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A New 1′-Methylenedisulfide Deoxyribose that Forms an Efficient Cross-Link to DNA Cytosine-5 Methyltransferase (DNMT)

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Although only four bases, adenine, guanine, cytosine, and thymine, encode all genetic information in DNA, there is a heritable "fifth" base, 5-methylcytosine, which can induce epigenetic changes that alter chromatin structures. Methylation at the 5-position of cytosine in a CpG dinucleotide is catalyzed by a conserved group of proteins called DNA cytosine-5 methyltransferases (DNMTs) by using *S*-adenosyl-methionine (SAM) as the cofactor (Figure 1A). This modification has a profound effect on gene expression. Many cancer cells are characterized by abnormal DNA methylation. Repetitive DNA sequences and some genes are hypomethylated and transcriptionally active, whereas many genes are hypermethylated and transcriptionally inactive.[1] Inhibition of human DNMTs has been shown to be an effective strategy to treat various cancers.[1e,f]

Despite our understanding of the DNA methylation status of many genes in cancer, we know little about which DNMTs mediate these effects at particular promoters, and even less about the protein complexes in which these enzymes function. In order to identify particular DNMTs, the design of a new probe that can efficiently trap these enzymes on specific DNA sequence is necessary. Although probes such as 5-azacytidine (5-Aza-dC) and 5-fluorocytidine (5-FdC), **F**, have been extensively used to trap DNMTs onto DNA (Figure 1, parts B and C),[2] both probes bear some shortcomings. The activation of the C5 carbon of the target cytosine in 5-FdC is an inefficient, slow process, and 5-Aza-dC can be hydrolyzed. Taking advantage of DNMTs' invariant active-site Cys residue and their base-flipping property,[3] we report here a new disulfide-based DNA probe, **B**, (Figure 1D and Figure 2) which provides a much faster trapping of DNMTs to DNA.

The 1′-methylenedisulfide deoxyribose probe was designed to trap DNMTs on DNA through formation of a disulfide bond with the protein's active-site Cys residue. Even though disulfide-based probes have been used to trap other proteins to DNA,[4] this probe is the first one designed to trap DNMTs. The synthetic scheme of the corresponding phosphoramidite is shown in Scheme 1. Briefly, Hoffer's chlorosugar (3,5-di-*O*-toluoyl-α,β−1-chloro-2-deoxy-D-ribofuranose)[5a] and β-cyanosugar (3,5-di-*O*-toluoyl-β−1-cyano-2-deoxy-D-ribofuranose)[5b] (**1**) were prepared as described previously. This compound was then converted to the 3′- phosphoramidite derivative **7** in 8 steps.

Using solid-phase DNA synthesis phosphoramidite **7** was incorporated into an oligonucleotide to give ssDNA-1 (Figure 2A). MALDI-TOF MS of ssDNA-1 before and after *tris*(2-carboxyethyl)phosphine (TCEP) treatment validated the incorporation of **7** into DNA as well as the presence of the disulfide group in the oligonucleotide (Figure S13, Supporting Information). Annealing ssDNA-1 with bases G, A, T, and C opposite to the modified **B** gave double-stranded dsDNA-3, dsDNA-4, dsDNA-5, and dsDNA-6, respectively (Figure 2A). For the purpose of comparison, we also synthesized ssDNA-2 which contained commercially available 5-FdC (**F**). Double-stranded dsDNA-7 and dsDNA-8 were prepared

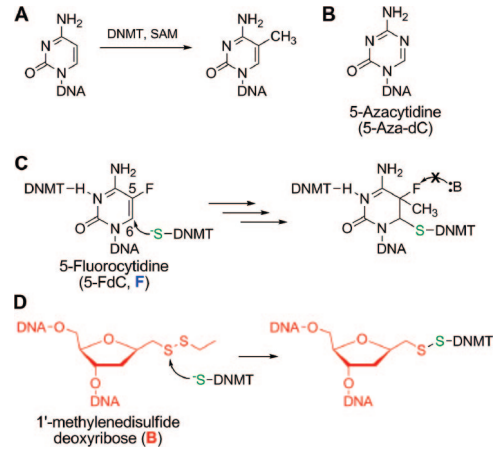

Figure 1. Probes used in trapping cytosine-5 methyltransferases (DNMTs). (A) Methylation of cytosine C5 by DNMTs using *S*-adenosyl-methionine (SAM). (B) 5-Azacytidine, a widely used DNMTs trapping probe. (C) Trapping of DNMTs using 5-fluorocytidine (**F**). (D) The 1′-methylenedisulfide deoxyribose (**B**) probe that can trap DNMTs through the active-site Cys residue.

with bases G and A opposite to **F** in the complementary strand, respectively (Figure 2A).

