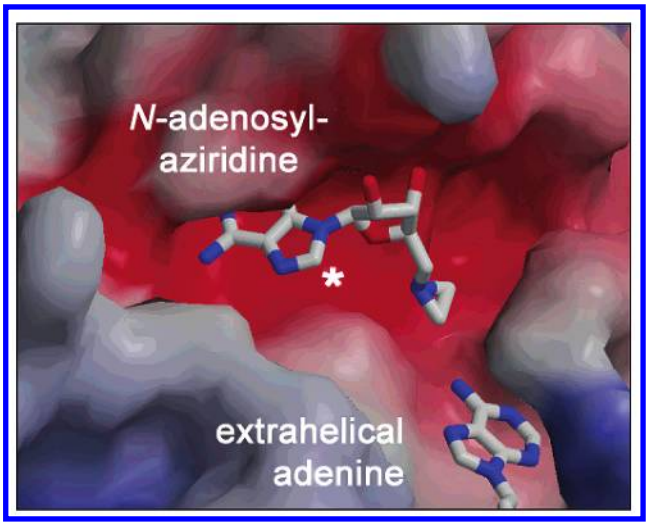


Figure 1. Model of *N*-adenosylaziridine (**2**) bound in the cofactor binding site of M·*Taq*I. The enzyme is shown as an electrostatic surface potential structure (red denotes negative potential, blue denotes positive potential, and white is neutral). The extrahelical target adenine residue and the cofactor are presented as stick models. The overall model was prepared by replacing the 2-aminoethylthio side chain of the bound cofactor analogue 5′-[2-(amino)ethylthio]-5′-deoxyadenosine from the ternary complex structure[12] with aziridine. The solvent-exposed 8-position of the cofactor adenine ring is indicated by a white star.

This prediction was tested by synthesizing the *N*-adenosylaziridine derivative **3** which contains the dansyl fluorophore connected to the 8-position via a diaminobutyl linker (Scheme 2). 8-Bromo-2′,3′-*O*-isopropylideneadenosine (**4**) was prepared according to a literature procedure[13] and treated with 1,4-diaminobutane to yield the primary amine **5**. Coupling of the dansyl fluorophore to the amine **5** was achieved by transient protection of the 5′-hydroxyl group using trimethylsilyl chloride followed by reaction with dansyl chloride. Activation of the 5′-hydroxyl group of the fluorescent nucleoside **6** using mesyl chloride led to mesylate **7** which was deprotected in aqueous formic acid. Finally, nucleophilic substitution of the deprotected mesylate **8** with aziridine yielded the fluorescent *N*-adenosylaziridine derivative **3**.

Binding of the Fluorescent *N*-Adenosylaziridine Derivative 3 to M·*Taq*I. Binding of **3** to M·*Taq*I was investigated by direct fluorescence titration. To a buffered solution of **3** increasing amounts of M·*Taq*I were added, and the dansyl fluorescence was monitored. With rising protein concentrations the fluorescence intensity of the dansyl group increased about 2.5-fold until saturation was observed (data not shown). Fitting the real solution of the quadratic equation for one binding site[14] to the obtained titration data yielded a dissociation constant K_D of 0.47 $\pm$ 0.05 μM. In comparison with the natural cofactor AdoMet (**1**), which binds to M·*Taq*I with a K_D-value of about 2.0 μM,[11] the fluorescent *N*-adenosylaziridine derivative **3** binds about 4-fold better to M·*Taq*I. This result is in line with our design and shows that modification of the 8-position of the cofactor **2** does not hinder but, in contrast, enhances binding to the enzyme. Since **3** is more hydrophobic than AdoMet (**1**), this enhanced binding affinity might not result from an additional specific interaction of the dansyl group with the enzyme but could well be the consequence of a hydrophobic effect.

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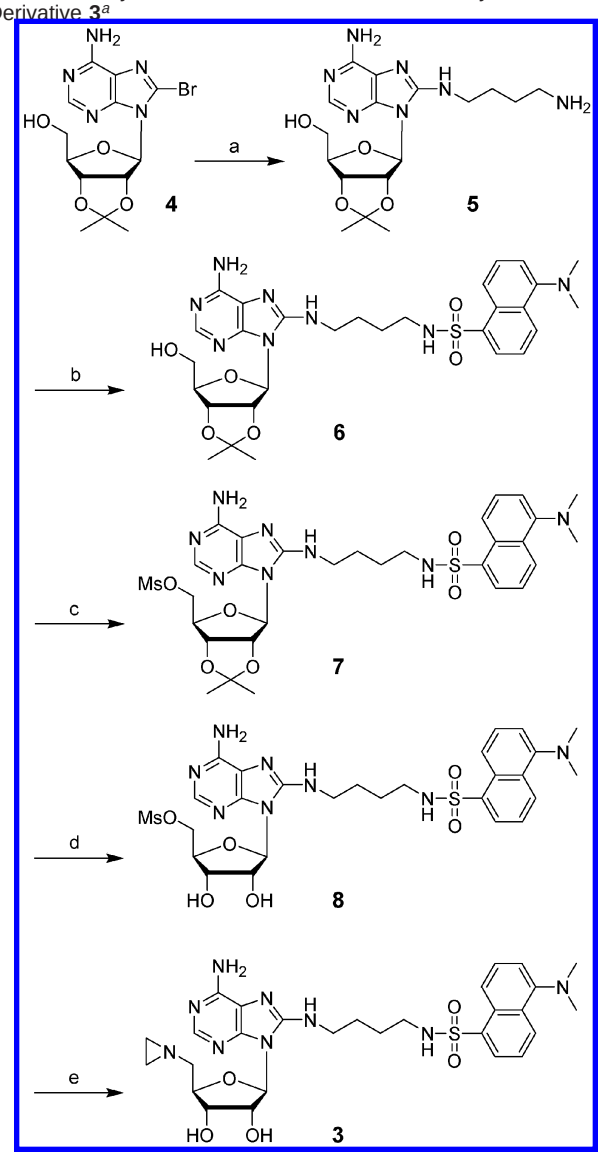
Scheme 2. Synthesis of the Fluorescent *N*-Adenosylaziridine Derivative **3**[a]

