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Selective covalent binding of a positively charged water-soluble benzoheterocycle triosmium cluster to single- and double-stranded DNA

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This paper is dedicated to Dick Fish in honor of his 65th Birthday and in recognition of his pioneering contributions to the field of Bioorganometallic Chemistry

Abstract

The water-soluble triosmium cluster $[Os_3(CO)_9(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-}H)(P(OCH_2CH_2N(CH_3)_3I)_3)]$ (**4**) was tested for its reactivity with plasmid DNA. In contrast to the band retardation previously observed with a related series of positively charged clusters, an intensification and retardation of three discrete bands was observed with increasing cluster concentration. In order to further investigate the apparent modification of DNA by **4**, its interaction with a 22-oligomer (sequence 5′-AGT TGT GGT GAC TTT CCC AGG C-3′) was examined. Incubation with this oligonucleotide (pH 7.4 in Tris–HCl buffer and 100 mM NaCl) followed by HPLC analysis revealed the formation of three dose dependent products assigned as covalent modifications at three sites of the oligonucleotide. Incubation of **4** with ^{32}P-ATP labeled oligonucleotide at the 5′-end followed by treatment with piperidine and comparison with the standard Maxam–Gilbert sequencing protocol products revealed only general background cleavage, indicating that the modification products are piperidine labile and suggesting that the modification involved formation of a Schiff base. An alternative approach was then pursued which involved annealing the **4**-oligonucleotide products with their complementary strand and treatment of the resulting duplex DNA-with the exonuclease, Exo III. This assay indicated three exonuclease stops, consistent with the three products observed by HPLC whose electrophoretic mobility approximately matched guanine containing fragments when compared with the Maxam–Gilbert sequencing lanes. Reduction of the **4**-oligonucleotide products with borohydride reducing agents, followed by treatment with piperidine, resulted in the formation of one product (by HPLC) with the same electrophoretic mobility as the AGTT fragment based on comparison with the Maxam–Gilbert sequencing lanes. This product most likely results from reduction of an initially formed Schiff base adduct (to the corresponding amine) with the guanine of the TGT fragment of the oligonucleotide, and corresponds to the most stable of the three Schiff base adducts detected by HPLC and by incubation with the exonuclease. The other two products are less stable and competitive reduction of the free aldehyde functionality on the cluster in equilibrium with these adducts precludes their detection after treatment with the reducing agents. The formation of the Schiff base adduct is further corroborated by the model reaction of $[Os_3(CO)_{10}(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-}H)]$ (**4′**) with acetylated guanine in nonaqueous solvents where disappearance of the aldehyde resonance and the appearance of several new resonances in the 6–9 ppm region of the ^{1}H NMR of the reaction mixture is noted.

Keywords: Osmium clusters; DNA; Bio-markers; Covalent binding; Schiff base

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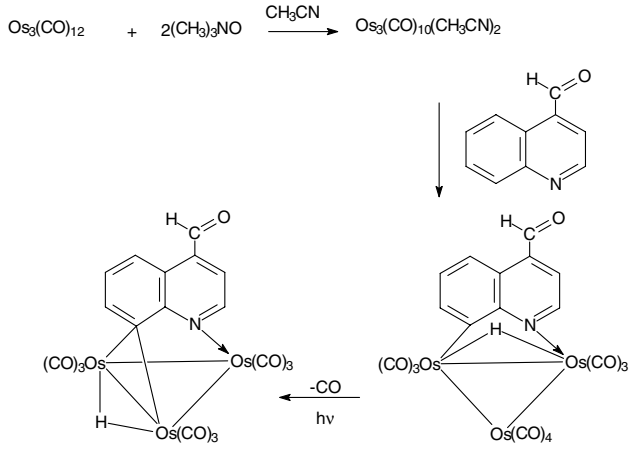
1. Introduction

