Synthesis, chemical and biochemical characterization of Ti^{IV} and V^{IV} complexes with anti-tumor activity

by

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Abstract

At the present time in which the metallocene dihalides have shown to possess antitumoral activity, scientists of all around the world have been working with the synthesis and structural modifications to improve their performance. This work was aimed to the synthesis and chemical/biochemical characterization of the titanium and vanadium complexes. Two complexes were synthetisized $Ti(maltolato)_2(OH)_2$ and $V(maltolato)_2Cl_2$, using as starting material titanocene and vanadocene dichlorides respectively. The characterization of both complexes was achieved using techniques as Infrared spectroscopy (IR), Nuclear Magnetic Resonance (NMR), Mass spectroscopy (MS), and Cyclic Voltammetry (CV) among others. Interaction studies between these complexes with apo-transferrin, using UV-VIS, showed that vanadium complex has the capacity to bind the biomolecule by donation of V(IV) to apo-transferrin bindin pocket. The titanium-maltol complex showed to be a robust one because does not donate the metal to apo-transferrin. Neither the titanium or vanadium complexes interact with the DNA molecule or oligonucleotide. Cytotoxic studies using HT-29 cancer cells showed that $Ti(maltolato)_2(OH)_2$ was the most active complex in comparison with other eight complexes including titanocene [1]. $Ti(maltolato)_2(OH)_2$ shows hydrolytically stability, with 120 hours of cytotoxic activity. Similar results were obtained using human epithelial adenocarcinoma cells (Caco-2 cells).

Resumen

Desde el momento en que los dihaluros de metalocenos mostraron tener capacidades antitumoral, científicos de todas partes del mundo se han dado a la tarea de llevar a cabo modificaciones y nuevas síntesis con el fin de optimizar el desempeño de estos. Este trabajo esta enfocado en la síntesis y caracterización química y bioquímica de complejos de titanio y vanadio. Dos complejos fueron sintetizados $Ti(maltolato)_2(OH)_2$ y $V(maltolato)_2Cl_2$, utilizando sus respectivos dialuros como material de partida. Entre las tècnicas utilizadas para la caracterización se encuentran: Resonancia Magnetico Nuclear (RMN), Spectroscopía de infrarrojo (IR), Espectroscopía de Masa (MS/MS), Voltametria cíclica (CV), entre otras. Se llevaron a cabo estudios de interacciòn con moléculas de importancia biologica tales como: apo-transferina y ADN (calf thymus DNA), utilizando UV-VIS. El complejo de Vanadio mostró tener la capacidad de donar el metal a la apo-transferina, mientras que el complejo de titanio no dona el metal, mostrando ser un complejo mas robusto. Datos similares fueron obtenidos con la molécula de ADN, no existe interacción apreciable entre estos complejos y la molécula de ADN. Estudios de citotoxicidad en células HT-29 demuestran que el complejo de titanio es mas activo que los ocho complejos utilizados en el estudio, incluyendo a titanoceno [1]. $Ti(maltolato)_2(OH)_2$ mostró actividad hasta por 120 horas lo que demuestra la gran estabilidad hidrolítica que este posee. Resultados similares fueron obtenidos utilizando células epiteliales humanas de adenocarcinomas colorectal (células Caco-2).

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To my son Eduardo Lamboy
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1. Introduction

Cancer is a disease that kills thousand of peoples around the world. The statistics of the American Cancer Society shows that the cancer is the 22.8% of all deaths, being the second cause of death in the world. Prostate, breast, lungs & bronchus, colon & rectum are the most common affected areas [2]. Basically, a cancer tumor is a group of cells that divide quickly and grows out of control. The word cancer is just a broad name for many different diseases. Whether the tumor is localized in an organ or is spread in the body (metastasis), it affects the body in different ways. There are some approach to treat the cancer, which one is appropriate depends on the age, gender, severity and tumor localization. These methods include radiation, chemotherapy, surgery and hormone therapy or the combination of them.

Radiation is one of the most used therapies to control or eliminate tissues cancerous cells. It consists of energy that is carried by waves or steam of particles that can alter the genetic code of the cell. This genetic code controls how a cell grows and divides in the body. This kind of therapy attacks reproducing cancer cells, but it can also affect reproducing cells of normal tissues. Each time radiation therapy is given, it involves a balance between destroying the cancer cells and sparing the normal cells. The damage to normal cells is what causes side effects. The longevity, nature, severity of side effects depends on the organs that receive the radiation, type of radiation, dose, fractionation, concurrent chemotherapy, and the patient. The most common side effects caused by this therapy include damage to the epithelial surface, inflammation of the soft tissue, fibrosis, hair loss, fatigue and death.

The most common therapy to treat cancer diseases is the chemotherapy. This therapy has been in use to treat this disease since early 1950s. The word chemotherapy was once used to refer to medicine for treating any disease. However, these days, chemotherapy is most often used to mean taking medicines, or drugs, to treat cancer. The patient might take these drugs before or after surgery, with radiation (x-rays) treatment or you might take the drugs by themselves. Although chemotherapy drugs have been tested and, extremely careful investigation show they work reducing the cell cancerous behavior, the secondary symptoms produced by this therapy are very dangerous.

The first metal complex used to treat the cancer was cis-diamminedichloroplatinum(II), known as cis-platin (Figure 1-1). This platinum based metal complex showed to possess antitumor activity in 1969 [3].

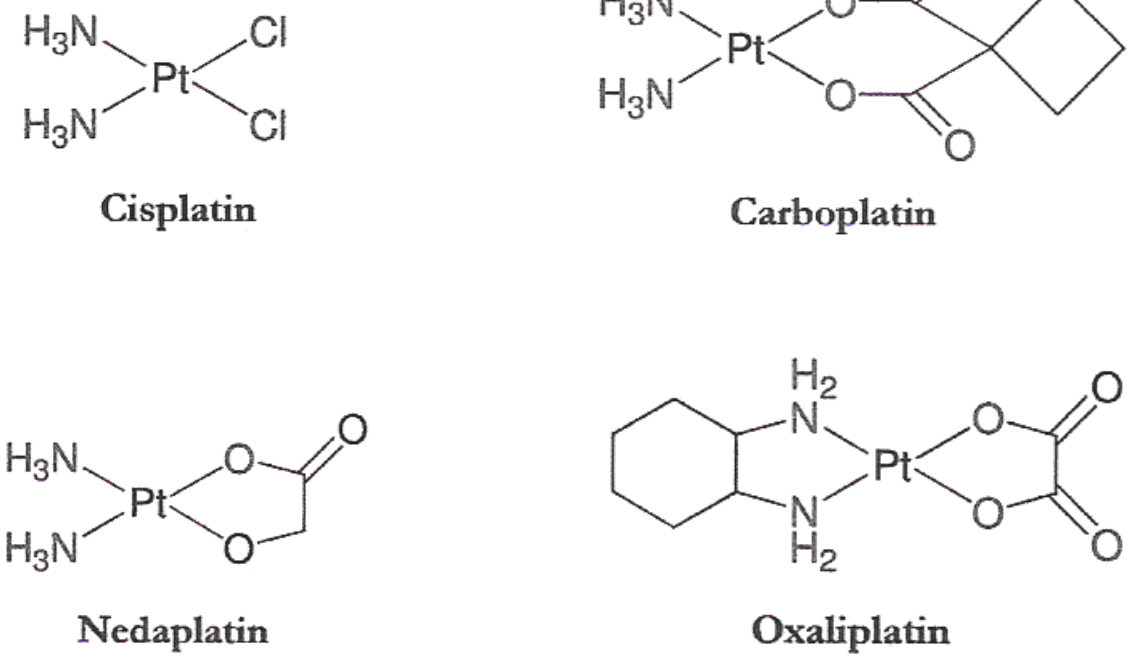


Figure 1-1: Structure of clinically used platinum (II) derivatives

This complex and its analogues have been used until the present, although it has toxic effect like nephrotoxicity and myelotoxicity [4-6]. The great disadvantage of platinum complexes toxic effects led to an increase in researchers focused to study complexes with an active metal center different to platinum (V, Nb, Mo, Ti), in order to find a solution to this proliferative disease. The publication in 1979 of titanocene (Cp_2TiCl_2) (Figure 1-2) by Köpf-Maier and Köpf [7] revealed the first metallocene with antitumor capacity, and provided an important point of departure for subsequent investigations. In general, these complexes showed antitumor activity against a wide variety of tumor cells among them Ehrlich ascites tumor, B16 melanoma, colon 38 carcinoma, Lewis lung carcinoma with less toxic effects than the well reputed cisplatin [3]. After these results, the structure modification of titanocene was pursued in order to elucidate structure-activity relationship. In addition, metallocene of general formula Cp_2MX_2 (M = Ti, V, Mo, Nb; X = halides, pseudo halides) have been synthesized and investigated for antitumor activity [4-6, 8, 9].

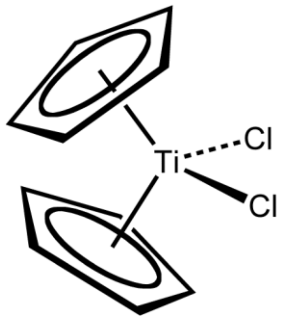


Figure 1-2: Structure of Titanocene Dichloride

The first non-metallocene complex to reach clinical trial was budotitane ($[CH_3CH_2O)_2(bzac)_2Ti(IV)]$) (Figure 1-3) [10]. Other cis-$[X_2(bzac)_2Ti(IV)]$ complexes have been investigated exhibiting similar biological activity as the ethoxide complex [11].

