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DNA Methyltransferase-Moderated Click Chemistry

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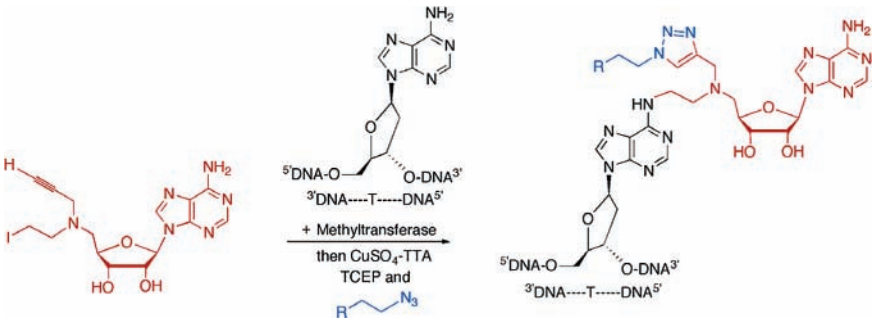
ABSTRACT



Biological methylation plays a vital role in regulatory mechanisms of gene transcription. Methylation of both promoter sequences within the genome, as well as protein substrates, has a profound impact upon gene transcription. Yet, few tools exist by which to identify sites of biological methylation in complex biological mixtures. We have generated a novel adenosine-derived *N*-mustard that serves as an efficient synthetic cofactor and allows for subsequent "click" chemistry involving the modified nucleic acid substrate.

Manifolds for the sequence-specific recognition and modification of DNA have proven to be vital for structural and functional studies of DNA, as well as the pursuit of rationally designed therapeutic agents. Successful strategies for sequence-selective targeting of DNA include triplex-forming oligodeoxyribonucleotides (ODNs), designer zinc finger proteins, and most notably, synthetic polyamides.[1] The mechanisms of recognition in each of these examples are unique but contingent upon a series of temporary interactions between the modifying agent and the substrate. We report here a method for covalent site-specific DNA alkylation mediated by methyltransferases (MTases) that enables subsequent chemoselective ligation via the Huisgen [2 + 3] cycloaddition.

DNA MTases combine sequence-specific recognition with covalent modification to act as molecular "third parties", directing delivery of the new alkyl group through association with individual target sequences. The transferred methyl group is derived from *S*-adenosyl-L-methionine (SAM).[2] SAM is routinely coined "mother nature's methyl iodide" for good reason. The agent is a potent and potentially nonspecific alkylating agent. However, under the direction of MTases, SAM is a highly selective alkylating agent capable of DNA, RNA, and protein modification and is an important biosynthetic tool for secondary metabolite production.[3] Indeed, SAM-dependent methylation of DNA and proteins is now a major theme in epigenomics. Understanding the role that biological methylation plays, particularly in gene transcription, has been hampered by a general lack of tools for identification of MTase substrates. The largest impediment arguably stems from the methyl group's relative absence of functionality, which renders it difficult to identify in complex biological environments.

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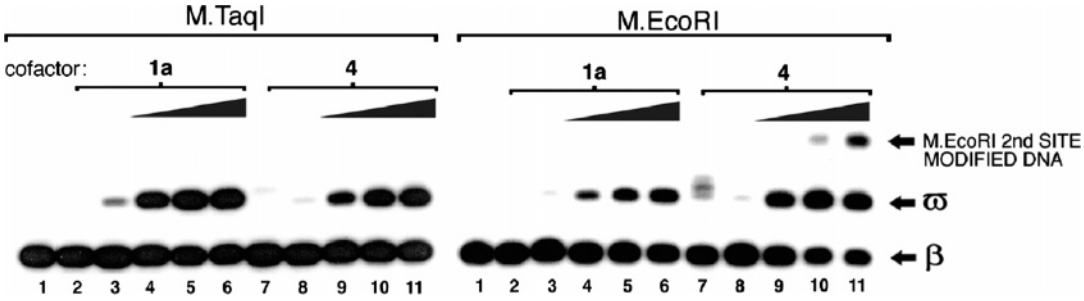
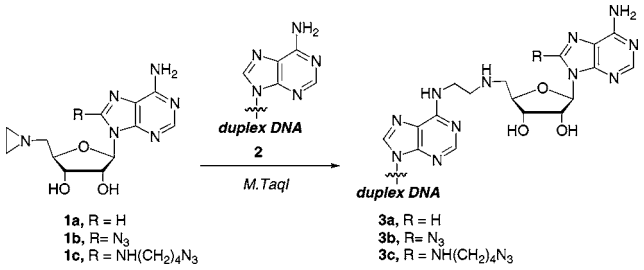