Since the discovery of *cis*-platin and the successful application of its cytotoxic activity as an anti-tumor agent, much attention has been devoted to covalent binding of metal complexes to DNA [1]. Recently, studies using organometallic compounds and polymetallic clusters have shown a variety of interactions with biological systems, such as enzymes, nucleic acids, and proteins [2–9]. These interactions have led to structural elucidation [2], enzyme inhibition [3], antiviral and antibiotic activity [4], and site specific recognition of mutations [5]. For example, water-soluble ruthenium clusters $[H_4\eta^6Ru_4(C_6H_6)_4][(BF_4)_2]$ and $[Ru_3(CO)_9(PTA)_3]$ (PTA = 1,3,5-triaza-7-phosphatricyclo[3.3.1.1]-decane) have been tested with plasmid DNA and the products have been resolved in an electrophoresis agarose gel, showing extensive cross-linking and evidence for intercalation [6]. Modified nucleotides bearing a tridentate iminodiacetic acid chelating ligands have been coordinated to complexes of general formula *fac*-$[M(H_2O)_3(CO)_3]+$ (M = ^{99m}Tc, Re) to form stable organometallic species in water which have been applied to the inhibition of human cytosolic thymidine kinase (hTK1) and where increases in the length of the linker between nucleotide and complex result in increased inhibition [7]. The water-soluble ruthenium–*p*-cymene complexes $[Ru(\eta^6\text{-}p\text{-cymene})X_2]_2$ (X = Cl, Br, I or NCS), $[Ru(\eta^6\text{-}p\text{-cymene})X_2(PTA)]$ (X = Cl, Br, I or NCS; PTA = 1,3,5-triaza-7-phosphatricyclo[3.3.1.1]decane) and $[H_4Ru_4(\eta^6\text{-benzene})_4]^{2+}$ have shown antibacterial activity toward *Escherichia coli*, *B. subtilis*, *P. aeruginus*, and antifungal activity toward *Candida albicans*, *Cladosporium resinae* and *Trichophyton mentagrophytes*, and this activity is ultimately linked to protein–cluster interactions rather than direct DNA binding and damage [4]. We have recently reported the interaction between water-soluble benzoheterocycle triosmium clusters of general formula $[Os_3(CO)_9(\mu\text{-}\eta^2\text{-L-H})(\mu\text{-H})(P(OCH_2CH_2N(CH_3)_3I)_3)]$ (L = 3-amino quinoline, (**1**); phenanthridine, (**2**); and 3amidobenzoyl quinoline, (**3**)) and the plasmid pUC19 in a 1% agarose gel. We observed band retardation, dependent on the structure of the benzoheterocycle bound to the triosmium cluster [8]. The rationale for using these particular triosmium clusters lies, in part, in the presence of the bridging hydride and the carbonyl ligands, which provide signals in NMR and IR regions, respectively; where most biomolecules are silent. This represents a distinct advantage over other mono- or polymetallic compounds, whose ligands generate signals overlapping with the ones from amino acid side chains or sugar moieties of nucleotides. Since the hydride chemical shift is sensitive to the changes in electron distribution between ligand and metal core, these clusters also represent good candidates for structural probes. The very long relaxation times of these hydride ligands also offer a reliable way of screening the binding of these systems to large biomacromolecules [9]. The presence of the trimetallic core additionally provides critical phase information for X-ray diffraction experiments by infusion of the water-soluble clusters into biomacromolecular crystals in an ordered manner as has been recently applied to the protein–RNA complex of Ribonuclease P [10]. The presence of the pseudo-nucleobase on the cluster and the synthetic procedures available for their modification offer a plethora of pathways to the design of sight specific binding to proteins and oligonucleotides via hydrogen bonding, intercalation and attachment of complementary peptide sequences [11]. Finally, the use of trimetallic and larger clusters as bio-markers holds out the possibility for direct observation of the binding site by electron microscopy on the supramolecular level as a complement to their use as structural probes on the molecular level [12]. One interesting feature of the electron-deficient precursor to these water-soluble clusters is that a 2-electron donor can alleviate the electron deficiency either at the benzoheterocycle or at the metal core based on the nature of the nucleophile. A carbanion will selectively react at the carbocyclic ring of the benzoheterocycle, while softer nucleophiles such as trivalent phosphorous and nitrogen, will selectively react at the metal core [9]. The application of metal clusters as biological markers requires water solubility. A phosphorous-based positively charged group coordinated to the metal core is therefore necessary in order to favor the interaction with the negatively charged DNA phosphate backbone. The presence of the heterocyclic nucleobase analogues in the clusters is essential for observing band retardation at the sub-millimolar level [8]. Since we observed that a quinoline triosmium cluster containing a 4-carboxaldehyde group readily forms a Schiff base with the primary amino groups of a silica polyamine composite material [13,14], we decided to test the same cluster with double- and single-stranded DNA for possible covalent binding to nucleophilic functionalities. Nucleophilic functionalities are present in the aromatic bases of DNA, and our previous observations on the binding affinities of the quinoline triosmium clusters suggested that the incorporation of the aldehyde functionality might further enhance reactivity and site selectivity by covalent binding to these nucleophilic functionalities.

2. Results

The electron-deficient precursor $[Os_3(CO)_9(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-H})]$ was synthesized by reaction of quinoline 4-carboxaldehyde with $Os_3(CO)_{10}(CH_3CN)_2$, followed by photolytic decarbonylation (Scheme 1) [9,11].

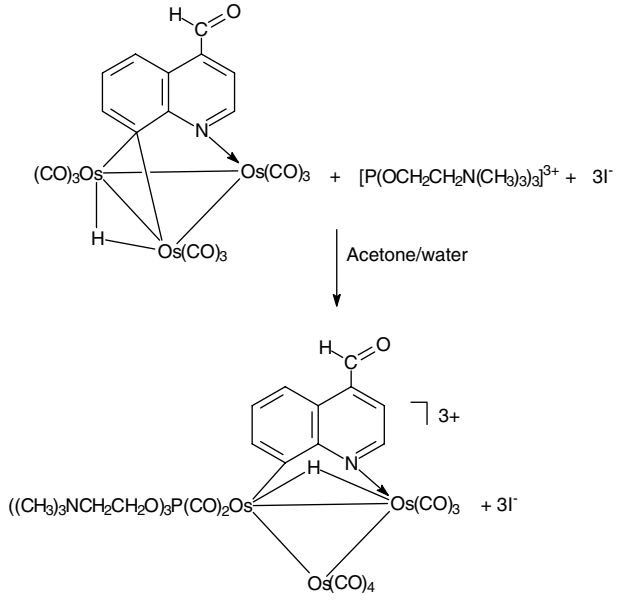
The water-soluble cluster $[Os_3(CO)_9(\mu\text{-}\eta^2\text{-}(4\text{-CHO})\text{-}C_9H_5N)(\mu\text{-H})(P(OCH_2CH_2N(CH_3)_3I)_3)]$ (**4**) was ob-



Scheme 1. Synthesis of $[Os_3(CO)_9(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-}H)]$.

tained in quantitative yield by reaction of the precursor with one equivalent of tris-(2-trimethylammonium iodide)-ethylphosphite in a mixed system of acetone and water (Scheme 2) [8]. The structure proposed for **4** is based on ^{13}C NMR, IR, and elemental analysis and by analogy with previously reported complexes, whose X-ray structure has been determined [8].

