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Application of *N*-Halogeno-*N*-sodiobenzenesulfonamide Reagents to the Selective Detection of 5-Methylcytosine in DNA Sequences

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Supporting Information

ABSTRACT: To surmount the challenges of the locus determination and accurate quantification of 5-methyl-2′-deoxycytidine (^{5Me}dC) in DNA fragments that contain multiple ^{5Me}dC residues, we designed and synthesized two *N*-halogeno-*N*-sodiobenzenesulfonamide reagents that provide a new chemical method for probing ^{5Me}dC in DNA sequences. When the strategy we provided was combined with *β*-glucosyltransferase, ^{5Me}dC could be distinguished from 5-hydroxymethyl-2′-deoxycytidine (^{5hm}dC) and deoxycytidine (dC) through the introduction of a glucose moiety to the hydroxyl group of ^{5hm}dC.

5-Methylcytosine (5mC) is an essential epigenetic modification that frequently appears in CpG sequences and acts as an important factor in the silencing of genes.[1] In recent years, with the development of epigenetics, high importance has been attached to the selective detection of 5mC in genes, given the strong correlation of this genetic modification with various aspects of gene control, such as gene regulation, genomic imprinting, and X chromosome inactivation,[2] among other effects. It has been reported that a high level of 5mC at CpG islands within promoters and the global hypomethylation of genomic DNA, which induces gene instability, can produce the activation of oncogenes and the high occurrence of various diseases.[3−5] Thus, identification of the 5mC level and status in genes is important for the early detection and treatment of many tumors. However, because of the subtle differences between deoxycytidine (dC) and 5-methyl-2′-deoxycytidine (^{5Me}dC), distinguishing ^{5Me}dC from dC is a difficult and challenging task.

To date, many initiatives have attempted to address this challenge. Various methods based on restriction enzymes,[6] methylation-specific PCR amplification,[7] photooxidation,[8] and DNA photoligation[9] have been developed for the detection of 5mC. Meanwhile, other chemical methods involving the conversion of either dC or ^{5Me}dC residues in DNA have been proposed. For instance, the Maxam−Gilbert chemical modification was applied to identify the ^{5Me}dC residues indirectly by means of the interference of the methyl group in the reaction with hydrazine.[10] Moreover, OsO_4 has been assessed as a potential reagent for differentiating between ^{5Me}dC and dC because OsO_4 reacts differently to the distinct nucleophilicities of the double bonds in ^{5Me}dC and dC.[11,12] However, the OsO_4 technique cannot address situations involving the oxidation of thymidines. Other combinations, such as the V_2O_5/LiBr or $NaIO_4$/LiBr pairings, have partially solved the aforementioned detection difficulties, but the sensitivity of these assays requires additional improvement.[13] Even the single molecule real time sequencing (SMRT) technique, which can directly detect DNA methylation without bisulfite conversion, possesses the problem of a high error rate.[14,15] In addition, traditional detection methods such as bisulfite sequencing[16,17] are time-consuming and cumbersome. The other reported methods for detecting ^{5Me}dC are either instrument-intensive or limited by their sensitivity and accuracy. In view of the aforementioned problems with traditional sequencing methods, it is vitally important to develop a rapid and sensitive chemical method for the accurate quantification of the cytosine methylation status of genomic DNA.

The apparent differences between ^{5Me}dC and dC are the nucleophilicity of the double bond in the pyrimidine rings and the steric hindrance that is caused by the methyl group of ^{5Me}dC; this hindrance led us to identify and characterize a new specific base-sensitive compound for the quantitative conversion of ^{5Me}dC or dC. Sharpless and co-workers have reported that chloramine-T (TsN−ClNa)[18−20] can serve as an excellent nucleophile for attacking the double bond of olefins and their derivatives, producing outstanding yields, particularly for substrates with electron-donating groups. The similarity of dC and ^{5Me}dC residues to olefin derivatives prompted us to design and synthesize various chloramine-T derivatives to assess their activities toward the specific substrates dC and ^{5Me}dC.

