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Novel water-soluble triosmium heterocyclic clusters as selective markers for biomacromolecules

Fabrizio Spada

The University of Montana

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Novel Water-Soluble Triosmium Heterocyclic Clusters as Selective

Markers for Biomacromolecules

By

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Presented in partial fulfillment of the requirements

for the degree of

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2004

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8-17-04

Date
Fabrizio Spada, Ph.D., August, 2004

Novel Water-Soluble Triosmium Heterocyclic Cluster as Selective Markers for Biomacromolecules.

Chairman: Edward Rosenberg.

The clusters \([\text{Os}_3(\text{CO})_9(\mu-\eta^2-(L-H))(\mu-H)L]L'(L = 3\text{- amino quinoline}, \quad L' = P(\text{C}_6\text{H}_5\text{SO}_3\text{Na})_3\quad [\text{P}]), \quad 1; \quad L = 3\text{- amino quinoline}, \quad L' = P(\text{OCH}_2\text{CH}_2\text{NMMe}_3)_2\quad [\text{P'}], \quad 2; \quad L = 3\text{- (2-phenyl acetimido) quinoline}, \quad L' = P^+, \quad 3; \quad L = \text{phenanthridine}, \quad L' = P^+; \quad 4; \quad L = 3\text{-quinoline carboxaldehyde}, \quad L' = P^+, \quad 5; \quad L = \text{quinoxaline}, \quad L' = P^+, \quad 6; \quad L = \text{quinoxaline}, \quad L' = P^+, \quad 7; \quad L = 5,6\text{-benzoquinolone}, \quad L' = P^+, \quad 8; \quad L = \text{quinoxaline}, \quad L' = \text{PPh}_3, \quad 9; \quad L = \text{2-methyl benzimidazole}, \quad L' = P^+, \quad 10; \quad L = 4\text{-quinoline carboxaldehyde}, \quad L' = P^+; \quad 11; \quad L = \text{2-methyl benzimidazole}, \quad L' = P^+, \quad 12)\) have been synthesized. The structures of 1 and 2 in aqueous solution have been characterized by \(^1H, \quad ^{13}C\) VT-NMR and LC-TOF-MS. The interactions of 1-5 with DNA have been studied using plasmid relaxation test in a 1% agarose gel. Band retardation was observed for the clusters 2-5, but not for 1. The relative binding affinities are 2<3~4 and all three showed greater binding affinities than the reference cluster \([\text{Rh}_3(\mu_5-S)\quad (\eta^2\text{-Me}_5\text{C}_5)\quad (\text{BF}_4)_2]\). These results suggest a relationship between the heterocyclic ligand and the binding affinity to DNA. The cluster 5 was tested for covalent binding to DNA because discrete retarded bands were observed in a plasmid relaxation assay.

The oligonucleotide KDSGTl (oligonucleotide sequence: 5'-AGT TGT GGT GAC TTT CCC AGG C-3') incubated with 5, and the products were detected by HPLC under physiological conditions. After labeling at the 5'-end with \(^32\text{P-ATP}\) and annealing with the complementary strand, KDSGTl was treated with the exonuclease Exo III. the multiple binding sites did not align with the Maxam-Gilbert sequencing products. Sequencing experiments in the presence of NaBH\(_4\) showed cleavage only at a specific guanidine (5'-AGT TGT GGT GAC TTT CCC AGG C-3'), supporting the hypothesis of Schiff base formation.

The electrochemical properties of 1-12 in water were also tested. Compound 6 undergoes a reversible 1e reduction in water.

The interactions of the positively and negatively charged clusters with bovine serum albumin have been investigated using T\(_1\) and T\(_2\) for the hydride resonances. Binding occurs with distinctly different correlation times \(\tau_c\).

The cluster 1 showed the highest inhibition of the tumor enzyme telomerase \textit{in vitro}. All the clusters tested with MCF-7 tumor cells only showed growth inhibition with continuous presence of triosmium cluster in the medium, with no evidence of cellular uptake.
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and my dear friends,  
For their continuous support throughout this adventure:  
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But always remind who I really am. Grazie.
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1.1 From coordination chemistry to bioinorganic chemistry.

Since the discovery of its cytotoxic and antitumor activity, cisplatin has been the drug of choice for the treatment of testicular and ovarian cancer. It successfully crosses the cellular membrane, accumulating in the cell and the nucleus: approximately 1% of the intracellular cisplatin reacts with genomic DNA yielding a variety of intra- and interstrand adducts, the most common being an intrastrand cross-link between adjacent guanines [1].

Cisplatin treatment results in inhibition of DNA replication, RNA transcription, arrest at the G2/M phase of the cell cycle, and programmed cell death (apoptosis) [2-4].

This breakthrough discovery led to an entirely new research area for inorganic chemists: bioinorganic chemistry.

To this day, bioinorganic chemistry is a highly interdisciplinary field of science, with roots coming both from research in inorganic chemistry focused on metal ions in biological systems, and from research in the biological sciences,
focused on metal centers that play critical roles in biological structure, function, or in medicine.

Research in bioinorganic chemistry promotes interactions and the exchange of ideas among synthetic chemists, biochemists, biophysicists, spectroscopists, theoretical chemists, molecular biologists, toxicologists, environmental scientists, medical researchers, and clinicians.

A significant example is represented by a handful of promising small molecules that have been reported to cleave DNA hydrolytically [5]. Almost all these artificial nucleases rely on transition metals or lanthanide metals for hydrolysis of the DNA backbone. Stable Co(III) complexes have been demonstrated to promote DNA hydrolysis through Lewis acid activation of the phosphate and nucleophilic attack by a coordinated hydroxide.

Model studies with crystallographically characterized dinuclear metal complexes emphasize the advantages of placing two metal centers together for cooperative activation of a substrate [6].

The ability to cleave DNA upon binding allows the use of radiolabeling techniques to determine the binding site, in order to design artificial nucleases with selectivity for a specific nucleotide sequence.

An alternative approach consists of coupling the metal complex to an oligopeptide, which can impart or modulate the selectivity for a specific sequence in DNA [7] (Figure 1.1).
One important result observed with hydrolysis studies on a 42-mer double-stranded DNA (labeled strand, 5'-CGA TGC GTA CGC GGT ATA TGC GGC ATA TGC GAA CTA TGG CAG-3') is the presence of double bands for the highly resolved products at the bottom of the gel consistent with formation of both 3'-phosphate termini and slower moving 3'-phosphoglycaldehyde termini.

The termini have been identified by comparing the migration of product bands with the migration of Maxam-Gilbert bands and dephosphorylated Maxam-Gilbert bands.
The conjugate in Figure 1 has also shown to convert supercoiled plasmid pBR322 to nicked and linear forms in the presence of Zn$^{2+}$ [8]: the metallointercalator, the tethered peptide, and the divalent cation are all essential for efficient cleavage activity, while if one of the components are absent, only a modest increase of nicked plasmid is observed.

An interesting examples of a multifunctional ruthenium(II) complex of general formula $[(\eta^6\text{-arene})\text{RuCl}(X)(Y)]$ (X, Y are monodentate or chelating ligands) features all the essential elements to provide base and sequence recognition for DNA binding [9]: a hydrophobic ligand that can π-stack with the nucleobase, a hydrogen binding donor/acceptor group and a labile ligand that can be displaced to covalently link the complex to the nucleotide (Figure 1.2).

![Figure 1.2: structure of $[(\eta^6\text{-Bip})\text{Ru(en)}\text{Cl}]^+$](image)

The binding sites of the complex $[(\eta^6\text{-Bip})\text{Ru(en)}\text{Cl}]^+$ (bip= biphenyl, en= ethylenediamine) with several nucleobase derivatives were identified by $^1\text{H}$-
NMR and $^{31}$P-NMR pH titrations, and each adduct was characterized by pK$_a$ values derived from the pH titration curves (Figure 1.3).

![Reaction Diagram]

**Figure 1.3: Reaction of $[(\eta^6\text{-Bip})\text{Ru(en)Cl}]^+$ with 5'-GMP.**

Purines exhibit competition for binding to the N7 versus the N1, with a slight preference for the N7 position in the case of metal ions. This is certainly the case for Pt(II) and Pd(II) complexes, which allow the observation of the dichotomy between N1 and N7 because the metal ion is both diamagnetic and, on the NMR scale, in slow exchange between the two sites.

Hydrogen bonding and nonbonding repulsive interactions play a critical role for the selective recognition of nucleobases by $[(\eta^6\text{-Bip})\text{Ru(en)Cl}]^+$: for G, N7
is the favored site for Ru(arene) binding. One of the (en) NH is involved in a strong hydrogen-bonding interaction with C6O, with a N···O distance of 2.8 Å and N-H···O angle of 163°. The ability of the NH proton of ethylenediamine to act as an H-bond donor toward an exocyclic oxo group but not toward an amino group appears to play an important role in controlling site recognition of the nucleobases by these Ru(arene) anticancer agents. Also in enzymatic digestion experiments, it was found that the binding of [(η^6-p-cymene)Ru(en)Cl]^+ to a 14-mer oligonucleotide occurred specifically at guanine sites [10].

In the light of these results, the same compound was tested with the human ovarian cancer cell line A2780: the cells were plated and then incubated with the Ru(I) complex. After removal of the complex, the cells were counted, and the IC$_{50}$ (as the concentrations at which cell growth is 50% inhibited) were determined. The compound in question caused growth inhibition with IC$_{50} = 6$ μM: although this is an order of magnitude less than cisplatin (IC$_{50} = 0.5$ μM), the potency is similar to that of the anticancer drug carboplatin [10].

Electroactive complexes are also used for elucidating the mechanism of oxidation in biological molecules. The complex Ru(bpy)$_3^{2+}$ has been employed in a system developed for measuring the rates of electron transfer from guanine in small quantities of oligonucleotides or polymers to weakly bound metal complexes.

The electron-transfer rate constants for oligonucleotides containing adjacent guanines have been determined by digital simulation of cyclic
voltammograms of Ru(bpy)$_3^{2+}$ in the presence of the oligonucleotides (bpy = 2,2'-bipyridine). Sequences containing an isolated guanine (in a 5'-AGT segment) give a rate constant of $1.4 \times 10^5$ M$^{-1}$s$^{-1}$, while sequences with containing a 5'-GG segment give an overall rate constant of $7.5 \times 10^5$ M$^{-1}$ s$^{-1}$.

This approach involves the acquisition of the cyclic voltammogram of Ru(bpy)$_3^{2+}$ at metal oxide electrodes with or without DNA. In the presence of DNA, the metal complex catalyzes the oxidation of guanine. The results show that the rate of oxidation of the 5'-guanine in a GG doublet is enhanced by a factor 12 compared to isolated guanine, which implies a potential for the stacked 5'-guanine of 0.95 V [11].

1.2 From organometallic chemistry to bio-organometallic chemistry.

Bioorganometallic chemistry, a nascent area of organometallic chemistry, has now become an important subtopic, in a manner similar to that for bioinorganic chemistry as a subtopic of inorganic chemistry.

Unlike the many enzymatic inorganic complexes found necessary to sustain life on earth, bioorganometallic complexes, those with a definite metal-carbon bond, are rarely seen in life-sustaining processes.

An important exception is methylcobalamin, one of the very few natural coenzymatic organometallic complexes that has been shown to exist, which contains a discrete CH$_3$-Co bond. Among other roles, its biomethylation of
environmentally important metals, such as $\text{Hg}^{2+}$, $\text{As}^{3+}$ and $\text{Sn}^{4+}$, provide the toxic-to-man $\text{CH}_3\text{HgX}$, $(\text{CH}_3)_3\text{AsX}$, and $\text{CH}_3\text{SnX}$ complexes.

In contrast with bioinorganic chemistry, which has developed a robust synthetic aspect focused on biomimetic models of active enzyme sites and their functional chemistry, recent studies in biorganometallic chemistry have focused more on structural aspects of organometallic complexes that contain bioligands and that have been evaluated as pharmaceuticals for cancer therapy, probes for biosensors, and novel supramolecular structures for molecular recognition studies, to name several representative examples [12-15].

It is also worth reporting that the first International Symposium on Bioorganometallic Chemistry (ISBOMC '02) was convened in Paris on July 18-20, 2002 and will meet every two years in different global venues.

An example of an organometallic complex as a biological probe is the use of ferrocene as a probe for estradiol [16].

Monitoring levels of estradiol is important for assessing ovarian functions and monitoring follicular development for assisted-reproduction protocols, but the traditional radioimmunoassay techniques are nowadays displaced by photochemical sensor-based assays. Furthermore, estradiol is also used to quantify the level of specific estrogen receptors in target organs such as the uterus, as well as in certain breast cancers [17]. Electrochemical detection coupled with an HPLC system have been used as simple analytical technique.
that approaches pM or fM sensitivity for the hormone, provided that the redox-active ferrocene unit is bound to estradiol (called FcEE).

Relative binding affinity measurements show that the modified hormone is still able to react with estrogen receptor, and since the ferrocenium derivatives are well-known for exhibiting antitumor activity [18, 19], the ferrocene-labeled estradiol unit could act as a carrier for the potentially active ferrocene unit, transferring it into cells [20] via the estrogen-receptor system (Figure 1.4).

The modified hormone exhibits a reversible cyclic voltammetry wave at +485 mV and an irreversible wave at ~+1.2 V, due to the ferrocene unit and the OH groups of the steroidal skeleton, respectively.
The cross-reaction rate of this complex for antibodies specific to estradiol was found to be 40%, showing that the complex is still well recognized by the specific antibodies.

The relative binding affinities (RBA) for the α and β subtypes of the estrogen receptor are 28% and 37% at 0° C, respectively, indicating that the modified hormone retains very good affinity for the receptor, in spite of the ferrocenylethynyl entity.

Carbonyl metallo immunoassay (CMIA) adopts the same approach, but it is a competitive immunoassay between the analyte and a metal carbonyl tracer, usually a dicobalt hexacarbonyl complex coupled with analyte of interest [21].

The enzyme is immobilized with the tracer on the surface of the wells of a microplate: after exposure to the analyte, the free tracer is extracted with the suitable solvent and then quantified by FT-IR in order to determine the amount of substrate in the sample solution (Figure 1.5).

![ClN
NHR N »
H Co 2 (CO) g](image)

Figure 1.5: structure of the dicobalt hexacarbonyl tracer of atrazine.

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Labeling of protein and analytes can be combined with medically important radioactive elements ($^{99m}$Tc, $^{188}$Re, $^{186}$Re) or with third row transition metals, in order to use radiolabeling techniques and electron diffraction microscopy for the analysis of protein structure and properties.

In addition to the substantial electronic density of third row transition metals, their presence allows the determination of phase information for protein diffraction data, since it is often the bottleneck of the method when solving completely new structures. Most of the experimental methods available to the protein crystallographer are based on the preparation of heavy-atom derivatives or derivatives including anomalous scatterers [22], which often lack specificity for a given target protein site and do not form covalent adducts [23].

Esters of N-hydroxysuccinimide were first introduced for peptide synthesis in the early sixties because they readily acylate primary amines to give amides in high yields [24]. Aminolysis of these esters (Figure 1.6) is notably much faster than hydrolysis, enabling acylation to be achieved even in aqueous solutions (pH ≥ 7).
Figure 1.6: Aminolysis and hydrolysis of esters of N-hydroxysuccinimide.

This property has led to the development of numerous radio-halogenation reagents, the most popular being the Bolton-Hunter reagent N-succinimidyl 3-(4-hydroxyphenyl)propionate [25]. The transition organometallic complexes N-succinimidyl esters of a variety of metals (Cr, Mn, Fe, Co, Ru, $^{99}$Tc, Mo, W, re, Os, Ir) have been synthesized [26], and the characterization of the protein conjugates has been readily performed by IR spectroscopy of the $\nu_{C=O}$ bands of the metal carbonyl moieties. In particular, the highly luminescent bipyridyl rhenium tricarbonyl N-succinimidyl ester in Figure 1.7 has been prepared for peptide/protein labeling [27].
Figure 1.7: Re(I) Organometallic ester of N-hydroxysuccinimide.

The main targets of such reagents are lysine groups, which can form a covalent amide bond with the organometallic complex. Cysteines and histidines may also be targeted, if N-maleimides or dienyl iron tricarbonyl are used, respectively [26].

1.3 Reference for Chapter 1


Chapter 2

Synthesis and Characterization of Novel Water-soluble Heterocycle Triosmium Clusters.

2.1 Synthesis of water-soluble clusters with general formula \([\text{Os}_3(\text{CO})_9(\mu-(L-H))(\mu-H)(\text{P}(\text{OCH}_2\text{CH}_2\text{N(CH}_3)_3\text{)}_3)]\).

Extensive research has been carried out in our group on the electron deficient coordination of quinoline and related benzo-heterocycles to a triosmium nonacarbonyl or decacarbonyl frame, because bonding of these biomedically important heterocycles imparts unique properties unobtainable with the free ligand. The three-center, two-electron bond between ligand and metals in the nonacarbonyl system imparts otherwise impossible regio- and stereoselective chemistry on the heterocycle, with the result of yielding important precursors for neurotransmitter inhibitors, especially since such bonding is available for a wide variety of heterocycles that can be coordinated in this fashion [1].
Certain complexes of heteroaromatics have long been used for their somewhat selective binding to DNA, where photolytically induced electron transfer results in oxidative cleavage of the bio-macromolecule [2, 3].

In those complexes, however, the positive charge of the metal is an adequate water-solubilizing feature to render the entire coordination complex stable in aqueous solutions.

The first challenge in our research was to provide the triosmium nonacarbonyl heterocycle cluster with the suitable water-solubilizing group.

In order to accomplish our goal, we decided to take advantage of the unique chemistry that the electron deficient nonacarbonyl complexes exhibit. The electron deficiency may be relieved at the heterocycle with a hard
nucleophile (typically a carbanion) or at the metal core with a softer nucleophile, such as a N-containing ligand or a trivalent phosphorus derivative, such as a phospine or phosphite (Figure 2.2) [1].

\[
\text{Os}_3(\text{CO})_9(\mu_3-\eta^2-(L\cdot-H))(\mu-H) + L \rightarrow \text{Os}_3(\text{CO})_9(\mu_3-\eta^2-(L\cdot-H))(\mu-H) + L
\]

**Figure 2.2: Coordination of a soft, 2-electron donor at the metal core.**

We began our studies with the synthesis of water soluble phosphine derivatives of the previously reported \([\text{Os}_3(\text{CO})_9(\mu_3-\eta^2-(L\cdot-H))(\mu-H)]\) (L=3-amino quinoline) [4]. This complex was chosen because it contains a good hydrogen bond donor-acceptor that could enhance binding to biomacromolecules and because the amino group is amenable to further modification (Figure 2.1).