Figure 1. Denaturing polyacrylamide gel electrophoresis (DPAGE) of DNA alkylation reactions with synthetic oligonucleotides and **4** (relative to **1a**). β = native unmodified DNA, and ϖ = DNA alkylated with **1a** or **4**. Each series of reactions/lanes 1−11 are as follows: 1, DNA-only control; 2, DNA + 100 μM **1a** (no enzyme added); 3, DNA + 1 μM **1a** + M.TaqI (to 1 μM); 4, DNA + 10 μM **1a** + M.TaqI; 5, DNA + 50 μM **1a** + M.TaqI; 6, DNA + 100 μM **1a** + M.TaqI. Lanes 7−11 were same as 2−6 except that alkyne cofactor **4** was used. Right-hand panel lanes are identical in loading to left-hand loadings with the exception that M.EcoRI was used instead of M.TaqI. Please note that for all reactions, the concentration of enzyme used was 1 μM. Controls accounting for cofactor solvent and/or appropriate MTase did not result in electrophoretically retarded materials (data not shown).

Aziridine adenylates **1a**−**c** take part in MTase-dependent DNA alkylations (Scheme 1).[4−6] Moreover, azido adenylates

Scheme 1. 5′-Aziridine Adenylates as MTase-Dependent DNA-Modifying Agents



1b and **1c** allow the conversion of DNA MTases into azidonucleoside transferases.[6] Substrates of azidation are the same as those ordinarily acted upon by MTases. Unlike the methyl group, azides provide a chemically unique handle to which other probes (radioisotopes, affinity matrix handles, etc.) can be linked under biologically amenable conditions.[7] The sequence selectivity of DNA azidation is accomplished by DNA MTases, and substrates modified with either **1b** or **1c** can be elaborated postenzymatically via Staudinger ligation chemistry.[6]

Aziridines **1a**−**c** are believed to undergo quaternization of the 5′ amine followed by MTase binding, delivery to the site of methylation, and subsequent aziridinium ring opening with concomitant substrate alkylation. Initial generation of the positively charged aziridinium is consistent not only with activation toward nucleophiles but also cation−π interactions between cofactor and MTase, which are a trademark of SAM−MTase complexes.[8] We sought to exploit this phenomenon in the design of a novel synthetic cofactor devoid of the inherently labile aziridine moiety of **1a**−**c**. Hydrochloride salt **4** was envisioned to rapidly form aziridinium **5** in situ, thus avoiding synthetic difficulties associated with intact aziridines. This intermediate was expected to be more reactive *and* more amenable to MTase-promoted chemistry by virtue of 5′ amine quaternization via aziridination instead of a potentially reversible protonation (as has been proposed for materials such as **1a**−**c**). Iodide **4** was designed with the propargyl substituent to allow for possible bioconjugation of the DNA substrate postalkylation.

In evaluating the hypothesis that **4** (and related agents) could serve as an effective cofactor for MTases, we were initially highly skeptical due to the unknown impact of the alkynyl substituent upon cofactor:MTase interactions. Indeed, **4** has so far proven to be completely devoid of DNA-damaging activity in the presence of the cytosine C5 MTase M.HhaI. In contrast, we have found that **4** is highly amenable to use by two different N6 adenine MTases.