Our initial studies with **4** followed the protocol used with **1**–**3** where the plasmid DNA, pUC19, was incubated with varying doses of each cluster in the series. Complexes 1–3 contain heterocyclic rings capable of interactions with DNA by intercalation and/or hydrogen bonding as well the expected electrostatic attraction. The plasmid, pUC19 was incubated with a concentration gradient of **4** (1, 2, 3 and 4 μM) under physiological conditions (10 mM Tris–HCl buffer, pH 7.4, NaCl 100



Scheme 2. Synthesis of $[Os_3(CO)_9(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-}H)(P(OCH_2CH_2N(CH_3)_3I)_3)]$ (**4**).

mM) at ~37 °C for 16 h and then analyzed by agarose gel electrophoresis. In these previous studies, band retardation was clearly detectable at the micromolar level and helped to confirm a structure-binding affinity relationship for the series of benzoheterocycle triosmium clusters clearly based on the intercalating ability of the heterocycle (i.e., **3** > **2** > **4**) [8]. In the case of **1**, however, the gel revealed a pattern of three discrete bands whose intensity increased with increasing cluster concentration (Fig. 1). The highest concentration caused precipitation, due to the non-specific salt effect observed with all related clusters tested so far [8]. Three discrete bands are also seen in the control lane and likely correspond to the three possible forms of the plasmid: super coiled, circular and linear. However, the three discrete bands in the cluster treated lanes have slightly different electrophoretic mobility and are dose dependent in intensity. Overall this suggests that addition of **4** to the plasmid may be increasing the relative amounts of circular and linear DNA relative to the super coiled form by a stronger, or at least different, interaction than with **1**–**3** where only a progressive retardation and smearing of a single super coiled plasmid DNA was observed [8]. Such speculation is dangerous as the increased band intensity of the slower moving bands could also be due to inconsistencies in loading of the agarose gel. The main point to be made here is that the behavior of **4** with the plasmid is distinctly different than that of **1**–**3**.

This unique behavior of the aldehyde functionality with plasmid DNA prompted us to investigate the interaction of **4** with a smaller single-stranded 22-*mer*-oligonucleotide (5′-AGT TGT GGT GAC TTT CCC AGG C-3′). Binding to such an oligomer would allow the use of ^{32}P-radiolabeling techniques in order to identify the site of binding. We thus repeated the binding experiments duplicating the conditions used with the plasmid and analyzed for product formation by HPLC. Direct injection of the reaction mixture onto an anion-exchange HPLC column achieved the separation of the modified oligonucleotide from the excess of positively charged cluster, with no significant loss of DNA. Product formation is dose-dependent and complete conversion to three overlapping product bands is observed in the presence of a 10-fold excess of cluster (Fig. 2).

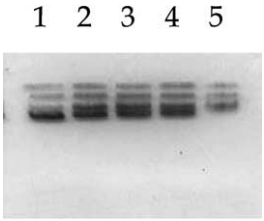


Fig. 1. Plasmid relaxation test of pUC19 and (**4**). Lane 1, control; lane 2, 2 μM cluster concentration; lane 3, 4 μM cluster concentration; lane 4, 6 μM cluster concentration; lane 5, 8 μM cluster concentration.

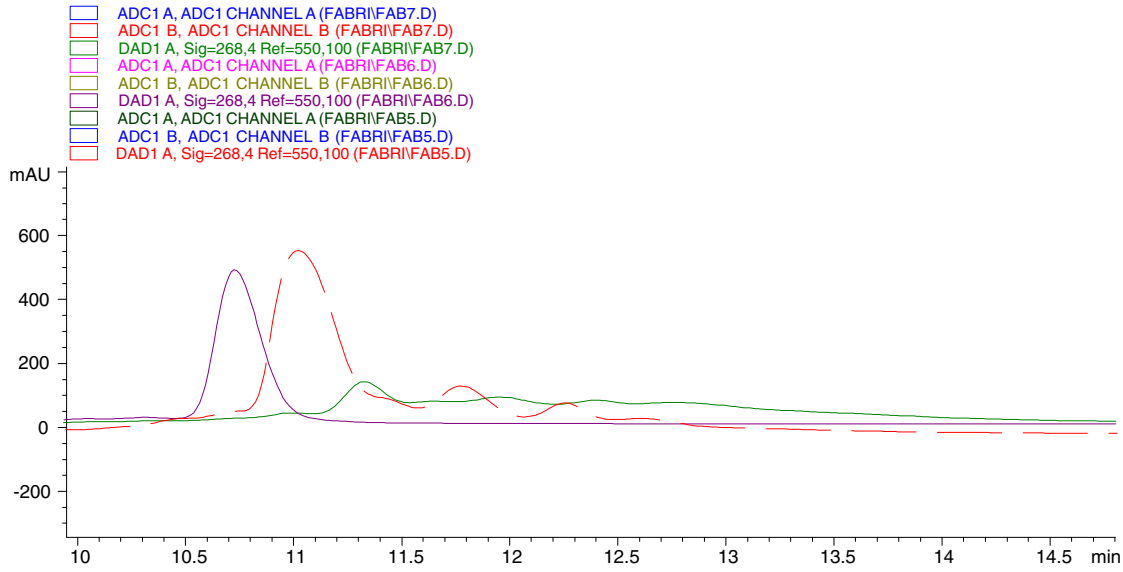


Fig. 2. Product detection by HPLC after direct injection in the anion exchange column: unmodified KDSGT1 (purple profile); equimolar ratio of KDSGT1 and [4] (red profile); 10-fold excess of [4] (green profile).

