Synthesis and characterization of the 5-methyl-2′-deoxycytidine glycol–dioxoosmium–bipyridine ternary complex in DNA†

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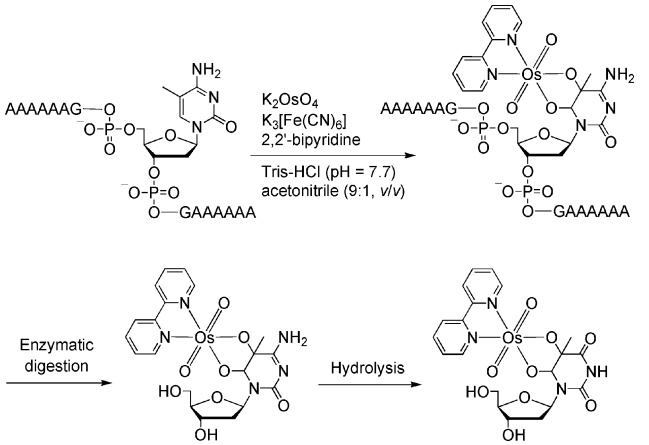
It has been established that approximately 80% of a 5-methyl-2′-deoxycytidine glycol–dioxoosmium(VI)–bipyridine ternary complex, which is known to be produced as one of various consequences of oxidative damage of DNA and is formed in a key step of a recently developed DNA methylation detection method, has the 5*R*,6*S* configuration.

Attack by oxidants is a major source of environmental damage to DNA and has been extensively studied *in vitro*.[1] Pyrimidines methylated at C5, such as thymine and 5-methylcytosine, are well known to be oxidized to the 5,6-glycol form by oxidation of a C5–C6 double bond with osmium tetroxide.[2–5] A combination of potassium osmate(VI), potassium hexacyanoferrate(III), and 2,2′-bipyridine also efficiently oxidizes 5-methylcytosine (M) in DNA, resulting in the formation of a stable methylcytosine–osmium–bipyridine ternary complex.[6] The formation of this complex is of great value as a pivotal reaction for a convenient method for sequence-specific detection of DNA methylation.[7–9] However, the absolute structure of the resulting ternary complex is still unclear, although the structure of the complex has been discussed by Behrman *et al.* at a nucleobase level using oxidation with osmium tetroxide.[3] Characterization of the absolute configuration of the ternary complex being generated in DNA will strongly support the design of improved molecular devices for methylation analysis. We herein report determination of the absolute structure of the osmium-centered complex produced by oxidation of 5-methyl-2′-deoxycytidine in DNA.

The ternary complex at 5-methylcytosine in DNA was synthesized by oxidation of an M-containing oligodeoxyribonucleotide (ODN), 5′-d(AAAAAAGMGAAAAAA)-3′, in a solution of 5 mM potassium osmate(VI), 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, 1 mM EDTA in 100 mM Tris-HCl buffer (pH 7.7) and 10% acetonitrile (Scheme 1). The starting ODN was completely consumed by reaction at 50 °C for 1 h, and converted into a product that appeared as a single peak in HPLC analysis.[10] The addition of the metal complex onto DNA was confirmed by MALDI-TOF mass analysis ([(M − H)⁻], calc.: 5067.54; found: 5067.92).[6] The ODN with an osmium-centered complex was digested to the nucleoside level using a mixture of snake venom phosphodiesterase, nuclease P1, and alkaline phosphatase, and then the nucleosides produced were analyzed by HPLC analysis (Fig. 1a). An HPLC profile monitored at 313 nm showed the existence of two sharp peaks with an area ratio of 79 : 21.



Scheme 1 Formation of a stable methylcytosine–osmium–bipyridine ternary complex in DNA and its degradation by enzymatic digestion and hydrolysis.