Figure 1 shows that the activity of **4** closely parallels that of **1a** with both M.TaqI and M.EcoRI. To evaluate M.TaqI activity, the ODN 5′-TGAATC**TCGA**GCACCC-3′ was 5′-end labeled with ^{32}P, gel purified, and annealed to complementary ODN 3′-AAACTTAG***AGCT***CGTGGG-5′. M.TaqI ordinarily methylates each adenine N6 (italicized) within the palindromic sequence shown in bold. Evaluation of M.EcoRI activity called for 5′-end labeling of 5′-TGAAT**GAATTC**-GACCC-3′ followed by gel purification and annealing to the complement 3′-AAACTTA**CTTAAG**CTGGG-5′. M.EcoRI ordinarily methylates each adenine N6 (italicized) within the bold-faced palindrome. Duplex substrates were incubated with the corresponding enzyme and either the aziridine **1a**

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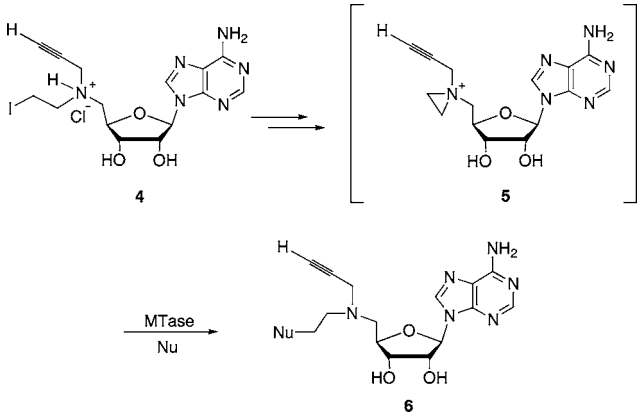
or hydrochloride **4**. Incubations were then quenched via addition of tRNA (40 μg/reaction) and repetitive EtOH precipitation. Notably, a second, more slowly moving DNA adduct is observed in reactions of M.EcoRI. This observation is consistent with work previously reported by Jeltsch and co-workers in which M.EcoRI was found to display significant promiscuity.[9] The precise origin and sequence selectivity of modification of this very slow mobility adduct is under active investigation.

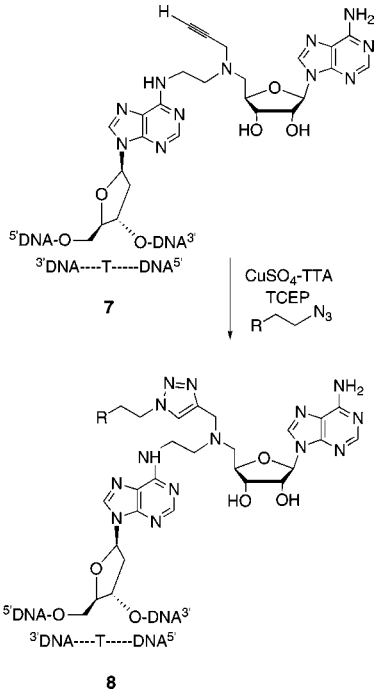
Scheme 2. Proposed Aziridinium Ion Formation



To ensure that the regiochemistry of DNA alkylation with **4** is the same as that normally observed for SAM-dependent methylation, we performed experiments involving premethylated substrates (Supporting Information). Alkylation reactions involving **4** were carried out on duplexes enzymatically methylated with SAM prior to treatment with fresh MTase and **4**. Confirmation that methylation of both M.TaqI and M.EcoRI sequence-bearing duplexes had taken place was accomplished by subjection of these DNAs to TaqI and EcoRI restriction endonucleases. DNAs subjected to SAM and MTase failed to act as sites of endonucleolytic cleavage; all other DNAs did, thus, confirming the integrity of substrate methylation. Similarly, methylated duplexes failed to undergo reaction when presented with fresh MTase and 50 μM **4**. That substrate DNA methylation precluded alkylation by **4** indicates that MTase-driven substrate alkylations by **4** and SAM share the same regiochemistry. This efficient and predictable site-specific alkylation of nucleic acids paved the way for novel elaboration of the modified sites by virtue of the propargyl substituent of **4**.