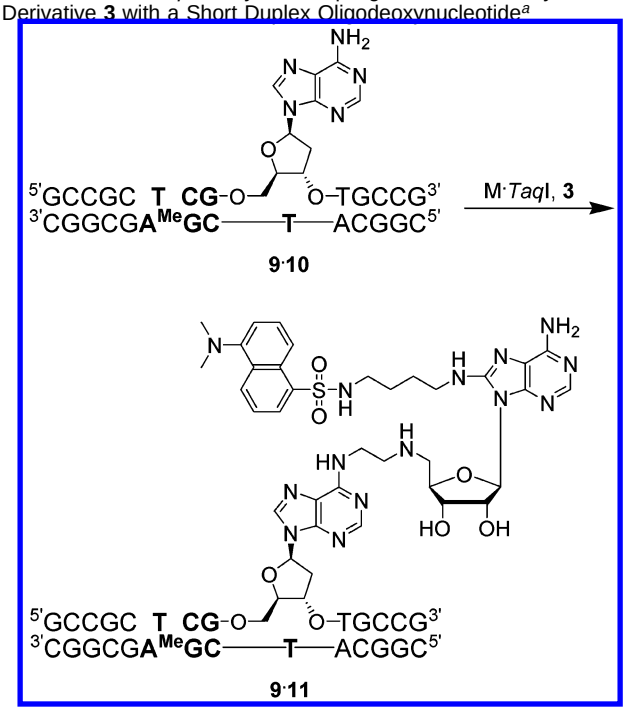


[a] (a) 1,4-Diaminobutane, TEA, 99%. (b) 1. Trimethylsilyl chloride, pyridine. 2. Dansyl chloride, 30%. (c) Mesyl chloride, TEA, DMAP, 43%. (d) Formic acid, H_2O (1:1), 55%. (e) Aziridine, EDIA, 36%.

Labeling of a Short Duplex Oligodeoxynucleotide. Following the binding study we tested whether the fluorescent *N*-adenosylaziridine derivative **3** functions as a cofactor for the DNA MTase M·*Taq*I (Scheme 3). A short hemimethylated duplex oligodeoxynucleotide (**9·10**, 14 base pairs) containing the 5′-TCG**A**-3′ recognition sequence of M·*Taq*I was prepared by annealing strand **9** with the methylated complementary strand **10** containing N6-methyl-2′-deoxyadenosine (A^{Me}) instead of 2′-deoxyadenosine within the recognition sequence. Although M·*Taq*I also alkylates nonmethylated recognition sequences (see labeling of plasmid DNA below) such a hemimethylated duplex better resembles the natural hemimethylated substrate which is formed after semiconservative DNA replication.

The duplex **9·10** was analyzed by anion-exchange HPLC (Figure 2A) and eluted after 23.2 min, and no additional peak was observed, indicating that complete hybridization had occurred. After incubation of the duplex **9·10** with stoichiometric amounts of M·*Taq*I and excess of the fluorescent *N*-adenosylaziridine derivative **3** the coupling reaction was monitored by

Scheme 3. M·*Taq*I-Catalyzed Coupling of the *N*-Adenosylaziridine Derivative **3** with a Short Duplex Oligodeoxynucleotide[a]



[a] The recognition sequence of M·*Taq*I is printed in bold face (A^{Me} = N6-methyl-2′-deoxyadenosine).

anion-exchange HPLC. Almost complete conversion to a new fluorescent product with a retention time of 8.0 min was observed (Figure 2B). In the absence of M·*Taq*I no conversion of the duplex **9·10** could be detected (data not shown), indicating that the observed reaction is enzyme-catalyzed. Interestingly, the fluorescent product duplex **9·11** eluted in complex with M·*Taq*I which was inferred from the small retention time and a reduced UV-absorption ratio (260 to 280 nm, not shown). Such a stable complex formation is not surprising because the M·*Taq*I-catalyzed reaction should lead to a covalently linked product which should bind much more tightly to the enzyme than the individual products of the natural methyl-group transfer reaction. The fluorescent duplex **9·11** was released from the complex by fragmentation of M·*Taq*I with proteinase K. As can be seen from the anion-exchange chromatogram shown in Figure 2C, the fluorescent peak at 8.0 min disappeared completely upon proteinase K treatment, and the fluorescent duplex **9·11** eluted with a retention time of 27.5 min. For further characterization the duplex **9·11** was isolated and treated with an enzyme mix consisting of DNaseI, phosphodiesterase from *Crotalus durissus*, phosphodiesterase from calf spleen, and alkaline phosphatase. The DNA fragmentation reaction was analyzed by reverse-phase HPLC (Figure 2D) and revealed besides the deoxynucleosides dC, dI (formed from dA by contaminating adenosine deaminase activity), dG, T, and dA^{Me} a new compound eluting after 47.8 min. This new compound was isolated and detected as positively charged ion at *m*/*z* 863.1 by electrospray ionization mass spectrometry (ESI-MS). The observed mass is in good agreement with the calculated molecular mass (863.4) of a protonated, with **3** modified 2′-deoxyadenosine (dA^{Dans}). This analysis demonstrates that the fluorescent *N*-adenosylaziridine derivative **3** acts as a new cofactor for M·*Taq*I and can be used for fluorescence labeling of DNA.