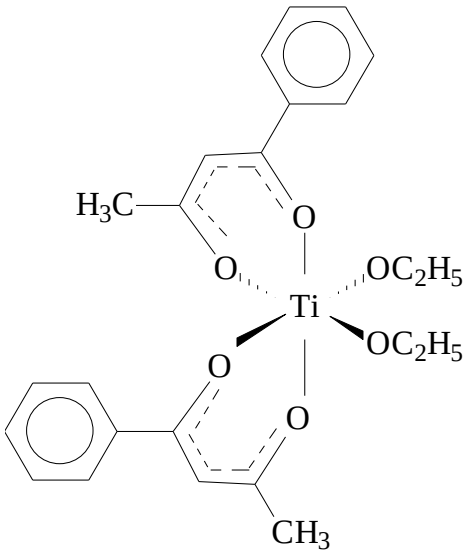


Figure 1-3: Structure of budotitane ([$CH_3CH_2O)_2$(bzac)$_2$Ti(IV)])

As mentioned above, the biological activity of these complexes was tested against different cancer cells, but the mechanism of action of these complexes at cellular level is not well understood. There are various hypotheses about the targets and possible carrier of the complexes into the cells. Nucleic acid was proposed as a target, and then DNA synthesis was interrupted. Another hypothesis, using apo-transferrin as carrier agent, has been proposed by Sadler and coworker [12-15]. Transferrin is an 80 kDa glycoprotein that can bind iron reversibly. It is known that transferrin carries iron (III) in blood at pH of 7.4 and delivers to cells and releases it at pH of 5.5. Transferrin also transport and deliver another metal ions as (Ga(III) and Ru(III)) to cancer cells [13]. This protein has been used on the treatment of many diseases, not only as metal transporter. For instance, Atransferrinemia is a disease characterized by anemia, iron overload. An infusion of apo-transferrin is used to treat this condition.

This investigation pursued the rational design of more efficient antineoplastic agents using titanium and vanadium as metal centers and maltol (figure 1-4) as ligand.

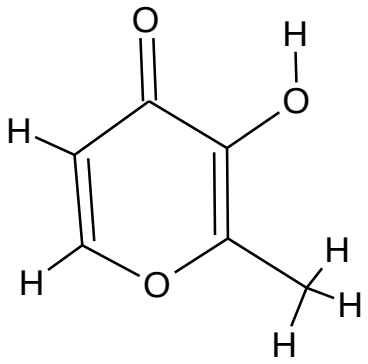


Figure 1-4: Structure of maltol (3-hydroxy-2-methyl-4-pyrone)

Maltol is a non-toxic compound that has different bioinorganic applications: (i) iron-maltol complexes are used as treatment of iron deficiency anemia; (ii) it has been examined as a possible chelating agent for lowering aluminum levels in the body; (iii) display potent insulinomimetic properties and; (iv) molybdenum-maltol complexes showed significant improvements in postischemic cardiac function [16]. Maltol belongs to the family of pyrone. It showed to be useful to the synthesis of metal complexes with vanadium and molybdenum [16, 17]. Interest in maltol arises from its ability to deprotonate readily (pKa for Hma= 8.38) and to act as an anionic chelating, bidentate O,O` (hydroxy and the ketone moieties) ligand toward a number of biologically active metals [16].

1.1 Objective

The proposed research was aimed to the synthesis, chemical and biochemical characterization of Ti(IV) and V(IV) complexes containing maltol as ligand, and the evaluation of the biological activity in tumor cells. The final objective is the rational design of metal complexes with enhanced antitumor activity. In order to accomplish these goals, the following objectives were pursued:

1. To characterize new Ti(IV) and V(IV) complexes using spectroscopic techniques such as UV, NMR, EPR and IR spectroscopies, electrochemical methods such as cyclic voltammetry and solid state structure determination by X-ray diffraction methods.
2. To study the stability and decomposition patterns of the complexes in aqueous solutions.
3. To study the interactions of Ti(IV) and V(IV) complexes with biologically important molecules such as Calf-Thymus DNA and human serum transferrin, using cyclic voltammetry and UV-Vis spectroscopy.
4. To evaluate the cytotoxic activity of titanium and vanadium complexes on tumor cells HT-29 human colon cancer.

1.2 Previous Works

1.2.1 Metallocenes dihalides as antitumor agents

Metallocene dihalides are a group of organometallic complexes synthesized more than five decades ago [18]. In its early stages, the use of these complexes was almost limited to the catalytic reaction. The publication of the biological activity of titanocene dichloride by Petra Köpf-Maier and Harmut Köpf in 1979, produced another important point of departure for the metal based antitumoral drugs [5]. Titanocene was the first metallocene to show antitumor activity. In the following years, other metallocene of general formula Cp_2MX_2 (M= V, Nb, Mo; X= halides and pseudohalides) have been tested for antitumor activity [3-6]. Of all metallocenes tested, titanocene exhibited the better antitumor activity against Ehrlich ascites tumor, B16 melanoma, colon 38 carcinoma, and Lewis lung carcinoma with no evidence of nephrotoxicity and myelotoxicity [3]. Due these encouraging results, titanocene dichloride reached clinical trials to threat a wide range of tumor cells. Its major difficulty or problem to overcome is the limited solubility and low stability in water.

After these initial optimistic results, the structure modification of the titanocene dichloride has been pursued by many researchers. These modifications go from the replacement of the chloride ligands with biologically important molecules to the modification or functionalization of cyclopentadienyl rings. Our research group has reported the characterization of the titanocene complexes with thionucleobases. The substitution of the chloride groups for thionucleobases was aimed to the understanding of the interaction between titanocene and modified RNA/DNA bases, as these interactions could have some implications in its mechanism of action [19]. Also, thionucleobases have

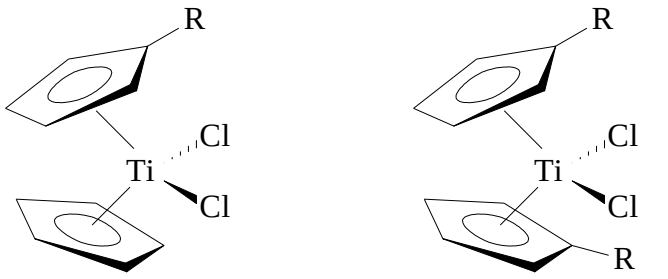
antitumoral activity on their own therefore, the use of two antineoplastic agents in the same compound could in principle yield species with enhancement antitumor activity.

The stability studies of these complexes in DMSO and DMSO-water (at low pH, 3) showed noticeable difference between Ti-thionucleobases interaction. The Ti-thionucleobase interactions are more stable in DMSO-water solution than in DMSO. The addition of NaCl to the medium does not change the rate of thionucleobase loss (dissociation). This behavior implies that the chloride does not have a major effect on the decomposition pattern. The kinetic pattern of the cyclopentadienyl was opposite to the behavior showed for the thionucleobases [19]. That is, the rate of Cp loss decreases by the presence of sodium chloride in the medium.

Chávez-Gil et. al. reported the synthesis and electrochemical characterization of a series of molybdenocene complexes using thionucelobase/thionucleoside ligands instead of chlorides groups. Cyclic voltammograms of these complexes were obtained in order to compare the electrochemical behavior with molybdenocene dichloride. All the complexes possess reversible redox waves consistent with a one electron redox process for the $Mo^{V/IV}$ couple, analogous to molybdenocene dichloride. The most important feature of these complexes is that they possess water solubility. This overcomes the problem of low water solubility encountered in the metallocenes [20].

Other areas of research have been developed recently. For instance, many researchers have focused their efforts in the functionalization of cyclopentadienyl rings, in order to provide either higher hydrolytic stability or enhanced antitumor properties [21]. These modifications involved the incorporation of electron donating or withdrawing groups. Earlier studies showed that the use of electron donating group diminished the

cytotoxicities of the compounds synthesized [22]. This is not an unexpected observation because the electron donating group would diminish the Lewis acidity character of the metal center. Using this rationale, the synthesis of new complexes employing electron withdrawing group became a target. Following this approach our research group reported the synthesis and characterization of functionalized titanocene dichloride. Gao et. al. reported the first detailed synthetic route for $(Cp\text{-}CO_2CH_2CH_3)CpTiCl_2$ and provided an alternate route for the synthesis of $(Cp\text{-}R)_2TiCl_2$ complexes. The carboethoxy-Cp functionalization provided complexes with citotoxicity comparable to titanocene dichloride, while the carbomethoxy functionalization proved to be non-active at the time interval studied [21].



IIa, R = CO_2Me IIIa, R = CO_2Me

IIb, R = CO_2Et IIIb, R = CO_2Et

Figure 1-5: General structure of ring-substituted titanocene dichlorides

Potter et. al. synthesized a new series of ten water soluble titanocene dichloride derivatives of the types $[Cp(\eta^5\text{-}C_5H_4R)TiCl_2$ and $(\eta^5\text{-}C_5H_4R)_2TiCl_2$ where R= cyclic pyrrolidylium, morpholinium and picolylium groups. They reported that dicationic titanocene dichloride derivatives showed greater potencies than the corresponding

monocationic derivatives containing the same functionalized ring plus one unsubstituted Cp ring [22]. Apparently the water solubility and dicationic nature of the titanocenes have important roles in their anticancer activity perhaps as a result of enhanced interaction with biomolecule such as DNA.

The idea of functionalization of the Cp rings has been extended to vanadocene dichloride. Gleeson et. al. reported the synthesis and characterization of three complexes of vanadocene with both cyclopentadienyl functionalized with a methoxybenzyl groups. The cytotoxic activities of these complexes were tested against pig kidney epithelial cells (LLC-PK) and compared with their titanocene counterpart. All of the vanadocene functionalized complexes showed to possess better antitumoral activity than titanocene functionalized complexes [23].