In order to understand the impact of charge on the aqueous behavior of a given water soluble cluster we reacted the electron deficient quinolyl cluster with the commercially available, negatively charged water solubilizing phosphine \([P(\text{C}_6\text{H}_4\text{SO}_3\text{Na})_3]\), and with the positively charged \([P(\text{OCH}_2\text{CH}_2\text{NMe}_3)]_3\] which was synthesized by reaction of \([P(\text{OCH}_2\text{CH}_2\text{NMe}_2)]_3\) with methyl iodide.
The latter was chosen because its positive charge is equal in magnitude to the negative charge on [P(C₆H₄SO₃Na)₃] and because its charge should be independent of pH. Both ligands react quantitatively with the deep green quinolyl complex (λ~ 380 nm in the visible UV region) when an aqueous solution of the phosphine is added to a methanol or acetone solution of the cluster to yield [Os₃(CO)₉(μ-η²-(L-H))(μ-H)L'] (L = 3-amino quinoline, L' = P(C₆H₄SO₃Na)₃, 1; L = 3-amino quinoline, L' = P(OCH₂CH₂NMe₃)₃, 2, according to the reaction scheme in Figure 2.2).

The resulting yellow orange products (λ~ 650 nm) were characterized by LC-MS, ¹H and ¹³C NMR and elemental analysis. The LC-electrospray - MS shows the singly, doubly and triply charged parent ions in the negative ion spectrum for 1 at 1464, 730 and 470 amu (calculated MW= 1535.27 g/mol) (Figure 2.3).

![Figure 2.3: LC-electrospray-MS of the negatively charged cluster [1].](image-url)
The corresponding positive ions were found for 2 at 1565, 717 and 417 amu (calculated MW= 1706.04 g/mol) (Figure 2.4).

The mass spectrum identifies both compounds as the desired water-soluble adducts, along with the $^1$H and the elemental analyses. The unusual aspect of the major peaks observed in the spectrum is due to the fact that osmium has 7 stable isotopes, $^{184}$Os (0.018%), $^{186}$Os(1.59%), $^{187}$Os(1.64%), $^{188}$Os(13.3%), $^{189}$Os (16.1%), $^{190}$Os (26.4), and $^{192}$Os (41.0%), which complicate the spectrum interpretation.

The ESI-MS shows singly, doubly and triply charged ions in the negative spectrum for 1 at 1464, 730 and 470 amu all showing the isotopic distribution patterns associated with the triosmium cluster fragments. The peaks centered at 1464 corresponds $[M^3+ 2Na^+ - CO]^{-}$, the peak at 730 corresponds $[M^3+ Na^+ -
CO\(^2\)- and the peak at 470 corresponds \([M^{3+} \text{ CO}]^3\). The corresponding positive ion cluster fragments for 2 are observed at 1565, \([M^{3+} + 2I]^+\), 717, \([M^{3+} + 1]^2+\) and 417 amu \([M^{3+} - 2\text{CO}]^3+\).

The \(\text{^1H}\) NMR of 1 and 2 both show doublet hydride resonances at -12.03 ppm (\(J_{ph} = 16\) Hz) (figure 2.6) and -12.97 ppm (\(J_{ph} = 16\) Hz) (figure 2.5). The magnitude of these coupling constants suggests that the phosphine is located on the same osmium atom as that of the hydride.

![Figure 2.5: Hydride resonance for cluster 2.](image)

The \(\text{^{13}C}\) NMR of an aqueous solution of a \(\text{^{13}CO}\)-enriched sample of 2 in the carbonyl region (Figure 2.5) reveals a pattern of carbonyl resonances that is very similar to that observed for the corresponding triphenylphosphine-quinoline adduct whose solid state structure has been determined [1, 5].
Both show two phosphorous-coupled resonances of relative intensity one at 185.6 ppm ($J_{PC}= 7.9$ Hz) and 184.3 ppm ($J_{PC}= 5.0$ Hz) for 2 and at 185.64 ppm ($J_{PC}= 6.2$ Hz) and 182.5 ppm ($J_{PC}= 4.6$ Hz) for the corresponding quinoline triphenylphosphine adduct. Both show a set of partially overlapping singlet resonances with a total relative intensity of six in the region 177.4 ppm to 181.3 ppm. Based on this data we propose structures for both 1 and 2 where the phosphine is located on carbon bound osmium cisoid to the hydride ligand (as reported in Figure 2 for the structure of the adduct).

The $^1$H and $^{13}$C NMR resonances for 1 and 2 are significantly broadened in aqueous solution suggesting that these compounds form micelles or related aggregates in solution.
This is particularly true for the aromatic resonances of the heterocyclic ligand (Figure 2.8). In the case of 1, a slow isomerization process is observed (over the course of 24 h) as evidenced by the fact that the initially observed doublet hydride resonance at -12.03 ppm gradually decreases in intensity and new sharp doublet resonances at -12.25 ppm and -12.65 ppm and are observed along with a broad singlet resonance appears at -15.6 ppm (Figure 2.6).

![Figure 2.7: ^H-NMR in D_2O of [1] (detail of the hydride region).](image)

This can be attributed to a dissociative isomerization to yield the possible regioisomers of the phosphine adduct (Figure 2.7).

![Figure 2.8: isomerization of [2] in aqueous solution, L= P(C_6H_4SO_3Na)_3.](image)
An analogous series of isomers has been previously observed in related triosmium clusters [6]. When aqueous solutions of relatively high concentrations of 1 are allowed to stand at room temperature, the dark green $[\text{Os}_3(\text{CO})_9(\mu_3-\eta^2-\text{C}_9\text{H}_7\text{N})(\mu-\text{H})]$ gradually precipitates. This observation seems to corroborate the proposed dissociative isomerization and seems to indicate that the bulky triphenylphosphine would rather aggregate with itself than with the complex 1.

In sharp contrast, 2 showed no tendency to isomerize in aqueous solution. Lowering the pH from 7 to 5 leads to significant sharpening of the aromatic heterocycle ligand resonances (Figure 2.8).

![Figure 2.9: NMR of the aromatic resonances of 2 at 400 MHz in D$_2$O at pH=5 (top); NMR of the aromatic resonances of 2 at 400 MHz in D$_2$O at pH=7 (bottom).](image)

The pK$_a$ of the anilinic nitrogen is approximately 4.6 therefore changing the pH from 7 to 5 lowers the free amine to protonated amine ratio from 200 to 1 to 2 to 1. This increase in the degree of protonation is sufficient to decrease the level of aggregation by placing like charges on the heterocyclic ligand and the metal core. Interestingly, although the aromatic signals are significantly
broadened in 1 and 2, the hydride resonances are relatively sharp. This is probably due to the relatively long relaxation times of the hydrides in these complexes compared with the aromatic protons [7].

Prior work has shown that extending the aromatic ring system from two to three rings greatly improves its ability to intercalate into the major groove of DNA [2, 3]. Therefore we chose to extend the aromatic ring system in 2 by tethering a benzoyl group to the 3-amino quinoline and by utilizing the tricyclic ring system phenanthridine. Both of these systems reacted quantitatively with P(OCH₂CH₂NMe₃)₃ to provide the desired water soluble clusters [Os₃(CO)₉(μ-
η²-(L-H))(μ-H)L] (L = 3- benzamido quinoline, L' = P(OCH₂CH₂NMe₃)₃, 3; L=phenanthridine, L' = P(OCH₂CH₂NMe₃)₃, 4, Figure 2.9). Their ¹H NMR indicated that they are isostructural with regard to the location of the phosphine relative to 2.
2.2 Conclusions.

A new class of triosmium clusters has been synthesized and characterized: the water-soluble products show adequate solubility and stability in aqueous solutions to make them good candidates for interactions with biomacromolecules.

The possibility to further modify the heterocycle either extending it or improving its flexibility also represents an important feature that can be exploited when designing the appropriate cluster for the target macromolecule.
The presence of like charges on different parts of the same molecule increases the concentration of single-solvated molecules versus the aggregates that results from the nature of the water-soluble adduct. The non-polar metal carbonyl frame and the heterocycle are inherently insoluble in aqueous solutions, while the positively charged phosphine or phosphite are well solvated in water. The amphipathic molecule obtained is adequately soluble, but aggregation at physiological pH is a feature of our clusters that will be difficult to avoid.

2.3 Experimental Section

2.3.1 Materials and general considerations.

The complexes, \([\text{Os}_3(\text{CO})_9(\mu-\eta^2-(L-H))(\mu-H)L])\) (L=3-amino quinoline, phenanthridine) were synthesized according to literature procedures [8, 9]. NMR spectra were obtained on a Varian Unity Plus 400 MHz spectrometer and IR spectra were obtained on a Thermo Nicolet 633 FT-IR. Elemental analyses were performed by Schwarzkopf Analytical Labs, Woodside, New York. Osmium carbonyl was purchased from Strem Chemical and used as received; 3-amino quinoline, benzoyl chloride, and phenanthridine were purchased from Aldrich and used as received. \(\text{P(O(CH}_2\text{)}_2\text{N(CH}_3\text{)}_2\text{)}_3\) was purchased from United Organometallics and used as received.
2.3.2 Synthesis of tris-(2-trimethylamino iodide)ethyl phosphite

In a round-bottom (RB) flask, 5.22 mL of P(O(CH₂)₂N(CH₃)₂)₃ (13 mmol) were dissolved in 20 mL of 95 % Ethyl Alcohol at ~0°C in an ice bath. Through a dropping funnel, 4.12 mL (27 mmol) of Methyl Iodide were added drop wise to the solution in the flask. A white precipitate is formed and then 60 mL of diethyl ether were added to precipitate the alkylated product from the solution. The precipitate is vacuum filtered and dried overnight under vacuum. Yield: approx. 11.2 g (~89.6%). (MW=739 g/mol).

¹H-NMR (in D₂O), δ: 4.26ppm (m, 2 H), 3.56ppm (t, 2 H), 3.08ppm (3 H).

Elemental analysis (calculated): C 25.30%, H 5.80%, N 5.5%.

Elemental analysis (actual): C 25.08%, H 5.87%, N 5.7%.

2.3.3 Synthesis of [Os₃(CO)₉(μ-η²-(3-NH₂)C₉H₅N)(μ-H)P(C₆H₄SO₃Na)₃] (1)

50 mg of 3-amino quinoline complex (0.051 mmol) were placed in a round-bottom (RB) flask and dissolved in ~10 mL of methanol (or acetone), then an equimolar amount of P(C₆H₄SO₃Na)₃ (MW=568.4 g/mol), approx. 30 mg, was dissolved in a few drops of water and then added to the aqueous solution of the cluster. The reaction is quantitative and yields ~80 mg (100%) of [Os₃(CO)₉(μ-η²-(3-NH₂)C₉H₅N)(μ-H)(Na₃[PC₆H₄SO₃]₃)] (1) (MW=1535.27).
IR (CO region): 2006 cm⁻¹ (s), 2139 cm⁻¹ (m), 2169 cm⁻¹ (w), 2194 cm⁻¹ (w),
2236 cm⁻¹ (m), 2273 cm⁻¹ (sh), 2297 cm⁻¹ (s), 2318 cm⁻¹ (s) and 2339 cm⁻¹ (s).

¹H-NMR data (D₂O), δ: 9.29 (s, 1H), 8.13 (d, 1H), 7.43 (s, 1H), 7.36 (m,
12H), 7.08 (d, 1H), 7.02 (t, 1H), -13.01 (d, 1H, JₚH=16.0 Hz).

Elemental analysis (calculated): C 28.16%, H 1.3%, N 1.8%.
Elemental analysis (actual): C 28.53%, H 1.94%, N 1.82%.

2.3.4 Synthesis of [Os₃(CO)₉(μ-η²-(3-NH₂)C₉H₅N)(μ-H)(P(OCH₂CH₂N(CH₃)₃)₃)]
(2)

In a RB flask, 50 mg of [Os₃(CO)₉(μ-H)(3-NH₂)C₉H₅N)(μ-H)] (0.050 mmol)
were dissolved it in ~10 mL of methanol (or acetone), then an equimolar amount
of [P(O(CH₂)₂N(CH₃)₃)₃], 38.2 mg, was dissolved in a few drops of water and
then added to the cluster solution. The reaction is quantitative and yields ~88 mg
(100%) of yellow product (MW=1705.87).

IR (CO region): 2013 cm⁻¹ (s), 2139 cm⁻¹ (m), 2172 cm⁻¹ (w), 2205 cm⁻¹ (w),
2228 cm⁻¹ (sh), 2275 cm⁻¹ (sh), 2317 cm⁻¹ (s), 2342 cm⁻¹ (s), 2358 cm⁻¹ (s).

Elemental analysis (calculated): C 23.00%, H 2.35%, N 4.1%.
Elemental analysis (actual): C 22.84%, H 2.13%, N 3.97%.

H-NMR data (D₂O), 8: 9.30ppm (s, 1 H), 8.1ppm (d, 1 H), 7.4ppm (s, 1 H),
7.12ppm (d, 1 H), 7.02ppm (t, 1 H), 2.9ppm (s, 27 H), 3.4ppm (m, 6 H), 3.07ppm
(s, 3 H), 3.04 (s, 3 H), -12.97ppm (d, 1 H, JₚH = 16 Hz)).
C-NMR data (D$_2$O), $\delta$: 177.1 ppm (s), 178.2 ppm (s), 179.2 ppm (s), 180.2 ppm (s), 181.1 ppm (s), 184.2 ppm (t, $J_{PC}=5.0$ Hz), 185.1 ppm (t, $J_{PC}=7.9$ Hz), 186.1 ppm (s).

2.3.5 Synthesis of [Os$_3$(CO)$_9$(μ$_3$-η$^2$-(3-NH-CO-CH$_2$-C$_6$H$_5$)C$_3$H$_5$N)(μ-H)]

Os$_3$(CO)$_9$(μ$_3$-η$^2$-(3-NH$_2$)C$_3$H$_5$N)(μ-H), 50 mg (0.050 mmol) was dissolved in ~ 10 mL of dry tetrahydrofuran. Triethyl amine, 7 mL, and then 6.6 mL of phenyl acetyl chloride were added, both in stoichiometric amounts. The reaction was complete when triethyl ammonium chloride, the reaction byproduct, precipitated in the solution. After filtration, the solvent is rotary evaporated and the product recovered as a green solid. The reaction was made quantitative by adding a 10% excess of trimethylamine, which could be easily removed during the solvent evaporation step.

IR (hexane, CO region): 2072 cm$^{-1}$ (m), 2040 cm$^{-1}$ (m), 2011 cm$^{-1}$ (s), 2001 cm$^{-1}$ (s), 1978 cm$^{-1}$ (sh), 1962 cm$^{-1}$ (s), 1953 cm$^{-1}$ (s), 1926 cm$^{-1}$ (s).

$^1$H-NMR, (D$_2$O), $\delta$: 9.7 ppm (s, 1 H), 8.7 ppm (d, 1 H), 8.6 ppm (s, 1 H), 8.4 ppm (dd, 1 H), 7.34 ppm (m, 5 H), 7.2 ppm (t, 1 H), -11.97 ppm (s, 1 H).

Elemental analysis (calculated): C 28.78 %, H 1.29 %, N 2.58 %.

Elemental analysis (actual): C 29.01 %, H 1.40 %, N 2.61 %.
2.3.6 Synthesis of \[\text{[Os}_3\text{(CO)}_9(\mu-\eta^2-(3-\text{NH-CO-CH}_2\text{-C}_6\text{H}_5)\text{C}_9\text{H}_5\text{N})(\mu-\text{H})} \]
\(\text{(P(OCH}_2\text{CH}_2\text{N(CH}_3\text{)}_3\text{I})_3]\) (3)

50 mg of \(\text{Os}_3\text{(CO)}_9(\mu_3-\eta^2-(3-\text{NH-CO-CH}_2\text{-C}_6\text{H}_5)\text{C}_9\text{H}_5\text{N})(\mu-\text{H})\) (0.050 mmol) are dissolved in \(\sim\) 10 mL of methanol (or acetone) and then a stoichiometric amount of \(\text{P(OCH}_2\text{CH}_2\text{N(CH}_3\text{)}_3\text{I})_3\) is dissolved in a few drops of water and then added to the cluster solution. The reaction is quantitative.

IR (\(\text{D}_2\text{O, CO region}\)): 2086 cm\(^{-1}\) (w), 2073 cm\(^{-1}\) (w), 2042 cm\(^{-1}\) (sh), 2020 cm\(^{-1}\) (sh), 1996 cm\(^{-1}\) (s), 1974 cm\(^{-1}\) (s), 1959 cm\(^{-1}\) (s), 1938 cm\(^{-1}\) (s).

H-NMR, (\(\text{D}_2\text{O}\)), \(\delta\): 9.30 ppm (s, 1 H), 8.1 ppm (d, 1 H), 7.4 ppm (s, 1 H), 7.34 ppm (m, 5 H), 7.12 ppm (d, 1 H), 7.02 ppm (t, 1 H), 2.9 ppm (s, 27 H), 3.4 ppm (m, 6 H), 3.07 ppm (s, 3 H), 3.04 ppm (s, 3 H), -12.97 ppm (d, 1 H, \(J_{PH} = 15.9\) Hz).

Elemental Analysis (calculated): C 28.97 %, H 3.23 %, N 3.83 %.

Elemental analysis (actual): C 28.52 %, H 3.33 %, N 3.85 %.

2.3.7 Synthesis of \[\text{[Os}_3\text{(CO)}_9(\mu-\eta^2-C_{13}\text{H}_8\text{N})(\mu-\text{H})} \]
\(\text{(P(OCH}_2\text{CH}_2\text{N(CH}_3\text{)}_3\text{I})_3]\) (4)

The same procedure as for \(\text{Os}_3\text{(CO)}_9(\mu-(3-\text{NH}_2)\text{C}_9\text{H}_5\text{N})(\mu-\text{H})\text{(P(OCH}_2\text{CH}_2\text{N(CH}_3\text{)}_3\text{I})_3}\) was used.

IR (CO region): 2075 cm\(^{-1}\) (w), 2073 cm\(^{-1}\) (w), 2038 cm\(^{-1}\) (sh), 2025 cm\(^{-1}\) (sh), 1991 cm\(^{-1}\) (s), 1967 cm\(^{-1}\) (s), 1961 cm\(^{-1}\) (s), 1945 cm\(^{-1}\) (s)
H-NMR, (D₂O), 8: 9.45 ppm (d, 1H), 8.91 ppm (d, 1H), 8.31 ppm (d, 1H), 7.92 ppm (s, 1H), 7.41 ppm (dd, 1H), 7.36 ppm (dd, 1H), 7.16 ppm (dd, 1H), 7.10 ppm (d, 1H), 2.9 ppm (s, 27 H), 3.4 ppm (m, 6 H), 3.07 ppm (s, 3 H), 3.04 ppm (s, 3 H), -11.89 ppm (JₚH=15.87 Hz).