Gel sequencing experiments were carried out by dilution of the 20 mM stock solution of **4** to 1 mL of 20 μM and then volumes ranging from 0.5 to 1.5 μL were added to a 20 μL reaction volume of the 5′-radiolabeled oligonucleotide. In order to detect the position of the lesion, we first treated the reaction products with piperidine and then compared the products with the standard Maxam–Gilbert sequencing protocol [15]. This standard approach is based on the hypothesis that the formation of a strong interaction between the cluster and DNA bases would weaken the bases' N-glycosidic bond and result in strand cleavage when exposed to piperidine. However, in the present case one might expect that the aldehyde would bind to exocyclic aromatic amine groups on the DNA bases by Schiff base formation and that addition of piperidine would result in competitive Schiff base formation with the aliphatic amine groups of the piperidine. Indeed, this is apparently what occurred because no site specific cleavage products are observed and only random cleavage of the oligonucleotide is seen as evidenced by the observation of no matching bands with Maxam–Gilbert sequencing lanes (Fig. 3).

Based on this result one could propose that the interaction of the cluster with the 22-*mer* was piperidine labile or that the lesions and modifications induced by the cluster do not result in a labile N-glycosidic bond [15,16]. We therefore decided to change the binding assay methodology and use the exonuclease Exo III, isolated from yeast [16]. The most interesting property of this enzyme is that its phosphatase activity stops wherever a lesion or a modification is present in double stranded DNA. Incubation with the cluster was carried out at 92 °C for 30 min., the same conditions that favor the formation of a Schiff base between piperidine and the reducing end of deoxyribose under alkali strand-cleavage conditions. The reason for changing these conditions was based on the hypothesis that a piperidine-labile Schiff base was indeed formed between the cluster and the DNA bases. The oligonucleotide was then annealed with its complementary strand because the enzyme specificity requires duplex DNA. The reac-

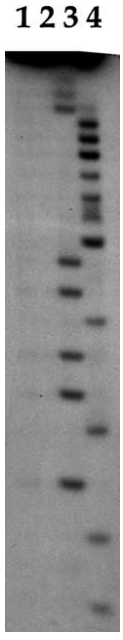


Fig. 3. Sequencing gel of 22-*mer* KDSGT1 after incubation with $[Os_3(CO)_9(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-}H)(P(OCH_2CH_2N(CH_3)_3I)_3)]$ (**4**) and treatment with piperidine. Lane 1, control; lane 2, KDSGT1 and (**1**); lane 3, Maxam–Gilbert AG sequencing product; lane 4, Maxam–Gilbert CT sequencing product.

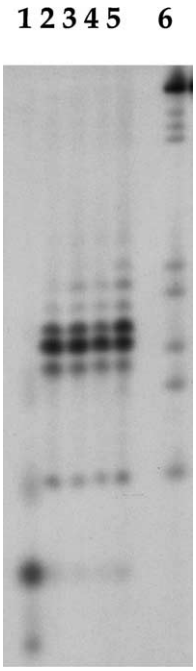


Fig. 4. Sequencing gel of 22-*mer* KDSGT1 after incubation with $[Os_3(CO)_9(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-}H)(P(OCH_2CH_2N(CH_3)_3I)_3)]$ and digestion with Exo III. Lane 1, control; lane 2, 250 nM cluster concentration; lane 3, 375 nM cluster concentration; lane 4, 500 nM cluster concentration; lane 5, 750 nM cluster concentration; lane 6, Maxam–Gilbert AG sequencing products.

tion was carried out and incubated with Exo III at 37 °C for 5 min. The gel showed a pronounced band in the control lane but in a different position than the bands in the lanes corresponding to cluster-treated oligonucleotide, consistent with further digestion of the unmodified oligonucleotide and formation of shorter strands.

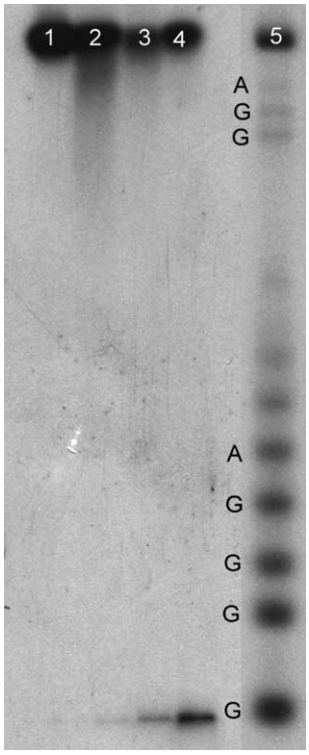


Fig. 6. sequencing gel of 22-*mer* KDSGT1 after incubation with $[Os_3(CO)_9(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-}H)(P(OCH_2CH_2N(CH_3)_3I)_3)]$ and reduction with $NaBH_4$. Lane 1, control; lane 2, 500 nM cluster concentration; lane 3, 500 nM cluster concentration,100 mM $Na(CN)BH_3$; lane 4, 500 nM cluster concentration, 100 mM $NaBH_4$; lane 5, Maxam–Gilbert AG sequencing products.

A final experiment was carried with Exo III and the modified KDSGT1 at 37 °C for 1 h. The control lane shows a product due to the incomplete enzyme digestion, but the stops in the lanes corresponding to clus-

