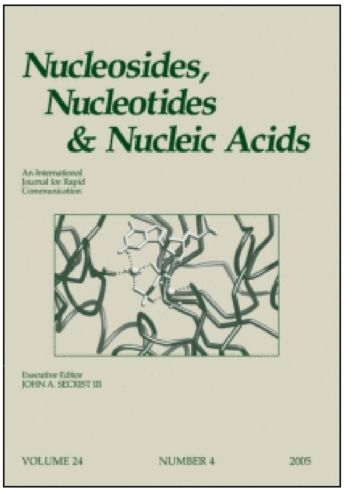

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5-METHYLCYTOSINE-SELECTIVE OSMIUM OXIDATION

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□ *Methylcytosine was efficiently and easily modified through osmium oxidation. Methylcytosine-selective oxidation is applicable to detection of the cytosine methylation status at a specific site of a long sequence using the formation of a bulge structure by hybridization with a guide DNA.*

Keywords 5-Methylcytosine; osmium oxidation; fluorescence

INTRODUCTION

5-Methylcytosine (M) is a common modified nucleobase, which frequently appears in genomic DNA, and plays a key role in epigenetic events, which strongly affect the control of gene expression and cell differentiation.[1] Analysis of the cytosine methylation status of a gene is very important for understanding the expression mechanism of genetic information. However, to distinguish M from C is not easy. For evaluation of the methylation status of genes, several methods showing a C-positive and M-negative signal have been used, such as a cleavage assay with methylation-insensitive restriction enzymes[2] and bisulfite DNA sequencing.[3] In these cases, primer design and complete modification of DNA are very significant in order to avoid false-positives for M and thus to obtain reliable results. The existence of a more rapid and selective chemical reaction capable of distinguishing between M and C would be promising as a useful method for efficiently analyzing the status of cytosine methylation at a specific site in a gene.

We report M-selective oxidation and its application to epigenotyping.[4] M was efficiently and easily modified through osmium oxidation. M-selective oxidation is applicable to detection of the cytosine methylation status at a specific site of a long sequence using the formation of a bulge structure by hybridization with a guide DNA.

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MATERIALS AND METHODS

Osmium Oxidation of Oligodeoxynucleotides (ODNs)

^{32}P-5′-End-labeled ODN to be examined was incubated in a solution of 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, 1 mM EDTA in 100 mM Tris-HC1 buffer (pH 7.7) and 10% acetonitrile at 0°C for 5 minutes. A reaction mixture was ethanol-precipitated with the addition of 15 μL of 3 M sodium acetate (pH = 5.0), 10 μL of salmon sperm DNA (1 mg/mL), and 1 mL of cold ethanol. The precipitated ODN was resolved in 50 μL of 10% piperidine (*vlv*), heated at 90°C for 20 minutes evaporated by vacuum rotary evaporation to dryness, and then resuspended in 5–20 μL of 80% formamide loading buffer. The samples (1 μL, 3–10 kcpm) were loaded onto 15% denatureing 19:1 acrylamide-bisacrylamide gel containing 7 M urea, electrophoresed at 1900 V for approximately 1 hour.

Product Analysis

The reaction mixture was prepared in a 50 μL total volume containing 100 μM 5′-d(AAAAAAGMGAAAAAA)-3′, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, 1 mM EDTA, 100 mM Tris-HCI buffer at pH 7.7 and 10% acetonitrile. After addition of 25 μL of 10 mM potassium osmate solution, the sample solution was incubated at 37°C for 6 hours. After desalting by Micro Bio-Spin Chromatography Columns, osmate complex was purified by reverse phase HPLC on a 5-ODS-H column. The purified osmate complex was treated with sodium sulfite for 3 hours at 50°C. The resulting ODN was purified by HPLC. The molecular weight of ODN obtained by MALDI-TOF mass measurement was in good agreement with the expected values.

RESULTS AND DISCUSSION

We initially prepared a ^{32}P-labeled oligodeoxynucleotide, 5′-^{32}P-d(AAAAAAGNGAAAAAA)-3′ (**ODN(N)**, N = M or C), that possessed a single pyrimidine base. **ODN(N)** was added to a mixture (Os-mix) of 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), and 100 mM bipyridine in 100 mM Tris-HCl buffer (pH = 7.7), 1 mM EDTA, and 10% acetonitrile, and then the reaction mixture was incubated at 0°C for 5 minutes. The oxidized strand was cleaved at a damaged pyrimidine base with hot piperidine (90°C, 20 minutes), and the products were analyzed as a band for a shortened strand using polyacrylamide gel electrophoresis. The result for the reaction of **ODN(N)** is shown in Figure 1. The strand cleavage at C of **ODN(C)** was negligible (lane 1), whereas **ODN(M)** was sequence-selectively