Elemental analysis (calculated): C 27.22%, H 3.05 %, N 3.17 %.

Elemental analysis (actual): C 27.00%, H 3.03%, N 3.21%.

2.4 References for Chapter 2


Chapter 3

DNA Binding Affinities of Water-soluble Triosmium Heterocycle Clusters.

3.1 Plasmid Relaxation Test.

After the candidate clusters were synthesized and characterized, experiments were undertaken to establish the nature of their DNA interaction and the best way to assay their binding.

Previous studies have shown that one of the most sensitive ways to detect small molecules-DNA interactions was by measuring electrophoretic mobility of circular double stranded DNA [1-3].

Plasmids are circular fragments of double stranded DNA genetically engineered for cloning purposes: they often contain a multicloning site, where different recognition sequences for various endonucleases allow microbiologists to open the plasmid (also called vector for their role as vehicles of foreign DNA)
in a specific fashion in order to insert a particular DNA fragment corresponding to a specific protein sequence that must be expressed. Usually, the plasmid is then inoculated into a competent single-cell organism and replicated by its genetic machinery. The plasmid itself may also be replicated in this fashion, and then recovered by selective precipitation from the cell lysate, since it is so much smaller than the organism’s genomic material.

Typically, a plasmid is present as a mixture of different physical forms: supercoiled and relaxed; supercoiled plasmid DNA is a more compact form, obtained by topoisomerases that twist the two strands of the DNA duplex with respect to each other: this creates strain that is removed by the circular structure by wrapping on itself. When one strand only of the DNA is cut, the strain created by topoisomerases is relieved and a simple open circular form is obtained (Figure 3.1).

![Relaxed plasmid and Supercoiled plasmid](image)

**Figure 3.1: Cartoon Representations of Supercoiled and Relaxed Plasmid DNA.**

Increased levels of relaxed plasmid are observed when the molecule interacting with it causes cleavage of one strand only, allowing the other strand to freely rotate, in fact unwinding the supercoiled structure.
In an electrophoresis apparatus, it is possible to distinguish between the two forms: although the ratio of mass over charge is the same for the same plasmid, the more compact form of supercoiled plasmid travels into an agarose gel matrix faster than the corresponding relaxed form.

Because of this peculiar property, plasmid has also been used as a sensitive tool to detect the interaction of small molecules with DNA. By monitoring the relative ratio of supercoiled to relaxed, it is possible to compare the binding affinities of different compounds. Certain coordination complexes can cause cleavage in both strands, forming linear DNA [3], which migrates in the agarose gel matrix between the supercoiled and the relaxed forms.

A more general, retardation effect is also possible, when no cleavage is observed but binding causes slower migration in the gel, due to higher mass or lower net charge, increasing the electrophoretic mobility.

The interaction of low oxidation state transition metal clusters with DNA has been evaluated with this particular assay: the compound [H4Ru4(C6H6)4][BF4]2, in particular, completely retards the DNA, and the product is visible in the loading well [2].

Another cluster tested, Ru9(CO)9(PTA)3 (PTA = 1,3,5-traza-7-phosphatrircyclo[3.3.1.1]decane), made the plasmid migrate as a smudged band running between the positions of supercoiled and open circular DNA.

The effects are attributed either to intercalation or covalent binding to the DNA, possibly cross-linking different positions in the nucleic acid sequence.
One obvious limitation of this assay is the fact that the nature of the interaction observed cannot be unequivocally determined based simply on the position of the retarded bands. The main advantage, on the other hand, is that the assay is very sensitive, allowing the use of very small amounts of DNA and test compound to assess whether an interaction occurs.

Furthermore, DNA precipitation in the wells does not indicate extensive cross-linking or covalent binding, unless other assays are designed to actually prove it. In our case, the plasmid relaxation assay represents an excellent tool to screen a large number of compounds quickly for even small molecule-DNA interactions.

The Plasmid Relaxation Test was conducted using commercially available pUC19 plasmid DNA. Upon incubation with the cluster, the super-coiled form can unwind to the open circular shape if one strand is cut ("nicked") or, if double strand cleavage occurs, it can open to linear double stranded DNA. Any modification in shape and/or charge can be accurately detected in a 1% agarose gel and by subsequent gel staining with ethidium bromide.

A concentration gradient of 3 different water-soluble clusters was incubated with the plasmid in order to understand whether any interaction occurred. The incubation time was 1 hour at 37° C and the different concentrations were run against 2 controls, one being the untreated plasmid and the other being the linear DNA obtained by digestion of the plasmid with the restriction enzyme BamHI.
As expected, the negatively charged cluster \([\text{Os}_3(\text{CO})_9(\mu-\eta^2-(\text{L-H}))((\mu-\text{H}))\text{L}')]\) (L = 3- amino quinoline, L' = P(C_6H_{12}SO_3Na)_3, 1 showed no evidence of binding to the plasmid due to the repulsive interaction with the negatively charged DNA backbone. In order to examine the strictly electrostatic component of cluster binding the model compound \([\text{Rh}_3(\mu_3-S)_2(\eta^5-\text{Me}_5\text{C}_3)_3]^{2+}\) was tested first as its tetrafluoroborate salt [4]. Band retardation was observable at 375 \(\mu\text{M}\) and increased until 1 mM when precipitation of the DNA cluster complex in the well occurred (Figure 3.2).

![Figure 3.2: Ethidium bromide stained plasmid relaxation test in a 1% agarose gel incubated with various concentrations of \([\text{Rh}_3(\mu_3-S)_2(\eta^5-\text{Cp}^\text{3})(\text{BF}_4)_2]\).](image)

The complex \([\text{Os}_3(\text{CO})_9(\mu-\eta^2-(\text{L-H}))((\mu-\text{H}))\text{L}]\) (L = 3- amino quinoline, L' = P(OCH_2CH_2NMes)_3, 2 was then tested in an identical manner. Interaction is again observed in the form of band retardation with respect to the unaltered
DNA. This means that the DNA mass/charge ratio has changed upon incubation with the cluster. Significant band retardation was observable at 41 μM and precipitation occurred at 50 μM (Figure 3.3). This represents an approximately 9 fold increase in binding affinity relative to [Rh₃(μ₃-S)₂(η⁵-Me₅C₃)₃]²⁺.

The "dangling phenyl ring", water-soluble cluster, [Os₃(CO)₉(μ-η²-(3-NH₂)C₅H₅N)(μ-H)(P(OCH₂CH₂N(CH₃)₃)₂)] (L = 3- benzamido quinoline, L' = P(OCH₂CH₂NMe₃)₃, 3, exhibited an even stronger binding affinity than 2. Band retardation was observable at 8.2 μM and precipitation was complete at 33 μM representing an approximately 5-fold increase in binding affinity relative to 2 (Figure 3.4).
Finally, we tested the binding affinity of the phenanthridine complex 
\[ \text{Os}_3(\text{CO})_9(\mu-\eta^2-(3-\text{NH COCH}_2\text{C}_6\text{H}_3)\text{C}_9\text{H}_5\text{N})(\mu-H)(\text{P(OCH}_2\text{CH}_2\text{N(CH}_3)_3\text{I})_3) \] (3).

which has a more extensive ring system than 2 but a more rigid fused three-ring system relative to 3 and therefore may interact with DNA in a different fashion. The relaxation test results are shown in the figure below. The degree of band retardation observed at 41.2μM for 2 and at 8.2μM for 3 in the previous experiments is now present at the concentration of 24.7μM (Figure 3.5). Precipitation was apparently complete at 33 μM, which is very similar to the observed results for complex 3.
The plasmid relaxation test was chosen for the bioassay of 1-4 for multiple reasons: the high sensitivity of the test allows working at very low concentrations of DNA and cluster; facile DNA detection can still be obtained at those concentrations by staining the gel with ethidium bromide after electrophoresis, thereby avoiding competition between the dye and the cluster.

The primary objective of these experiments was to see if there was a relationship between DNA affinity and the structure of the heterocycle ligand bound to the cluster.

Using the plasmid DNA, whose structure is invariant, optimizes the chances of detecting a structural relationship, in contrast to using, for example, calf thymus DNA, which is more heterogeneous.

Our results do not allow an unequivocal characterization of the interaction observed, but an important point can be made: whatever interaction
is occurring upon incubation, the cluster concentration at which it can be observed is different for each cluster. As the charge on the water-soluble ligand is constant in all the experiments with complexes 2 and 3, the heterocycle must contribute to the observed effect because the heterocycle is the only part of the cluster that changes in the experiment.

As the most reactive cluster features a dangling "arm" comprised of a phenyl ring connected to the heterocycle by a peptide linkage, we believe that the presence of a flexible ligand remote enough from the bulky triosmium frame is a critical factor in enhancing the ability of the cluster to adapt and bind to the DNA structure.

This assay represents a useful tool to detect DNA-small molecule interaction at micromolar concentrations, and is a first necessary step to understand whether a particular triosmium cluster is a satisfactory candidate for DNA binding.

3.2 Binding of $[\text{Os}_3(\text{CO})_9(\mu-\eta^2-(L-H))(\mu-H)L']$ ($L = 3$- amino quinoline, L' = P(C$_6$H$_4$SO$_3$Na)$_3$) [1] to an RNA-enzyme complex for X-ray structure determination.

The negatively charged water-soluble cluster 1 was used to resolve the X-ray structure of the RNA-ribonuclease $p$ complex whose crystals were already available. The cluster was allowed to diffuse into the crystals from an aqueous solution in order to determine whether the cluster selectively binds to specific
regions of the crystallized complex. Binding of the cluster did not disrupt the crystallinity of the biologic complex, and the unit cell shows the presence of diffraction spots due to cluster molecules regularly positioned into the lattice (Figure 3.6).

Figure 3.6: Unit cell of the RNA-enzyme complex after binding of 1 to a specific RNA-ribonuclease p complex. Patterson map is provided as courtesy of Kevin Compher, Kevin Jude, and David Christianson, of the University of Pennsylvania.

Due to the limits of resolution attained at this moment (7-10 Å), only the general shape of the nucleic acid-RNAse complex is discernible; further efforts
to increase the resolution to 3-5 Å are necessary to obtain a clear picture of the amino acid side chains.

This preliminary result represents an important proof of concept for the entire project that this dissertation focuses on, since selective binding of a triosmium cluster to a specific part of a biomacromolecule is necessary to acquire crucial phase information for structure determination.

3.3 Experimental section

3.3.1 Synthesis of [Rh₃(μ₃-S)₂(η⁵-Me₅C₅)₃][BF₄]₂.

The synthesis was carried out according to the published procedure [4].

3.3.2 Plasmid relaxation test

pUC19, 0.6 µL (0.6 µg), was suspended into 20 µL of 10 mM Tris-HCl pH=7.4, 100 mM NaCl and then incubated with the reported concentration gradient of water-soluble cluster at 37° C for 1 hour. Right before loading in a 1% agarose gel, 2 µL of glycerol loading buffer were added. The gel was run for 5 hour at ~37 mV. The gel was subsequently soaked in a 1% solution (v/v) of ethidium bromide in double distilled water and destained in the same water overnight to obtain better light contrast and band definition. The bands were then visualized on a Transilluminator (UVP, Inc.) and photographed on a Polapan 665 Polaroid film with an exposure of 15 sec.
3.4 References for Chapter 3


Chapter 4

Covalent binding of a Positively Charged Water Soluble Benzoheterocycle Triosmium Cluster to single- and double-stranded DNA.

4.1 Covalent Modifications of DNA.

Covalent modifications of DNA caused by inorganic or organic agents, in particular metals, have proven to be a valuable structural tool [1, 2].

The mechanism of DNA scission by the tetrahedral 2:1 (1,10-phenanthroline) copper (I) complex consists of oxidation of the deoxyribose moiety using hydrogen peroxide as an essential coreactant: the principal products are free nucleic acid base, 5-methylenefuranone, and 3'- and 5'-phosphorylated termini. By performing a series of experiments with different labeled solvents (H$_{2}^{16}$O and H$_{2}^{18}$O) and coreactant (H$_{2}^{16}$O$_{2}$ and H$_{2}^{18}$O$_{2}$), H$_{2}^{18}$O is indicated as the sole source of the carbonyl oxygen in 5-methylenefuranone: based on this and other evidence, the proposed mechanism includes attack of water on a positively charged intermediate resulting from the oxidation of the DNA bound copper complex by hydrogen peroxide. An important feature of
this mechanism is that the phosphodiester backbone is cleaved prior to attack of water [1].

The complex [Rh(phen)₂phi]³⁺ and its derivatives bind tightly in the major groove of DNA through intercalation of the phi ligand. After irradiation with a Hg/Xe lamp for 8 minutes in the presence of DNA, the complex cleaves the DNA at the 5’- position with respect to the intercalation site, indicating access through the major groove [2].

In order to increase or modify the sequence selectivity of the complex, triplex-inducing oligonucleotides have been coupled to the Rh intercalator and larger planar aromatic ligands have been coordinated (such as 5,6-chrysenequinone diimine) in order recognize base mismatches in DNA [3, 4].

Another characterized metal–DNA interaction has been the effect of Cr (VI) on nucleic acids has been an area of intensive study, due to its relationship to Cr(VI) induced cancers [5].

In particular, the oxidation product of guanine commonly referred to as 8-oxoG has been accepted as an important biological marker in order to understand the metabolic pathway that eventually leads to further modifications and promotion of cancer in cells [6].

The use of metal complexes or clusters as markers for DNA represents a parallel approach that involves the binding of one or more metal centers to DNA at a specific position in the sequence.
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 [7], we decided to test the same cluster with double- and single-stranded DNA for possible covalent binding to nucleophilic functionalities. These 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.

4.2 Synthesis and binding affinity of \([\text{Os}_3(\text{CO})_9(\mu-\eta^2-(4-\text{CHO})\text{C}_9\text{H}_5\text{N})(\mu-\text{H})\text{P}(\text{OCH}_2\text{CH}_2\text{N}(\text{CH}_3)_3\text{I})_3)](5)\).

The electron-deficient precursor \(\text{Os}_3(\text{CO})_9(\mu_2-\eta^3-(4-\text{CHO})\text{C}_9\text{H}_5))(\mu-\text{H})\) was synthesized by reaction of quinoline 4-carboxaldehyde with \(\text{Os}_3(\text{CO})_{10}(\text{CH}_3\text{CN})_2\), followed by photolytic decarbonylation, as reported for the clusters in chapter 1 [8].

The water-soluble cluster \([\text{Os}_3(\text{CO})_9(\mu-\eta^2-(4-\text{CHO})\text{C}_9\text{H}_5\text{N})(\mu-\text{H})\text{P}(\text{OCH}_2\text{CH}_2\text{N}(\text{CH}_3)_3\text{I})_3)](5)\) was obtained 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, according to the general reaction scheme reported for clusters 1, 2, 3, and 4.
We first repeated the plasmid relaxation tests with pUC19, because the assay's high sensitivity has been an excellent tool for detecting metal complex binding to DNA.

In such studies, the plasmid pUC19 was incubated with a concentration gradient of 5 (1 μM, 2 μM, 3 μM and 4 μM) under physiological conditions (10 mM Tris-HCl buffer pH=7.4, NaCl 100 mM) at ~37°C for 16 h. (overnight) and then analyzed by agarose gel electrophoresis.

In the case of 5, however, the gel revealed a pattern of 3 discrete bands whose intensity increased with increasing cluster concentration (Figure 4.1), and the bands also migrated slower in the gel with increasing cluster concentration. The highest concentration caused precipitation, due to the non-specific salt effect, which is always present and minimized by running the gel in 0.5X TB buffer.

![Fig. 4.1: Plasmid relaxation test of pUC19 and (1). 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.](image)

All the lanes, including the control, show the presence of discrete bands, but these bands seem to run closer to each other and progressively slower with
increasing addition of 5: most importantly, no smearing is present, indicating a
discrete addition of cluster molecules onto pUC19, because a non-covalent
interaction would appear in the agarose gel as a smeared band, just like we
obtained with the previously tested clusters and pUC19.

The farthest traveling band in the gel corresponds to super-coiled
plasmid, immediately followed by the relaxed form: since both bands are
present in the control lane, they are probably due to naturally occurring plasmid
relaxation upon freezing-thawing cycles.

The observed increase in band intensity with increased cluster
concentration may be the result of inconsistencies in loading of the agarose gel
thus shedding doubt on the conclusions drawn form the observations described
above.

This different behavior of the aldehyde functionality with plasmid DNA
prompted us to investigate the interaction of 5 with a smaller single-stranded
twenty-two-oligonucleotide, of sequence 5'-AGT TGT GGT GAG TTT CGC
AGG C-3'. Evidence of binding to such an oligomer would allow the use of
radiolabeling techniques (32P) in order to identify the site of binding.

We repeated the experiment and duplicated the conditions used with the
plasmid test and separated the products by HPLC. Using polyacrylamide-
packed P6 columns to purify the 22-mer after incubation with 5 before injection
onto the HPLC column was not successful, as most of the DNA was retained in
the P6 column and lost.
Direct injection of the reaction mixture onto the anion-exchange HPLC column achieved the separation of the modified oligonucleotide from the excess of positively charged cluster, with no DNA loss. Product formation is dose-dependent and maximized in the presence of a 10-fold excess of cluster (Figure 4.2).

![Graph](image)

**Fig. 4.2:** Product detection of KDSGT1 and [5] by HPLC after direct injection onto an anion exchange column: unmodified KDSGT1 (purple profile); equimolar ratio of KDSGT1 and [5] (red profile); 10-fold excess of [5] (green profile).

### 4.3 Sequencing of covalently modified 22-mer single-stranded DNA.

The gel sequencing experiments were carried out by dilution of the 20 mM stock solution of 5 to 20 μM and then volumes ranging from 0.5 μL to 1.5 μL were added to a 20 μL reaction volume of the 5'-radiolabeled oligonucleotide KDSGT1 (sequence 5'-AGT TGT GGT GAG TIT GGG AGG G-3'), maintaining the 10-fold excess of cluster that optimized product detection by HPLC.
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. The reason for using this approach was supported by the hypothesis that the Schiff base formed between cluster and DNA bases would labilize the bases' N-glycosidic bond and result in strand cleavage when exposed to alkali. To our surprise, no cleavage products were detected in the gel, only non specific background cleavage was observed (Figure 4.3).