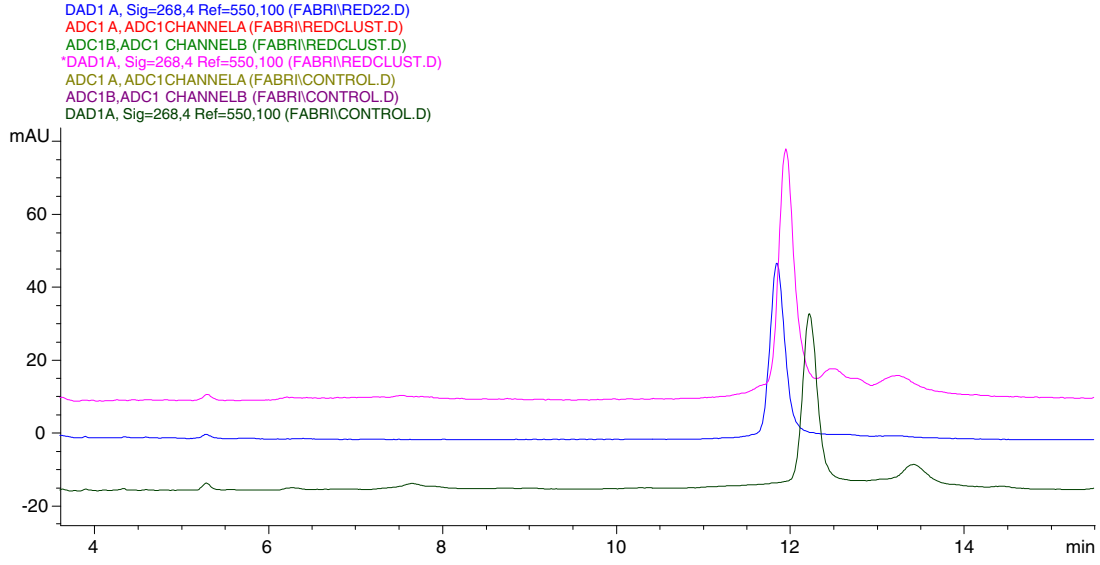


Fig. 5. HPLC chromatogram of KDSGT1 and [4] in presence of $NaBH_4$; unmodified KDSGT1 (blue profile); KDSGT1 and [4] after reduction with $NaBH_4$ (purple profile); KDSGT1 and $NaBH_4$ (green profile).

ter-treated oligonucleotide indicate a modification in the DNA sequence that blocked enzyme activity (Fig. 4).

Because of the extreme sensitivity of the enzyme to modifications in the DNA sequence and structure, even low ratios of **4** to KDSGT1 caused stops in the nuclease activity of Exo III. Thus at the lowest cluster concentration (250 nM), there is only a 2.5-fold excess of cluster per DNA, but this is still sufficient to block enzyme activity; the highest concentration (750 nM) corresponds to a 7.5-fold excess of cluster. Although the stops are evident in the gel, the partial digestion products formed do not line up with the products in the sequencing lanes from the Maxam–Gilbert protocol. It is likely that this is caused by the different termini expected from the two different methods of cleaving the DNA: a 3′-phosphate terminus for the Maxam–Gilbert products and 3′-phosphoglycaldehyde terminus for the Exo III partial digestion products. In an effort to determine the position of binding in the 22-oligonucleotide, we decided to use a trapping technique that has proven successful for the enzyme hOGG1 and covalently modified positions in a DNA strand. The reducing agent sodium borohydride [$NaBH_4$] can reduce the Schiff base formed between an essential lysine residue in the enzyme and the reducing end of the deoxyribose sugar in the modified nucleotide [17]. Since we also propose the formation of Schiff base between the aldehyde functionality on the cluster and a suitable nucleophile on the DNA strand, we decided to utilize the same approach and repeat the reaction followed by incubation with a 100 mM concentration of [$NaBH_4$]. We first repeated the HPLC analysis of the reaction products, and observed a different profile with respect to the three different products observed in the absence of the reducing agent. The three peaks observed in the previous chromatogram are now only two peaks, but one peak also appears in the sample of KDSGT1 treated only with [$NaBH_4$], indicating that one major product seems to be produced once the cluster–DNA adduct is incubated with the reducing agent (Fig. 5).

In light of this result, we repeated the sequencing gel work in the presence of [$NaBH_4$], and then the reduction products were treated with hot piperidine, in order to form cleaved DNA strands identifiable in a denaturing polyacrylamide sequencing gel. The gel showed only one major product, corresponding to a particular guanine in the sequence (Fig. 6).

Although it is not possible to speculate about the nature of the partial digestion products formed by Exo III, the number of stops obtained and their patterns logically suggest the presence of multiple binding sites on