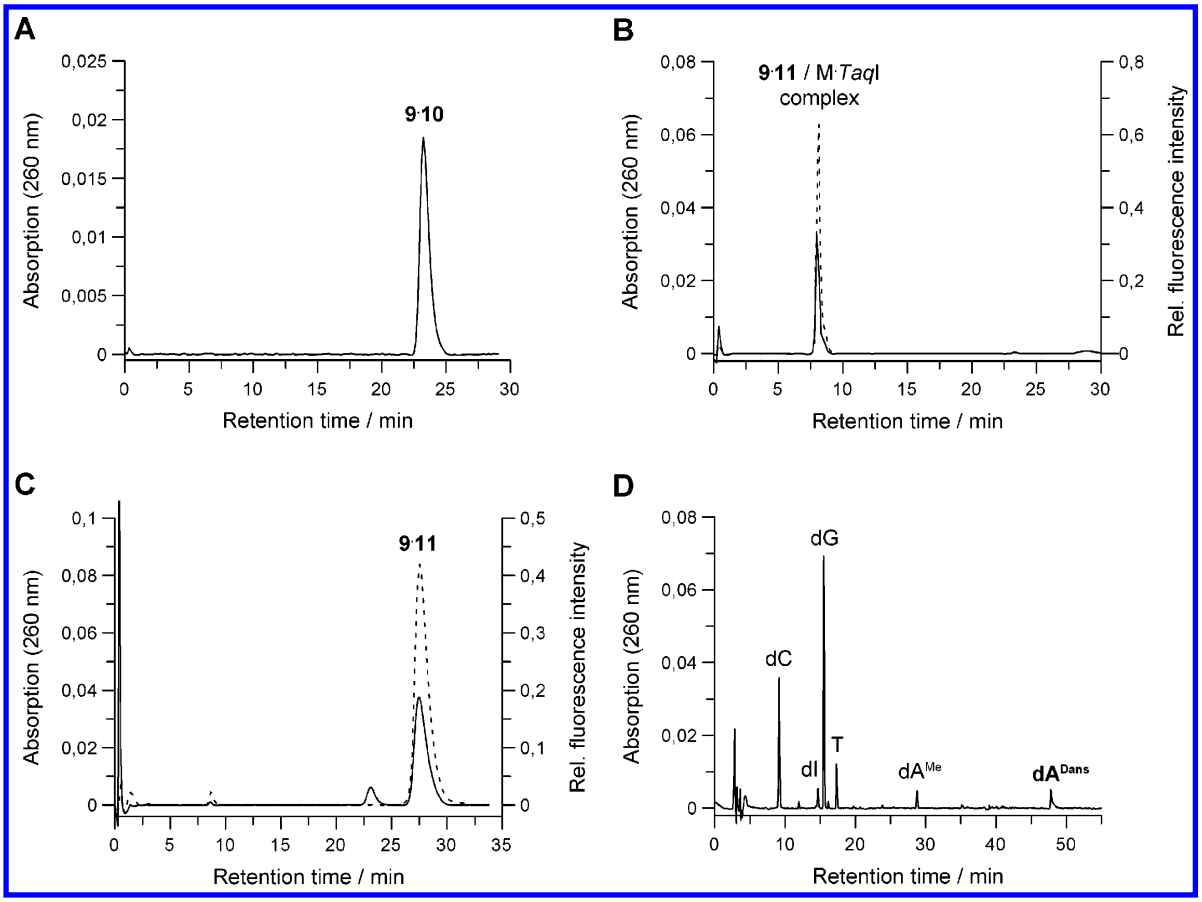


Figure 2. Labeling of the short duplex oligodeoxynucleotide **9·10** (10 μM) with the new fluorescent cofactor **3** (20 μM) and M·*Taq*I (10 μM). (A) Anion-exchange HPLC of the starting material **9·10**. (B) Analysis of the coupling reaction (analytical scale) after 15 h at 37 °C by anion-exchange HPLC (solid line: UV-detection; dotted line: fluorescence detection, $\lambda_{ex} = 330$ nm, $\lambda_{em} = 551$ nm). (C) Analysis of the coupling reaction (preparative scale) by anion-exchange HPLC after additional proteinase K treatment (37 °C for 1 h; solid line: UV-detection, dotted line: fluorescence detection, $\lambda_{ex} = 330$ nm, $\lambda_{em} = 551$ nm). (D) Reverse-phase HPLC analysis of the product duplex **9·11** after enzymatic fragmentation with DNaseI, phosphodiesterase from *Crotalus durissus*, phosphodiesterase from calf spleen and alkaline phosphatase (37 °C for 20 h). In addition to the nucleosides dC, dI, dG, T, and dA^{Me}, the dansylated nucleoside (dA^{Dans}) was observed (dI was formed from dA by contaminating adenosine deaminase activity present in the commercial phosphodiesterase from calf spleen).