Fig. 4.3: Sequencing gel of KDSGT1 after incubation with 5 and treatment with piperidine. Lane 1: control; lane 2: KDSGT1 and (5); lane 3: Maxam-Gilbert AG sequencing product; lane 4: Maxam-Gilbert CT sequencing product.
Based on this result it may be concluded 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 [9, 10]. We therefore decided to change the assay and use the exonuclease Exo III, isolated from yeast [9]. 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 alkaline strand-cleavage conditions. The reason for changing these conditions was supported by 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 requires duplex DNA. The reaction was carried out and incubated with Exo III at 37° C for 5 minutes. The gel showed a pronounced band in the control lane, due to enzymatic digestion, but in a different position than the bands in the lanes corresponding to cluster-treated oligonucleotide, consistent with digestion of the unmodified oligonucleotide to a shorter strand. 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 cluster-treated
oligonucleotide indicate a modification in the DNA sequence that blocked enzyme activity (Figure 4.4).

Fig. 4.4: Sequencing gel of 22-mer KDSGT1 after incubation with [5] 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: Maxaam-Gilbert AG sequencing products.
Because of the extreme sensitivity of the enzyme to modifications in the DNA sequence and structure, small ratios of 5 to KDSGT1 caused stops in the nuclease activity of Exo III: at the lowest cluster concentration (250 nM), there is only 2.5 fold excess of cluster per DNA, but it is still adequate to block enzyme activity; the higher concentration (750 nM) corresponds to a 7.5-fold excess.

Although the stops are evident in the gel, the partial digest products do not line up with the products in the sequencing lanes from the Maxam-Gilbert protocol.

It is highly possible that different termini are involved, in particular a 3' phosphate terminus for the Maxam-Gilbert products and 3' phosphoglycaldehyde terminus for the Exo III partial digestion products.

Another possibility is that cluster molecules are bound to KDSGT1 past the position at which the exonuclease stops: since every cluster molecule changes mass and charge of the oligonucleotide, the electrophoretic mobility will significantly change as well.

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 (NaBH₄) can reduce the Schiff base formed between an essential Lysine residue in the enzyme ad the reducing end of the deoxyribose sugar in the modified nucleotide (Figure 4.5) [11].

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Figure 4.5: NaBH₄ trapping of hOGG1-DNA adduct.

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 NaBH₄.

We first repeated the HPLC analysis of the reaction products, and observed a different profile with respect to the three different products observed in absence of the reducing agent (Figure 4.6).

Figure 4.6: HPLC chromatogram of KDSGT1 and [5] in presence of NaBH₄; unmodified KDSGT1 (blue profile); KDSGT1 and [5] after reduction with NaBH₄ (purple profile); KDSGT1 and NaBH₄ (green profile).
Compared with the previous chromatogram only two peaks are now observed, but one peak also appears in the sample of KDSGT1 treated only with NaBH₄, indicating that only one major product seems to be produced once the cluster-DNA adduct is incubated with the reducing agent (Figure 4.6).

In light of this result, we repeated the sequencing gel work in presence of NaBH₄, 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 finally showed only one major product, corresponding with a particular guanine in the sequence (Figure 4.7).

![Figure 4.7: sequencing gel of KDSGT1 after incubation with NaBH₄ and reduction with NaBH₄. 1: control; 2: 500 nM cluster concentration; 3: 500 nM cluster concentration, 100mM Na(CN)BH₃; 4: 500 nM cluster concentration, 100mM NaBH₄; 5: Maxaam-Gilbert AG sequencing products.](image)

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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 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 lack of important sequence binding information.

The fact that reduction of the cluster-DNA adduct by NaBH₄ 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 5.

4.4 Conclusions.

The band retardation in the plasmid relaxation test suggests that this new cluster does bind to the super-coiled structure of pUC19, but the absence of smearing indicates that it does not bind in the same dynamic fashion as the previous clusters, 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 the other clusters tested so far, in which the heterocycles could at best partially intercalate or form hydrogen bonds. 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 oligomer occur, since discrete peaks are clearly distinguishable rather than a continuous distribution of products.

Most importantly, when HPLC analyses were performed on clusters 2, 3, and 4 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. This result clearly demonstrates the nature of the interaction of 5 with the oligonucleotide relative to the other clusters tested so far. Because of the anion exchange column used, this result clearly indicates a weaker interaction and the formation of products that do not survive 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 displacement of the modified base and 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 5 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 5 and KD5GT1 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'-32P-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 dimension of the lesion, the enzyme may stop its phosphatase activity at the nucleotide in the 5' position from the actual modification [9].

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 5.

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. Assuming that the enzyme stops one nucleotide before the actual lesion, as reported in the case of adenine dimers induced by UV light [9], it could be inferred that the binding sites correspond to two adjacent guanines underlined in bold in the sequence (5'-AGT TGT GGT GAG TTT CGC AGG G-3'). However, it is quite risky to base such a conclusion on a fairly questionable result, because sequencing gels rely on the accurate match of cleavage products with known fragments produced by known chemical modifications.

Later results indicate the formation of only one product after reduction, and the binding site corresponds to a single guanine positioned 2 nucleotides 5'-to the adjacent guanines we first considered as binding site (5'-AGT TGT GGT GAC TTT CCC AGG C-3').

It therefore seems 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 5 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 5 poses a
problem when the modified oligos are separated in a denaturing polyacrylamide gel: partial loss of cluster due to those 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 first one being uncharged phosphoglycaldehyde and the second one bearing a phosphate group.

In the presence of a reducing agent, the C=N double bond is reduced to a single C-N bond that is 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 Brønsted 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.
Figure 4.8: Alkaline cleavage of methylated guanine in presence of piperidine.

After reduction of the proposed Schiff base by NaBH₄, 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 reduction of the aldehyde functionality on the benzoheterocycle is always competing with the reduction of C=N, and the cluster is present in large excess, it is conceivable that reduction of the cluster would shift the equilibrium toward the Schiff base formation toward the reagents, reverting the covalent products back to the unmodified oligonucleotide (Figure 4.9). Furthermore, the more labile Schiff bases will be depleted first, leaving only the thermodynamically most stable one available for reduction.
Figure 4.9: Competitive reduction of the aldehyde functionality and the Schiff base.

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 guanidine heterocycle to a quite 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; because of the increased instability, the unbound cluster is more susceptible to being reduced instead of the C=N moiety, overall depleting the fraction of cluster molecules bound to those nucleotides at any one time. 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.

4.5 Experimental Section

The water solubilizing phosphite ligand P(OCH₂CH₂N(CH₃)₃I)₃ was synthesized according to published literature procedures [12]. The cluster Os₃(CO)₉(μ₃-η²-(4-CHO)C₆H₅N)(μ-H) was synthesized by published literature procedure [13]. 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. ³²P-γ-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 Shwarzkopf Microanalytical Laboratories, Woodside, NY. Infrared spectra were run on a Thermo Nicolet 633 FT-IR and NMR spectra were run on a Varian Unity Plus 400MHz.

4.5.1 Synthesis of Os₃(CO)₉(μ-η²-(4-CHO)C₆H₅N)(μ-H)
(P(OCH₂CH₂N(CH₃)₃I)₃) (5).

20 mg (0.02 mmol) of [Os₃(CO)₉(μ₃-η²-(4-CHO)C₆H₅N)(μ-H)] was dissolved in a minimum amount of acetone and then 15 mg (0.02 mmol) of
P(OCH₂CH₂N(CH₃)₃l)₃ dissolved in a few drops of deionized water was added to the acetone solution while stirring. The dark green solution turns amber instantly. The reaction was quantitative and yielded a dark amber solid product, after rotary evaporation of the solvent, Os₃(CO)₉(µ-η²-(4-CHO)C₉H₅N)(µ-H)(P(OCH₂CH₂N(CH₃)₃l)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.

IR (νCO, H₂O): 2093 cm⁻¹ (w), 2042 cm⁻¹ (m), 1991 cm⁻¹ (s), 1955 cm⁻¹ (sh), 1926 cm⁻¹ (sh).

¹H-NMR (D₂O, water suppressed, δ): 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), 3.04 ppm (s, 3H), 2.92 ppm (s, 27 H), -13.13 ppm (d, 1H, J_HP= 16.1 Hz).

Elemental analysis (calculated): H 3.05%, C 25.84%, N 3.27%.

Elemental analysis (actual): H 2.85%, C 25.76%, N 3.35%.

4.5.2 Plasmid Relaxation Test.

pUC19, 0.6 µL (0.6 µg), was suspended into 20 µL of 10 mM Tris-HCl pH=7.4 100 mM NaCl and then incubated with a gradient of water-soluble cluster (2 µM-4 µM-6 µM-8 µM) at 90° C for 30 min. Right before loading in a 1% agarose gel, 2 µL of glycerol loading buffer were added. The gel was run for 1 hour at ~90 mV. The gel was stained with a 5% solution of ethidium bromide in double distilled water for 30 minutes and then washed in the same water.
overnight. The bands were then visualized on a Transilluminator (UVP, Inc.) and photographed on a Polapan 665 Polaroid film with an exposure of 15 sec.

4.5.3 HPLC Analysis of Products.

The unmodified and modified KDSGT1 oligonucleotides were purified using a Dionex Nucleopac PA100, 4 mm x 250 mm anion exchange column employing a linear gradient from 90% mobile phase A (10% aqueous acetonitrile) and 10% mobile phase B (1.5M 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.

4.5.4 Sequencing gels.

Radio-labeled KDSGT1, 0.0198 ng, was suspended in 20 μM of 10 mM Tris-HCl buffer pH=7.4 100 mM NaCl, and incubated with a gradient of cluster concentration (250 nM- 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. When alkaline-labile cleavage site 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 mixture was then annealed to give the double-stranded
oligonucleotide. The reaction volume was brought to 50 µL with Exo III buffer
(Tris-HCl pH=7.4, 50 mM Mg²⁺). Exo III was then added (0.5 units), incubated at
37° C for 1 hour, and deactivated by heating at ~90° C for 10 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 x
50 cm denaturing (7 M urea) polyacrylamide gel. Electrophoresis was carried
out at 1500 V and 24 mA with 1 x 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 (10X) NaN₃ 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 minutes. The solutions were evaporated to dryness and
then washed with 20 µL of distilled water twice before adding the formamide
loading buffer.
4.6 References for chapter 4.


4. Nunez, M.E.N., K.T., Gianolio, D.A., McLaughlin, L.W., Barton, J.K.,

   1315-1322.


12. Rosenberg, E.S., F.; Sugden, K.; Martin, B.; Gobetto, R.; Milone, L.; Viale,
Chapter 5.

Redox Active Water-soluble Triosmium Clusters and their Interaction with Proteins.

5.1 Introduction

UV-visible spectroscopy has been often employed as a valuable technique to detect or quantify transition metal complexes or organometallic compounds once they bind to biomacromolecules, because they possess absorption and/or emission spectra in spectroscopic regions where most biomacromolecules are silent. Another research area that has developed incredibly sensitive techniques for the detection of metal containing biomolecules is electrochemstry: by measuring signature reduction or oxidation waves, picomoles of electroactive metals can be detected.

Furthermore, the ability to monitor changes in the signature waves due to binding allows the collection of important structural information about the electronic communication between the metal center and the macromolecule.
Since all the triosmium clusters tested for binding to DNA are electroactive, we envision the possibility of studying the adducts by electrochemical assays.

For this reason, we have become interested in the electrochemistry of aqueous solutions of the complexes illustrated in Fig. 1, modified with water solubilizing ligands. We placed ligands bearing positive \( \text{L}^+ = [\text{P(OCH}_2\text{CH}_2\text{NMe}_3)]^3 \) or negative \( \text{L}^- = [\text{P(C}_6\text{H}_4\text{SO})_3\text{Na}]_3 \) charges on the metal core to provide water solubility.

![Structures of the family of electron deficient benzoheterocycle triosmium clusters.](image)

All of the compounds proved to be electrochemically active but in most of the complexes, electrochemically reversible but chemically irreversible reductions were observed. The phenanthridine, 5,6-benzoquinoline electron deficient complexes (Fig. 5.1) and one electron precise decacarbonyl clusters, \([\text{Os}_3(\text{CO})_{10}(\mu-\eta^2\text{-Bz})(\mu-\text{H})](\text{HBz} = \text{quinoxaline})\) (Eq. (1)) show electrochemically and chemically reversible \(1e^-\) reductions. The corresponding stable monoanion

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radicals have been studied in detail both from the experimental as well as from the theoretical point of view [1, 2].

The remote location of the hydride signal in these compounds and their long relaxation times make these clusters, with their bio-designed ligands excellent candidates for probing small molecules large molecule interactions.

5.2.1 Electrochemical behavior of [Os₃(CO)₉(μ-η²-Bz)(μ-H)(P(OCH₂CH₂NMe₃)₃I₃)] (6, HBz = quinoxaline) in aqueous media.

The complex, [Os₃(CO)₉(μ-η²-Bz)(μ-H)(P(OCH₂CH₂NMe₃)₃I₃)](6, HBz = quinoxaline, Fig. 5.2) shows a reversible 1e⁻ reduction. An aqueous solution of 6 (pH = 4-5) shows a reversible half wave potential at -0.62 V vs. SCE at a scan rate of 500 mV/s. The free ligand, quinoxaline, shows irreversible electrochemical behavior on the cyclic voltammetry time scale in both aqueous and non-aqueous media [3, 4]. Slight variations in the value of this half wave potential and the observation of a second partially overlapping wave at more negative potentials prompted us to make a more detailed investigation of 6 in aqueous media.
Fig. 5.2: Structures of the water-soluble clusters.

Polarography of 6 in water/0.1 M KCl or phosphate buffer shows two overlapping cathodic waves. Half wave potentials of both waves are dependent on the pH value, which indicates participation of protonation equilibria in the reduction process. The wave at more positive potential decreases in height on going from acidic to alkaline solution but the overall height of the overlapped waves remains constant up to pH=10. Controlled potential coulometry performed either at the potential of the limiting current plateau of the first wave

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or at the potential behind the second wave gives approximately the same value of the charge consumption: 1.5 F/mol.

Control of the pH can be achieved by means of a H₃PO₄/CH₃COOH/H₃BO₃ buffer solution. At pH values lower than 7, CV performed on a glassy carbon (GC) electrode shows that the first reduction at less negative potentials is reversible on the CV time scale (Fig. 5.3), at scan rates as low as 50 mV/s.

Fig. 5.3: Cyclic voltammetry of buffered solutions of 6 at 200 mV/s, GC electrode, at values of pH of 2.2, 3.0 and 4.3.
The second reduction process overlaps with the first, as the pH values are raised by stepwise addition of a concentrated solution of NaOH (Fig. 5.4).

![Cyclic Voltammetry of buffered solutions of 6 at 200 mV/s, GC electrode, at values of pH of 6.1, 8.7 and 11.0.](image)

**Fig. 5.4. Cyclic Voltammetry of buffered solutions of 6 at 200 mV/s, GC electrode, at values of pH of 6.1, 8.7 and 11.0.**

Also, the intensity of both cathodic peaks is pH dependent, and the decrease in current associated with the first reduction peak correlates with an increase of current for the second reduction peak. This can be easily visualized qualitatively by square wave voltammetry (Fig. 5.5).

These results lead to the conclusion that the reduction processes in both waves proceed with proton consumption and lead to the same reduction products. This interpretation is supported by polarographic monitoring in non-buffered solutions during electrolysis and formation of hydroxyl ions as detected
by the appearance of an anodic wave on the mercury electrode at ca. 0.08 V (vs. SCE). Using a standard addition of NaOH it could be determined that the consumption of one electron results in the formation of one hydroxyl ion [2].

Fig. 5.5: Square Wave Voltammetry (SWV) of buffered solutions of 6 at 30 Hz, at values of pH of 2.0, 4.1 and 6.25.

The more positive reduction wave, which is larger in acidic solutions, can be ascribed to the reduction of the protonated complex and the second wave to the reduction of non-protonated species followed by protonation of the reduced form. This is supported by the fact that the aromatic proton resonances of 6 sharpen and show increasing down field shifts as acid is added to a solution of 6 in D$_2$O. The sharpening is an indication of a decreasing tendency to aggregate as positive charge is placed on the aromatic ring [2]. The hydride signal located at -12.97 ppm is not pH dependent; no other hydride resonance appears during the
whole titration ruling out the possibility of a protonation process at the metal triangle. Lowering the pH from 7 to 1.4 results in small downfield shifts for the aromatic resonances with the signal at 9.7 ppm attributable to the proton adjacent to the uncoordinated nitrogen undergoing the largest downfield shift due to aromatic ring protonation at that atom.

That 1.5 F/mol are consumed as observed by coulometry could be the result of competitive follow-up reactions of the reduced, protonated complex; hydrogen evolution with regeneration of the oxidized form of the complex and a reaction leading to degradation of the cluster structure.

The pH dependence of the polarographic half-wave potential in the case of the one-electron reduction process \((\text{Ox} + 1e^- \rightleftharpoons \text{Red})\), with protonation of both oxidized and reduced forms \((\text{Ox} + H^+ \rightleftharpoons \text{OxH}^+ \text{ and } \text{Red} + H^+ \rightleftharpoons \text{RedH}^+)\), should follow the equation (assuming equal diffusion coefficients) [5, 6]:

\[
E_{1/2} = E_0 + \frac{RT}{F} \ln \left( \frac{K_a}{K_a'} \right) + \frac{RT}{F} \ln \left( \frac{(K_a' + [H^+])}{(K_a + [H^+])} \right)
\]

(1),

where \(K_a, K_a'\) are the equilibrium dissociation constants of the protonated oxidized and reduced forms respectively:

\[
K_a = \left[ \frac{[\text{Ox}] [H^+]}{[\text{OxH}^+]} \right], \quad K_a' = \left[ \frac{[\text{Red}] [H^+]}{[\text{RedH}^+]} \right].
\]

Three limiting cases can be considered:
1. In very acid solutions, where $[H^+] >> K_a, K_a'$, the half-wave potential is independent of pH and is shifted versus standard potential according to relation $E_{1/2} = E_0 + (RT/F) \ln (K_a/K_a')$

2. In the pH range $pK_a < pH < pK_a'$, i.e. $K_a >> [H^+] >> K_a'$, the equation (1) is simplified to $E_{1/2} = E_0 + (RT/F) \ln (K_a/K_a') + (RT/F) \ln ([H^+] / K_a)$ (2), i.e. at 25°C:

$$E_{1/2} = E_0 + 0.059pK_a' - 0.059pH$$

and a negative potential shift of 59 mV/pH is expected

3. In alkaline solutions, $[H^+] << K_a, K_a'$, the reduction proceeds at the standard potential, $E_{1/2} = E_0$.

The experimentally observed potential shift is linear in the measured range of pH 2 to 10 and slopes 61 mV/pH were observed. Therefore, from the theoretical relations given the dissociation constants $K_a \sim 10^{-2}$ and $K_a' \sim 10^{-10}$, for oxidized and reduced complex respectively can be estimated (Fig. 5.6).