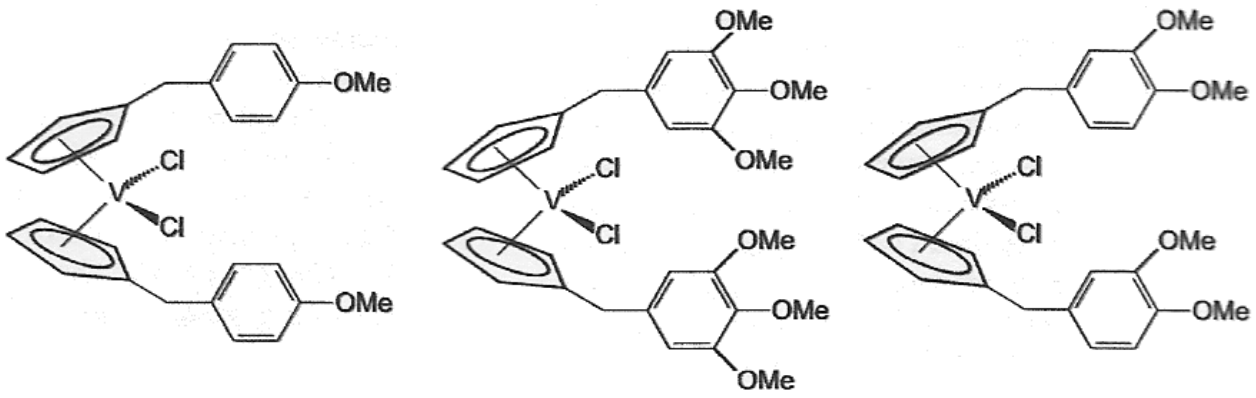


Figure 1-6: Structures of benzyl-substituted vanadocene dichloride derivatives

1.2.2 Non-metallocene complexes as antitumor agents

The first non-metallocene complex to reach clinical trial was budotitane ([$(CH_3CH_2O)_2(bzac)_2Ti(IV)$]) and was found to be active against a wide variety of ascites and solid tumors [3]. Other cis-[$X_2(bzac)_2Ti(IV)$] complexes were investigated exhibiting similar antitumor activity as the ethoxide complex. Several diacidobis(β-diketonato)metal(IV) complexes with M = Ti, Zr, Hf, have also shown to possess antitumor activity [16]. These complexes exhibited antitumor properties against animal tumor such as Ehrlich ascites tumor, Stockholm ascites tumor, sarcoma 180 ascitic tumor and to solid tumor such as Walker 256 carcinoma and MAC 15A colon carcinoma [11, 24, 25]. Marginal response was observed for leukemias P388 and L1210. Interestingly, the complexes containing titanium were the most active agents. In particular dichlorobis(1-phenylbutane-1,3-dionato)titanium(IV), [$Ti(bzac)_2Cl_2$], and diethoxybis(1-phenylbutane-1,3-dionato)titanium(IV), [$Ti(bzac)_2(OEt)_2$], were the most active species. It is worth mentioning that [$Ti(bzac)_2(OEt)_2$] is more active against colon tumor than 5-fluorouracil and underwent clinical trials [11, 24, 25]. Its major difficulty or problem to overcome is the limited solubility and low stability in water.

As we have presented thus far, it is very interesting to notice that titanocene dichloride and [$(CH_3CH_2O)_2(bzac)_2Ti(IV)$] (budotitane) are two different compounds with very similar biological activity against cancer. Thus, the right combination of ligands around Ti(IV) can indeed provide the antiproliferative properties necessary to be a good anticancer agent, regardless whether the species is an inorganic or organometallic compound.

1.2.3 Interaction with biomolecules

The interaction of metallocenes and its analogous with biologically important molecules has been studied by different groups of investigation. Although the mechanism of action of these complexes at cellular level is not well understood, some molecules have been implicated in the transport and target of the metal complexes. The hypothesis of human serum transferrin, an iron transport agent, as carrier agent of Ti(IV) to the target places has been explored by Sadler and co-workers [26]. Guo et. al. showed that the interaction of titanocene dichloride with apo-transferrin form Ti_2-transferrin species. Binding studies of Ti_2-transferrin with ATP have shown that transferrin could transfer Ti(IV) to ATP at pH of 5.5 inside the cell by endocytosis. Thus, transferrin acts as mediator for titanium delivery into tumor cells [12]. Cardona et. al. worked with the interaction between human apo-transferrin and titanocene, and titanocene-aminoacid complexes. The study suggests that $Ti^{(IV)}$ was loaded into the iron binding sites of the protein, regardless of the ancillary ligands (aminoacid or chlorides). The studies were supported with analytical methodologies that includes NMR, UV-VIS and ICP-AES [26].

More recent studies demonstrated that transferrin-enriched environment does not improved the cytotoxic behavior of some titanium complexes [1]. Hernández et. al. investigated the cytotoxic properties of Ti(IV) complexes on HT-29 cells using MTT assay, and established that it is highly probable that transferrin has only a stabilizing role in a hydrolytically hostile environment such as plasma [1]. Interestingly, the three titanocene-aminoacid complexes evaluated transferred the $Ti^{(IV)}$ to the transferrin [1]. Studies showed that transferrin also binds vanadocene. Nishida et. al. investigated the uptake of $V^{(IV)}$ ion of vanadocene dichloride by apo-tranferrin using ESR spectroscopy.

The ESR does not showed signals corresponding to the free vanadocene dichloride, suggesting that transferrin had an interaction with the complexes. Also albumin was tested using the same methodology and showed similar results [27].

Vera et. al. studied the interactions of titanocene and molybdenocene with DNA using ICP-AES. They reported an analytical methodology that resembles the cell environment and determined that 90% of titanium was bound to DNA after 46 hours in contrast with less than 5% bound for molybdenum [28]. Also the interactions of molybdenocene with nitrogen bases using spectroscopy and electrochemical methods have been studied, showing that molybdenocene has higher affinity for guanine followed by adenine and pyrimidine [29].

The general review presented here, provided an important guide and motivation to pursue our goals with this investigation. This research was aimed to the rational design of metal complexes with enhanced antitumor activity. The task to find ligand that could act as inert molecules and can stabilize the active species is a conclusive step. Maltol was selected as ligand. This molecule is an approved food additive and a naturally occurring compound. The most important characteristic of maltol is it biologically friendly and non-toxic molecule.

2. Materials and Methods

2.1 Complex Synthesis:

The Schlenk line method or vacuum gas manifold method was developed by Wilhelm Schlenk [20]. Figure A shows the Schlenk line used to perform the complexes syntheses. The system described in this method consists of a dual manifold with several ports. One manifold is connected to a source of purified inert gas, while the other is connected to a high vacuum pump. An oil gas bubbler is used to vent the inert gas line. Usually a liquid nitrogen trap is used to avoid the pump contamination with solvent vapors and/or gaseous reaction products. Since Schlenk lines are useful for manipulating air sensitive compounds, we selected this method to perform our syntheses which must be done in an oxygen free environment.

2.1.1 Synthesis of Titanium-Maltol complex

The reaction was carried out in an oxygen free environment, using a blanket of pre-purified nitrogen. In order to obtain this condition, 80.0-100.0 mL of double distilled and deionized water was boiled for fifteen to twenty minutes and placed in a 100.0 mL three neck flask and purged with nitrogen. The reagents Cp_2TiCl_2 and maltol were obtained from Aldrich and Sigma-Aldrich, respectively. Both reagents were used just as received. 0.200 g ($1.586x10^{-3}$ mol) of maltol and 0.200 g ($8.033x10^{-4}$ mol) of Cp_2TiCl_2 were deoxygenated with nitrogen for approximately five minutes, prior to mixing in water. The reagents amounts could varied slightly between samples, but the quantities were adjusted always to obtained a maltol:titanocene ratio of 2:1. The reagents were

added to the deoxygenated water and sodium hydroxide (NaOH) was used to adjust the pH at approximately 5.5. A continuum stirring of the mixture was required for up to 24 hours to ensure the maximum reaction yield. The reaction mixture changes in color (red to yellow) in the first ten to fifteen minutes, indicating the synthesis progression. The reaction was ended when a yellow precipitated was observed, approximately 24 after its initiation. The solution was filtered using a microfiltration kit. Several washes with purified water followed by microfiltrations were performed to eliminate as much contaminants and un-reacted reagents as possible. Finally a thin yellow powder-like solid was obtained (80%yield). The collected material was dried in vacuo and placed in a sealed bottle. To avoid the sample from absorbed water from the environment, the sample was stored in a dehumidifier.