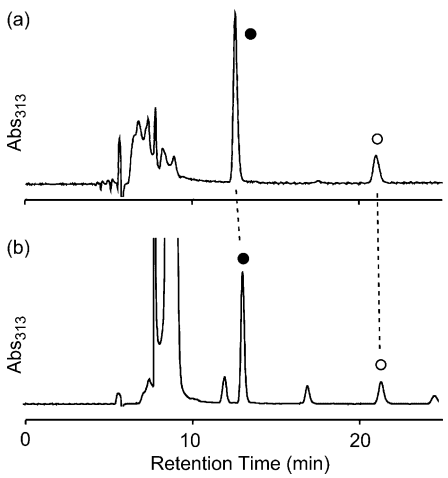


Fig. 1 Product analysis for osmium complexation to 5-methylcytosine. The reaction samples were analyzed by HPLC on an ODS column (elution with a solvent mixture of 0.1 M triethylammonium acetate, pH 7.0, 7% acetonitrile over 30 min at a flow rate of 3.0 mL min^{-1}). Peak signals were detected using absorption at 313 nm. Shaded and open circles show the major and minor isomeric complexes, respectively. (a) HPLC profile of enzymatically digested products of osmium-treated ODN 5′-d(AAAAA-AGMGAAAAAA)-3′. (b) HPLC profile of the crude products synthesized by osmium complexation of 5-methyl-2′-deoxycytidine.

The ternary complex was prepared from 5-methyl-2′-deoxycytidine using the complex-forming protocol for ODN, and then analyzed by HPLC. Two peaks overlapping the peaks observed in Fig. 1a were detected with an 80 : 20 area ratio in the

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HPLC profile including several other small peaks (Fig. 1b),[11] and analyzed by NMR. As a result, both products were identified as the stereoisomers of the osmium-centered complexes including 5-methyl-2′-deoxycytidine glycol and a bipyridine ligand. In addition, the spectra of the products exhibited an NOE signal between a methyl group at C5 and a hydrogen atom at C6 of the methylcytosine moiety, suggesting that the products were an osmate ester with either the (5*R*,6*S*)- or the (5*S*,6*R*)-glycol.

For a more detailed structural analysis, the ternary complex from 5-methyl-2′-deoxycytidine was hydrolyzed according to the protocol reported by Behrman in which the osmium–bipyridine complex of the 1,5-dimethylcytosine base was deaminated into the corresponding thymine derivative in aqueous media.[3] The two isomeric complexes obtained from 5-methyl-2′-deoxycytidine were left in water at 50 °C for three days. A new peak, which seems to be the deamination product, was observed in HPLC analysis for each isomeric complex (Fig. 2). These hydrolyzed products were identified using the osmium complex of thymidine, which was prepared by the protocol described above. The HPLC analysis for the reaction products from thymidine showed two peaks with an area ratio of 81 : 19 (see ESI†), which overlapped the hydrolyzed products of the ternary complexes obtained from 5-methyl-2′-deoxycytidine. The major product was crystallized in water and the structure was determined by X-ray crystallography (Fig. 3).[12] The complex had a slightly distorted octahedral geometry with coplanar glycol oxygens (O(4) and O(5)) and bipyridine nitrogens (N(3) and N(4)). The two Os–O double bonds (Os(1)–O(8) and Os(1)–O(9)) were *trans*, and tilted 3–6° to the bipyridine side. The structure of thymine glycol in the complex was the 5*R*,6*S* configuration. This configuration is the same as the structure of the major isomer of thymine glycol predicted from previous NMR studies.[13–15] In addition, the torsion angle between the thymine ring C(2)–C(3) and C(4)–N(1) is 49.0°, and thus the oxidized thymine base no longer has a planar structure. The *N*-glycosyl bond is still in an *anti* conformation, and the puckering of the ribose ring also remains in the C2′-*endo* conformation. As a result of X-ray crystallography, the major complex obtained from 5-methylcytosine in DNA can be concluded to be a dioxoosmium(VI) complex with (5*R*,6*S*)-methylcytosine glycol. The minor complex, which has the *syn* configuration at C5 and C6 and the same mass as the major complex, is thus a diastereomeric isomer containing (5*S*,6*R*)-methylcytosine glycol. The predominant formation of the (5*R*,6*S*)-glycol is probably due to the larger contribution of the configuration of the nucleoside rather than that of the whole structure of the DNA strand, because the ratio of the major and minor stereoisomers was approximately 80 : 20 both for 5-methylcytosine in a DNA strand and for 5-methyl-2′-deoxycytidine. A bulging-out C5′ of 5-methyl-2′-deoxycytidine, which includes an *anti*-formed glycosyl bond and a C2′-*endo* ribose ring, should determine the direction of attack of an active osmium–bipyridine complex to the C5–C6 double bond.