![Fig. 5.6: Dependence of the polarographic half-wave potential of the reduction wave of (6) on pH; experimental values (■) and calculated (Eq. 1) curve for $pK_a = 2$, $pK_a' = 10$.](image)
The plotting of Fig. 5.6 as an S curve instead of a straight line is based on the calculated values of $E_{1/2}$ at high pH using the above analysis. Unfortunately, it was not possible to check this because of precipitation in the solutions examined above pH=10. In any case, the estimates of $K_a$ and $K_a'$ are taken from the linear region and should be not be significantly impacted by this approximation.

The splitting of the polarographic wave into two overlapping waves and the variation of their heights with pH can be ascribed to an insufficiently mobile equilibrium due to a low value for the rate constant of protonation of the complex in its oxidized form [1].

The cyclic voltammetry measurements are in agreement with the above-proposed mechanism. The reduction in the first wave appears as a reversible cathodic/anodic peak couple. The reoxidation after the cathodic scan going to the second reduction peak proceeds only at the first, more positive wave due to a fast protonation/slow deprotonation of the reduced cluster.

The polarographic limiting current behind the reduction wave(s) significantly decreases (by ca. 30%) between the pH values 11 to 12 and the solution color changes from red to green. Obviously, this unknown transformation of the complex stops the side reaction of proton reduction, which increases the polarographic current in more acidic solutions.
5.2.2 Electrochemical behavior of \([\text{Os}_3(\text{CO})_9(\mu-\eta^2-\text{Bz})(\mu-\text{H})(\text{P(OCH}_2\text{CH}_2\text{NMe}_3)_3\text{I}_3)]\) (6, HBz = quinoxaline) in non-aqueous media

In CH\textsubscript{3}CN/0.1M tetrabutylammonium hexafluorophosphate 6 exhibits two polarographic reduction waves, \(E_{1/2} = -1.52\) and -2.01 V (vs. FeCp\textsubscript{2}/FeCp\textsubscript{2}\textsuperscript{+}), of approximately the same height. The limiting current both behind the first and second wave grows irregularly which indicates formation of decomposition products from the reduced complex. Cyclic voltammetry shows reversibility of the first reduction at higher scan rates (5V/s). The second reduction appears to be irreversible with only a small anodic counter peak.

Coulometry gives 1e\textsuperscript{-}/mol for the first wave and after the second 1e\textsuperscript{-} reduction both waves disappear and several new waves of decomposition products appear at more negative potential (which can be reduced with further charge consumption). Reduction directly at the potential behind the second wave gives 2-2.5 F/mol of consumption and a precipitate is formed in the solution. This rather complex electrochemical behavior aside, it is important to note that for complex 6 the initial 1e\textsuperscript{-} reduction gives a more stable reduction product at less negative potentials in aqueous media rather than in non-aqueous media.
5.2.3 Electrochemical behavior of \([\text{Os}_3(\text{CO})_9(\mu-\eta^2-\text{Bz})(\mu-\text{H})(\text{L}^+ \text{or L}^-)]\) (HBz=quinoxaline, L\(^-\) 7; HBz = phenanthridine, L\(^+\), 4; HBz = 5,6-Benzoquinoline, L\(^+\), 8; HBz = 3-NH\(_2\)-quinoline, L\(^+\), 2; HBz = 3-NH\(_2\)-quinoline, L\(^-\), 1 and the neutral analog \([\text{Os}_3(\text{CO})_9(\mu-\eta^2-\text{Bz})(\mu-\text{H})\text{L}]\) (HBz=quinoxaline, L=[P(C\(_6\)H\(_5\))]\(_3\) \(\text{L}^+=[[\text{P(OCH}_2\text{CH}_2\text{NMe}_3)]_3}\) and L\(^-\)=[P(C\(_6\)H\(_4\)SO\(_3\))_3Na\(_3\)]\)

Complex 7 in dimethylformamide solution is reduced in a one-electron reversible polarographic wave at -1.74 V (vs. FeC\(_{20}/FeC\(_{20}^+\)). Reversibility was confirmed by cyclic voltammetry and the charge consumption by controlled potential coulometry. In contrast, the complexes 4-2 in acetonitrile or dimethylformamide solutions do not exhibit any anodic counter peak on cyclic voltammetry within the range of scan rates employed (0.05 - 40 V/s), although the corresponding 1e\(^-\) polarographic waves appear as reversible (7) or semi-reversible (slightly decreased slope due to slower electrode reaction; 2,4,8). This indicates a fast chemical reaction following the charge transfer. Instability of the primary reduction products and a conversion to electro-inactive (non-oxidizable) species is also observed in bulk electrolysis experiments. Reduction of the complex (1) is irreversible both in polarography and voltammetry.

The behavior of the complexes 1-7 in aqueous solution seems to be analogous in some respects to the behavior of 6. The polarographic reduction potentials of 1 - 7 in polar organic solvents and in aqueous solution are given in Tables 5.1 and 5.2. Two overlapping polarographic waves are shifted to negative
potential with increased pH values, thus suggesting a consumption of protons in the reduction process. A reversible electrochemical response is observed only with 7, at the more positive wave, but only at very fast scan rates. In the case of complexes 1-4 the reduction processes are irreversible and furthermore, the waves are ill defined, either obscured by polarographic maxima or overlapping with strongly growing current which may originate from hydrogen evolution, catalyzed by the complexes or by decomposition fragments from them. It should be noted that in general the reduction potentials of all the clusters was less negative in water than in non-polar solvents.

Table 5.1. Polarographic half-wave potential in non-aqueous media, in V vs. FeCp₂/FeCp₂

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>$E_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(6)</td>
<td>CH₃CN</td>
<td>-1.52</td>
</tr>
<tr>
<td></td>
<td>DMF</td>
<td>-1.56</td>
</tr>
<tr>
<td>(7)</td>
<td>DMF</td>
<td>-1.74</td>
</tr>
<tr>
<td>(4)</td>
<td>CH₃CN</td>
<td>-1.89</td>
</tr>
<tr>
<td>(8)</td>
<td>CH₃CN</td>
<td>-1.81</td>
</tr>
<tr>
<td>(2)</td>
<td>CH₃CN</td>
<td>-1.98</td>
</tr>
<tr>
<td>(1)</td>
<td>DMF</td>
<td>-2.24</td>
</tr>
</tbody>
</table>
Table 5.2. Polarographic Half-wave Potential in Aqueous Media, in V vs. SCE

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH</th>
<th>$E_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(6)</td>
<td>4.5$^a$</td>
<td>-0.62</td>
</tr>
<tr>
<td></td>
<td>8.7$^a$</td>
<td>-0.85</td>
</tr>
<tr>
<td>(7)</td>
<td>4.5$^a$</td>
<td>-0.71</td>
</tr>
<tr>
<td></td>
<td>8.7$^a$</td>
<td>-0.90</td>
</tr>
<tr>
<td>(4)</td>
<td>4.5$^a$</td>
<td>-1.09</td>
</tr>
<tr>
<td></td>
<td>8.7$^a$</td>
<td>-1.32</td>
</tr>
<tr>
<td>(8)</td>
<td>8.7$^a$</td>
<td>-1.45$^c$</td>
</tr>
<tr>
<td>(2)</td>
<td>8.7$^a$</td>
<td>-1.20$^c$</td>
</tr>
<tr>
<td>(1)</td>
<td>b</td>
<td>-1.65$^c$</td>
</tr>
</tbody>
</table>

$^a$ phosphate buffer $^b$ 0.1M Tetrabutylammonium chloride (TBACl) $^c$ ill defined

This is most likely due to the higher dielectric constant and corresponding lower resistivity of the aqueous solutions employed which contained the high salt concentrations required by the buffer system employed. As expected, the positively charged clusters 6 and 2 with the same heterocycle as the negatively charged species 7 and 1 are reduced at less negative potentials. More interestingly, the positively charged clusters 2-4 are reduced at more negative potentials than the negatively charged 7 indicating that it is the nature of the heterocycle rather than the overall charge on the cluster that determines the reduction potential. This is further corroborated by the electrochemical behavior.
of the neutral analog, 9, which shows a reversible 1e− reduction at -1.84V vs. FeCp2/FeCp2+ in CH2Cl2.

As expected the reduction product is less stable than the corresponding decacarbonyl as evidenced by decreasing current of the half wave with decreasing scan rate. The first reduction potential of 9 is slightly more negative than that of 6 and 7 measured in more polar organic solvents. The limited solubility of 9 in DMF prevented a direct comparison with 6 and 7 but the similarity of the potentials relative to the other complexes certainly supports the idea that the nature of the heterocycle and the substitution of phosphine for CO (i.e. the corresponding decacarbonyl has a reversible wave at -1.16 V in CH2Cl2 vs. FeCp2/FeCp2+) are the dominant influences on the reduction potential in these complexes rather than the charge on the complex.

5.2.4 Interaction of the Water Soluble Clusters with Albumin

When a 0.001 M solution of the negatively charged cluster [Os3(CO)9(μ-η2-Bz)(μ-H)L−] (HBz = 2-methylbenzimidazole, L− = [P(C6H4SO3)3Na3], 10) is titrated with successively increasing amounts of albumin the cluster’s 1H NMR signals progressively broaden. The resonances are detectable until the ratio of albumin/cluster = 0.04, at higher protein concentrations, the signals become too broad to be observed (Fig. 5.7).
Fig. 5.7: The $^1$H NMR spectrum of [10] in the presence of various Albumin to cluster ratios measured in water at 600 MHz.

Similar behavior is observed for complex 1 and for [Os$_3$(CO)$_9$(μ-η$^2$-Bz)(μ-H) L·] (HBz = quinoline-4-carboxaldehyde, L· = [P(C$_6$H$_4$SO$_3$)$_3$Na$_3$], 11). The latter complex was investigated because it was thought the aldehyde group might covalently attach to the primary amines of the protein and thus show different behavior than the other clusters where only an electrostatic and or π-π stacking interactions are possible. The longitudinal ($T_1$) and transverse ($T_2$) relaxation times of the hydride signal before and after the addition of albumin at two different ratios have been measured. The results of these measurements for the three clusters are summarized in Table 3. In all three cases the $T_1$ increases while the $T_2$ decreases.
Table 5.3. Transverse ($T_2$) and Longitudinal ($T_1$) Relaxation Times for the Negatively Charged Clusters $[\text{Os}_3(\text{CO})_9(\mu-\eta^2-\text{Bz})(\mu-\text{H})(\text{P(C}_6\text{H}_4\text{SO}_3)_3\text{Na}_3)]^a, b$

A. HBz = 2-Me-benzimidazole, 10

<table>
<thead>
<tr>
<th>Albumin / cluster</th>
<th>$T_1$ (s)</th>
<th>$T_2$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.44 / 1.42</td>
<td>358 / 383</td>
</tr>
<tr>
<td>0.02</td>
<td>2.05 / 2.14</td>
<td>67 / 53</td>
</tr>
<tr>
<td>0.04</td>
<td>2.03</td>
<td>$\geq34$</td>
</tr>
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</table>

B. HBz = 3-NH$_2$-quinoline, 1

<table>
<thead>
<tr>
<th>Albumin/Cluster</th>
<th>$T_1$ (s)</th>
<th>$T_2$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.45 / 1.50</td>
<td>325 / 704</td>
</tr>
<tr>
<td>0.02</td>
<td>1.59 / 1.47</td>
<td>92 / 92</td>
</tr>
<tr>
<td>0.04</td>
<td>1.74</td>
<td>50</td>
</tr>
</tbody>
</table>

C. HBz = quinoline-4-carboxaldehyde, 11

<table>
<thead>
<tr>
<th>Albumin / cluster</th>
<th>$T_1$ (s)</th>
<th>$T_2$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.52 / 1.52</td>
<td>641 / 487</td>
</tr>
<tr>
<td>0.02</td>
<td>1.75</td>
<td>200</td>
</tr>
<tr>
<td>0.02 after 90°</td>
<td>1.93</td>
<td>120</td>
</tr>
</tbody>
</table>

$^a$The two values refer to the two components of the doublet

$^b$Values reported with an error of $\pm10\%$
These data can be interpreted in terms of the expected relationship between rotational correlation time and the spin lattice relaxation time $T_1$ (Fig. 5.8) [7]. According to this relationship there is a minimum value for $T_1$ that corresponds to a specific rate of molecular rotation where energy transfer is most efficient.

![Fig. 5.8. The relationship between the rotational correlation time ($\tau_C$) and the longitudinal ($T_1$) and transverse ($T_2$) relaxation times.](image)

Thus, increases in the correlation time $\tau_C$ can result in either an increase or a decrease in $T_1$ depending on whether or not a decrease in the molecular rotation increases the efficiency of energy transfer to the lattice. In the case of the negative clusters 1, 10 and 11 adduct formation would slow molecular tumbling leading to the conclusion that the correlation is on the positive slope side of the $T_1$ curve and that therefore the correlation time of the hydride has significantly increased [8]. We do not know what the effect of the phosphine and of the
heterocycle on $T_1$ is, but the relatively large increase in $\tau_C$ is consistent with adduct formation. As expected the increase in $\tau_C$ is always associated with a decrease in $T_2$. In the particular case of 11 the albumin cluster solution was heated to 90 °C for one hour to see if evidence of covalent bonding of the cluster to the protein could be observed [9]. As can be seen from Table 3 there was an increase in $T_1$ but it is not large enough to attribute it to irreversible covalent binding of the cluster to the protein [10]. In a separate experiment it was shown that at elevated temperatures 11 isomerizes to a hydride with a different chemical shift and this most likely accounts for the change in $T_1$.

In order to be sure that the variations observed in the relaxation rates are due to a real interaction with the protein and not simply to the increased viscosity of the solution after albumin addition, the measurements have been repeated on 1 in the presence of different quantities of polyethylene glycol (PEG, M. W. = 1500), which should enhance viscosity presumably without interacting with the cluster. Significantly, no appreciable line broadening of the hydride resonance is observed throughout the concentration range examined and the changes in $T_1$ and $T_2$ are relatively small compared with those observed with albumin (Fig. 5.9).
Fig. 5.9: Plot of the variation in $T_1$ and $T_2$ of [1] with added polyethylene glycol (PEG)

The vertical line in Fig. 5.9 represents the molar concentration ratio where PEG/cluster $= 0.04$ the value at which maximum yet observable line broadening is seen with albumin. It seems that up to this value (and even for higher concentration), no effect on the relaxation is observed and this means that the variations found in the albumin case are due to the interaction between the cluster and the protein. No significant line broadening of the hydride resonance is observed with the positively charged clusters $[\text{Os}_3(\text{CO})_9(\mu-\eta^2-\text{Bz})(\mu-\text{H})L^+](\text{HBz} = 2\text{-Me-benzimidazole, } L^+= [\text{P(OCH}_2\text{CH}_2\text{NMe}_3)_3]$, 12, HBz = quinoline-4-carboxaldehyde, $L^+= [\text{P(OCH}_2\text{CH}_2\text{NMe}_3)_3]$, 5) at albumin/cluster ratios lower than 0.04, while in the negative case broadening is extensive at ratios $= 0.02$. At ratios of 0.1 the negatively charged cluster hydride resonances in 6, 10 and 11 are broadened into the base line while in the case of 12 and 5 the hydride resonance is still relatively sharp (Fig. 5.7 and Fig. 5.10).
Fig. 5.10: The $^1$H NMR spectrum of [12] in the presence of various albumin to cluster ratios measured in water at 600 MHz.

The $T_1$ measurements were carried out on 12 and 5 but at higher albumin concentrations. The results are summarized in Table 4 and it can be seen that here, the addition of albumin to the cluster solution results in shorter $T_1$ values although the $T_1$ for the cluster itself is not very different than that found for the negative clusters. Considering that there are probably many clusters bound to the albumin at any one time the apparently weaker interaction with the protein would place fewer clusters on the albumin at any one instant leading to more rapid tumbling of the protein and placing this cluster protein system on the negative sloping part of the $T_1$ curve (Fig. 5.8). The trend for $T_2$ is the same found for the negative cluster as expected. The same behavior was observed for 5 as expected (Table 4).
Table 5.4. Transverse (T₂) and Longitudinal (T₁) Relaxation Times for the Positively Charged Clusters [Os₃(CO)₉(μ-η²-Bz)(μ-H)(P(C₆H₄SO₃)₃Na₃)]ᵃᵇ

A. HBz = 2-Me-benzimidazole, 12

<table>
<thead>
<tr>
<th>Albumine / cluster</th>
<th>T₁ (s)</th>
<th>T₂ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.90 / 2.07</td>
<td>310 / 324</td>
</tr>
<tr>
<td>0.04</td>
<td>0.77 / 0.68</td>
<td>53 / 48</td>
</tr>
<tr>
<td>0.1</td>
<td>0.57</td>
<td>≈32</td>
</tr>
</tbody>
</table>

B. HBz = quinoline-4-carboxaldehyde, 5

<table>
<thead>
<tr>
<th>Albumine / cluster</th>
<th>T₁ (s)</th>
<th>T₂ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.93 / 2.02</td>
<td>312 / 216</td>
</tr>
<tr>
<td>0.02</td>
<td>1.40 / 1.58</td>
<td>147 / 98</td>
</tr>
</tbody>
</table>

ᵃThe two values refer to the two components of the doublet
ᵇValues reported with an error of ± 10 %

5.3 Conclusions

The reversible electrochemistry observed for compounds 6 and 7 is the first example of this type of behaviour for organometallic cluster complexes in water and holds out the possibility that electrochemical monitoring of cluster binding interactions with biomacromolecules is a realizable goal. The overall trends in the electrochemical data suggest that complexes that show reversible electrochemical behaviour in non-aqueous solvents can be carried into water even when modified with relatively highly charged phosphine ligands. Although the electrochemistry for the remaining compounds is irreversible, the
overall trends in the redox data reveal that the nature of the heterocycle rather than the overall charge on the complex determines the reduction potential of the water-soluble cluster species. The significance of this result is that redox active cluster containing heterocyclic complexes can be designed as electrochemical probes with either positively charged water solubilizing ligands for binding to DNA [11] or with negatively charged ligands for binding to proteins with basic amino acids on the protein surface.