2.1.2 Synthesis of Vanadium-Maltol complex

The reaction took placed in an oxygen free environment. In order to obtain this condition 80.0-100.0 mL of double distilled and deionized water was boiled for 15-20 minutes and placed in a 100.0 mL three neck flask and purged with nitrogen. The reagents Cp_2VCl_2 and maltol were obtained from Aldrich and Sigma-Aldrich, respectively. Both reagents were used just as received. 0.200g ($1.586x10^{-3}$ mol) of maltol and 0.200g ($7.935x10^{-4}$mol) of Cp_2VCl_2 were also purged with nitrogen for approximately five minutes. The reagents amounts could varied slightly between samples, but the quantities were adjusted always to obtained a maltol:vanadocene ratio of 2:1. The reagents were added to the deoxygenated water in the “three neck flask”. Approximately 1.0-1.5 mL of NaOH 1M (one equivalent per maltol) was added to the

synthesis. A continuum stirring of the mixture was required for up to 24 hours to ensure the maximum reaction yield. Similar to the synthesis described above, in the first 10-25 minutes the reaction mixture changed in color from green to dark brown, indicating the synthesis progression. The reaction was terminated 24 hours after it was initiated, following the same procedure as with the titanocene-maltol synthesis. The formation of dark-brown solution and the lack of any reagent residue in the bottom of the flask were indicatives of an adequate synthesis evolution. The solution was placed in a rotovapor (Büchi Rotavapor R-200 with a Büchi Heating Bath B-490) to eliminate as much water as possible. A brown granular solid was obtained. The collected material was exposed to continuum vacuum overnight for further drying and placed in a sealed bottle. To avoid the sample from absorbed water from the environment, the sample was stored in a dehumidifier.

2.2 Biological samples

2.2.1 Buffer preparation

A Tris-HCl was used to dilute most of the biological samples. Usually, one liter of 0.1 M Tris-HCl / 10 mM NaCl / 100 mM $NaHCO_3$ were prepared using distilled and deionized. The buffer was prepared by dissolving 15.76g of Tris-HCl, 0.5840g of sodium chloride and 8.401g of sodium bicarbonate in distilled/deionized water in a 1.0 L volumetric flask. Constant stirring was required to ensure the whole solid dilution. NaOH 1.0 M was used to adjust the solution to physiological pH (~7.5). This buffer was stored at 5 °C.

2.2.2 Apo-transferrin solutions

Apo-transferrin was purchased from Sigma Aldrich. Further purification of the product was performed. It was washed several times with a solution of KCl (0.10 M) and centrifuged to eliminate impurities with centricon microfilters (Millipore Company). Approximately 9.0 mg of filtered apo-transferrin was dissolved on 5.0 mL Tris-HCl buffer solution to make a concentrated (stock) solution.

A dilution of stock apo-protein solution was required to perform further experiments. The solution concentration obtained was approximately 1.0 X 10^{-5} M. The concentration of the protein was determined using the Beer-Lambert equation ($A=\varepsilon bC$) where A is the absorbance at certain wavelength, b is the width in centimeter of the quartz cell and ε is the absorptivity coefficient. The absorption band at 280 nm was used to performed the calculation which has a $\varepsilon = 93{,}000\ M^{-1}\ cm^{-1}$ and the cell width was 1 cm.

2.2.3. Oligonucleotides/DNA Binding Studies

Two types of molecules were selected to perform the binding studies. Primarily, an oligonucleotide sequence of CGCATATATGCG was used to determine whether or not the complexes can engage in any type of binding interaction. Secondly, a mixture of calf-thymus DNA/complex was made to obtain further information, more complex binding interactions that cannot be observed in oligonulceotides. The oligonucleotide sequence was obtained from BIOSYNTHESIS Co. A deuterated Tris buffer was made in 2.0 mL of a 9:1 H_2O:D_2O solvent. Approximately, 0.0037 g of oligonucleotides was added to the 2.0 mL buffer. The solution was analyzed immediately by NMR.

Calf-thymus DNA sample was obtained from Sigma. The sample was diluted in 10.0 mL without further purification.

2.3 Complex Structural Analysis

2.3.1 Infrared Spectroscopy

Infrared spectroscopy was performed to determine functional groups on the complex. If the synthesis was successful we expect to be able to identify some of the characteristic vibrations of the starting complex (parent compound) as well as new vibrations for the functional groups incorporated (for vanadium) or the complete replacement of the starting vibrations by a completely new vibration spectrum (for titanium). A FT-IR Spectometer PARAGON 1000 PC (Perkin Elmer) was used to perform this analysis. Data collection was performed using the Spectrum RX (v3.02) software.

To prepare a compressed KBr disk of the sample for the IR analysis, approximately 250.0 mg of dry IR grade KBr are placed in an agate mortar. The solid must be grid to ensure that no solid crystals remain in the sample. Approximately 1.0-2.0 mg of the complex was added to the KBr and grinded until the sample was evenly distributed. A small amount of the sample was used to make the pellet.

The IR spectrophotometer was calibrated with air and then a KBr tablet was analyzed to be used as blank. The KBr-sample disk was placed in the spectrophotometer and 32-64 acquisitions were done depending of the noise observed in the spectra.

2.3.2 Elemental analysis

Following the complex formation determination, a detailed analysis of the elements present in the complex was required. An elemental analysis experiment was done in order to obtain the atomistic description. These kind of analyses are performed by combustion using automatic analyzers, pyrolysis and/or flask combustion followed by ion chromatography. All analysis is percent by weight determinations.

The elemental analysis was performed at the Atlantic Microlab Inc. in Georgia. To perform the elemental analysis 5.0-10.0 mg of the dried complex were send to the laboratories to ensure that the analysis can be properly performed.

2.3.3 Electrochemistry

Since the complex prepared is an organometallic complex it was necessary to determine the redox behaviour of the metal center. The electrochemical characterization was performed in a BAS CV-50W voltammetric analyzer. Dichloromethane was used as solvent. Tetrabutylammonium tetrafluoroborate ($[NBu_4][BF_4]$) was used as supporting electrolyte. This electrolyte was recrystallized from methanol and dried in vacuo prior to use. The experiments were performed with a three electrode configuration: a glassy carbon working electrode, a platinum wire counter electrode (Pt-wire), and a non aqueous silver wire in contact with a ~0.1 M $[NBu_4][BF_4]$ in dichloromethane separated from the bulk solution by a fine glass frit as pseudo–reference electrode. The internal reference was $[Fe(C_5H_5)_2]^{0/+}$ and the potentials were quote relative to SCE. A cleansing of the

work electrode with alumina slurry and deionized water was done previous each experiment.

The samples were prepared by dissolving 0.0033 g of the complex in a 10.0 mL of ~0.1 M $[NBu_4][BF_4]$ in dichloromethane. A sweep between 1.9 and -2.0 mV was performed for each sample.

2.3.4 Nuclear Magnetic Resonance

The complex obtained in titanium-maltol synthesis was highly soluble in DMSO. Since Nuclear Magnetic Resonance (NMR) is a well known technique to elucidate the structure of molecules in solution, it was used as a first approach to determine the structure of the synthesized complex. A 500 MHz (11.74 Tesla magnetic field) Bruker Advance 500 instrument equipped with vibrations absorbing legs, three channels of detection and, two probes (a broadband detection and a QXI-4 inverse detection) was used for the NMR analysis. The equipment has a VT unit controller for temperature control. The acquisition parameters used for the ^{1}H and ^{13}C experiments were 1200-1400 transients and zgpr pulse program, for water suppression. Tetramethylsilane (TMS) and 3-(trimethylsylil) propanesulfonic acid (DSS) were used as internal references.

Approximately one milligram of the complex was weighed and dissolved in 1.0 mL of deuterated DMSO-d_6 or D_2O. The approximate solution concentration of titanium-maltol complex was $3.01x10^{-3}$ M.

2.3.5 Electron Paramagnetic Resonance

The complex obtained in vanadium-maltol synthesis was analyzed with Electron Paramagnetic Resonance (EPR). EPR spectra were recorded with an Elexsys E 500 spectrometer with an ER 4122SHQE resonator. Magnetic fields were measured with an E036 TM Teslameter. Solution samples were acquired with ER 106FC-Q flat cells. Simulations were optimized with XSophe version 1.114.

2.3.6 Crystallography

One of the physical properties observed in the synthesized complexes was the formation of an amorphous precipitate in aqueous solutions. Several crystallization techniques were used to obtain complex crystals that diffract, to be able to determine its structure by X-ray Crystallography. Primarily, a saturated solution of the complex was placed in a flask and dried by evaporation using a flow of nitrogen for up to two weeks. During the process small crystals were formed. Some of these crystals were removed and placed in a new saturated solution of the complex. The inclusion of these small crystals to the solution facilitated further crystal formation by means of eliminating the nucleation step which is the limiting step in this process. This procedure is known as seeding and leads to the formation of larger crystals than the evaporation technique.

All the crystals were grown in our facilities; however the crystallographic determination was performed at University of California, San Diego, Department of Chemistry and Biochemitry. An orange plate cut to the 0.30 X 0.20 X 0.20 mm in size was mounted on a cryoloop with Paratone oil. Data was collected in a nitrogen gas

stream at -173^{o}C on a Bruker Smart system. Data collection was 99.6% complete to 25^{o} in θ. The data was integrated using the Bruker SAINT software program. Crystal to detector distance was 60 mm and exposure time was 10 s per frame using a scan width of 0.5 °. The structure was solved by direct methods and all non-hydrogen atoms were refined anisotropically by full-matrix least-squares (SHELXL-97).

2.3.7 Mass Spectroscopy

Although, the determination of the structure of the complexes was performed by X-ray crystallography in the solid state, species present in solution were determined by Mass spectrometry. A high performance ESI-Ion Trap MSn Bruker Daltonics Esquire 6000 system was used to make the mass spectral experiments. The type of ionization used to perform the analysis was the electron spray positive ion. The system was equipped with an Agilent electrospray (ESI) ion source which provides appropriate sensitivity and reproducibility to our experiments.

The analysis was performed with a sample of approximately 0.0010 g of the complex was dissolved in 5.0 mL of a mixture of water:methanol (1:1). The complex solution concentration was 100 ppm.