Bioconjugation via alkyne moieties is ideally suited to the Huisgen [2 + 3] cycloaddition. This abiotic chemoselective ligation of azides and alkynes affords highly stable triazole moieties under biologically relevant conditions.[10] The reaction has been extensively used in a number of biological motifs; the most effective incarnation calls for the use of Cu(I) and a unique tris-triazaloylamine (TTA) ligand along with either ascorbate or tris-carboxyethylphosphine (TCEP) as reductants for $CuSO_4$. The number of examples with nucleic acids is still relatively small presumably because of the well-known role that Cu(I) can play in Haber−Weiss redox cycling to produce hydroxyl radical.[11,12] However, it was not obvious that the Cu(I)−TTA complex can promote Haber−Weiss redox cycling. Moreover, if Cu(I)−TTA does permit hydroxyl radical formation, it is likely that *t*-BuOH present in the reaction would immediately sequester radical intermediates, thus circumventing DNA strand scission. As such, we envisioned the scenario in Scheme 3 where DNA

Scheme 3. Proposed Huisgen Cycloaddition with Alkyne Modified DNA



lesion **7** could render triazole **8** following reaction with alkyl azides.

The viability of the coupling indicated by Scheme 3 is supported by Figure 2. DNA modified with **4** (in an M.EcoRI-dependent fashion) underwent extremely facile coupling to azide **9** under aqueous conditions.[13] The slow mobility adduct formed (χ) is proposed to be the result of Huisgen [2 + 3] cycloaddition since the product is not apparent in reactions lacking alkyne or azide. This is best reflected in comparing lanes 3 to 5 and then 5 to 6 for both timepoints. Subjection of DNA modified with **1a** to azide, a 1:1 complex of Cu−TTA, and TCEP afforded no new radiolabeled product. The same conditions applied to DNA modified with alkyne **4** rendered the new DNA product noted

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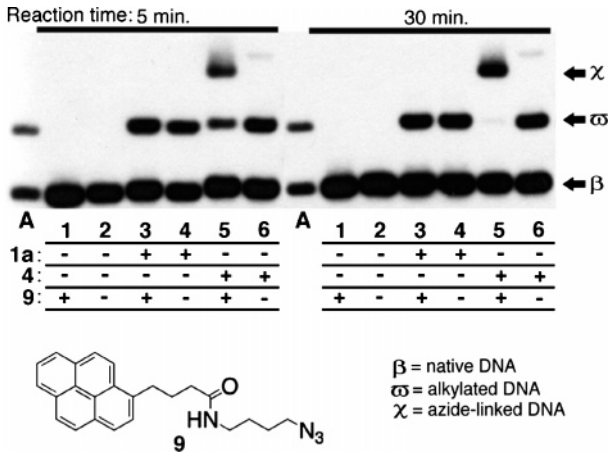

Figure 2. DPAGE analysis of click reactions between pyrene azide **9** and synthetic oligonucleotides modified either with **1a** or **4**. Lane A is a DNA−**4** conjugate control. Lanes 1 and 2 are reactions of DNA treated with M.EcoRI but no cofactor. Lanes 3 and 4 are reactions involving DNA subjected to M.EcoRI and **1a**. Lanes 5 and 6 are reactions using DNA treated with M.EcoRI and alkyne cofactor **4**. Reactions assayed in lanes 1−6 all contained 80 nM DNA duplex, 20 mM $NaHCO_3$, 1 mM $CuSO_4$:TTA complex, 2 mM TCEP, and 5% *t*-BuOH. All reactions were conducted at room temperature, and the time of reaction is noted above each series of lanes. Reactions monitored in odd-numbered lanes contained 2.5 mM azide **9**; even-numbered lanes/reactions lacked azide. In this manner, it was deduced that the generation of slow mobility material designated with χ is azide dependent.