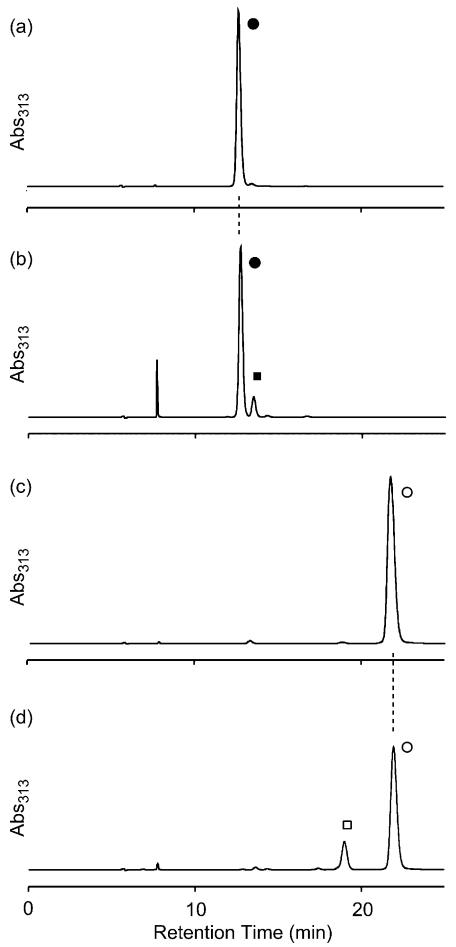

Fig. 2 Product analysis for hydrolysis of the osmium complexes obtained from 5-methyl-2′-deoxycytidine. The reaction samples were analyzed by HPLC under the condition described in Fig. 1. Shaded and open circles show the major and minor isomeric complexes before hydrolysis, respectively. Shaded and open squares show new products after hydrolysis of each complex. (a) Before hydrolysis of the major isomeric complex. (b) After incubation of the major isomeric complex for three days at 50 °C. (c) Before hydrolysis of the minor isomeric complex. (d) After incubation of the minor isomeric complex for three days at 50 °C.

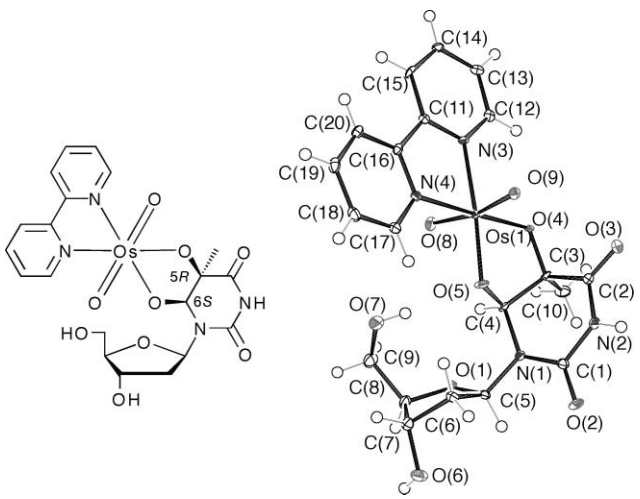

Fig. 3 A view of the molecular structure of the (5*R*,6*S*)-thymidine glycol–dioxoosmium(VI)–bipyridine ternary complex as the major isomeric complex, showing the atom-labelling scheme. Displacement ellipsoids are drawn at the 50% probability level.

In conclusion, we have synthesized a DNA strand containing a 5-methyl-2′-deoxycytidine glycol–dioxoosmium–bipyridine ternary complex and determined its absolute structure. Approximately 80% of the complex was a dioxoosmium(VI) complex

with a (5*R*,6*S*)-glycol structure. Characterization of the structure of the osmium-centered complex in DNA discloses one of the mechanisms of oxidative damage occurring in a DNA strand and would strongly support new drug design for efficient DNA methylation analysis.

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10 The peaks at 7–10 min are a mixture of bipyridine and its derivatives.
11 Three small peaks in Fig. 1b correspond to bipyridine complexes. These peaks were observed for the osmium complexation of nucleosides because the reaction mixtures were analyzed without any salt removal process.
12 The 5-methyl-2′-deoxycytidine glycol–dioxoosmium–bipyridine complex does not crystallize in water. CCDC reference number 665206. For crystallographic data in CIF or other electronic format see DOI: 10.1039/b716400a.
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