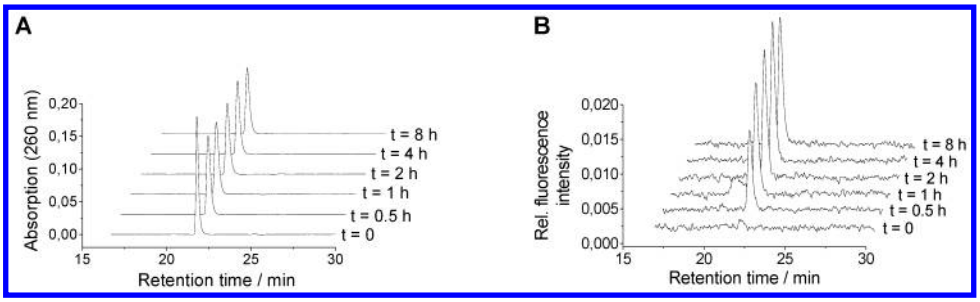


Figure 3. Labeling of pUC19 plasmid DNA (28 nM, four M·*Taq*I recognition sites) with the new fluorescent cofactor **3** (20 μM) and M·*Taq*I (133 nM) at 65 °C analyzed by anion-exchange HPLC. (A) UV-detection. (B) Detection of the dansyl fluorescence ($\lambda_{ex} = 330$ nm, $\lambda_{em} = 551$ nm). Since fluorescence detection was performed after UV-detection, a small delay of the retention time compared to A was observed.

Labeling of pUC19 Plasmid DNA. We also tested whether the new fluorescent cofactor **3** in combination with M·*Taq*I can be used to label long DNA molecules. The plasmid pUC19 which contains four recognition sites for M·*Taq*I was incubated with **3** and M·*Taq*I and the labeling reaction followed by anion-exchange HPLC (Figure 3). The plasmid eluted after 21.9 min (UV-detection), and the integration of the corresponding signal was constant over the incubation time of 8 h (Figure 3A). Only a small broadening of the peak shape was observed during the first 2 h. Most interestingly, detection of the dansyl fluorescence revealed that the plasmid was fluorescently labeled with time, and completion of the reaction was reached after 4 h (Figure 3B). Labeling of plasmid DNA was faster than labeling of the short duplex **9·10** because of the higher incubation temperature. In a parallel experiment where M·*Taq*I was omitted, labeling of the plasmid was not observed (data not shown). This result demonstrates that the labeling reaction is enzyme-catalyzed and not a result of a direct reaction between **3** and DNA. To investigate the specificity of the labeling reaction we performed a control experiment in which pUC19 was first methylated using M·*Taq*I and the natural cofactor AdoMet (**1**), isolated, and then incubated with the fluorescent cofactor **3** and M·*Taq*I. Since

M·*Taq*I catalyzes the methylation of each adenine residue within the palindromic 5′-TCG**A**-3′ recognition sequence only once, methylation of the recognition sequences in pUC19 should block further alkylation by M·*Taq*I and **3**. Anion-exchange HPLC analysis of this control experiment revealed no fluorescence signal for the plasmid (data not shown). From this result we conclude that the sequence specificity of M·*Taq*I is not lowered with the new fluorescent cofactor **3**. Given the high sequence specificity of M·*Taq*I (the second-best found site is methylated about 100000-fold slower than the canonical recognition site by M·*Taq*I; unpublished result), DNA labeling with M·*Taq*I should be highly specific.

Conclusions and Outlook

This work illustrates the successful design of the new fluorescent MTase cofactor **3** which is sequence-specifically coupled with DNA using the DNA MTase M·*Taq*I and provides a novel method for sequence-specific labeling of large DNA fragments. It is likely that derivatives of **3** containing other reporter groups instead of the fluorescent dansyl group will also function as cofactors for M·*Taq*I and could be used for sequence-specific labeling of DNA with a variety of reporter groups. Furthermore, preliminary experiments in our laboratory indicate that *N*-adenosylaziridine (**2**) is also a cofactor for N4- and C5-cytosine DNA MTases. Thus, other DNA MTases with different recognition sequences might also use the new fluorescent cofactor **3**, provided that, as in M·*Taq*I, the 8-position of the cofactor adenine ring is accessible from the solvent. Inspection of the known three-dimensional structures of other DNA MTases, such as M·*Hha*I,[15] M·*Pvu*II,[16] DpnM,[17] M·*Rsr*I,[18] and M·*Mbo*II,[19] in complex with AdoMet (**1**) or adenosine derivatives reveals that this appears to be the case in most DNA MTases. Thus, the new fluorescent cofactor **3** or derivatives with other reporter groups could be useful for labeling a wide variety of DNA sequences. This novel technique named **s**equence-specific **m**ethyltransferase-**i**nduced **l**abel**ing** of **DNA** (SMILing DNA) could find interesting applications in functional studies of DNA modifying enzymes, molecular biology, DNA-based medical diagnosis, and nanobiotechnology.

Experimental Section

Materials. 8-Bromo-2′,3′-*O*-isopropylideneadenosine (**4**) and aziridine were prepared according to literature procedures.[13,20] (CAUTION: Aziridine is hazardous and should be handled with care.) 1,4-Diaminobutane was purchased from Aldrich, and dansyl chloride, trimethysilyl chloride, mesyl chloride, and *N*-ethyldiisopropylamine were purchased from Fluka. All reagents were of p.a. grade. Dry solvents were either purchased or dried using common laboratory techniques.[21] The DNA MTase M·*Taq*I was prepared as described before.[12] Proteinase K was obtained from Merck, and DNase I (grade II), alkaline phosphatase, phosphodiesterase from *Crotalus durissus* and from calf spleen were from Roche Diagnostics. Oligodeoxynucleotides **9** and **10** were synthesized by MWG-Biotech, Ebersberg, Germany, and pUC19 plasmid DNA was purchased from New England Biolabs.