Here we report on the results obtained for these derivative compounds, *N*-sodio-*N*-chloro-*p*-nitrobenzenesulfonamide (**1**) and *N*-sodio-*N*-bromo-*m*-nitrobenzenesulfonamide (**2**) (Figure 1). By combining the results obtained using both of these compounds, we can accurately identify the number and loci of ^{5Me}dC residues in DNA sequences. This novel indirect detection method is capable of overcoming the problems discussed above and can detect multiple ^{5Me}dC residues and loci at a time, satisfying the need for the reliable detection of ^{5Me}dC.

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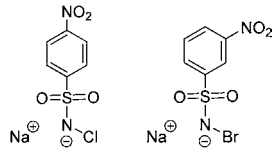

Compound **1** Compound **2**

Figure 1. Structures of the two compounds we designed and synthesized.

At first, we prepared oligodeoxynucleotide 1 (ODN 1), a short sequence of VHL tumor-suppressor gene containing both dC and ^{5Me}dC residues.[21] ODN 1 was subjected to two different treatments, each of which was expected to target different base residues selectively, allowing us either directly or indirectly to distinguish between dC and ^{5Me}dC. Both treatments employed a mixture of 100 mM Tris-HCl buffer (pH 5.0), 2 mM **1** or **2**, and 50% acetonitrile. The difference between the two treatments was that the reaction with **1** was performed with 100 μM I_2 and incubated at 60 °C for 1 h, whereas the other reaction was performed under milder conditions, proceeding at 50 °C for 10 min without any catalyst. As shown in Figure 2a, it was expected that ODNs would be converted into two products after the treatments with **1** or **2**. After desalting, the obtained DNA products were treated with hot piperidine (90 °C, 30 min) to induce cleavage at damaged pyrimidine bases, and the calculations of bond energies in the pyrimidine rings were performed [see the Supporting Information (SI)] to study the possible mechanism (Table S1 in the SI). The final results were analyzed through denaturing polyacrylamide gel electrophoresis (PAGE); the electrophoresis results are depicted in Figure 2b. In lane 1 of this figure, the results of the reaction with **1** can be observed; this compound presented excellent selectivity, producing an obvious cleavage at the dC site of the tested oligodeoxynucleotide. By contrast, in the reaction with **2** (Figure 2b, lane 2), two products generated by cleavage at the dC and ^{5Me}dC sites of the target DNA sequence can clearly be observed. Impressively, both compounds demonstrated high selectivity, with negligible reaction at the vulnerable dT and dG sites[13,22] of the DNA. Moreover, by subtracting the lane 1 results from the lane 2 results, we could indirectly obtain an accurate quantification of the number and loci of the ^{5Me}dC residues in the tested DNA; thus, this method possesses the potential to detect ^{5Me}dC residues in a convenient and precise way.