The significance of the reported changes in $T_1$ of the hydride resonance on binding to albumin demonstrates that this NMR resonance, which is completely separated from those associated with biomacromolecules, can be used as a qualitative screen for cluster bio-macromolecule interactions and that the direction of this change is understandable in terms of the expected protein–cluster electrostatic interactions. Thus, that the negatively charged clusters appear to bind more tightly than their positive analogues is not surprising in light of the fact that albumin is rich in positively charged amino acids [12]. The combination of reversible redox behavior, direct visualization with electron microscopy and the availability of a readily observable NMR probe (the chemically inert hydride) promises to make the class of clusters reported here unique tools for probing the structure and behaviour of a wide range of biomacromolecules. What needs to be done now is to develop electrochemically active clusters that have high binding constants to specific amino acid or nucleic acid residues. This will require developing cluster functional groups and tethers
that have specific and perhaps covalent interactions with given nucleic acid or peptide sequences. These studies are underway in our laboratories.

5.4 Experimental

5.4.1 General

The benzoheterocycle triosmium complex \([\text{Os}_3(\text{CO})_9(\mu_2-\eta^2-\text{Bz-H})(\mu-\text{H})]\) (HBz = quinoline-4-carboxaldehyde) was prepared according to published literature procedures [1]. The water soluble phosphine derivatives 4, 2, 1 and 5 and the positively charged phosphine ligand, \([\text{P}(\text{OCH}_2\text{CH}_2\text{NMes})_3]\), were synthesized according to published literature procedures [8, 9]. Osmium carbonyl was purchased from Strem Chemicals and used as received. Sodium triphenylphosphane sulfonate was purchased from Aldrich and used as received.

5.4.2 Electrochemical Measurements

An EG&G Princeton Applied Research Potentionstat/Galvanostat Model M273, connected to a PC with EG&G Princeton M270 software, was used. The working electrodes were a dropping mercury electrode (DME) and a glossy carbon electrode (GCE); the reference electrode was a self-made calomel saturated electrode, the counter electrode was a platinum wire. The GCE was polished with wet alumina before use. All solutions used for electrochemical
measurements solutions were deoxygenated with an argon purge and were kept under argon flux during the measurements. THF was freshly distilled from benzophenone ketyl and CH₂Cl₂ from phosphorus pentoxide. The pH-measurements were performed with an Amel 334-B pH-meter. Aqueous electrochemical measurements were performed using the following buffer: 0.72 mL of 80% H₃PO₄, 0.57 mL of glacial CH₃COOH, 0.62 g of H₃BO₃ dissolved in water to make 250 mL of stock solution. By adding controlled amounts of 4 M NaOH, pH values between 1.8 and 12.0 can be obtained [10, 13]. Electrochemical measurements in organic solvents were done using 0.1 M tetrabutylammonium hexafluorophosphate as the supporting electrolyte. In aqueous solutions, measurements were made against a SCE reference electrode. The non-aqueous reduction potentials are reported with reference to the ferrocene/ferrocenium ion redox couple in the appropriate solvent.

5.4.3 NMR Measurements

The NMR spectra were recorded on Varian Unity Plus 400 MHz, JEOL EX 400 and Bruker Avance 600 MHz spectrometers. The NMR solvents (Aldrich) were dried over activated molecular sieves (Type 4A). Chemical shifts were referenced internally relative to the residual protons in the deuterated solvents used. The 2D NMR measurements were performed using 1024 data points in t₂ and 256 in t₁ with a pulse repetition of 3 s. Non-selective inversion recovery was used to obtain ¹H T₁ values. Samples for T₁ measurements were prepared in the
absence of O₂ by using standard freeze-pump-thaw techniques. Temperature calibration was carried out with a methanol standard ¹H thermometer. Errors in the reported T₁ and T₂ values were estimated to be in the range of ±10 %. Samples for the experiments with albumin were prepared by dissolving 1 mg (0.001 mmol) of cluster in 0.5 mL of D₂O (no buffer). Precipitation does not occur at any albumin concentration. Clusters used: 3-aminouquinoline negative, 1, 2-Me-benzimidazole negative, 10, 2-Me-benzimidazole positive, 3, quinoline-4-carboxaldehyde negative, 11, quinoline-4-carboxaldehyde positive (not completely water-soluble), 5.

5.4.4 Synthesis of the Water Soluble Clusters [Os₃(CO)₉(µ-η²-Bz)(µ-H) (L⁺ or L⁻)] (HBz = Quinoxaline, L⁺, 6, L⁻, 7; 5,6-Benzquinoline, L⁺, 8; 2-MethylBenzimidazole, L⁺, 12, L⁻, 10 and Quinoline-4-carboxaldehyde, L⁻, 11 (L⁺ = [P(OCH₂CH₂NMe₃)₃] L⁻ = [P(C₆H₄SO₃)₃Na₃].

In a 25 mL round bottom flask 50 mg of [Os₃(CO)₉(µ-η²-Bz)(µ-H)] (0.05 mmol) were dissolved in 10 mL of methanol (or acetone), then an equimolar amount of L⁺ (38 mg) or L⁻ (30 mg) dissolved in a few drops of water was added to the cluster solution. The reaction is quantitative and yields 80-88 mg (100%) of yellow to orange product as a precipitate. Analytical and spectroscopic data of the compounds are reported below.

**Compound 6**: Anal. Calcd for [C₃₂H₄₅N₅O₁₂Os₃P₁₃]: C, 22.7, H 2.70, N 4.15 %. Found: C, 22.01; H, 2.79; N, 3.84 %. IR (ν CO) in D₂O: 2094 (m), 2052 (s), 1998

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(s), 1937 (sh) cm	extsuperscript{-1}.\textsuperscript{1}H-NMR in D\textsubscript{2}O: 89.41 (br, 1H), 8.40 (br, 1H), 8.33 (d, 1H), 7.51 (dd, 2H), 3.40 (m, 6H), 3.07 (s, 3H), 3.04 (s, 3H), 2.90 (s, 27H) and -13.15 (d, 1H, J\textsubscript{PH}=16.47 Hz).

**Compound 7:** Anal. Calcd for [C\textsubscript{35}H\textsubscript{18}N\textsubscript{2}O\textsubscript{18}O\textsubscript{3}P\textsubscript{3}Na\textsubscript{3}]: C, 27.65, H 1.85, N 1.19 %. Found: C, 27.03; H, 1.09; N, 1.84 %. IR (v CO) in D\textsubscript{2}O: 2089 (sh), 2046 (sh), 1965 (w), 1926 (s) cm\textsuperscript{-1}.\textsuperscript{1}H-NMR in D\textsubscript{2}O: 89.47 (s, 1H), 8.22 (s, 1H), 7.73 (d, 1H), 7.60 (m, 6H), 7.2 (s, 6H), 7.03 (d, 1H), 6.75 (s, 1H) and -12.26 (d, 1H, J\textsubscript{PH}=16.09 Hz).

**Compound 12:** Anal. Calcd for [C\textsubscript{37}H\textsubscript{48}N\textsubscript{4}O\textsubscript{12}O\textsubscript{3}P\textsubscript{3}]: C, 24.82, H 2.78, N 3.23 %. Found: C, 24.36; H, 2.03; N, 3.25 %. IR (v CO) in D\textsubscript{2}O: 2090 (w), 2076 (sh), 2045 (m), 2010 (sh), 1971 (s), 1938 (sh) cm\textsuperscript{-1}.\textsuperscript{1}H-NMR in D\textsubscript{2}O: 89.40 (br, 1H), 8.92 (br, 1H), 8.55 (s, 1H), 8.35 (br, 1H), 7.80 (br, 1H), 7.58 (br, 1H), 7.41 (br, 1H), 7.11 (br, 1H), 3.40 (m, 6H), 3.07 (s, 3H), 3.04 (s, 3H), 2.90 (s, 27H) and -13.07 (d, 1H, J\textsubscript{PH}=16.7 Hz).

**Compound 8:** Anal. Calcd for [C\textsubscript{32}H\textsubscript{46}N\textsubscript{5}O\textsubscript{1}O\textsubscript{3}O\textsubscript{3}P\textsubscript{3}]: C, 22.93, H 2.74, N 4.18 %. Found: C, 22.29; H, 3.60; N, 4.18, %. IR (v CO) in D\textsubscript{2}O: 2087 (w), 2041 (m), 1997 (s), 1949 (sh) and 1921 (sh) cm\textsuperscript{-1}.\textsuperscript{1}H-NMR in D\textsubscript{2}O: 87.40 (t, 1H), 6.90 (d, 1H), 6.80 (d, 1H), 3.40 (m, 6H), 3.07 (s, 3H), 3.04 (s, 3H), 2.90 (s, 27H) and -13.00 (d, 1H, J\textsubscript{PH}=16.88 Hz).

**Compound 10** (in the hexahydrate form): Anal. Calcd for [C\textsubscript{35}H\textsubscript{19}N\textsubscript{2}O\textsubscript{16}O\textsubscript{3}P\textsubscript{3}Na\textsubscript{3}]: C 26.83, H 1.99, N 0.85 %. Found: C 26.97, H 1.99, N 0.82 %. IR (v CO) in D\textsubscript{2}O: 2087 (w), 2043 (m), 1998 (s), 1982 (sh) and 1954 (sh) cm
\(^1\)H-NMR in D\(_2\)O: 87.60 (t, 1H), 7.50 (d, 1H), 7.40 (m, 12H), 7.20 (d, 1H), 2.40 (s, 3H) and -12.00 (d, 1H, \(J_{PH}=16.37\) Hz).

**Compound 11:** Anal. Calcd for [C\(_{37}\)H\(_{19}\)NO\(_{19}\)Os\(_3\)PS\(_3\)Na\(_3\)]: C 25.84, H 3.05, N 3.27 %. Found: C 25.76, H 2.85, N 3.35 %. IR (ν CO) in D\(_2\)O: 2088 (w), 2045 (m), 1991 (s), 1955 (sh) and 1926 (sh) cm\(^{-1}\). \(^1\)H-NMR in D\(_2\)O: 89.90 (s, 1H), 9.45 (d, 1H), 8.93 (d, 1H), 7.80 (d, 1H), 7.36 (m, 12H), 7.30 (d, 1H), 7.02 (t, 1H) and -12.00 (d, 1H, \(J_{PH}=16.22\) Hz).

5.4.5 **Synthesis of [Os\(_3\)(CO)\(_9\)(μ-η\(^2\)-Bz)(μ-H)L] (HBz = Quinoxaline, L = PPh\(_3\) 9)**

In a 25 mL round bottom flask 50 mg of [Os\(_3\)(CO)\(_9\)(μ-3-η\(^2\)-Bz)(μ-H)] (HBz = quinoxaline, 0.05 mmol) were dissolved in 10 mL methylene chloride then an equimolar amount of PPh\(_3\) (13 mg) was added to the cluster solution. The reaction was quantitative and yielded ~60 mg (100%) of red product after evaporation of the solvent and crystallization from CH\(_2\)Cl\(_2\)-hexane.

**Compound 9:** Anal. Calcd for [C\(_{32}\)H\(_{21}\)N\(_2\)O\(_{9}\)Os\(_3\)P]: C, 34.57, H 1.73, N 2.30 %. Found: C, 34.38; H, 1.68; N, 2.29, %. IR (ν CO) in CH\(_2\)Cl\(_2\): 2089 (s), 2048 (s), 2010 (s), 1992 (m) 1962 (m) and 1929 (w) cm\(^{-1}\) UV/Vis in CH\(_2\)Cl\(_2\) (\(λ_{max}\)): 395 and 530 nm. \(^1\)H-NMR in CD\(_2\)Cl\(_2\): 89.21 (d, 1H), 8.45 (d, 1H), 7.85 (d, 1H), 7.31 (m, 4H), 7.20 (m, 12H), and -11.92 (d, 1H, \(J_{PH}=15.63\) Hz).
5.5 References for chapter 5.

1. Rosenberg, E., Rokhsana, D., Nervi, C., Gobetto, R., Milone, L., Viale, A.,

2. Nervi, C., Gianolio, R., Milone, L., Viale, A., Rosenberg, E., Rokhsana, D.,

3. Rosenberg, E., Abedin, J.Md., Rokhsana, D., Osella, D., Milone, L., Nervi,

4. Abedin, M.J., Bergman, B., Holmquist, R., Smith, R., Rosenberg, E.,
   Ciurash, J., Hardcastle, K.I., Roe, J., Vasquez, V., Roe, C., Kabir, S.E., Roy,


6. Leek, A.A., Polarographic Behavior of Coordination Compounds: In Progress in


8. Rosenberg, E., Spada, F., Sugden, K., Martin, B., Gobetto, R., Milone, L.,

   Submitted for publication.

10. Heyrovsky, J.K., J, in Principles in Polarography, C.A Sc., Editor. 1965:
    Prague.

12. Peters, T., *All about Albumin: Biochemistry, Genetics and Medical Applications.*

Chapter 6

Applications of Water-soluble Triosmium Clusters as Selective Inhibitors for Telomerase Activity.

6.1 Introduction.

Telomerase is a ribonucleoprotein polymerase that maintains the length of telomeric DNA by adding hexameric units (TTAGGG in humans). The telomeric stabilization contributes to the preservation of unlimited cell proliferation potential. Telomerase is a multimeric enzyme and consists of different components: the RNA subunit hTERC (human telomerase RNA component), the catalytic subunit hTERT (human telomerase reverse transcriptase), and associated proteins [1].

In most human tumors a strong telomerase up-regulation has been demonstrated, thus suggesting that telomerase inhibition could be a highly specific approach for preventing tumor cell metastases [2]. Nowadays, several compounds have shown interesting results in inhibition of telomerase. Among these some cis-Pt(II)-complexes, which most likely target the nucleobases of the
RNA component of the enzyme have been shown to be effective inhibitors of telomerase [3]. In particular, we have found that the water-soluble cis-\([\text{Pt(Cl)}_2(\text{py})(5\text{-SO}_3\text{H-isoquinoline})]\) compound achieved the best results in terms of effective telomerase inhibition and low non-specific cytotoxicity, an important requirement for the long-term treatments associated with anti-telomerase therapy [4]. Interestingly, simple quinoxaline derivates have also been reported to be telomerase inhibitors leading to cellular senescence of human cancer cells, but with a clearly different mechanism of action [5].

The water-soluble organometallic clusters which are the subject of this study contain a nucleobase-like ligand, such as quinoline, and a charged phosphine or phosphite ligand, both coordinated to a chemically inert, triosmium-nonacarbonyl-hydrido core. This differs from cisplatin-like drugs because they are not electrophilic and are not alkylating agents.

Provided that the clusters approach telomerase enzyme by virtue of some nonspecific interaction, their steric encumbrance could interfere with the catalytic activity of the enzyme telomerase. Through a collaboration with the research group of Dr. Domenico Osella in Italy, the telomerase-inhibiting activity of the clusters \([(\mu\text{-H})\text{Os}_3(\text{CO})_9(L)(\mu_3-\eta^2-(Q-H))]\), where \(L = [\text{P}(C_6\text{H}_4\text{SO}_3\text{Na})_3]\) or \([\text{P}(\text{OCH}_2\text{CH}_2\text{NMMe}_3)_3]\) and \(Q = \text{quinoline, 3-amino quinoline, quinoxaline, or phenanthridine}\), have been studied both in a cell-free biochemical system and in breast cancer MCF-7 cells. The clusters are here renumbered 1a-4b for simplicity (Figure 6.1).
6.2 Telomerase Inhibition.

In the cell-free biochemical assay, all the negatively charged clusters 1a-4a were able to inhibit telomerase activity in a roughly dose-dependent manner in the $10^{-7}$ to $10^{-5}$ M concentration range. The results were calculated with respect to the untreated control, and corrected for the possible interference of clusters on the
amplification step (as described in the Experimental section) (Figure 6.2). The most active compound was the 3-amino quinoline derivative 2a (58% inhibition of telomerase at 10^{-5} M).

![Figure 6.2: Changes in telomerase activity of semi-purified enzyme in response to incubation with clusters 1a-4a at three concentrations. Telomerase activity was measured by the TRAP assay as described in the experimental section. The effects of clusters under investigation on Taq polymerase, were also evaluated and the activity values were corrected for these interferences. The data are expressed as the mean of three independent experiments ± SD.](image)

The amino-group in 2a probably enhances the hydrogen bond donor-acceptor interaction with the target macromolecules. On the contrary, 4a, which contains the potentially intercalating phenanthridine-group did not exhibit remarkable activity. Interestingly, the positively charged homologues 1b-4b were
found to be totally inactive under the same experimental conditions, and these compounds were not further investigated. These data clearly suggest that the first interaction of clusters 1a-4a with telomerase is electrostatic in nature, and concerns the positively charged portion of the multimeric enzyme.

This rules out the RNA component hTERC as the site of inhibition (due to its negatively charged phosphate backbone) and suggests that the polymerase hTERT component, which is rich with positively charged side chains, is the component where inhibition of the enzyme is operative. In fact, in hTERT telomerase reverse transcriptase there is in excess of 86 positively charged residues (mainly arginine and lysine groups) [6]. After electrostatic attraction and H-bonding interaction of amino group, the bulkiness of clusters may be the main factor that interferes with the enzyme activity.

Log-phase growing MCF-7 cell monolayers were treated for 24 hours with $10^{-7}$ to $10^{-5}$ M 1a-4a clusters, and the residual telomerase activity was evaluated after extraction and semi-purification of the enzyme. The inhibition was very low (if any), not significantly different from experimental error (Figure 6.3).
Figure 6.3: Decrease in telomerase activity after 24 hour treatment of the MCF-7 cells with different concentrations of clusters 1a-4a. Telomerase activity was detected by TRAP assay as described in materials and methods. The data are expressed as the mean of 3 independent experiments ± SD.

This striking difference may indicate difficulty in crossing the cell membrane, entering the cell, and, finally, reaching the nucleus, where the telomerase enzyme is active. Difficulty in crossing the cell membrane is understandable for such bulky and triply charged clusters. This problem has been already documented for polyoxometalate (POM) clusters that were able to inhibit immunodeficiency virus (HIV) protease [7]. These large clusters with a minus-seven charge establish that electrostatic interactions with the positively charged ammonium groups of HIV protease lysine residues are very important in enzyme inhibition. Biologists are currently concerned about whether and how these POMs can be internalized into the cells [8].
6.3 Viability and Uptake

The effects of clusters la-4a on viability of MCF-7 cells were determined after 24 h treatment and 48 h drug-free recovery in the concentration range of $10^{-9} - 10^{-4}$ M. The percentage of growth inhibition was plotted against the concentration of the clusters in the complete medium, and IC$_{50}$ values in μM (defined as the concentration of drug required to reduce 50% cell viability respect to the untreated control) were calculated for la-4a clusters as well as for cisplatin as reference (Table 6.1). The cytotoxicity of la-4a clusters is fairly high and similar to that of cisplatin.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>21±2</td>
</tr>
<tr>
<td>2a</td>
<td>9±1</td>
</tr>
<tr>
<td>3a</td>
<td>48±3</td>
</tr>
<tr>
<td>4a</td>
<td>4±0.5</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>28±1</td>
</tr>
</tbody>
</table>

Table 6.1: cytotoxicity of la-4a clusters as IC$_{50}$ (in μM).