General Procedures. All chemical reactions were carried out in oven-dried glassware under an argon atmosphere, except for the deprotection of the nucleoside **7**. DC-alumina plates Sil-G60/UV254 (Merck) were used for TLC. Compounds were visualized by treatment with a solution of ammonium molybdate (15 g) and cerium(IV) sulfate (0.4 g) in 10% sulfuric acid (300 mL) and subsequent heating with a heat gun. Flash chromatography was carried out using Merck silica gel 60 (40−63 μm). HPLC was performed using a Beckman System Gold equipped with a programmable solvent module 125, a diode array detector module 168, an analogue interface module 406, and a Shimadzu spectrofluorometric detector RF-551. NMR spectra were obtained using a Bruker AX 500 (500 and 125.7 MHz, for ^{1}H and ^{13}C, respectively). $CDCl_3$ ($\delta_H = 7.24$ and $\delta_C = 77.0$) or [d_6]DMSO ($\delta_H = 2.49$ and $\delta_C = 39.5$) was used as solvent. Assignment of ^{13}C signals are based on ^{1}H, ^{13}C-correlated 2D-NMR and on ^{13}C-DEPT spectra. Electrospray ionization mass spectra (ESI-MS) were obtained using a Finnigan LCQ connected to a nano-electrospray ion source.[22] Measurements were carried out in the positive ion mode. The samples were dissolved in CH_3OH/water (1:1 v/v) containing 5% formic acid. UV-absorption measurements were performed in water using a Varian Cary 3E spectrometer. Fluorescence data were collected with a SLM-Aminco AB2 spectrofluorometer. The excitation and emission bandwidths were adjusted to 2 and 8 nm, respectively.

8-Amino[1″-(4′-aminobutyl)]-2′,3′-*O*-isopropylideneadenosine (5). To a solution of 8-bromo-2′,3′-*O*-isopropylideneadenosine (**4**) (628 mg, 1.6 mmol) in dry DMSO (10 mL) were added dry triethylamine (2.26 mL, 16.3 mmol) and 1,4-diaminobutane (0.82 mL, 8.1 mmol). The solution was stirred at 110 °C and the reaction progress monitored by TLC. After 4 h the solvent was removed under reduced pressure. The residue was dissolved in water (50 mL) and the pH adjusted to 5.3 with acetic acid (0.1 M). The crude product was purified by cation-exchange chromatography (Dowex 50 × 4, H^+-form, 100 g, elution with 600 mL of water followed by 1000 mL of 1 M potassium hydroxide). Fractions containing the product were extracted with chloroform. The organic layers were combined, and the solvent was removed under reduced pressure to yield compound **5** (639 mg, 99%) as a white solid (R_f 0.44, butanol/acetic acid/water 12:3:5): ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.33 (s, 3H; acetonide-H), 1.48−1.55 (m, 2H; linker-H), 1.61 (s, 3H; acetonide-H), 1.64−1.70 (m, 2H; linker-H), 2.66−2.73 (m, 2H; linker-H), 3.33−3.42 (m, 2H; linker-H), 3.77−3.91 (m, 2H; 5′-H), 4.28−4.30 (m, 1H; 4′-H), 4.99 (dd, $^3J = 2.7$, 6.3 Hz, 1H; 3′-H), 5.08 (dd, $^3J = 4.8$, 6.3 Hz, 1H; 2′-H), 5.39 (s, br, 2H; 6-NH_2), 6.15 (d, $^3J = 4.5$ Hz, 1H; 1′-H), 6.55−6.60 (m, 1H; 8-NH), 8.10 (s, 1H; 2-H); ^{13}C NMR (125.7 MHz, $CDCl_3$): δ 25.30 (q; acetonide-CH_3), 25.73 (t; linker-C), 27.42 (q; acetonide-CH_3), 29.60 (t; linker-C), 40.46 (t; linker-C), 42.69 (t; linker-C), 61.17 (t; 5′-C), 80.59 (d; 3′-C), 82.19 (d; 2′-C), 84.48 (d; 4′-C), 89.21 (d; 1′-C), 114.50 (s; acetonide-$\underline{C}(CH_3)_2$), 117.68 (s; 5-C), 149.49 (d; 2-C), 149.95 (s; 8-C), 151.68 (s; 4-C), 151.72 (s; 6-C); ESI-MS *m*/*z* (relative intensity): 394.3 (25) $[M + H]^+$, 222.3 (100) [adenine + aminobutyl + $H]^+$.

8-Amino[1″-(*N*″-dansyl)-4″-aminobutyl]-2′,3′-*O*-isopropylideneadenosine (6). To a solution of amine **5** (104 mg, 0,26 mmol) in dry pyridine (7 mL) was added slowly trimethylsilyl chloride (0.07 mL, 0.53 mmol) at 0 °C, and the solution was stirred at room temperature for 1 h. Subsequently, dansyl chloride (103.8 mg, 0.37 mmol, dissolved in 3 mL of dry pyridine) was added, and the solution was stirred at room temperature for 4 h. The progress of the reaction was monitored by TLC. After complete conversion the solution was treated with water (5 mL) at 0 °C, and the solvent was removed under reduced pressure.