by χ. Similarly, subjection of DNA modified with **4** to reaction conditions lacking **9** failed to render χ. We believe this to be the first entirely solution-phase[11a] example of nucleic acid-based click chemistry that does not call for extensive heating[11b] or templating effects.[11c] It is significant that during the course of EtOH precipitations following cycloaddition, we did not observe any isotope loss to supernatant, consistent with little or no Cu(I)-mediated DNA strand scission. This, in combination with the appearance of very clean DNA bands, suggests that, indeed, DNA can serve as an effective participant in Cu(I)-catalyzed click chemistry. We have also noted that ascorbate can be effectively substituted for TCEP without appreciable nucleic acid degradation (data not shown).

Coupling of alkyne-modified DNA and **9** proceeded very quickly as evidenced by the amount of product observed in lane 5 of Figure 2. Preliminary experiments (data not shown) suggest this is largely a function of Cu(I) availability. For solubility reasons, we generated a 1:1 complex of TTA ligand and $CuSO_4$ prior to cycloaddition. This contrasts with most click chemistry examples in which TTA and a Cu(I) source are added independently. In our hands, independent addition of $CuSO_4$ and TTA had a profoundly detrimental impact on coupling efficiency relative to the use of premixed $CuSO_4$:TTA.

The generality of the cycloaddition is depicted in Figure 3. Azides **10** and **11** were prepared with the expectation that size and charge differences would result in visible mobility

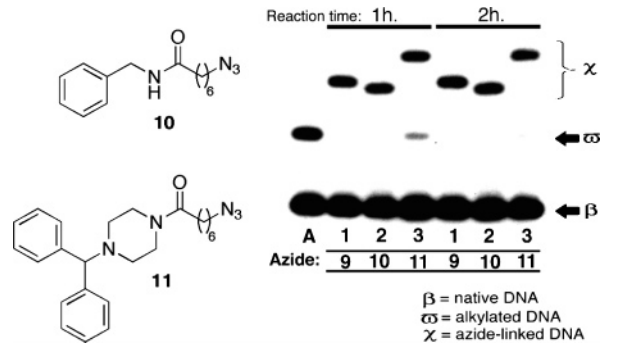

Figure 3. DPAGE analysis of click reactions of **4**-modified DNA and azides **9**−**11**. Lane A contained DNA−**4** conjugate standard, and lanes 1−3 contained reactions of **4**-modified DNA with 2.5 mM **9**, **10**, and **11**, respectively

differences among the variously modified DNAs. Subsequent click reactions performed with **10** and **11** versus **9** (all other factors the same) afforded products again denoted by "χ" in lanes 1−3. Particularly striking is that coupled product bearing the piperazine moiety of **11** is significantly more reduced in electrophoretic mobility than are products formed with **9** or **10**. Protonation of the piperazine nitrogen during electrophoresis is the likely cause of this difference. Comparison of the same reactions carried out for shorter times revealed very similar rates of product formation (data not shown). Importantly, these results refute the possibility that the data from Figure 2 were dependent upon the pyrene moiety of **9** and potential redox activities of the polycyclic moiety.

These data highlight two important concepts. First, synthetic cofactors need not be restricted only to 5′-aziridines, which have proven to be rather elusive synthetic targets. By demonstrating the viability of *N*-mustards such as **4**, we hope to hasten the rate at which these agents undergo further development as biochemical tools. In this vein, it is significant that **4** has been used very effectively to protect linearized pUC19 DNA from R.TaqI digestion following brief treatments with M.TaqI (unpublished data). *Thus, MTase-dependent delivery of **4** to DNA is not restricted to small duplexes.* Second, DNA can take part in click chemistry, and synthetic cofactor **4** represents a novel means by which to perform such chemistry in a sequence-selective manner.

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Supporting Information Available: Experimental procedures and characterization for substances **4** and **9**−**11** and biological protocols relating to Figures 1−3. This material is available free of charge via the Internet at http://pubs.acs.org.

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