2.3.8 Ultraviolet and visible spectroscopy

In order to determine if the complexes interact with oligonucleotides and/or DNA molecules an analysis using UV-Vis spectroscopy was performed. The oligonucleotide sequence used was CGCATATATGCG, while the Calf-thymus DNA was selected to performed the DNA interaction experiment. Primarily, a detailed spectral analysis of the oligonucleotides/DNA solution was done, followed by a titration with the complex. To

achieve this task a Perkin Elmer Lambda Bio 20 double beam instrument was used. Primarily, the determination of the biological samples concentration was done followed by carrying out of the titration studies. All the interactions were performed in a 1.0 cm quartz cell. A nitrogen gas system and a temperature controller were connected to the spectrophotometer. Data collection was performing using the Winlab software.

A titration of apotransferrin (1.0×10^{-5} M) was performed to determine the apo-transferrin-metal interaction. The experiment consisted of sequential additions of complex aliquots (~2.0×10^{-4} M) to a transferrin sample until a ratio of 4:1 complex:transferrin was reached. Usually, the titration was performed during a period of 8 hours. Each aliquot was added at 30 minutes intervals. UV-Vis scans were made every 30 minutes. Data was collected at 242, 280, 295 and 321 nm. The pH was measured before and after each complex addition to ensure it remained constant during the whole titration process.

2.3.9 Cytotoxicity

The cytotoxicity experiments were performed using the MTT assay, that is a laboratory test and a standard colorimetric assay by which cell growth is monitored. It is one of the most used tests to determine the cytotoxicity of potential medicinal complexes and other materials of biological relevance. The mechanism of the test goes as follow: yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan in the mitochondria of living cells. The reduction of the complex only takes place if the mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of living cells. A solution is added to dissolve the insoluble purple formazan product into a colored solution. The most common solvents

are dimethyl sulfoxide or an acidified ethanol solution. The absorbance of the solution obtained can be quantified by means of its UV-Vis absorbance. Thus, by measuring the solution absorbance at a certain wavelength with a UV-Vis spectrophotometer it is possible to obtain the product concentration. The absorption maximum is highly dependent on the solvent employed. In this experiment it was required to obtain various cell populations to be exposed to the complexes or to serve as control groups. Minimal modifications were made to the MTT assay to perform the cytotoxicity analysis of the synthesized complexes on the human colon cancer cell line (HT-29). Titanocene dichloride and titanium-maltol complexes were re-tested in concentrations that ranged from 120-2500 μM at 72 hours [1]. A time interval of 96 hours was used to titanocene dichloride due to the longer intracellular activation period. Dose-response curve were constructed to illustrate the results.

3. Results and Discussion

3.1 Synthesis of $Ti(maltolato)_2(OH)_2$ and $V(maltolato)_2Cl_2$ complexes

The synthesis of $Ti(maltolato)_2(OH)_2$, eq. 1, was carried out in an oxygen free environment by reacting Cp_2TiCl_2 with maltol in a 1:2 ratio in water. We used anaerobic conditions in order avoid the formation of TiO_2 and other titanium oxides. Both, Cp_2TiCl_2 and maltol were mixed in an aqueous solution at a pH of 5.5. The reaction mixture changed from red to yellow, indicating that the reaction was completed. The yellow precipitate obtained was slightly soluble in water. Attempts to dissolve

$Ti(maltolato)_2(OH)_2$ in water at pH of 7.4 and above led to the formation of a tetrameric species $[Ti_4(maltolato)_8(\mu\text{-}O_4)]$ as identified by x-ray crystallography.

$$4Cp_2TiCl_2 + 8maltol \xrightarrow[pH\ 5.4]{H_2O} 4Ti(maltolato)_2(OH)_2 \xrightarrow[pH\ 8.4]{} [Ti_4(maltolato)_8(\mu\text{-}O_4)] \quad \text{eq.1}$$

Similar to the synthesis of $Ti(maltolato)_2(OH)_2$, the synthesis of maltol-vanadium was carried out in a nitrogen atmosphere at 2:1 ratio, equation 2. One equivalent of NaOH 1.0M was added to the reaction mixture to deprotonate the maltol molecule. The lack of any residue at the bottom of the flask and the change in color of the solution from green to dark brown were indicative of the reaction completion. A dark brown granular solid was obtained when the liquid of the solution was removed using a rotoevaporation

$$Cp_2VCl_2 + 2\ maltol \xrightarrow{H_2O} V(maltolato)_2(Cl)_2 \qquad \text{eq. 2}$$

In order to investigate in further details the physical and biological properties of these complexes, a solubility test was performed.

3.2 Solubility properties of Ti(IV) complex

One of the important steps in the design of the anticancer complexes is the solubility properties, in particular in aqueous and physiological environments. The purification and formulation phase of the complexes depend of the solubility behavior of the complexes. The principal objective of the investigation is the synthesis of the antitumoral that could be compatible with the physiological conditions. Solubility test results for $Ti(maltolato)_2(OH)_2$ are summarized in **Table 3-1**. In general,

$Ti(maltolato)_2(OH)_2$ complex was soluble in DMSO, methanol and dichloromethane. The solubility in water was partially, however the increasing in pH to 8.4 promote that the complex reach the expected solubility but it undergo tetramerization leading to the formation of $[Ti_4(maltolato)_8(\mu\text{-}O_4)]$. At nearly physiological condition this complex was stable without any notable decomposition in a period of ten days. Solvent like acetonitrile, chloroform and acetone also were tested with unsuccessful results.

Solvent	Soluble	Partially soluble	Insoluble
Dimethyl sulfoxide	X		
Dimethyl formamide		X	
Methanol	X		
Water		X	
Acetonitrile			X
Chloroform			X
Dichloromethane	X		
Acetone			X

Table 3- 1: Solubility test for $Ti(maltolato)_2(OH)_2$ complex

3.3 ^{1}H NMR Characterization

Figure 3-1 shows the ^{1}H NMR spectrum of the cis:trans isomeric mixture of $Ti(maltolato)_2(OH)_2$ complex in a ratio of 4:1 in DMSO-d_6. The spectrum shows two set of doublets at 6.7 ppm and 8.4 ppm corresponding to the H(5) and H(6), also two broad

signals at 6.6 ppm and 8.3 ppm corresponding to the OH protons are evident. The addition of D_2O into the DMSO-d_6 solution produces the disappearance of these broad signals, indicating that these signals belong to exchangeable OH protons **Figure 3-2**. In addition, the spectrum shows two broad signals at 6.2 ppm and 8.0 ppm that belong to the other isomer. To assign signals on the ^1H NMR spectral data, we should consider few structural criteria. For the cis isomers, the limited symmetry of the complex conferred by two chelating maltolate and two mutually cis-hydroxo ligands, makes the metal center chiral. Given the fact that we observe two sets of resonance for the maltolato and hydroxo ligands at different ratios, we can envision the presence of two different isomers with C_2 axis or plane of symmetry. The two possibilities are: a mixture of two cis isomers IA and IB (containing the maltol methyl groups syn and anti to each other) **Figures 3-3, 3-4** or a mixture of cis IA and trans isomers IIA and IIB **Figures 3-5, 3-6.** Extrapolating from the X-ray data (vide infra) and IA should exist in solution. This isomer has a C_2 rotation axis. Therefore, in the ^1H NMR spectrum, in DMSO-d_6, it should have four signals: two doublets corresponding to H-6 and H-5 and two singlets, one from the CH_3 group and one for the OH. Isomer IB lacks of a C_2 rotation axis or plane of symmetry. Thus, two set of H-5 and H-6 signals should be observed (the maltol are not magnetically equivalent) as well as $2CH_3$ and 2OH signals. Since this is not the case, isomer IIA or IIB should be other isomer present in the mixture. The information of the isomers was corroborated lately by the use of ESI-MS spectroscopy and x-Ray diffraction techniques.

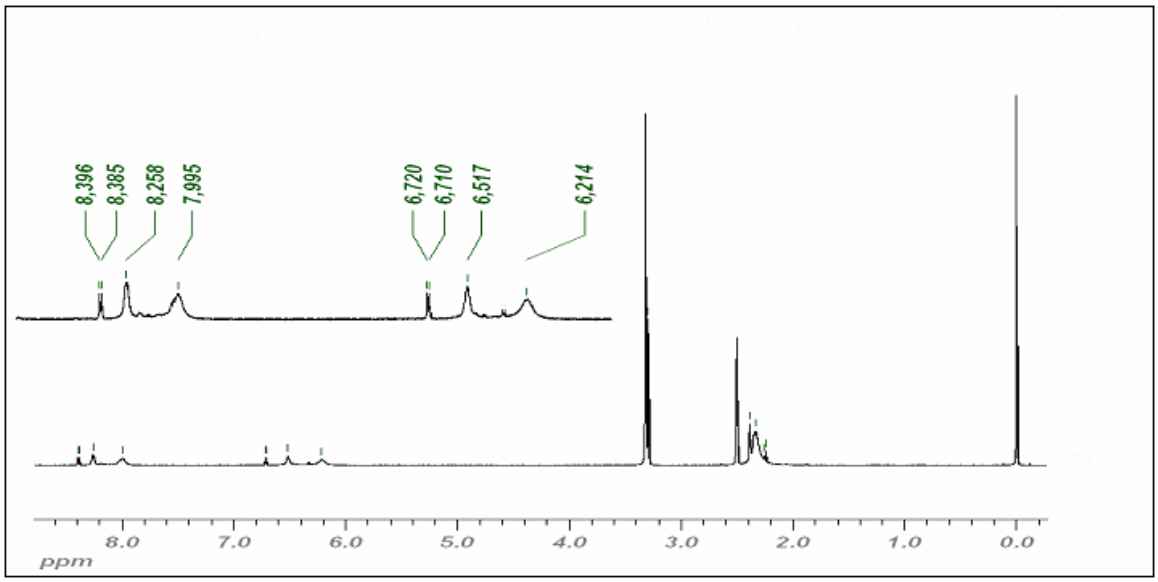


Figure 3-1: The 500-MHz 1H NMR spectrum of Ti(maltolato)$_2$(OH)$_2$ in DMSO-d$_6$ at room temperature