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The crude product was purified by column chromatography (silica gel, 40 g, elution with 5% methanol in methylene chloride) to give nucleoside **6** (50 mg, 30%) as a yellow fluorescent solid (R_f 0.54, 10% methanol in methylene chloride): ^{1}H NMR (500 MHz, [d_6]DMSO): δ 1.29 (s, 3H; acetonide-H), 1.39−1.43 (m, 2H; linker-H), 1.47−1.50 (m, 2H; linker-H), 1.53 (s, 3H; acetonide-H), 2.78−2.82 (m, 8H; linker-H and $N(CH_3)_2$), 3.16−3.24 (m, 2H; linker-H), 3.50−3.58 (m, 2H; 5′-H), 4.12−4.14 (m, 1H; 4′-H), 4.94 (dd, ^3J = 2.7, 6.1 Hz, 1H; 3′-H), 5.33 (dd, ^3J = 3.7, 6.1 Hz, 1H; 2′-H), 5.41−5.44 (m, 1H; 5′-OH), 6.01 (d, ^3J = 3.5 Hz, 1H; 1′-H), 6.49 (s, br, 2H; 6-NH_2), 6.85 (t, ^3J = 5.0 Hz, 1H; 8-NH), 7.22 (d, ^3J = 7.5 Hz, 1H; arom-H), 7.54−7.61 (m, 2H; arom-H), 7.87−7.90 (m, 1H; $NHSO_2$), 7.90 (s, 1H; 2-H), 8.08 (d, ^3J = 7.2 Hz, 1H; arom-H), 8.30 (d, ^3J = 8.5 Hz, 1H; arom-H), 8.43 (d, ^3J = 8.5 Hz, 1H; arom-H); ^{13}C NMR (125.7 MHz, [d_6]DMSO): δ 25.42 (q; acetonide-CH_3), 26.00 (t; linker-C), 26.96 (t; linker-C), 27.33 (q; acetonide-CH_3), 41.92 (t; linker-C), 42.43 (t; linker-C), 45.21 (q; $N(CH_3)_2$), 61.40 (t; 5′-C), 81.14 (d; 3′-C), 81.50 (d; 2′-C), 85.29 (d; 4′-C), 87.85 (d; 1′-C), 113.38 (s), 115.24 (d; arom-C), 117.24 (s), 119.29 (d; arom-C), 123.72 (d; arom-C), 127.92 (d; arom-C), 128.31 (d; arom-C), 129.26 (s), 129.48 (d; arom-C), 136.27 (s), 148.89 (d; 2-C), 149.30 (s), 151.20 (s), 151.50 (s), 152.58 (s); ESI-MS *m*/*z* (relative intensity): 627.1 (100) $[M + H]^+$, 455.2 (8) [adenine + linker + dansyl + H]$^+$.

8-Amino[1″-(*N*″-dansyl)-4″-aminobutyl]-2′,3′-*O*-isopropylidene-5′-*O*-mesyladenosine (7). To a solution of nucleoside **6** (181 mg, 0.32 mmol) and (dimethylamino)pyridine (40 mg, 0.32 mmol) in dry methylene chloride (20 mL) was added dry triethylamine (1.1 mL, 8.0 mmol) and the solution cooled to 0 °C. Mesyl chloride (200 μL, 2.6 mmol) was added, and the solution was stirred for 30 min. The reaction was quenched with a cold, saturated sodium hydrogencarbonate solution (5 mL). The solution was extracted three times with cold chloroform (10 mL), the organic phases were combined, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (silica gel, 40 g, elution with 3% methanol in methylene chloride) to give nucleoside **7** (96 mg, 43%) as a yellow fluorescent solid (R_f 0.55, 10% methanol in methylene chloride); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.37 (s, 3H; acetonide-H), 1.45−1.48 (m, 2H; linker-H), 1.59−1.61 (m, 5H; linker-H and acetonide-H), 2.85 (s, 6H; $N(CH_3)_2$), 2.96 (s, 3H; SO_2CH_3), 2.98−3.02 (m, 2H; linker-H), 3.32−3.36 (m, 2H; linker-H), 4.33−4.43 (m, 3H; 5′-H and 4′-H), 5.03 (m, 1H; 3′-H), 5.52 (dd, ^3J = 2.5, 6.5 Hz, 1H; 2′-H), 6.04 (d, ^3J = 2.5 Hz, 1H; 1′-H), 6.13 (s, br, 2H; 6-NH_2), 6.91 (t, ^3J = 5.8 Hz, 1H; 8-NH), 7.13 (d, ^3J = 7.3 Hz, 1H; arom-H), 7.43 (t, ^3J = 8.2 Hz, 1H; arom-H), 7.50 (t, ^3J = 7.9 Hz, 1H; arom-H), 8.10 (s, 1H; 2-H), 8.23 (d, ^3J = 7.0 Hz, 1H; arom-H), 8.37 (d, ^3J = 8.5 Hz, 1H; arom-H), 8.51 (d, ^3J = 8.6 Hz, 1H; arom-H); ^{13}C NMR (125.7 MHz, $CDCl_3$): δ 24.62 (q; acetonide-CH_3), 25.30 (t; linker-C), 26.89 (t; linker-C), 27.04 (q; acetonide-CH_3), 37.50 (q; SO_2CH_3), 41.58 (t; linker-C), 42.70 (t; linker-C), 45.44 (q; $N(CH_3)_2$), 68.38 (t; 5′-C), 80.10 (d; 3′-C), 82.11 (d; 2′-C), 83.29 (d; 4′-C), 88.63 (d; 1′-C), 115.16 (d; arom-C), 118.94 (d; arom-C), 123.23 (d; arom-C), 128.20 (d; arom-C), 129.70 (d; arom-C), 130.37 (d; arom-C), 149.78 (d; 2-C), 151.84 (s), 152.41 (s); ESI-MS *m*/*z* (relative intensity): 705.3 (70) $[M + H]^+$, 609.7 (100) [cyclonucleoside + H]$^+$.