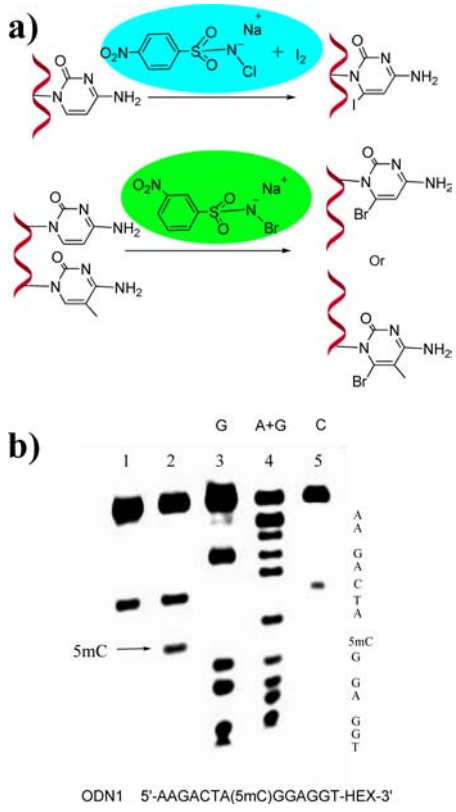

Figure 2. (a) Proposed products of reactions of ODNs **1** (blue) and **2** (green). (b) Sequence of ODN 1 and PAGE analysis of this ODN after relevant treatments. Lane 1: labeled DNA was incubated at 60 °C for 1 h in a mixture of 100 mM Tris-HCl buffer (pH 5.0), 100 μM I_2, 2 mM **1**, and 50% acetonitrile, giving a clearly visible cleavage at the dC site. Lane 2: two cleavage products at the dC and ^{5Me}dC sites were observed when labeled DNA was incubated in a solution containing 2 mM **2**, 100 mM Tris-HCl buffer (pH 5.0), and 50% acetonitrile at 50 °C for 10 min. Lanes 3–5: Maxam–Gilbert G, A + G, and C sequencing lanes.

The sensitivity and accuracy of this method were tested later by applying this method to longer sequences containing multiple dC and ^{5Me}dC residues. All of these prepared ODNs were short sequences of VHL tumor-suppressor gene. Denaturing PAGE analyses of ODN 4 containing five ^{5Me}dC residues after treatment with **1** and **2** using the protocol described above are depicted in Figure 3a, which clearly indicates the number and exact loci of the ^{5Me}dC residues in the tested DNA sequence without producing any additional cleavage at the dT and dG sites. To ensure the selective cleavage of dC and ^{5Me}dC a longer sequence (50 mer) with eight ^{5Me}dC residues (ODN 5; Figure 3b), a lower concentration of **2** (250 μM) and a lower pH (3.0) were adopted. PAGE analyses of other strands with different numbers of ^{5Me}dC residues after cleavage by **1** and **2** and hot piperidine treatment are presented in Figure S1 in the SI. When the strategy was performed directly on the double-stranded DNA (dsDNA), we still observed the same piperidine-sensitive cleavage sites compared with single-stranded DNA (ssDNA). Thus, this method can also be used to identify the amount and loci of ^{5Me}dC in dsDNA (Figure S2).

The methylation-selective methods that have previously been reported in the literature lack the ability to discriminate between 5-hydroxymethylcytosine (5hmC) and 5mC,[23] which limits their applications with respect to the detection of methylation. To confirm that our approach demonstrates a preference for ^{5Me}dC, synthetic 24-mer single-stranded ODN 3′ containing two dC, two ^{5Me}dC, and one ^{5hm}dC was designed. Another single-stranded DNA, ODN 3, which had the same sequence as ODN 3′ except that the ^{5hm}dC residue was replaced with another ^{5Me}dC residue, was chosen as a control. Applying the method provided, we observed a relatively weaker band at the site of ^{5hm}dC from this comparative examination. However, the difference in steric hindrance due to ^{5Me}dC and ^{5hm}dC is not sufficient for the discrimination of ^{5m}dC and ^{5hm}dC using this method. Thus, we introduced a glucose moiety to the hydroxyl group of ^{5hm}dC by using β-glucosyltransferase (β-GT)[15,24] to increase the steric hindrance of ^{5hm}dC further. As shown in Figure 4, the band corresponding to ^{5hm}dC completely disappeared in lane 4, indicating that the double

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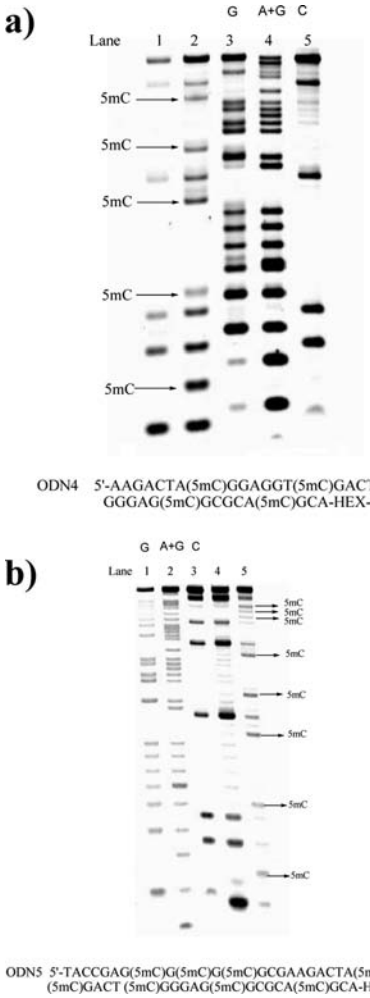