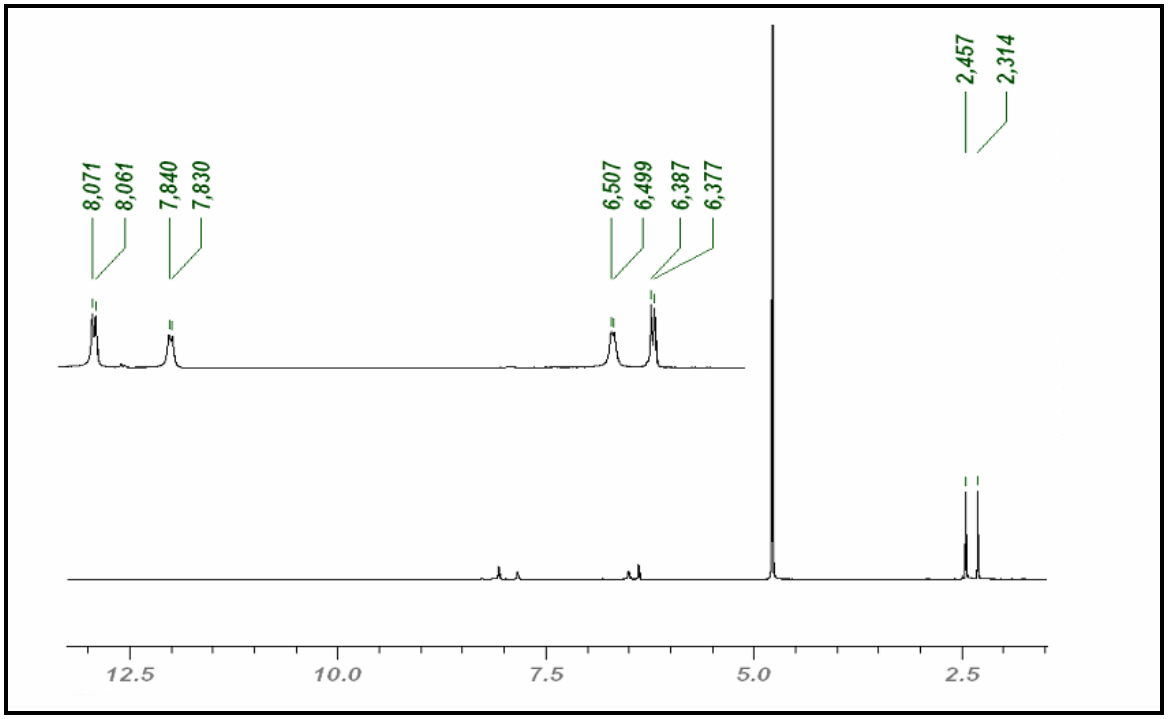


Figure 3-2: The 500-MHz 1H NMR spectrum of Ti(maltolato)$_2$(OH)$_2$ in DMSO-d$_6$ after the addition of D$_2$O to the sample solution at room temperature