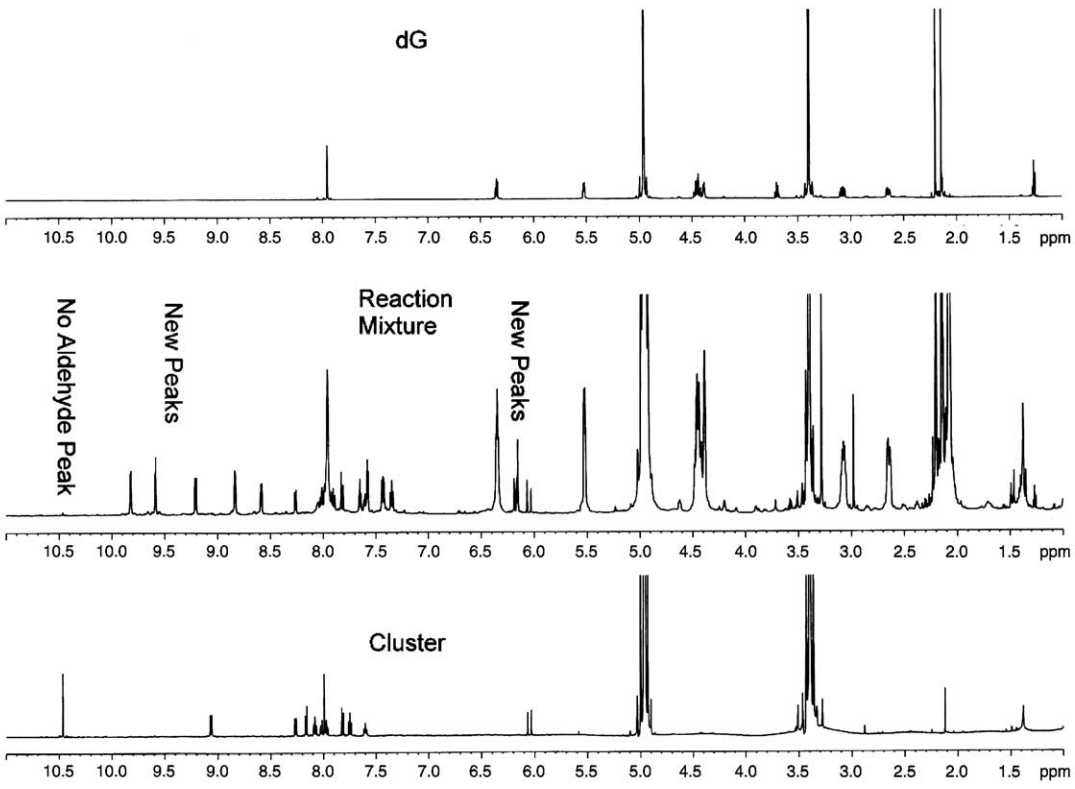
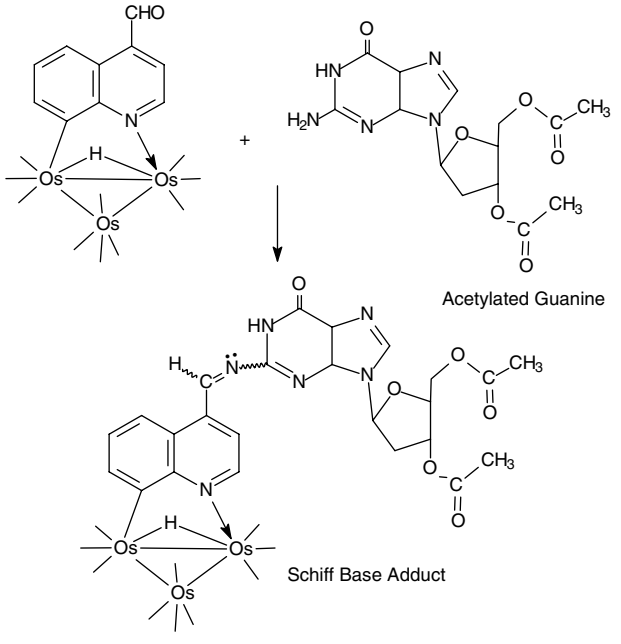


Fig. 7. (a) ^{1}H NMR of acetylated guanine in methanol-d_4 at 600 MHz. (b) ^{1}H NMR of (**4′**) in the presence of excess acetylated guanine in methanol-d_4 at 600 MHz. (c) ^{1}H NMR of (**4′**) in methanol-d_4 at 600 MHz.

the oligonucleotide, whose positions cannot be unequivocally determined because of the incorrect lineup of the enzyme digestion products with the cleavage products obtained by the Maxam–Gilbert protocol.

Furthermore, the lability of Schiff bases in aqueous solutions interferes with the electrophoretic mobility of the fragments, resulting in a lack of important sequence binding information. The fact that reduction of the cluster–DNA adduct by [$NaBH_4$] yields a single product, which then undergoes cleavage when treated with piperidine, indicates that one binding site in KDSGT1 is preferred out of the multiple ones observed in the Exo III experiments, consistent with the effect of different local environments in the oligonucleotide on the binding of **4**. Further evidence of the nature of the interaction of **4** with KDSGT1 comes from a modeling study based on the reaction of the analog of **4**, [$Os_3(CO)_{10}(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-}H)$] (**4′**) with acetylated guanine in CD_3OD. When a solution of **4′** is incubated with an excess of acetylated guanine for several hours and monitored by ^1H NMR the aldehyde resonance disappears and several new peaks appear in the aromatic region as well as two new peaks at ~6.1 ppm (Fig. 7). The C–H resonance of imines occurs over a wide range, from 6 to 9 ppm [19]. Some of the new peaks in the aromatic region can be attributed to shifted ring proton resonances of **4′** based on their coupling constants. Unfortunately, this adduct proved to be unstable on isolation which precluded full characterization by more thorough spectroscopic methods and elemental analysis. The structure proposed for the Schiff base adduct is shown in Scheme 3. The many new peaks seen in the ^1H NMR can be attributed to the existence of geometric isomers around the C=N bond.



Scheme 3. Proposed structure for the Schiff base adduct of [$Os_3(CO)_{10}(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-}H)$] (**4′**).

3. Discussion

The band retardation in the plasmid relaxation test suggests that this new cluster does bind to the supercoiled structure of pUC19, but the absence of smearing indicates that it does not bind in the same dynamic fashion as the previously studied clusters, **1**–**3**, which showed interaction with the plasmid as smeared retarded bands in an agarose gel. The fact that the observed discrete bands still migrate more slowly with increased cluster concentration without smearing clearly indicates that once the cluster binds to pUC19, it does not come off as easily as **1**–**3**, in which the heterocycles could at best partially intercalate or form hydrogen bonds with the plasmid. No cluster tested to this point had the appropriate functionality to covalently bind to DNA, only functional groups such as amino groups or aromatic rings were available to interact with pUC19. The data obtained by HPLC also corroborate the fact that discrete additions of cluster molecules to the oligonucleotide occur, since partially resolved peaks are clearly distinguishable rather than a continuous diffuse band. When HPLC analyses were performed on clusters **1**, **2** and **3** with KDSGT1, the cluster eluted right at the beginning of the chromatogram, and the oligonucleotide eluted at the same retention time as the unmodified 22-*mer*. Considering that an anion exchange column was used, this result clearly indicates a weaker interaction and the formation of products that do not endure chromatographic separation techniques. When sequencing techniques were used for determining the binding site, no strand cleavage was detected after treatment with piperidine. Piperidine is widely used in sequencing assays because of its ability to cause strand scissions in DNA by cleavage of the N-glycosidic bonds labilized by small molecules covalently linked to a specific position on the heterocyclic base. This results in the displacement of the modified base and the formation of a Schiff base with the reducing end of the deoxyribose ring, which ultimately results in strand cleavage. The fact that no lesions are detected in the presence of piperidine suggests that the products of the oligonucleotide with **4** are reversed in the presence of piperidine, or that piperidine competes for the aldehyde functionality in the cluster and then no cluster binds to DNA. In particular, the very bond formed between our cluster and the biomolecule may be sensitive to piperidine and be reversed when heated at 92 °C in 1 M piperidine for 30 min. In order to bypass the inconclusive results obtained with the piperidine treatment, we chose to assay the products of the reaction of **4** and KDSGT1 with exonuclease, Exo III. This exonuclease has phosphatase activity only on