Figure 3. (a) Sequence of ODN 4 and PAGE analysis of the reaction. Lanes 1 and 2: DNA was treated with **1** and **2**, respectively, under the same reaction conditions described in Figure 2. Lanes 3−5: Maxam−Gilbert G, A + G, and C sequencing lanes for ODN 4. (b) Sequence of ODN 5 and PAGE analysis of this ODN after the treatment described above. Lanes 1−3: Maxam−Gilbert G, A + G, and C sequencing lanes. Lane 4: ODN 5 was treated with **1** under the same reaction conditions as described above. Lane 5: ODN 5 was treated with a solution containing 250 μM **2**, 100 mM Tris-HCl buffer (pH 3.0), and 50% acetonitrile at 50 °C for 10 min.

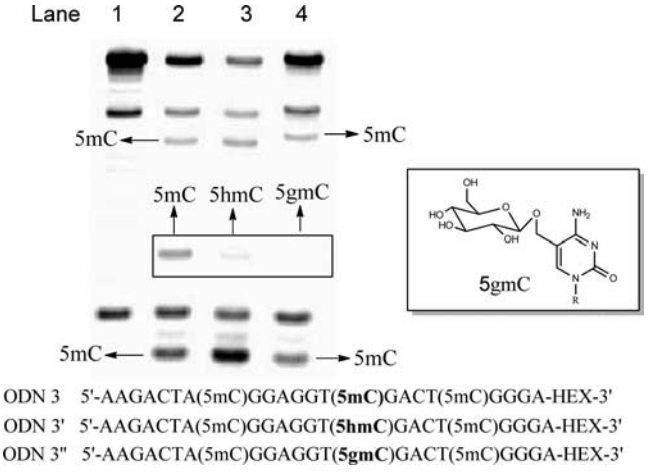

Figure 4. Sequences of single strands ODN 3, ODN 3′ and ODN 3″ and polyacrylamide gel electrophoresis (PAGE) analysis of these sequences. Lane 1, ODN 3′ was treated with **1**, indicating the locations of dC. Lane 2, ODN 3 was treated with **2** under the condition described above, demonstrating the amount and locations of dC and ^{5Me}dC, which was used as a comparation to lane 3 and lane 4. Lane 3, ODN 3′ that contain ^{5hm}dC residue was performed at the same condition as lane 2, demonstrating a relative weaker band corresponding to 5hmC. Lane 4, 5hmC was catalyzed to form 5gmC by β-glucosyltransferase and then the corresponding DNA 3″ was reacted with **2** under the same condition. No visible cleavage was observed at the site of 5gmC.

bond of ^{5hm}dC is protected from forming bromonium cation intermediate as a result of the increased steric effect.

Otherwise, excitingly, **1** demonstrates the particular ability to cleave dC quantitatively (lanes 1 in Figure 3a,b) without significant cleavage at any other residues. Therefore, this compound can offer another alternative for preparing C lanes. Furthermore, because of the toxicity and explosiveness of hydrazine, the Maxam−Gilbert method is a much more dangerous and cumbersome way to create C ladders than our one-step strategy.