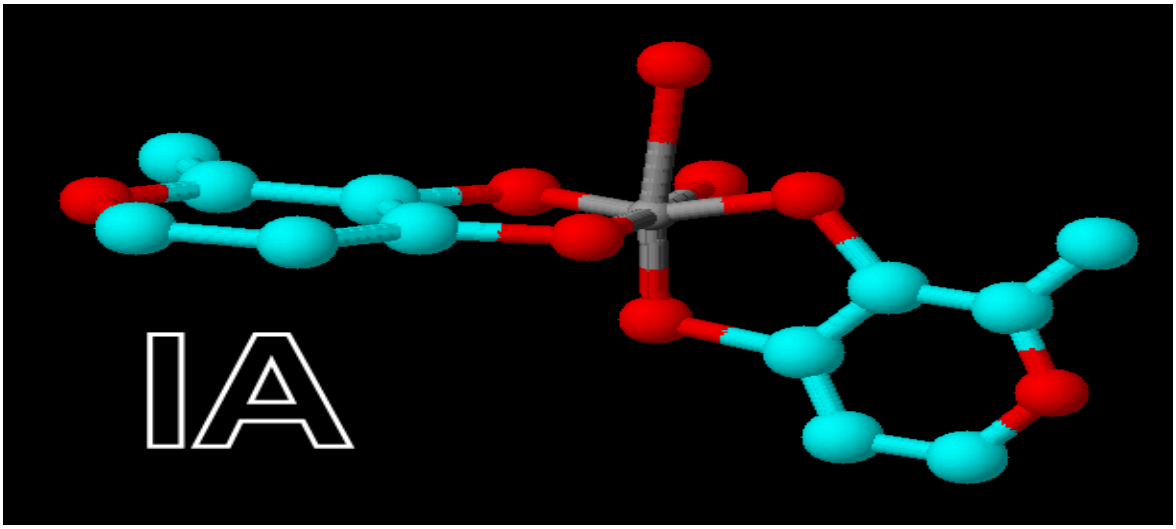


Figure 3-3: Cis-Ti(maltolato)$_2$(OH)$_2$ diastereisomer IA

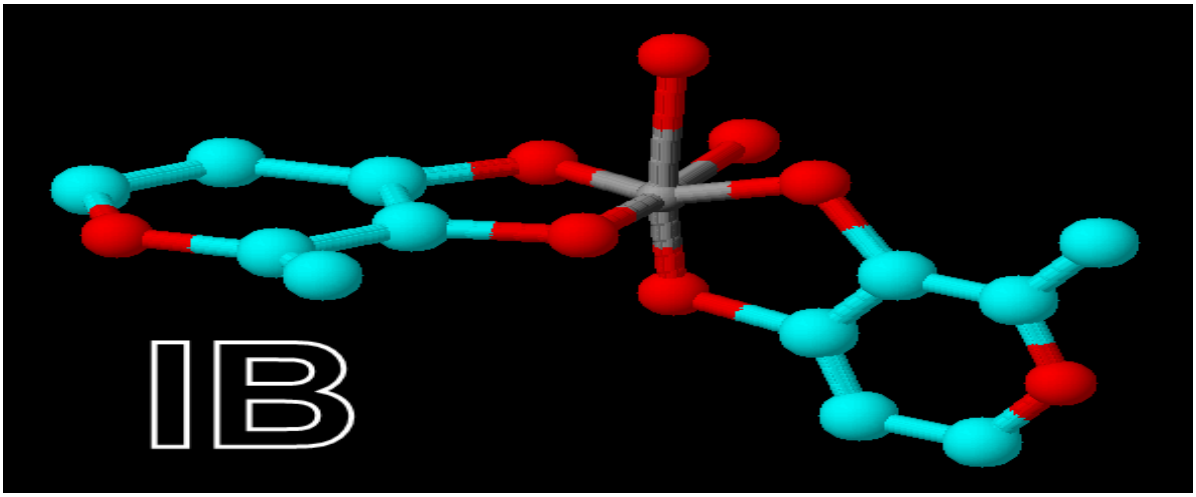


Figure 3-4: Cis-Ti(maltolato)$_2$(OH)$_2$ diastereisomer IB

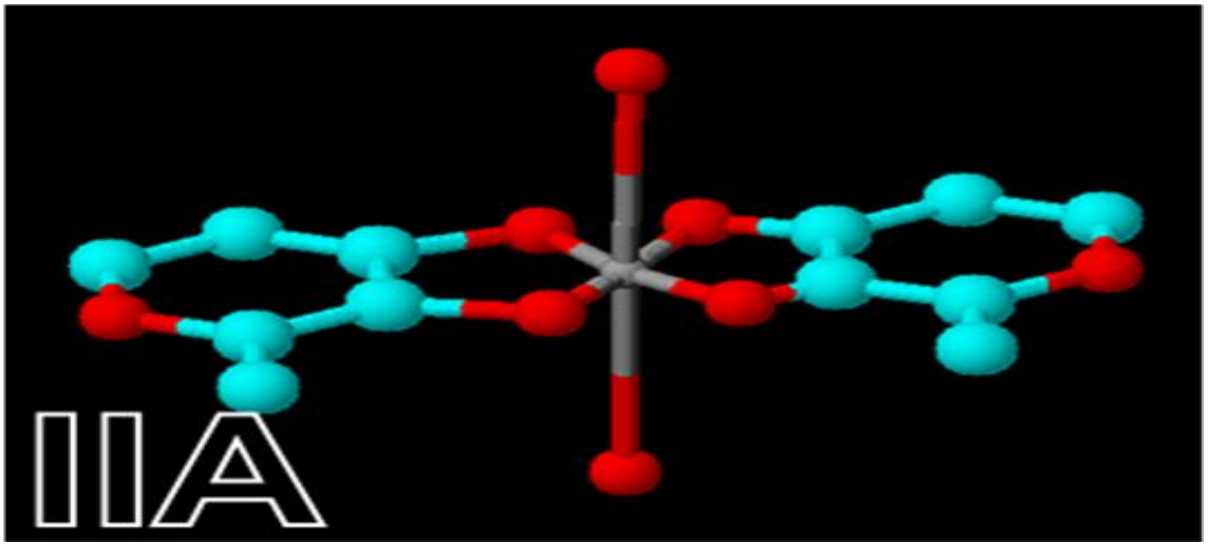


Figure 3-5: Trans-dihydroxobis(maltolato)titanium (IV) diasteisomer IIA

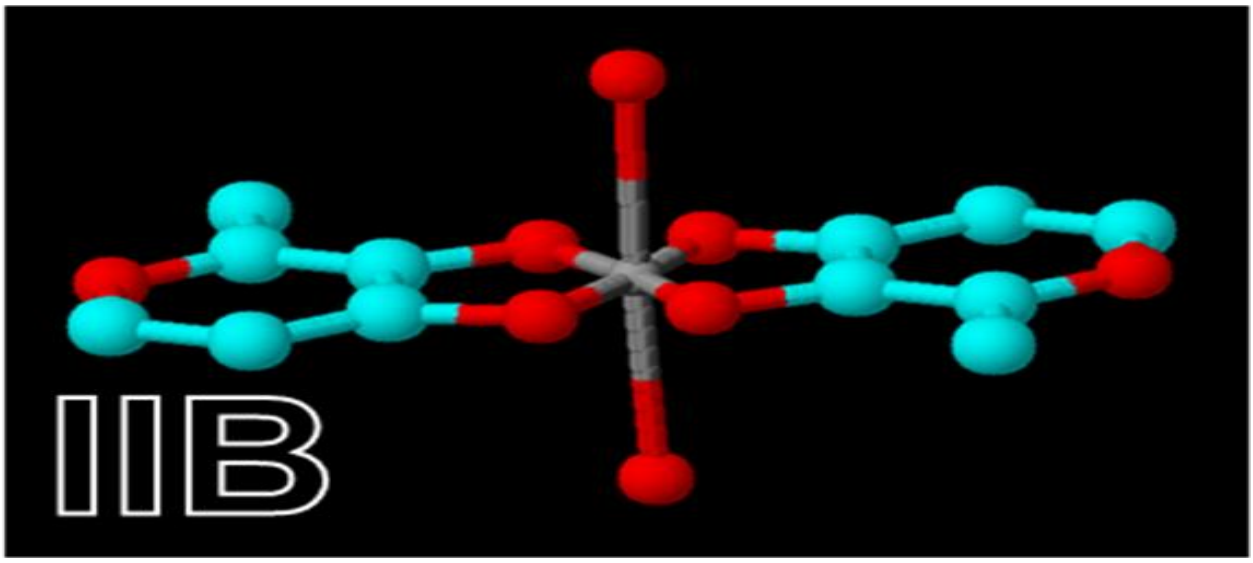


Figure 3-6: Trans-dihydroxobis(maltolato)titanium (IV) diasteisomer IIB

3.4 IR Characterization results

IR spectrum for Ti(maltolato)$_2$(OH)$_2$ shows four bands in the region of 1400-1620 cm^{-1}. The band at 1660 cm^{-1} assigned to υ(C=O) in free maltol is shifted to 1620 cm^{-1}

while the combination bands of the υ(C=O) and υ(C=C) vibration modes at 1626 cm^{-1} and 1565 cm^{-1} are shifted to 1585 cm^{-1} and 1518 cm^{-1}. Also, a broad band at 3396 cm^{-1} is attributed to the OH stretching.

3.5 Elemental Analysis results

In order to determine sample composition, analytical pure samples of Ti-maltolato were sent for elemental analysis to Atlantic Microlab. This assay corroborates the proposed formula for titanium complex. The experimental data (**Table 3-2**) showed that the complex has two maltol molecules and two OH groups per titanium.

Compound	Element	Theory (%)	Found (%)	
$Ti(maltolato)_2(OH)_2$	C	43.40	42.98	42.89
	H	3.64	3.60	3.59

Table 3- 2: Percent of composition of $Ti(maltolato)_2(OH)_2$

3.6 MS Characterization

Electrospray ionization mass spectrometry **(**ESI-MS) was used to confirm the proposed formula for titanium-maltolato complex. Using a mixture of H_2O/Methanol we were able to identify the predominant species in solution as $[Ti(maltolato)_2(OH)_2]\text{-}H]^+$ with a parent peak at 329 *m/z* (**Figure 3-7)**. Further fragmentation of this peak, using ion trap methodology, shows the loss of the OH ligand, **Figure 3-8.** The MS data combined

with elemental analysis provide evidence that the predominant species at low pH is monomeric.