To test the cross-linking efficacy of the new base probe **B** (in dsDNA) to DNMTs we used a bacterial methyltransferase M. *Hha*I from *Hemophilus hemolyticus*. M. *Hha*I methylates the internal C in the target sequence 5′-GCGC-3′. This sequence was incorporated into our synthetic DNA with the target cytosine replaced with either **B** or **F**.

Wild-type M. *Hha*I was overexpressed and purified,[6a] and its cross-linking to the modified DNA was tested. We incubated 3 equiv of DNA (3−8) with 1 equiv of M. *Hha*I at 16 °C for 0.25−16 h. The reaction was quenched with methyl methanethiolsulfonate, and the cross-linked products were examined using nonreducing SDS-polyacrylamide gel electrophoresis. To our delight, all the probes containing **B** cross-linked efficiently with M. *Hha*I as shown by the appearance of a new band with retarded mobility on the SDS-PAGE gel (Figure 2B).

Importantly, time-course experiments demonstrated that our probe **B** cross-linked to M. *Hha*I much faster than the commercially available **F** (compare gel analyses B and C to gel analysis D in Figure 2). Even after lowering the protein and DNA concentrations to 2.5 μM, respectively, and performing reaction on ice, the cross-linking of M. *Hha*I with the **B**-containing probe 3 completes within one minute (Figure 2C), while it takes 16 h to obtain comparable cross-linking with the **F**-modified probe. Cross-linking of M. *Hha*I to the **F**-modified DNA worked better when the complementary strand contains G opposite to **F** (Figure 2D, lanes 5 and 6), whereas for DNA containing **B** there seems to be less preference for the base opposite to **B** (Figure 2B, lanes 5−8). Since **B** cannot form canonical DNA base pairs, it may be preferentially recognized by DNMTs in an unstable base pair.

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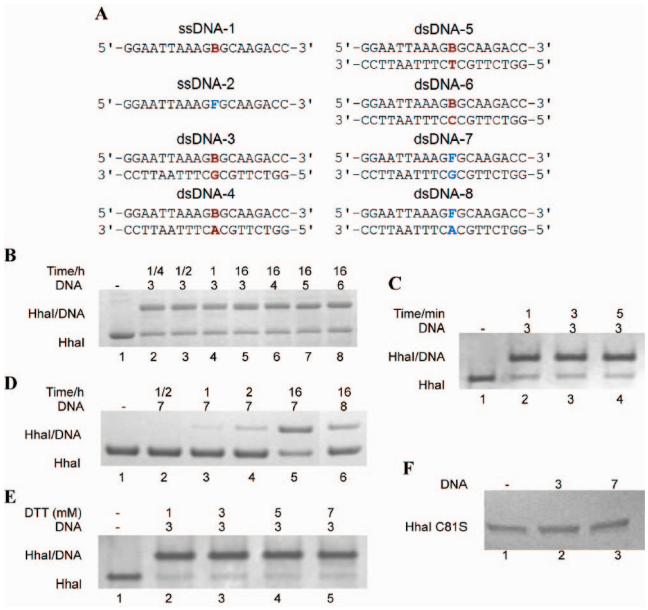
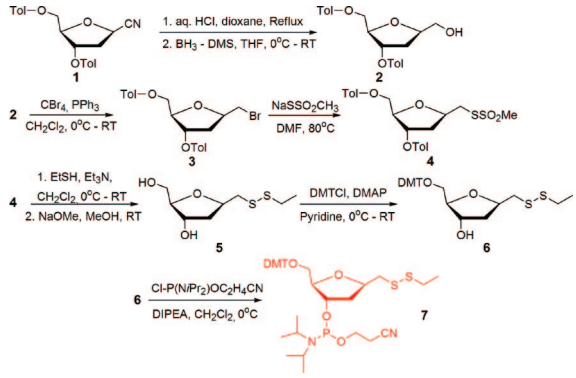