8-Amino[1″-(*N*″-dansyl)-4″-aminobutyl]-5′-*O*-mesyladenosine (8). Nucleoside **7** (96.2 mg, 0.14 mmol) was dissolved in aqueous formic acid (50%, 10 mL), and the resulting solution was stirred at room temperature for 4 d. After complete conversion the solvent was removed under reduced pressure, and remaining solvent was coevaporated with a mixture of water and methanol (1:1, 3 × 5 mL). The crude product was purified by column chromatography (silica gel, 15 g, elution with 10% methanol in methylene chloride) to give nucleoside **8** (49.2 mg, 55%) as a yellow fluorescent solid (R_f 0.23, 10% methanol in methylene chloride); ^{1}H NMR (500 MHz, [d_6]DMSO): δ 1.36−1.42 (m, 2H; linker-H), 1.47−1.53 (m, 2H; linker-H), 2.77−2.79 (m, 2H; linker-H), 2.81 (s, 6H; $N(CH_3)_2$), 3.07 (s, 3H; SO_2CH_3), 3.17−3.20 (m, 2H; linker-H), 4.01−4.04 (m, 1H; 4′-H), 4.33−4.38 (m, 2H; 5′-H), 4.44−4.47 (m, 1H; 3′-H), 5.08 (ddd = q, ^3J = 5.5 Hz, 1H; 2′-H), 5.37 (d, ^3J = 5.5 Hz, 1H; OH), 5.44 (d, ^3J = 5.5 Hz, 1H; OH), 5.72 (d, ^3J = 5.1 Hz, 1H; 1′-H), 6.48 (s, br, 2H; 6-NH_2), 6.78 (t, ^3J = 5.3 Hz, 1H; 8-NH), 7.24 (d, ^3J = 7.8 Hz, 1H; arom-H), 7.57 (t, ^3J = 8.3 Hz, 1H; arom-H), 7.61 (t, ^3J = 7.8 Hz, 1H; arom-H), 7.88 (s, 1H; 2-H), 7.95 (t, ^3J = 5.7 Hz, 1H; $NHSO_2$), 8.08 (d, ^3J = 6.9 Hz, 1H; arom-H), 8.28 (d, ^3J = 8.7 Hz, 1H; arom-H), 8.44 (d, ^3J = 8.7 Hz, 1H; arom-H); ^{13}C NMR (125.7 MHz, [d_6]DMSO): δ 27.24 (t; linker-C), 28.06 (t; linker-C), 37.91 (q; SO_2CH_3), 43.07 (t; linker-C), 43.58 (t; linker-C), 46.41 (q; $N(CH_3)_2$), 71.21 (t; 5′-C), 71.45 (d; 3′-C), 71.61 (d; 2′-C), 82.20 (d; 4′-C), 88.63 (d; 1′-C), 116.44 (d; arom-C), 118.76 (s), 120.46 (d; arom-C), 124.98 (d; arom-C), 129.16 (d; arom-C), 129.54 (d; arom-C), 130.34 (s), 130.39 (d; arom-C), 130.68 (s), 137.35 (s), 149.95 (d; 2-C), 150.78 (s), 152.66 (s), 153.06 (s), 153.73 (s); ESI-MS *m*/*z* (relative intensity): 665.6 (85) $[M + H]^+$, 687.4 (100) $[M + Na]^+$.

8-Amino[1″-(*N*″-dansyl)-4″-aminobutyl]-5′-(1-aziridinyl)-5′-deoxyadenosine (3). Nucleoside **8** (20 mg, 30 μmol) was dissolved in dry aziridine (1 mL) and *N*-ethyldiisopropylamine (350 μL) and the solution stirred at room temperature for 3 d. The reaction was monitored by analytical reverse-phase HPLC (Hypersil-ODS, 5 μm, 120 Å, 250 mm × 4.6 mm, Bischoff, Leonberg, Germany). Compounds were eluted with acetonitrile (0% for 5 min, followed by a linear gradient to 35% in 30 min and to 70% in 10 min) in triethylammonium acetate buffer (0.1 M, pH 7.0) and a flow rate of 1 mL/min. After the reaction was complete, the solvent was removed under reduced pressure, and the crude product was purified by column chromatography (silica gel, 2 g, elution with 10% methanol in methylene chloride) to give nucleoside **3** (6.7 mg, 36%) as a yellow fluorescent solid (R_f 0.23, 10% methanol in methylene chloride); ^{1}H NMR (500 MHz, [d_6]DMSO): δ 1.19−1.22 (m, 2H; aziridine-H), 1.32−1.34 (m, 2H; linker-H), 1.37−1.39 (m, 2H; linker-H), 1.59−1.61 (m, 2H; aziridine-H), 1.94 (dd, ^3J = 3.2 Hz, ^2J = 13.5 Hz, 1H; 5′−H_a), 2.74−2.79 (m, 2H; linker-H), 2.81 (s, 6H; $N(CH_3)_2$), 2.91−2.95 (m, 1H; 5′−H_b), 3.07−3.16 (m, 2H; linker-H), 3.94−3.96 (m, 1H; 4′-H), 4.19−4.21 (m, 1H; 3′-H), 4.63−4.67 (m, 1H; 2′-H), 5.20 (d, ^3J = 4.1 Hz, 1H; OH), 5.30 (d, ^3J = 6.8 Hz, 1H; OH), 5.90 (d, ^3J = 7.2 Hz, 1H; 1′-H), 6.42 (s, br, 2H; 6-NH_2), 7.23 (d, ^3J = 7.2 Hz, 1H; arom-H), 7.55−7.61 (m, 3H; arom-H and 8-NH), 7.87 (s, 1H; 2-H), 7.95 (t, ^3J = 5.6 Hz, 1H; $NHSO_2$), 8.08 (d, ^3J = 7.2 Hz, 1H; arom-H), 8.28 (d, ^3J = 8.6 Hz, 1H; arom-H), 8.43 (d, ^3J = 8.6 Hz, 1H; arom-H); ^{13}C NMR (125.7 MHz, [d_6]DMSO): δ 26.92 (t; aziridine-C), 27.43 (t; linker-C), 28.01 (t; linker-C), 30.02 (t; aziridine-C), 43.02 (t; linker-C), 43.65 (t; linker-C), 46.41 (q; $N(CH_3)_2$), 62.96 (t; 5′-C), 71.14 (d; 2′-C), 72.29 (d; 3′-C), 85.31 (d; 4′-C), 87.11 (d; 1′-C), 116.45 (d; arom-C), 118.20 (s), 120.45 (d; arom-C), 124.96 (d; arom-C), 129.16 (d; arom-C), 129.57 (d; arom-C), 130.00 (s), 130.36 (d; arom-C), 130.68 (s), 137.37 (s), 149.86 (d; 2-C), 151.49 (s), 152.42 (s), 152.66 (s), 153.43 (s); ESI-MS *m*/*z* (relative intensity): 612.7 (100) $[M + H]^+$.