To provide further confirmation of the mechanism of the selective transformation of dC and ^{5Me}dC, we analyzed the MALDI-TOF mass spectrometry data for an ODN with the same sequence as ODN 1 but without the fluorophore (Figure S11) after it had first been treated with **1** or **2** and then purified by HPLC (Figure S12). These data indicate that the possible products from the reaction are substitutions of hydrogen in dC or ^{5Me}dC residues; these substitutions could occur through the mechanisms we have proposed in Scheme S1 in the SI. In particular, we suggest that in the reaction with **1**, iodine reacts with **1** to produce sodium iodide and an I^+ source, which then reacts with cytosine to form an iodonium cation intermediate.[18,25] Because of the large atomic radius of iodine, I^+ has a preference for dC because of the steric hindrance created by the methyl group in ^{5Me}dC. Negligible cleavage of dT residues by **1** was observed for the same reason. However, the reaction with **2** can generate a Br^+ source through an uncatalyzed hydrolysis. Because of the different radii of iodine and bromine atoms, both ^{5Me}dC and dC can readily form a bromonium cation intermediate, but it is hard for ^{5gm}dC to form this intermediate because of its larger steric hindrance. Both I^+ and Br^+ ions barely attack dT residues because of their electron-deficient C5−C6 double bonds and the steric hindrance of the methyl group at their C5 sites.

Initially, various other Br^+ and I^+ sources were assessed for selectivity at the C5−C6 double bonds of dC and ^{5Me}dC. Like **1**, chloramine-T also possesses the ability to generate iodonium cation intermediates in a reaction catalyzed by I_2. However, because of the electron-donor group at the para position on the benzene ring of chloramine-T, the cleavage of dC by chloramine-T is relatively minimal, as observed in PAGE analyses that demonstrated a lower yield for the reaction with chloramine-T than the reaction with **1** (Figure S3). A variety of other catalysts (e.g., $CuCl_2$, CuCl, and $KOsO_4$) were tested, but no reactions could be observed at the sites of ^{5Me}dC or dC residues (Figure S4). Thus, it appears that the reaction of **1** at dC sites can be attributed to the formation of I^+. Meanwhile, a variety of substituted bromoamine-T derivatives were designed and screened with the goal of selecting the most sensitive one among the examined compounds. Several of the tested compounds, such as bromamine-B ($PhSO_2$−NBrNa), which contains no substituent on its benzene ring, also demonstrated

the ability to react with ^{5Me}dC and dC; however, some cleavage bands were relatively weaker when compared with **2**. Furthermore, the same results were obtained when the ODNs were treated with either CH_3PhSO_2−NBrNa or *p*-NO_2PhSO_2−NBrNa (Figure S5). In general, the results obtained above demonstrate that all of the bromamine-T derivatives basically have the ability to produce piperidine-sensitive cleavage sites at dC and ^{5Me}dC residues upon treatment under the optimum conditions. However, because of the weaker cleavage bands at some sites when these other derivatives were used, we chose the relatively better one, compound **2**.

Meanwhile, the influences of the pH and reaction temperature were also tested. The results show that the pH has little influence on the reaction with **1** but greatly influences the result of the reaction with **2** because of the hydrolytic process. In addition, the reaction temperature impacts both reactions. Lower temperature may lead to lower yields, while higher temperature can decrease the selectivity, producing unwanted cleavage bands at the sites of dT (Figures S6 and S7). Besides, the acetonitrile solvent was added to the solution to increase the nucleophilicity of the reaction.[19]

In summary, we have developed a novel indirect chemical method for distinguishing between cytosine and 5mC in DNA sequences. In addition, this method allows us to identify the number and loci of the ^{5Me}dC residues both in ssDNA and dsDNA accurately and efficiently by combining results obtained using the two compounds examined in this study. Furthermore, this strategy also enabled us to distinguish ^{5Me}dC from ^{5hm}dC by increasing the steric hindrance of 5hmC using β-glucosyltransferase.

■ ASSOCIATED CONTENT

Supporting Information

General methods; synthesis and characterization data; experimental details of Maxam−Gilbert methods for G, A + G, and C sequencing lanes; other PAGE analyses; ab initio calculations of the energies of C═C bonds in the pyrimidine rings; HPLC and MALDI-TOF MS data; and detailed proposed mechanisms of the reactions with **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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