We also measured the whole cellular content of osmium (intact cell with membrane, cytosol and nucleus) by ICP-MS, after treatment with 2a (used as the prototype of negatively charged clusters and because it is the most active).

Since the ICP-MS technique is at least one order of magnitude more sensitive than conventional atomic absorption spectroscopy (AAS), the cells can be
challenged with 2a at a concentration lower than its IC$_{50}$, and this allows one to follow the afflux/efflux process in long-term experiments (treatment time 1-72 h, concentrations 10$^{-7}$ and 10$^{-6}$ M; higher concentrations caused partial cell death).

The cellular uptake of cisplatin, after 10$^{-7}$ M treatment, is reported for the sake of comparison. Figures 6.4 and 6.5 show that the cluster uptake (pmol cluster / mg protein) and the cluster accumulation (intracellular concentration / medium concentration) in MCF-7 cells lines challenged with 2a at 10$^{-7}$ and 10$^{-6}$ M, respectively.

![Figure 6.4](image-url)

**Figure 6.4:** Uptake-time (upper) and accumulation-time (down) curves with exposure of 0.1 µM of cluster 2a to MCF-7 cells, after 24 h the treatment with cluster is maintained or replaced with fresh medium. Mean ± SD. The absence of error bar on any particular point indicates that the size of the calculated SD is smaller than the symbol used for that point.
For continuous treatment, the uptake and accumulation increases linearly, reaching an accumulation ratio of about 50 at $10^{-7}$M and 28 at $10^{-6}$M, after 72 hours.

![Graph of uptake-time and accumulation-time curves](image)

Figure 6.5: Uptake-time (upper) and accumulation-time (down) curves with exposure of 1 µM of cluster 2a to MCF-7 cells, after 24 h the treatment with cluster is maintained or replaced with fresh medium. Mean ± SD. The absence of error bar on any particular point indicates that the size of the calculated SD is smaller than the symbol used for that point.

A metallating agent like cisplatin, which into the cells, exhibits, after 24 hours of continuous incubation, a moderate accumulation ratio (4-5), that reaches a sort of plateau (48 h) and finally decreases after 72 hours. Moreover, cisplatin shows an accumulation ratio almost independent from its concentration in extracellular medium, indicating an ideal diffusion through the cell membrane [9].

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This is not the case for the osmium clusters, where almost linear uptake and efflux dependent on time of incubation or washing is observed. Furthermore, the increase in concentration dependent accumulation in the case of the clusters in the medium suggests a possible adhesion to the membrane instead of internalization. This hypothesis may explain the cytotoxicity of such clusters as the consequence of interference with the normal trafficking and functions of the membrane.

6.4 Experimental

6.4.1 Chemicals

The triosmium clusters $[(\mu-H)Os_3(CO)_9(L)(\mu_3-\eta^2-(Q-H))]$, where $L = [P(C_6H_4SO_3Na)_3]$ or $[P(OCH_2CH_2NMesI)_3]$ and $Q = $ Quinoline, 3-amino quinoline, quinoxaline, phenanthridine, 1a-4b were synthesized according to published procedures [10]. Their purity was verified by FT-IR, and multinuclear NMR spectroscopy and elemental analysis.

All reagents were purchased from Sigma. OsO₄ and K₂PtCl₄ were gifted from Johnson-Mattey (Reading, UK) and were employed for the synthesis of the starting materials Os₃(CO)₁₂ and cisplatin, respectively.
6.4.2 Cell culture

MCF-7 cells (human mammary adenocarcinoma) (ATCC HTB-22, American Type Culture Collection, Rockville, Md USA) were used for in vitro experiments. Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. Cells were cultured at 37°C in humidified 5% CO₂ atmosphere and cell monolayers were periodically screened for mycoplasma contamination. All reagents were obtained from Sigma (Sigma-Aldrich, USA).

6.4.3 Cell-free biochemical test

Exponentially growing MCF7 cells were lysed and telomerase extracted as described in the literature [11]. Briefly, cells were trypsinized and washed with cold buffer containing 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, sodium salt, pH 7.4], 1.5 mM MgCl₂, 10 mM KCl, and 1 mM DTT [dithiothreitol]. The pellets were then resuspended in a lysis buffer containing 10 mM Tris-HCl [tris(hydroxymethyl)aminomethane hydrochloride, buffered at pH 7.5], 1 mM MgCl₂, 1 mM EGTA [ethylenbis(oxyethylenenitrilo)tetraacetic acid], 0.5% CHAPS [3-[(3-
cholamidopropyl)dimethylammonio]-1-propane-sulfonate], 10% glycerol, 0.1 mM phenylmethanesulphonyl fluoride (PMSF), and 5 mM 2-mercaptoethanol.

Lysis was carried out for 30 minutes on ice and samples were separated at 20,000 x g for 30 min at 4° C. Protein content was determined using BCA Protein Assay (Pierce) [12] and aliquots, containing the active telomerase, were diluted in order to obtain a low chloride concentration (about 1 mM), compatible with intracellular concentration.

The enzyme present in this solution was challenged with the clusters under investigation at a final concentration ranging from 10^{-7} to 10^{-4} M. Incubations were carried out for 15 minutes at 23° C, to ensure the preservation of enzyme efficiency. After the treatment, the mixture was adjusted to obtain a final salt composition of: 20 mM Tris-HCl, pH 8.3; 1.5 mM MgCl₂, 63 mM KCl; 1 mM EGTA; 0.1 mg/ml BSA; and 0.005% Tween-20 (TRAP buffer). We included a true positive control (the untreated cell lysate), and an interference control (the cluster under study added only after this step, named elongation step).

The residual telomerase activity was determined following the standard procedures for TRAP assay (telomeric repeat amplification protocol, named amplification step).
6.4.4 Determination of the residual telomerase activity in cells

Sub-confluent cell monolayers were treated with the series of clusters at different concentration (10^{-9} - 10^{-5} M) for 24 h. Telomerase-containing extract was prepared as described in section 2.3. A range of 0.5 - 1 μg total cellular protein was analysed for the TRAP reaction.

6.4.5 Telomeric repeats amplification protocols

The residual telomerase activity was determined with two methods: the semi quantitative TRAP assay and real-time quantitative-TRAP [11]. In the traditional TRAP assay the elongation substrate for telomerase, the synthetic oligonucleotide TS (5'-AATCCGTCGAGCAGAGTT), was added to a mixture containing 1 μg of enzyme lysate (derived from cell-free biochemical assay or from lysate of the treated cells), 2U Taq polymerase (Roche, Germany), 50 μM dNTPmix, 2 μCi (α-32)dCTP (Amersham, UK) and TRAP buffer. The elongation step was conducted at 23 °C for 30 minutes. At the end of this step, the downstream primer CX (5’-GCGCGGCTACCTACCTACCTGCTTACCCTAACC) was added to the reaction buffer and the amplification of the hexameric elongation products was made by polymerase chain reaction, PCR, (30 cycles; 50° C annealing temperature). We included a true positive control (the untreated cell lysate), and an interference control (i.e. the cluster under study added only after
the elongation step. The inhibition of clusters 1a-4a on Taq polymerase was negligible. The amplification reaction products were resolved by non-denaturing 8% polyacrylamide electrophoresis. Densitometry was performed using a GS-250 Molecular Imager (BioRad) with BI screen type and Phosphor Analyst 1.1 program for densitometric analysis. Internal amplification control was included (ITAS, forward 5' - AATCCGTGCAGCAGGTTAAAAGGCGAGAAGCGAT, backwards 5' - ATCGCTTCTCGGCCTTTT) in the reaction and used for densitometric calculations.

The second method that we adopted employed a real-time PCR amplification protocol for telomerase products. The reaction mixture included 1X SYBR green buffer (Applera PE, Italy), 5 mM MgCl₂, 25 mM each dNTP, 2 U AmpliTaq Gold DNA-polymerase, 10 mM EGTA, 0.1 µg of each primers (TS 5' - AATCCGTCAGCAGGTT and ACX 5' - GCGCGG(CTTACC)₃CTAACC. The reaction was performed using an ABI Prism 7000 instrument (Applied Biosystems, USA). The experimental conditions were 25 °C for 20 minutes for the elongation step, 95 °C for 10 minutes for the activation of Taq, 95 °C for 20 seconds, 50 °C for 30 seconds and 72 °C for 90 seconds for denaturing, annealing and amplification, respectively.

The real-time fluorescence detection threshold was calculated as 10 standard deviations (SD) of the baseline measured between 3 to 15 cycles (ABI Prism 7000 SDS software). For amplification specificity at the end of 40 cycles a 20 minutes 60 °C to 94 °C dissociation cycle was performed (specific dissociation temperature = 76
°C). The untreated control gave a typical Ct (threshold cycle value) of 21 that corresponded to 100% telomerase activity, as measured at a relative fluorescence variation threshold of 0.03. A serial dilution of the untreated control (1, 0.5, 0.25, 0.1, 0.03, 0.012, 0.006, 0.0 micrograms of cell lysate) was adopted for standard curve determination (e.g. slope -2.069). The untreated control indicated the best performance of telomerase present in the lysate. An increase of Ct indicated a progressive reduction of telomerase activity, due to the action of the complexes directly on the semi-purified fraction or on the cells. Each sample was assayed in duplicate.

6.4.6 Cell viability assay

Viability was measured by using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [13], which evaluates the reduction of the tetrazolium salt by mitochondrial succinate dehydrogenase in metabolically active cells. Converted dye was dissolved in HCl-acidified isopropanol (0.1 N) and measured spectrophotometrically at 570 nm (background subtraction at 630 and 690 nm). All experiments were conducted at least in triplicate.

The results are presented as mean ± standard deviation (SD). The percentage of growth inhibition was plotted against concentration of the cluster in medium, and IC₅₀ value (defined as the concentration of drug required to reduce 50% cell viability with respect to untreated control) was calculated.
6.4.7 Platinum and osmium uptake and efflux.

Exponentially growing cells were treated with $10^{-7}$ M cisplatin or osmium clusters ($10^{-6}$ or $10^{-7}$ M). After 24, 48 and 72 h of continuous incubation, cell monolayers were washed 3 times with ice-cold saline solution (0.9 % NaCl) and harvested with trypsin for metal and protein determinations.

The experiments were repeated at least in triplicate.

In other experiments, in order to measure the drug efflux after 24 h of treatment at the same experimental conditions as above, the cells were rinsed quickly, twice with fresh complete medium at room temperature, and finally re-incubated for 24 or 48 h.

Mineralization was carried out with 65 % HNO$_3$ at 120 °C, the dry material was dissolved in 2% HNO$_3$ solution. The Pt or Os content was measured by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). The major isotopes of platinum and osmium were monitored at 195 and 190 m/z, respectively. The major isotope of indium (the internal standard) was monitored at 115 m/z. Cellular platinum or osmium levels are expressed as pmol complex / mg protein.

6.5 References for Chapter 6


12. Smith, P.K.K., R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H.,
Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C.,

Chapter 7

Future Research Directions.

7.1 Covalent Binding to proteins.

Other heterocyclic derivatives of quinoline have been considered as valid candidates for selective binding to proteins and DNA: the methyl imidate derivative of cymantrene ([η^5-cyclopentadienyl] tricarbonyl manganese), obtained through methanolysis of the corresponding nitrile, has been reported to couple with BSA (Bovine Serum Albumin) by binding to the lysine residues of the protein [1].

The adduct concentration in aqueous solutions has then been measured by AC voltammetry and the remarkable detection limit of 2 x 10^{-7} M BSA has been achieved.

By the same approach, the nitrile derivative of quinoline has been converted into the ethyl imidate (13) and then successfully coordinated onto the triosmium metal frame to yield the electron deficient cluster [Os_3(CO)_9(μ^3-η^2-C_{12}H_{11}NO)(μ-H)], (14) (Figure 7.1). Acid-catalyzed ethanolysis of 3-quinoline carbonitrile was carried out by dissolving the compound in a 50:50 mixture of ethanol and dioxane inside the inner finger of a water condenser: the outer part of the condenser was used to circulate water at 4 °C while gaseous HCl was

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bubbled in the inner finger for about 30 hours. The resulting hydrochloride was precipitated by adding diethyl ether to the solution and by washing the precipitate with more diethyl ether: the product was dried in vacuo over NaOH in order to remove eventual HCl present after washing.

![Chemical structure](image)

Figure 7.1: coordination of 13 onto the triosmium metal frame.

The solid-state structure of 14 has been determined in order to characterize the geometry of the imidate ligand: the structure is shown in figure 7.2 and selected bonds and angles are reported in table 7.1.

The bond distances of the metal frame indicate that the osmium triangle is isosceles, with the distance between the osmium atoms bridged by the hydride
shorter than the other metal-metal bond distances, 2.7566 Å, 2.7766 Å, and 2.7866 Å respectively.

The electron-deficient bond is also isosceles, with the two bond distances between C(8) of the carbocycle and the two osmium atoms practically equal, 2.266 Å and 2.292 Å respectively.

The angles C(9)-C(8)-Os(1) = 114.3° and C(9)-C(8)-Os(3) = 112.8° indicate that the heterocycle forms equal angles with the metal atoms it bridges, in fact bisecting the metal frame at a perpendicular angle with respect to the plane of the metals.

![Figure 7.2: X-ray structure of Os₃(CO)₉(μ₃-η²-C₉H₅N(3-C(NH)OCH₂CH₃))(μ-H)](image)

Figure 7.2: X-ray structure of Os₃(CO)₉(μ₃-η²-C₉H₅N(3-C(NH)OCH₂CH₃))(μ-H) [12]
The distances and angles reported indicate that the cluster has comparable structure to the one of the parent quinoline electron deficient cluster, in terms of structure of the triosmium plane and the relative position of the benzoheterocycle to the metal frame.

Interestingly, the crystal structure data report the presence of 3-ethyl ester group instead of the imino functionality in approximately 25% of the cluster molecules, probably due to further hydrolysis of the imino functionality to the corresponding ester. We were able to prepare very pure crystals of 12 in subsequent runs, and the presence of the ester can be detected by NMR.

The water soluble derivatives of 12 (using either P(C₆H₄SO₃Na)₃ or P(OCH₂CH₂N(CH₃)₃I)₃) are appropriate candidates for protein labeling in aqueous solutions, due to the useful electrochemical properties exhibited by similar water-soluble clusters and their demonstrated ability to bind to DNA and proteins discussed in the previous chapters.

The reported covalent labeling of BSA with the cymantrrene complex occurs in a pH 9 buffer, results in the displacement of the ethoxy group, and links the metal-containing label to the side chains of target amino acids. In the case of bovine serum albumin (BSA), the amino functionality of lysine side chains binds to the ethyl imidate to form a guanidinium group (Figure 7.3), which does not apparently alter the electrochemical properties of the complex in aqueous solutions [1].
Preliminary binding experiments with enkephalin and the negatively charged $\text{[Os}_3(\text{CO})_9(\mu-\eta^2-\text{C}_9\text{H}_5\text{N}(3-\text{C(NH)}\text{OCH}_2\text{CH}_3))(\mu-\text{H})(\text{P(C}_6\text{H}_4\text{SO}_3\text{Na})_3] [13]$ have been carried out and $^1\text{H-NMR}$ and $^1\text{H-NOESY}$ have been employed to monitor the reaction.

Enkephalin is an important oligopeptide involved in the transmission of neuronal signals in the brain, whose primary structure is Tyr-Gly-Gly-Phe-Met. It is a natural opioid substance and potent pain reliever found in very small concentrations in vivo and very few receptors are present in neural tissue. Labeling of this oligopeptide, along with other peptide neurotransmitters like endorphins, gonadotropin, and somatostatin may lead to the design of an appropriate bioassays for studying the binding of enkephalin to the receptor in living systems [2].

We have performed some preliminary tests with 13 and enkephalin: in a titration-like experiment, 1 mg of 13 was dissolved in 0.4 mL of D$_2$O at room temperature and then enkephalin was added until an equimolar amount was present.
Once the $^1$H-NMR revealed dramatic spectroscopic changes in terms of line broadening and chemical shift changes, we proceeded to collecting the $^1$H-NOE spectra.

Figure 7.4: $^1$H-NMR spectrum of the interaction between 13 and enkephalin at equimolar ratio.

The two dimensional $^1$H-NOE spectra show that, rather than covalent binding, aromatic $\pi$-stacking and H-bonding is observed, likely due to the lack of nucleophilic moieties on the aminoacids' side chains (Figure 7.4 and 7.5).
Figure 7.5: NOESY crosspeaks between the O-CH$_2$ of the imidate moiety in 13 and the phenylalanine and tyrosine aromatic resonances in Enkephalin.

In particular, the hydrogens on the tyrosine and the phenylalanine aromatic rings show NOESY cross peaks with the ethoxy imidate functionality, suggesting noncovalent binding but some type of complex formation that requires folding of the protein around the ligand of the cluster.
Figure 7.6: NOESY crosspeaks between the CH$_3$ of the imidate moiety in 13 and Enkephalin.

Based on the NOESY experiments, it appears that the interaction is largely due to partial stacking of the aromatic rings in tyrosine and phenylanaline with the quinoline heterocycle, and the interaction with tyrosine is enhanced by the possibility of hydrogen bonds between the phenolic hydroxyl and the ethoxy imidate functionality, as suggested by the NOESY crosspeaks.

It would be worth selecting a family of oligopeptides whose X-ray structure is not yet available and whose \textit{in vivo} biological functions are relevant,
then the appropriate residues could be covalently modified with the suitable cluster in order to obtain critical phase information for structure determination.

7.2 DNA binding studies.

Based on the selective binding observed with \([\text{Os}_3(\text{CO})_9(\mu_{\eta^2-}(4-\text{CHO})\text{C}_9\text{H}_5\text{N})(\mu_{\text{H}})(\text{P}(\text{OCH}_2\text{CH}_2\text{N}(\text{CH}_3)\text{Si})_3)]\) 5 and single-stranded DNA, there are a few experiments that represent a natural continuation of the work carried out thus far: double stranded DNA (namely KDSGT1 and its complementary strand KDSGT2) will be incubated with the cluster in order to understand the impact of different steric accessibility on the nucleophilic attack that leads to formation of the adduct.