double stranded DNA and excises one nucleotide at a time starting from the 3′-end. If the polynucleotide is 5′-^{32}P-labeled, the 5′-digestion products can be separated by electrophoresis in a denaturing polyacrylamide gel and visualized on a photographic film. Examples of Exo III assays in the literature show that, depending on the nature and the dimension of the lesion, the enzyme may stop its phosphatase activity one position removed from the actual modification [18]. The consistent pattern of partially digested products obtained in different experiments indicates that the cluster blocks the enzymatic activity of Exo III by binding to different positions in the oligonucleotide, hence multiple stops are observed in the gel across the range of concentrations of **4**. However, the positions modified by the cluster cannot be unequivocally determined through the Exo III digestion, since the lineup of the products with the Maxam–Gilbert sequencing lanes is not exact. After borohydride reduction only one product is observed after piperidine digestion, and the binding site corresponds exactly to a single guanine based on the exact match with AGTGT fragment in the Maxam Gilbert sequencing lane (5′-AGT T***G***T GGT GAC TTT CCC AGG C-3′). It is reasonable to propose that the cluster can bind to different guanines in a polynucleotide sequence, since it does not possess suitable features needed for sequence specificity or recognition; in this case, the Exo III experiments suggest binding of **4** to different guanines in the sequence, thus displaying multiple stops in the enzymatic activity. Unfortunately, the labile nature of the covalent bond formed between KDSGT1 and **4** poses a problem when the modified oligos are separated in a denaturing polyacrylamide gel: partial loss of cluster due to these conditions would change the fragments' electrophoretic mobility as they move through the gel, resulting in ambiguous positions in the gel with respect to the cleavage products obtained with the Maxam–Gilbert sequencing protocol. Furthermore, the different termini obtained by enzymatic cleavage and by Maxam–Gilbert sequencing protocols also influence the electrophoretic mobility, the former being uncharged phosphoglycaldehyde and the latter bearing a phosphate group. In the presence of a reducing agent, the C=N double bond is reduced to a single C–N bond, much more stable in water, and able to resist the piperidine treatment and denaturing conditions. The fact that piperidine preferentially cleaves certain positions in a DNA strand is usually due to a labilized N-glycosidic bond between a covalently modified nucleobase and its deoxyribose sugar. Piperidine is a Brönsted and a Lewis base, being able to form a Schiff base with the reducing end of the sugar once the modified base is no longer present. After reduction of the proposed Schiff base by $NaBH_4$, the position in KDSGT1 that is preferentially cleaved after treatment with piperidine is likely to be the most stable C=N formed out of the multiple binding sites observed in the Exo III experiments. Since the reduction of the aldehyde functionality on the benzoheterocycle is always competitive with the reduction of C=N bond, and the cluster is present in excess, it is conceivable that the reduction of the aldehyde functionality on the cluster would shift the equilibrium for Schiff base formation toward the reactants, reverting the covalent products back to the unmodified oligonucleotide. Thus the more labile Schiff bases will be depleted first, leaving only the thermodynamically most stable one available for reduction. The guanine to which the cluster preferentially binds is the only purine base that is not followed or preceded by another purine: the two thymidines that flank this particular guanine allow for reasonable exposure of the guanine heterocycle to a large molecule like the cluster. Our rationale for explaining why only one binding site is eventually trapped by the reducing agent of choice is that the steric encumbrance that the cluster encounters in all other guanine sites on the oligonucleotide is ultimately responsible for the increased lability of the Schiff bases formed. The final effect is that only the most thermodynamically stable Schiff base will be present in solution long enough to be reduced to a single C–N bond. Studies directed at the isolation of stable Schiff base adducts between complexes related to **4** and mononucleotides, as well as further molecular biology experiments to further define and extend the selective covalent binding interactions reported here are underway in our laboratories.

4. Experimental

The water-soluble phosphite ligand $P(OCH_2CH_2N(CH_3)_3I)_3$ was synthesized according to published literature procedures [8]. The clusters $[Os_3(CO)_9(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-}H)]$ (**4**) and $[Os_3(CO)_{10}(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-}H)]$ (**4′**) were synthesized by published literature procedures [9,11]. Acetylated guanine was synthesized according to published literature procedures [20]. The 22-*mer* KDSGT1 and the complementary strand KDSGT2 were purchased from Trilink and subsequently purified by HPLC. The enzyme Exo III was purchased from New England Biolabs. ^{32}P-ATP was purchased from Perkin–Elmer Life Sciences, Inc. Microbiospin 6 chromatography columns and 40% acrylamide/bis-acrylamide solutions were purchased from BioRad. Trizma base, ammonium persulfate and electrophoresis-grade urea were purchased from Sigma and used as received. Elemental analysis was carried out by Schwarzkopf Microanalytical Laboratories, Woodside, NY. Infrared spectra were run on a Thermo Nicolet 633 FT-IR and NMR spectra were run on Varian Unity Plus 400 MHz and Bruker Avance 600 MHz spectrometers.