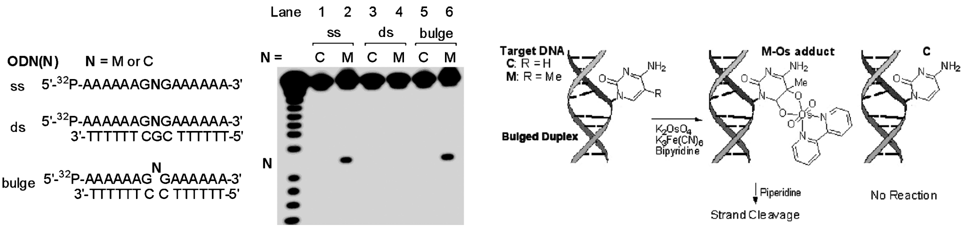

FIGURE 1 Sequence of ^{32}P-labeled **ODN(N)** and PAGE analysis of strand cleavage through Os oxidation and hot piperidine treatment. **ODN(N)** was incubated in a solution of 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, 1 mM EDTA in 100 mM Tris-HCl buffer (pH = 7.7) and 10% acetonitrile at 0°C for 5 minutes, and then treated with hot piperidine (90°C, 20 minutes). The products were analyzed using polyacrylamide gel electrophoresis. Lane 1, single strand, N = C; lane 2, single strand, N = M; lane 3, full-matched duplex, N = C; lane 4, full-matched duplex, N = M; lane 5, bulged duplex, N = C; lane 6, bulged duplex, N = M.

cleaved at M (lane 2). The MALDI-TOF MS data of **ODN(M)** treated with Os-mix suggested the formation of an adduct in which the osmium complex is attached to **ODN(M)** (Figure 2). This adduct was converted into a product in which M was oxidized to 5-methylcytosine glycol, when treated with sodium sulfite.[5]

On the other hand, the duplex formation strongly suppressed M oxidation. We examined the reaction for the hybrid of **ODN(M)** and the complementary strand 5′-d(TTTTTTCGCTTTTTT)-3′. The strand cleavage of the duplex was negligible, which is quite different from the effective cleavage of single-stranded **ODN(M)** (lanes 3 and 4). This weak reactivity is probably

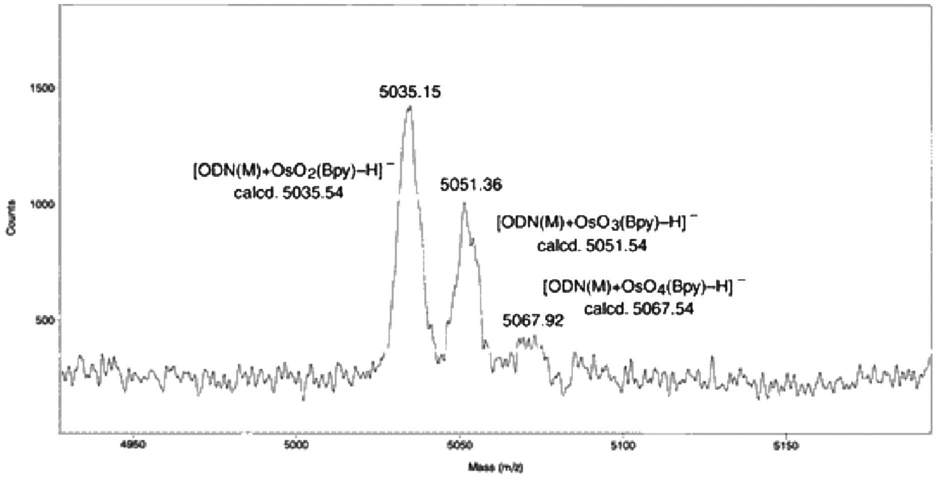

FIGURE 2 Mass spectrum of **ODN(M)**-Os adduct. The mass was determined with a MALDI-TOF MS (acceleration voltage 21 kV, negative ion mode) with 2′,3′,4′-trihydroxyacetophenone as matrix, using T 8-mer ($[M - H]^-$2370.61) and T 27-mer ($[M - H]^-$8150.33) as an internal standard. [(**ODN(M)**+OsO_4(Bpy)−H)$^-$], calcd. 5067.54, found 5067.92; [(**ODN(M)**+OsO_3(Bpy)−H)$^-$], calcd. 5051.54, found 5051.36; [(**ODN(M)**+OsO_2(Bpy)−H)$^-$], calcd. 5035.54, found 5035.15.

attributed to inhibition of the attack of an osmium complex on the π-orbital of a C5–C6 double bond by the base stacking of the duplex structure.

The oxidation efficiency of M-bulged duplex in Os-mix is quite different from that of C-bulged duplex. The oxidation of these bulged duplexes observed for the first one minute was fitted to a first-order rate equation. The calculated rate constants for M-bulged and C-bulged duplexes were 1.11×10^{-2} and 2.51×10^{-5} s^{-1}, respectively. Electron donation to a C5–C6 double bond by a methyl group strongly contributes to efficient reaction for the M base. It has been reported that the calculated and experimental ionization potentials of M are much lower than those of C.[6]

In conclusion, we have described the osmium oxidation, of M, M was efficiently and easily modified through osmium oxidation. M-selective oxidation is applicable to detection of the cytosine methylation status at a specific site of a long sequence using the formation of a bulge structure by hybridization with a guide DNA. This reaction will serve as an alternative method for the epigenotyping of a small amount of gene sample.

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