Figure 2. Cross-linking of a methyltransferase (M.) *Hha*I with various oligonucleotides. All the experiments contain 300 μM of SAM. Experiment C was carried out on ice, whereas all other experiments were carried out at 16 °C. All the reactions were quenched with 20 mM methyl methanethiolsulfonate, and SDS gel was run without DTT. (A) Oligonucleotides used in M. *Hha*I cross-linking; B indicates 1′-methylenedisulfide deoxyribose, F indicates 5-fluorocytidine. (B) Coomassie Blue-stained nonreducing SDS gel analysis of cross-linking between WT M. *Hha*I (10 μM) and DNA 3−6 (30 μM). The slow mobility band is the cross-linked complex band. Lanes 2−5 show the time-course experiments of the reaction between M. *Hha*I and DNA-3 at 16 °C. (C) Silver stained nonreducing SDS gel analysis of cross-linking between M. *Hha*I (2.5 μM) and dsDNA-3 (2.5 μM) after 1, 3 and 5 min on ice. (D) SDS gel analysis of the cross-linking between M. *Hha*I and DNA 7−8 (lanes 5−6); lanes 2−4 show the time course experiments. Even after 1 h incubation, we could barely observe cross-linking between DNA-7 and M. *Hha*I. (E) Silver-stained SDS gel analysis of the cross-linking reaction between M. *Hha*I (2.5 μM) and DNA-3 (2.5 μM) competed with reducing agent (DTT). (F) The C81S mutant M. *Hha*I did not show any cross-linking with DNA-3 and DNA-7.

Scheme 1. Synthesis of 1′-Methylenedisulfide Deoxyribose Phosphoramidite



We tested the cross-linking reaction between M. *Hha*I and DNA in the presence of varying amounts of DTT. Even with up to 7 mM of DTT, the cross-linking yield was not affected, highlighting the extremely high stability of the disulfide bond between the protein and the DNA probe (Figure 2E). The cross-linking is also selective to the **B**-containing probe as a different disulfide-modified DNA gave almost no cross-linking under the same reaction conditions (Figure S14, Supporting Information).

To confirm that the cross-linking is occurring to our probe through the active-site Cys81, we mutated this residue to Ser, and then overexpressed and purified the mutant enzyme.[6b] Both dsDNA-3 and dsDNA-7 failed to show any cross-linking with the mutant C81S M. *Hha*I (Figure 2F). This result suggests that the cross-linking between the wild-type M. *Hha*I and the **B**-containing DNA is occurring primarily through the active-site Cys81. The binding affinity of the mutant C81S M. *Hha*I to the **B**-containing dsDNA-3 in the absence of reducing agents was measured. K_d was determined to be ~20 μM (Figure S15, Supporting Information), which is higher than that reported between a 37 mer regular DNA and M. *Hha*I.[6c] To further validate that our probe works for cytosine-5 methyltransferases in general, we tested cross-linking between M. *Sss*I and our probes. A very good cross-linking yield was obtained between M. *Sss*I and dsDNA-3 (Figure S16, Supporting Information), further demonstrating that **B** can be a general and efficient cross-linker to trap cytosine-5 methyltransferases onto DNA.

In conclusion, we demonstrate here a newly designed 1′-methylenedisulfide deoxyribose that can efficiently trap DNMTs onto DNA. Considering the importance of DNMTs that control various biological processes, the development of this new probe will greatly aid the preparation of DNMT−DNA complexes for various studies. Although the current 1′-methylenedisulfide deoxyribose lacks base recognition by DNMTs and cannot be incorporated into DNA by polymerase, the fast and highly efficient cross-linking reaction will expedite the preparation of DNMT−DNA complexes for various applications. The probe may be incorporated into a specific sequence of promoter DNA for trapping and identifying DNMT that works on that sequence and the complex can be used as the bait to fish out partner proteins that help to direct DNA methylation of a specific promoter sequence. Beside proteomic studies, the high yields of DNMT-DNA complexes can be prepared for structural characterization as well. All these studies are currently undergoing in our laboratory.

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Supporting Information Available: Experimental details, Figures S13−S16. This material is available free of charge via the Internet at http://pubs.acs.org.

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