Furthermore, since the sequence TGT seems to be the preferred binding site for 5, the general sequence Pyr-G-Pyr will be tested, such as TGC, CGT, and CGC, in order to understand whether steric considerations are adequate to describe the selective binding observed.

The breakthrough results obtained by infusing a solution of \([\text{Os}_3(\text{CO})_9(\mu_{\eta^2-}(3-\text{NH}_2)\text{C}_9\text{H}_5\text{N})(\text{P}(\text{C}_6\text{H}_4\text{SO}_3\text{Na})]\) into a crystal of RNA-RNAse p are an important proof of concept for the entire project, since the anomalous diffraction pattern obtained at 7 Å resolution allows the collection of critical phase information for crystal structure determination of proteins [3].
The anomalous diffraction patterns in picture 3.6 in chapter 3 are due to the presence of cluster molecules orderly disposed in the crystal cell, indicating that the cluster does not disrupt the molecular disposition into the lattice.

The direct visualization of biomolecules by electron microscopy also represents an important challenge for this project, since the substantial electronic density around the metal centers would certainly make for a facile detection of the metal-biomolecule adduct.

Imaging of the cluster by TEM or SEM techniques would allow a direct correlation between cluster structure and binding selectivity by immediate localization of the binding site, and would also enhance the contrast necessary to actually study the three-dimensional structure of biomolecules, often almost transparent to electron scatter techniques because of the limited electronic density around light elements like carbon, nitrogen, phosphorus, and especially hydrogen.

It is possible to lay polynucleotides onto carbon nanotubes, which offer excellent contrast for the detection of binding sites by microscopy techniques: the cluster molecules bound to the polynucleotide can be clearly localized based on the efficient electron scattering.

Since the functions of RNA are much more dramatically driven by its three-dimensional structure, compared to DNA, the collection of structural data on RNA and RNAses is of paramount importance: laying RNA strands onto carbon nanotubes would represent a valid alternative to crystal structure
determination for a first analysis of the three-dimensional geometry. The design of a suitable triosmium cluster would enhance the chances to obtain critical structure information before engaging into growing crystals of the ribopolynucleotide of interest.

A few preliminary tests have been carried out in collaboration with Dr. Kurt Gekeler at the Gwang-Ju Institute of science and technology in Gwang-Ju, South Korea, but at the moment there are no definitive data to report.

7.3 NMR modeling of binding site.

Although the sequencing gels carried out in presence of NaBH₄ amply support the formation of a Schiff base, the synthesis of a model compound elucidating the bond formed between dG and 5 represents important final evidence. The possibility of obtaining an X-ray structure of the model compound along with a full characterization by NMR is a natural conclusion to this study of the reactivity of our candidate cluster with nucleic acids.

A task that seemed facile at first glance has posed a few problems when deciding the conditions under which the synthetic reaction ought to be performed. Most of the reactions reported in the literature on reductive aminations of carbonylic compounds require an acid catalyst, sometimes incompatible with the N-glycosidic bond of dG, which becomes unstable below pH~4 [4]. On the other hand, the very reaction we are going to model is not extremely efficient in aqueous solutions. The band in the sequencing gel in
This clearly indicates that reduction of the aldehyde strongly competes with the actual binding, reflecting an intrinsic instability of the Schiff base in water that cannot be avoided [4].

Given these limitations, a first experiment was carried out on the hydrophobic electron-precise decacarbonyl cluster [Os₃(CO)₁₀(μ-η²-(4-CHO)C₉H₅N)(μ-H)] and 3',5'-diacetyldeoxyguanosine, because the nucleoside is otherwise insoluble in practically any solvent but a 1M hydrochloric acid solution.

The main objective of this reaction was the direct observation of NMR peaks relative to the Schiff base: after stirring under an inert atmosphere in methanol, a ¹H-NMR of the crude reaction mixture revealed a dramatic decrease of the aldehyde peak and the appearance of several peaks at 6.2 ppm and the aromatic region at 9.6 and 9.9 ppm (figure 7.7). The pattern was consistent with the chemical shift of Shiff bases, considering the different geometric isomers that can be formed, cis and trans, referring to the possible relative orientations of the lone pair on the nitrogen and the hydrogen on the carbon (Figure 7.4).
Attempts to isolate the Schiff base adducts have failed due to the instability of the C=N functional group in the presence of silica gel and reverse phase silica gel. Reverse phase silica gel (RP-TLC) represents a valid alternative, since no polar groups such as hydroxyls are present on the surface, and separation can be obtained.

Based on the sequencing gel results and the preliminary NMR data, we propose the formation of a compound that binds the triosmium cluster to the deoxyguanosine through a C=N bond (figure 7.7).
The reduction of the Schiff base in situ, on the other hand, has not yielded the desired product because the reaction conditions reported in the literature suggest the use of the reagent sodium triacetoxyborohydride (NaBH(OOCCH$_3$)$_3$) in 1,2-dichloroethane. Unfortunately, 3',5'-diacetyldeoxyguanosine is not soluble in any solvent but methanol; furthermore, the presence of a 1.5-2 fold excess of acid reported in the synthesis does not help the stability of the nucleoside's N-glycosidic bond.

One last possibility is to carry out the reaction in water with NaBH$_4$ as reducing agent without any acid catalyst: if the yield is adequate, a full NMR characterization and a suitable crystal for X-ray diffraction studies ought to be possible.
7.4 Other potential cluster candidates.

Based on the properties of the water-soluble clusters in aqueous solutions, minimal aggregation is desirable to obtain the best interaction between clusters and biomolecules. In particular, the water-soluble cluster with the 3-amino quinoline ligand showed negligible aggregation at low pH because protonation of the amino functionality favored electrostatic repulsion between cluster molecules and minimized hydrophobic interaction.

Using the same approach, if like charges are placed in different parts of the water-soluble cluster, the benzo heterocycle can be modified to introduce intercalating moieties that can eventually lead to novel selectivity toward nucleic acids (figure 7.8).

Figure 7.9: target ligand for intercalating clusters.
Whether coordination of the quinoline ligand onto the metal frame occurs prior to or after the linkage to phenanthridine, the extended 3-ring system has the correct size and shape (also called "bite") to successfully intercalate in the major groove of double-stranded DNA. The water-soluble positively charged cluster of the target ligand possess all the suitable features to open up an entire new area of study that is likely to show different binding trends and different selectivity than the ones observed with any cluster tested so far.

The ligand 2,2′-diamino-1,1′-binaphtalene (H_{2}binam) is also a valid candidate for intercalation, since it has been already successfully coordinated onto a triosmium metal frame [5]. The electron deficient triosmium nonacarbonyl cluster is obtained, in which the amino nitrogen coordinates to an osmium center through its electron pair, similarly to the nitrogen in quinoline.

The advantage in using H_{2}binam is that two aromatic rings are available for intercalation and the amino functionality is also available for H-bonding patterns and sequence selectivity (figure 7.9).

Figure 7.10: structure of Os_{3}(CO)_{9}(\mu^{3}-\eta^{2}-Hbinam)(\mu-H).
The reactivity toward phosphines and phosphites must be studied in order to ascertain whether the water-soluble derivative can be synthesized, a necessary modification for any relevant study with biomacromolecules.

On the wake of the coordination exhibited by this compound, 2-aminoanthracene will also be coordinated onto the triosmium metal frame, hoping to obtain the same coordination as binam has.

The main difference would be a 3-ring fused heterocycle protruding from the cluster, able to intercalate into double stranded DNA. The different orientation of the anthracene ring would perhaps show different selectivity for intercalation, based on the space available for the three rings (figure 7.10).

![Figure 7.11: proposed structure for [Os₃(CO)₉(μ³-η²-C₁₆H₁₀N)(μ-H)]](image.png)

The reactivity of the electron-deficient precursor also must be tested toward two-electron donors such as phosphines and phosphites, in order to obtain the water-soluble adduct needed for bioassays.
7.5 Conclusions.

The water-soluble clusters that have been developed for the first time through this project really possess all the necessary features as successful candidates for interaction with biomacromolecules. Not only does the heterocycle allow complete versatility in terms of functional groups available for either H-bonding, covalent modification or intercalation, but the very water-solubilizing ligand imparts specific reactivity towards nucleic acids or proteins, for the positively charged and negatively charged trivalent phosphorus-based ligand respectively.

The dramatic change in activity and reactivity observed with different ionic groups on the triosmium frame indicates that the electrostatic interaction of the water-soluble clusters plays a fundamental role in the chemistry observed with DNA and proteins, allowing the selection of the target biomolecule even in the presence of an RNA-RNase complex. Depending on the charge of the cluster, binding can be targeted for the ribonucleic acid or the enzyme.

Once we ascertained that the bridging heterocycle is available for interaction with nucleic acids and proteins, H-bonding, covalent modification, and intercalation can be achieved by placement of the appropriate functional group on the heterocyclic ligand.

Whether the application is crystallographic structure determination, direct visualization by microscopic techniques, modification of neurotransmitting oligopeptides, or covalent modification of nucleic acids, the wide
range of regio- and stereo-selective reactions that can be performed on the triosmium clusters, either at the metal core or at the benzoheterocycle, translates into completely versatile and flexible structures that can be tailored to target the biomolecule of interest for structural and biochemical studies.

Coordination of extended fused heterocycle such as 2-aminoanthracene onto the metal frame will also allow the use of fluorescence techniques for monitoring intercalation by fluorescence experiments, adding an extremely useful technique to the already ample array of biochemical assays that have been used to elucidate the interaction of the clusters and nucleic acids.

7.6 Experimental Section.

7.6.1 Synthesis of quinoline 3-ethoxy imidate.

Using a Friedrichs water condenser, 1 g of quinoline 3-carbonitrile (6.5 mmol) was dissolved in a mixture of 20 mL of ethanol and 20 mL of dioxane in the cold finger: the outer part of the condenser was cooled to ~4°C by circulating water through a temperature-controlled bath. Meanwhile, gaseous hydrogen chloride was bubbled in the solution until the solid completely dissolved.

After 30 hours, diethyl ether was added to the solution in order to precipitate the product as the hydrochloride. The recovered solid (1.65 g, 92.7%) was washed with diethyl ether and then dried overnight under vacuum over potassium hydroxide.
The solid was dissolved in deionized water and the solution was brought to neutral pH by addition of aqueous sodium hydroxide, and then the solution was extracted twice with diethyl ether. The organic layer was dried over sodium sulfate and then rotary evaporated to yield 1.21 g of quinoline 3-ethoxy imidate (6.00 mmol).

\[ ^1H-NMR \text{ of the hydrochloride (H}_2O, \delta): 8.5 \text{ ppm (s, 1H), 7.9 \text{ ppm (d, 1H), 7.6 \text{ ppm (d, 1H), 7.4 \text{ ppm (m, 2H), 7.1 \text{ ppm (s, 1H), 4.5 \text{ ppm (q, 2H), 1.47 \text{ ppm (t, 3H).}}}} \]

Elemental analysis (calculated): H 5.16%, C 52.30%, N 10.21%, Cl 25.75%.

Elemental analysis (actual): H 5.17%, C 51.56%, N 9.96%, Cl 25.61%.

7.6.2 Synthesis of [Os$_3$(CO)$_9$(μ$_3$-η$^2$-(3-C(NH)OCH$_2$CH$_3$)C$_9$H$_5$N)(μ-H)], 12.

The cluster [Os$_3$(CO)$_9$(μ$_3$-η$^2$-(L-H))(μ-H)] (L = 3-(C(NH)OCH$_2$CH$_3$)C$_9$H$_5$N) was synthesized according to literature procedures [6, 7].

\[ ^1H-NMR \text{ (CDCl}_3, \delta): 9.74 \text{ ppm (d, 1H), 8.70 \text{ ppm (s, 1H), 8.55 \text{ ppm (s, 1H), 8.50 \text{ ppm (d, 1H), 7.28 \text{ ppm (t, 1H), 5.28 \text{ ppm (s, 1H), 4.50 \text{ ppm (q, 2H), 1.47 ppm (t, 3H), -12.05 ppm (s, 1H).}}}} \]

Elemental analysis (calculated): C 24.65%, N 2.75%, H 1.18%.

Elemental analysis (actual): C 24.60%, N 2.35%, H 1.02%.

IR (νCO, hexane): 2072 (m), 2040 (m), 2011 (s), 2001 (s), 1978 (sh), 1962 (s), 1953 (s), 1926 (s) cm$^{-1}$. 

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7.6.3 Synthesis of Os₃(CO)₉(μ-η²-(3-C(NH)OCH₂CH₃)C₉H₅N)(μ-H)

(P(C₆H₄SO₃Na)₃), 13.

The cluster Os₃(CO)₉(η-(3-C(NH)OCH₂CH₃)C₉H₅N)(μ-H)

(P(C₆H₄SO₃Na)₃) was synthesized according to literature procedures [8].

¹H-NMR (D₂O, water decoupled), δ: 9.85 (s, 1H), 8.5 (d, 1H), 8.10 (s, 1H), 8.00 (d, 1H), 7.65 (m, 12 H), 7.20 (t, 1H), 3.40 (q, 2H), 1.25 (t, 3H), -12.10 (d, 1H, JₚH = 16.05 Hz).

Elemental Analysis (Calculated): C 29.85%, N 1.79%, H 1.54%.

Elemental Analysis (Actual): C 30.01 %, N 1.70 %, H 1.48 %.

IR (vCO, D₂O): 2006 (s), 2139 (m), 2169 (w), 2194 (w), 2236 (m), 2273 (sh), 2297 (s), 2318 (s), 2339 (s) cm⁻¹.

7.6.4 Synthesis of 3',5'-diacetyl-2'-deoxyguanosine.

The synthesis of 3',5'-diacetyl-2'-deoxyguanosine was carried out according to the published literature [9].

7.6.5 Crystal Structure Analysis

A suitable crystal of 12 was coated with Paratone N oil, suspended in a small fiber loop and placed in a cooled nitrogen gas stream at 100 K on a Bruker D8 SMART 1000 CCD sealed tube diffractometer with graphite monochromated CuKα (1.54178Å) radiation. A full set of data was measured using a series of combinations of phi and omega scans with 10 s frame exposures and 0.3° frame
Data collection, indexing and initial cell refinements were all carried out using SMART software [10]. Frame integration and final cell refinements were done using SAINT software [11]. The final cell parameters were determined from least-squares refinement on 6141 reflections. The SADABS program was used to carry out absorption corrections [12].

The structure was solved using Direct methods and difference Fourier techniques (SHELXTL, V6.12) [13]. Hydrogen atoms were placed their expected chemical positions using the HFIX command and were included in the final cycles of least squares with isotropic U\textsubscript{ij} 's related to the atom's ridden upon. The C-H distances were fixed at 0.93 Å (aromatic and amide), 0.98 Å (methine), 0.97 Å (methylene), or 0.96 Å (methyl). The hydride, H (13), was positioned by using the XHYDEX program in the WinGX suite of programs [14]. All non-hydrogen atoms were refined anisotropically except for N(2), O(2) and C(21). Scattering factors and anomalous dispersion corrections are taken from the International Tables for X-ray Crystallography [15]. Structure solution, refinement, graphics and generation of publication materials were performed by using SHELXTL, V6.12 software. Additional details of data collection and structure refinement are given in Table 7.1.
Details

The side chain on the quinoline was disordered in that ~25% of the molecules had an oxygen attached to C(10) while ~75% of the molecules had the expected NH group.

Table 7.1. Selected bond lengths [Å] and angles [°] for fs01s.

<table>
<thead>
<tr>
<th>Bond</th>
<th>Length [Å]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Os(1)-C(8)</td>
<td>2.266(12)</td>
</tr>
<tr>
<td>Os(1)-Os(3)</td>
<td>2.7566(8)</td>
</tr>
<tr>
<td>Os(1)-Os(2)</td>
<td>2.7766(6)</td>
</tr>
<tr>
<td>Os(2)-N(1)</td>
<td>2.181(9)</td>
</tr>
<tr>
<td>Os(2)-Os(3)</td>
<td>2.7866(7)</td>
</tr>
<tr>
<td>Os(3)-C(8)</td>
<td>2.292(11)</td>
</tr>
<tr>
<td>C(5)-C(6)</td>
<td>1.372(15)</td>
</tr>
<tr>
<td>C(6)-C(7)</td>
<td>1.403(15)</td>
</tr>
<tr>
<td>C(7)-C(8)</td>
<td>1.383(15)</td>
</tr>
<tr>
<td>C(9)-N(1)</td>
<td>1.360(13)</td>
</tr>
<tr>
<td>C(21)-O(11)</td>
<td>1.115(14)</td>
</tr>
<tr>
<td>N(2)-H(10)</td>
<td>0.7700</td>
</tr>
<tr>
<td>Os(1)-Os(2)-Os(3)</td>
<td>59.406(18)</td>
</tr>
<tr>
<td>Os(3)-Os(1)-Os(2)</td>
<td>60.478(18)</td>
</tr>
</tbody>
</table>
Os(1)-Os(3)-Os(2)  60.115(18)
Os(1)-C(8)-Os(3)  74.4(3)
Os(3)-Os(1)-H(13)  41.9
Os(2)-Os(1)-H(13)  87.6
C(5)-C(6)-C(7)  121.5(10)
C(8)-C(7)-C(6)  122.6(10)
C(7)-C(8)-C(9)  116.5(10)
C(7)-C(8)-Os(1)  116.9(8)
C(9)-C(8)-Os(1)  114.3(8)
C(7)-C(8)-Os(3)  115.0(8)
C(9)-C(8)-Os(3)  112.8(7)
C(8)-Os(3)-Os(2)  77.6(3)
C(8)-Os(1)-Os(3)  53.2(3)
C(8)-Os(1)-Os(2)  78.2(3)
N(1)-Os(2)-Os(1)  84.3(2)

7.7 References for chapter 7.


3. K. Compher, K. Jude, D. Christianson, the University of Pennsylvania, 
Personal Communication.


5. Cabeza, J.A.d.S., I., del Rio, I., Garcia-granda, S., Riera, V., Sanchez-Vega, 

6. Abedin, M.J., Bergman, B., Holmquist, R., Smith, R., Rosenberg, E., 
Ciurash, J., Hardcastle, K.I., Roe, J., Vasquez, V., Roe, C., Kabir, S.E., Roy, 

7. Bergman, B., Holmquist, R.H., Smith, R., Rosenberg, E., Hardcastle, K.I., 

8. Rosenberg, E., Spada, F., Sugden, K., Martin, B., Gobetto, R., Milone, L., 

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