Fluorescence Titration. To a solution of cofactor **3** (1 μM, based on a molecular extinction coefficient ϵ^{335} = 4600 cm^{-1} M^{-1} for the dansyl group) in buffer A (20 mM Tris acetate, pH 7.9, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT and 0.01% Triton X-100) were added increasing amounts of M·*Taq*I in buffer A containing cofactor **3** (1 μM) at 25 °C, and the dansyl fluorescence intensity (λ_{ex} = 330 nm, λ_{em} = 551 nm) was monitored after each addition. Fitting the real solution of the quadratic equation for one binding site[14] to the titration data was performed with the data analysis program GraFit.[23]

Fluorescence Labeling of Duplex Oligodeoynucleotide 9·10 and Analysis of the Product Duplex 9·11. Molecular extinction coefficients at 260 nm of oligodeoynucleotides **9** and **10** were calculated according

(23) Leatherbarrow, R. J. 1992, *GraFit*, Version 3.0; Erithacus Software Ltd.: Staines, U.K.

to the nearest-neighbor method,[24] and concentrations were determined by UV spectroscopy. Annealing of the complementary strands **9** and **10** was performed by heating an equimolar solution to 95 °C for 2 min followed by slow cooling to room temperature.

A solution (500 μL) of duplex **9·10** (10 μM), cofactor **3** (20 μM), and M·*Taq*I (10 μM) in buffer B (20 mM Tris acetate, pH 6.0, 50 mM potassium acetate, 10 mM magnesium acetate and 0.01% Triton X-100) was incubated at 37 °C. After 15 h an aliquot was injected onto an anion-exchange HPLC column (Poros 10 HQ, 10 μm, 4.6 mm × 100 mm, Applied Biosystems), and compounds were eluted with potassium chloride (0.2 M for 5 min followed by linear gradients to 0.5 M in 5 min and then to 1.0 M in 30 min) in Tris hydrochloride buffer (10 mM, pH 7.0) at a flow rate of 4 mL/min.

The pH of the reaction solution was adjusted to 8.0 with a potassium hydroxide solution (10 M). Then, a solution (4 μL) of proteinase K (31 μg/μL) in Tris hydrochloride buffer (50 mM, pH 8.0) and calcium chloride (1 mM) was added and the mixture incubated at 37 °C for 1 h. The proteolytic fragmentation was monitored by anion-exchange HPLC as described above. For further characterization the duplex **9·11** was isolated by reverse-phase HPLC (conditions see synthesis of cofactor **3**, retention time: 25.0 min).

Enzymatic fragmentation of the purified duplex **9·11** (0.57 OD at 260 nm) in buffer (228 μL, 10 mM potassium phosphate, pH 7.0, 10 mM magnesium chloride) was carried out by adding DNase I (13.7 μL, 2.7 U), phosphodiesterase from *Crotalus durissus* (13.7 μL, 41 mU), phosphodiesterase from calf spleen (13.7 μL, 55 mU), and alkaline phosphatase (13.7 μL, 13.7 U). After incubation at 37 °C for 20 h an aliquot (100 μL) was injected onto the analytical reverse-phase HPLC column (see synthesis of cofactor **3**), and the products were eluted with an acetonitrile gradient (0 to 10.5% in 30 min followed by 10.5 to 28% in 10 min and 28 to 70% in 15 min) in triethylammonium acetate buffer (0.1 M, pH 7.0) at a flow rate of 1 mL/min.

Fluorescence Labeling of pUC19 Plasmid DNA. A solution of pUC19 DNA (28 nM, four M·*Taq*I recognition sites), cofactor **3** (20 μM), and M·*Taq*I (133 nM) in buffer B was incubated at 65 °C. Aliquots were withdrawn after different incubation times and injected onto an anion-exchange HPLC column (Nucleogen 4000-7 DEAE, 7 μm, 125 mm × 6 mm, Macherey-Nagel, Düren, Germany), and compounds were eluted with potassium chloride (0.3 M for 5 min followed by a linear gradient to 1.5 M in 30 min) in potassium phosphate buffer (30 mM, pH 6.5) containing acetonitrile (20%) at a flow rate of 1.5 mL/min.

Methylation of pUC19 Plasmid DNA. A solution of pUC19 DNA (66 nM), **1** (80 μM), and M·*Taq*I (7.5 nM) in buffer A containing bovine serum albumin (1 mg/mL) was incubated at 65 °C for 1 h. The methylated DNA was purified using the Qiagen PCR Purification Kit.

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