4.1. Synthesis of $[Os_3(CO)_9(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-}H)(P(OCH_2CH_2N(CH_3)_3I)_3)]$ (4)

20 mg (0.02 mmol) of $[Os_3(CO)_9(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)\text{-}C_9H_5N)(\mu\text{-}H)]$ were dissolved in a minimum amount of acetone and then 15 mg (0.02 mmol) of $[P(OCH_2CH_2N(CH_3)_3I)_3]$ were dissolved in a few drops of deionized water and added to the acetone solution while stirring. The dark green solution turns amber instantly. The reaction is quantitative and yields a dark amber solid product, after rotary evaporation of the solvent, $[Os_3(CO)_9(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-}H)(P(OCH_2CH_2N(CH_3)_3I)_3)]$ (34.4 mg, 100%). The cluster was dissolved in 1 ml of water to give a 20 mM stock solution for use in the DNA binding experiments. Elemental analysis (calculated): H 3.05%, C 25.84%, N 3.27%. Elemental analysis (actual): H 2.85%, C 25.76%, N 3.35%. IR (ν_{CO}, H_2O): 2093.52 cm^{-1} (w), 2042 cm^{-1} (m), 1991 cm^{-1} (s), 1955 cm^{-1} (sh), 1926 (sh) cm^{-1} ^1H NMR (D_2O, water suppressed, B): 10.6 ppm (s, 1H, CHO), 9.45 ppm (d, 1H), 8.93 ppm (d, 1H), 7.80 ppm (d, 1H), 7.31 ppm (d, 1H), 7.02 ppm (t, 1H), 3.47 ppm (m, 6H), 3.17 ppm (s, 3H),. 04 ppm (s, 3H), 2.92 ppm (s, 27H), −13.13 ppm (d, 1H, J_{HP} = 16.1 Hz).

4.2. Plasmid relaxation test

pUC19, 0.6 μL (0.6 μg), was suspended into 20 μL of 10 mM Tris–HCl, pH 7.4, in 100 mM NaCl and then incubated with a gradient of water-soluble cluster (2–4–6–8 μM) at 90 °C for 30 min. Right before loading in a 1% agarose gel containing ethidium bromide, 2 μL of glycerol loading buffer were added. The gel was run for 1 h at ~90 mV. The bands were then visualized on a Transilluminator (UVP, Inc.) and photographed on a Polapan 665 Polaroid film with an exposure of 15 s.

4.3. HPLC analysis of products

The unmodified and modified KDSGT1 oligonucleotides were purified using a Dionex Nucleopac PA100, 4 mm × 250 mm anion exchange column employing a linear gradient from 90% mobile phase A (10% aqueous acetonitrile) and 10% mobile phase B (1.5 M ammonium acetate, pH 6, in 10% acetonitrile) to 100% mobile phase B over the course of 22 min. Eluted nucleotides were monitored by diode array at 268 nm. The fraction containing the purified oligonucleotide was collected, EtOH precipitated, and evaporated to dryness.

4.4. Sequencing gels

Radio-labeled KDSGT1, 0.0198 ng, was suspended in 20 μM of 10 mM Tris–HCl buffer, pH 7.4, in 100 mM NaCl, and incubated with a gradient of cluster concentration (250–375–500–750 nM at ~90 °C for 30 min.). The solution volume was then brought to 40 μL, and a 10-fold excess of the complementary strand KDSGT2 was added for a final volume of 41 μL. The mixture was then annealed to give the double-stranded oligonucleotide. The reaction volume was then brought to 50 μL with Exo III and buffer (Tris–HCl, pH 7.4, 50 mM Mg^{2+}). Exo III was then added (0.5 units), incubated at 37 °C for 1 h, and deactivated at ~90 °C for 10 min. When the alkaline-labile cleavage sites on KDSGT 1 were analyzed, 100 μL of a 1.0 M solution of freshly prepared piperidine were added, followed by heating at 92 °C for 30 min.The solutions were evaporated to dryness and resuspended in 5 μL of 80% formamide loading buffer containing 0.05% xylene cyanol and bromophenol blue. Only 1 μL was loaded on a 20%, 0.4 mm thickness, 21 cm × 50 cm denaturing (7 M urea) polyacrylamide gel. Electrophoresis was carried out at 1500 V and 24 mA with 1 × TB as the running buffer. Visualization of the DNA cleavage products was carried out by autoradiography using Kodak X-Omat Ar-5 film. When the exonuclease was not employed, the reduction was carried out by preparing a fresh solution of 1 M (10×) [$NaBH_4$] and then by adding 2 μL to the solution after incubation with the cluster, and the annealing step was not carried out. Whether a reducing agent was employed or not, the solutions were evaporated to dryness and then resuspended in 100 μL of 1 M piperidine and heated at 92 °C for 30 min. The solutions were evaporated to dryness and then washed with 20 μL of distilled water twice before adding the formamide loading buffer.

4.5. Preparation of the NMR solution of $[Os_3(CO)_{10}(\mu\text{-}\eta^2\text{-}(4\text{-}CHO)C_9H_5N)(\mu\text{-}H)]$ (4′) with acetylated guanine

A solution of acetylated guanine in methanol-d_4 (3 mg, 0.01 mmol in 0.3 mL) was combined with a solution of **4′** in methanol-d_4 (1 mg 0.001 mmol in 0.3 mL) in a 5 mm NMR tube. The solution was allowed to stand for several hours and then the ^1H NMR was recorded. Attempts to separate the product formed from the excess acetylated guanine by chromatographic means failed and resulted in the decomposition of the product.

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