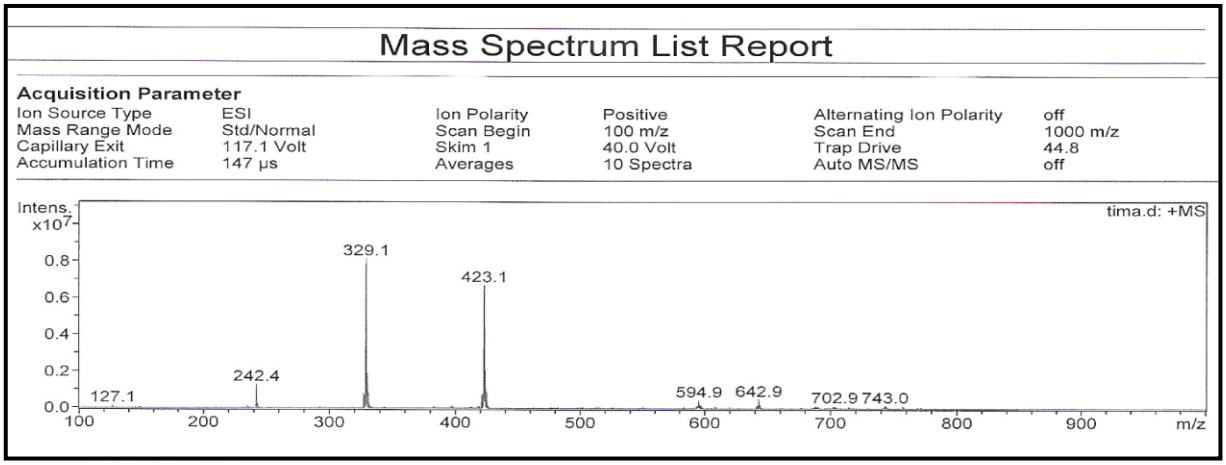


Figure 3- 7: MS spectrum for Ti(maltolato)$_2$(OH)$_2$

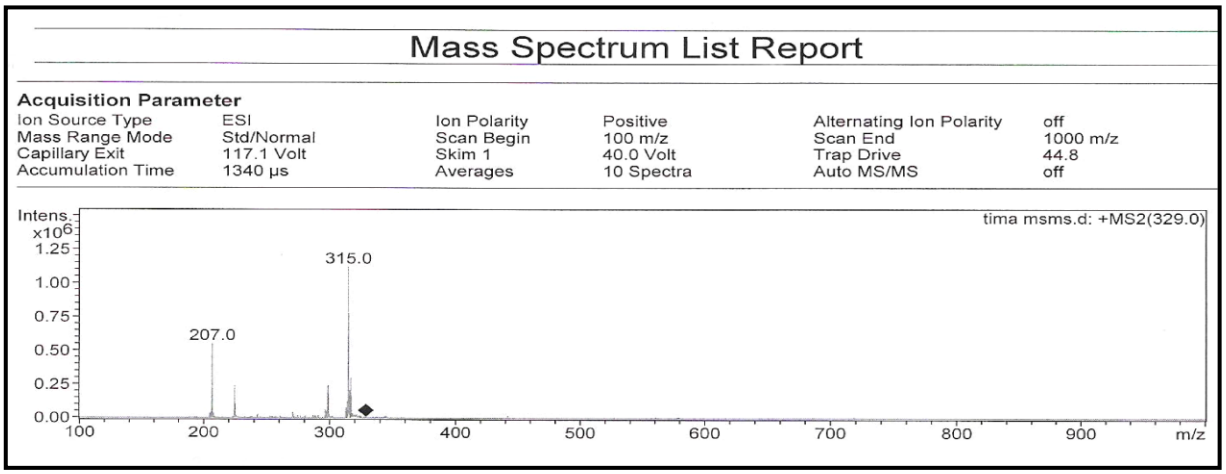


Figure 3- 8: MS spectrum for the fragmentation of the parent peak 329 m/z

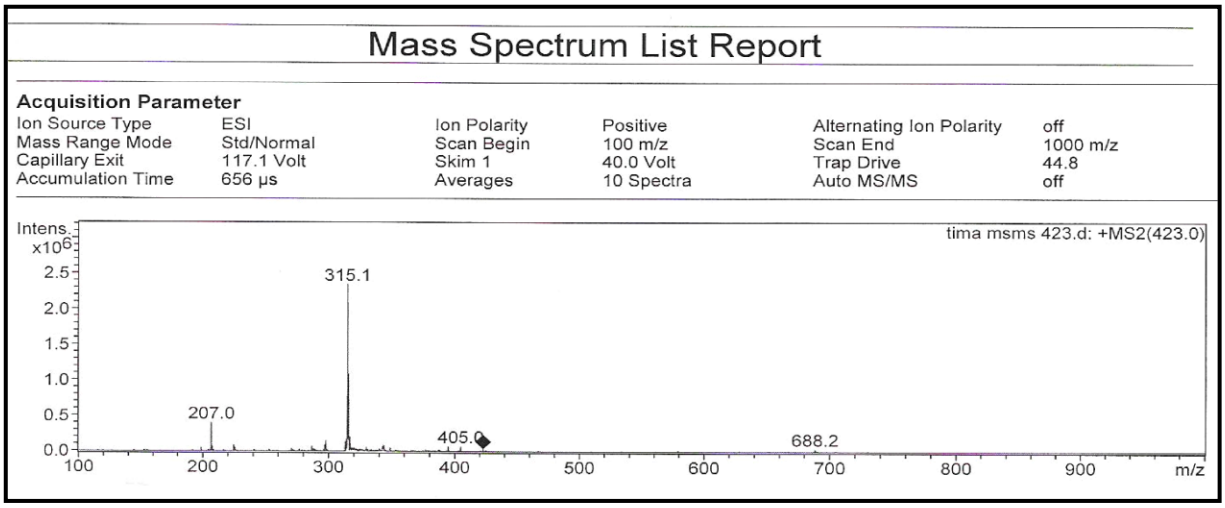


Figure 3- 9: MS spectrum for the fragmentation of the 423 m/z peak

3.7 Electrochemical Characterization

Electrochemical characterization of Ti(maltolato)$_2$(OH)$_2$ was performed using cyclic voltammetry. The electrochemical behavior of the complex in aqueous solution is a critical step in the characterization of anticancer metal-based drugs. Many metal-based drugs, under physiological conditions, might get involved in redox processes that cause damage to the cells. However, due to its low aqueous solubility, the cyclic voltammetry (CV) experiments were performed in CH_2Cl_2. In CH_2Cl_2/[NBu_4][BF_4], Ti(maltolato)$_2$(OH)$_2$ showed an irreversible cathodic wave potential of ($E_{red.}$) -1.36 V (**Figure 3-10**), suggesting irreversible reduction of Ti(IV)-Ti(III) while no anodic wave was observed up to the solvent-electrolyte discharge potential. As point of comparison, other Ti(IV)-maltol and Ti(IV)-guaiacol complexes with different nuclearity have been characterized by CV [30]. If we compare Ti(maltolato)$_2$(OH)$_2$ reduction potential with Ti(maltolato)$_2$(OEt)$_2$ (-1.0 V) and Ti(maltolato)$_2$(Cl)$_2$ (-1.55 V), it is clear that our subject complex resembles to their potentials whereas tetrameric species such as

$[Ti_4(guaiacolate)_8(\mu\text{-}O_4)]$ has a reduction potential of -1.94 V. Thus, this corroborates that $Ti(maltolato)_2(OH)_2$ is the species isolated at low pH. Attempts to characterize $[Ti_4(maltolato)_8(\mu\text{-}O_4)]$ failed since it is electrochemically inactive.

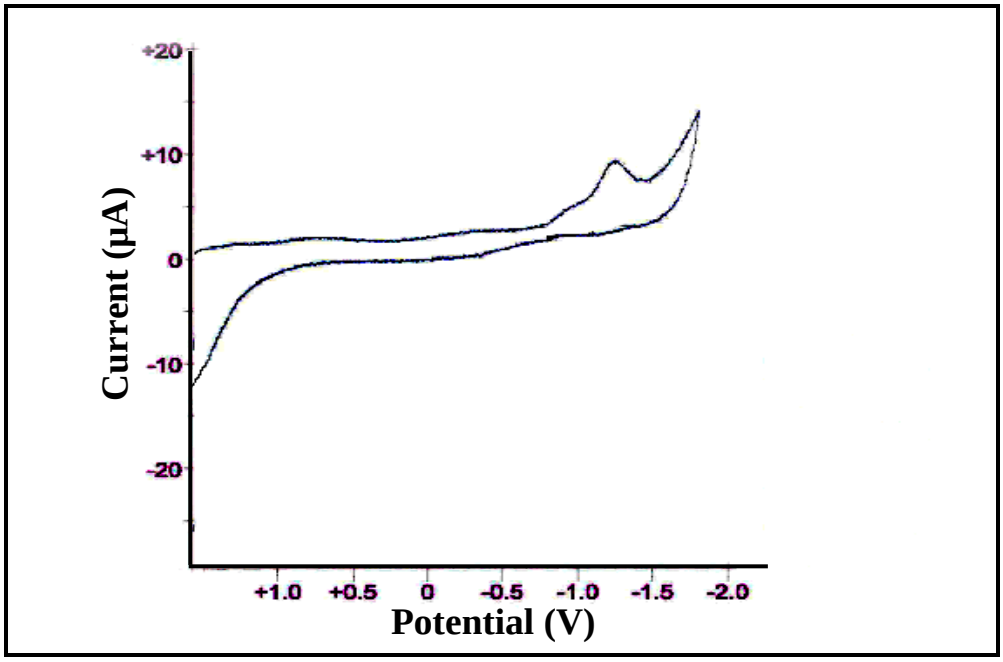


Figure 3-10: Voltammogram of the $Ti(maltolato)_2(OH)_2$ complex

3.8 X-Ray characterization of $[Ti_4(maltolato)_8(\mu\text{-}O_4)]$

X-Ray diffraction method is one of the most important methods, combined with NMR spectroscopy to characterize a metal complex. The method consists in the determination of the arrangement of atoms within a crystal, in which a beam of X-rays strike the crystal and produce a specific pattern. This specific pattern was collected and a three dimensional picture could be produced.

The formation of the amorphous material in the synthesis flask leads us to investigate different crystallization methods, in order to grow a crystal that could diffract. The evaporation method was the first attempt and small crystals were formed. The uses of seeding technique permit us to grow well shaped crystals and an orange plate of 0.30 x 0.20 x 0.20 mm in size were obtained, a suitable size to elucidate the structure. The complex has a tetrameric conformation with two maltolato molecules per titanium atom. The Ti-O(deprotonated) bonds (Ti-O(5) and Ti-O(2)) are trans to each other, with bond distances (Ti–O(5) 1.9583(17)Å and Ti–O(2) 1.9706(17)Å) substantially shorter than the Ti–O(ketonic) bonds (Ti–O(1) 2.1797(17)Å and Ti–O(4) 2.1495(17)Å). This demonstrates the greater coordination and donating ability of the hydroxyl oxygens compared to ketonic oxygens. Ti–O(oxo) bonds are almost identical, 1.799(16) and 1.8050(16)Å. The chelating angles (O(4)–Ti(1)–O(5), 76.75(7)˚ and O(1)–Ti(1)–O(2), 76.64(6)˚) are smaller than 90˚, reflecting the strain created by the five member ring chelate wheras the cis-O(25)–Ti(1)–O(28) angle (100.18(7)˚) shows a deviation of 10˚ from the octahedral angles. In terms of the maltolato ligand, there is a small lengthening in the C=O(ketone) bonds (C(1)–O(10) 1.266(3)Å and C(7)–O(4) 1.276(3)Å) compared to free maltol, 1.248–1.254Å [31]. Within the maltol ring, carbon–carbon bond distances ranged from 1.328 to 1.436Å. If we consider a typical single carbon–carbon bond with length of 1.54Å and a double bond with 1.32Å, it is evident that there is some degree of delocalization of the double bonds in the maltol ring. **Figure 3-11** shows the solid state structure of $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$.

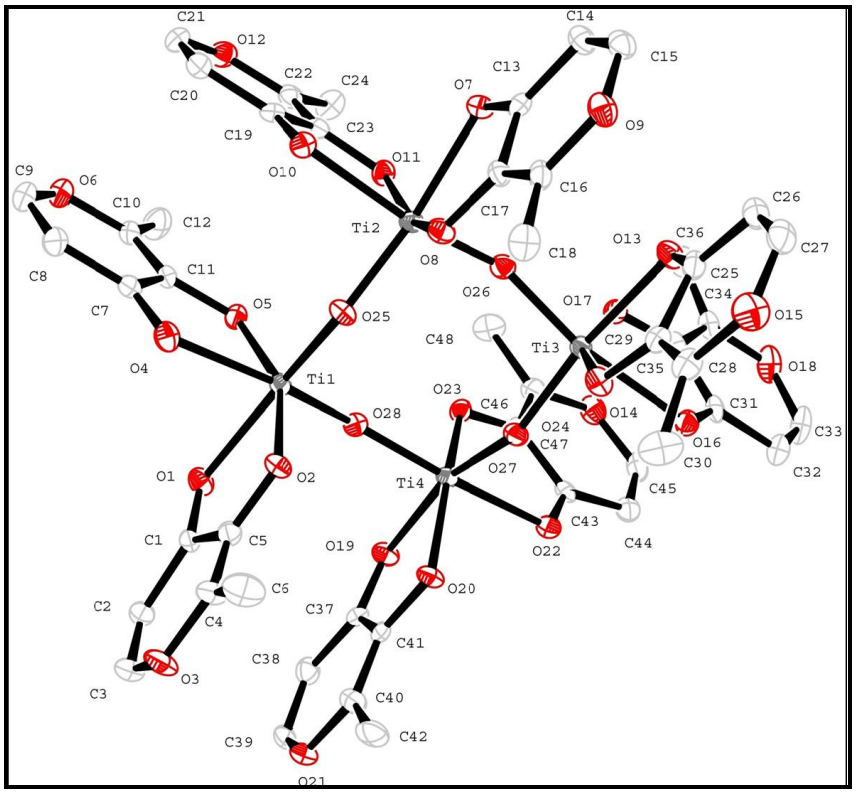


Figure 3-11: Solid state structure of $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$

4. Interaction studies

4.1 UV-Vis interaction results

The interaction of new metal complexes with biologically important molecules is of fundamental importance to understand the mechanism of action at molecular levels. Three biologically important molecules were studied: human serum transferrin, calf thymus DNA and an oligonucleotide of sequence CGCATATATGCG. The selection of these molecules was based on the fact that up to day the proposed mechanisms suggest the transport of the metal complex by carrier protein (transferrin) and coordination with DNA as probable route to reach and kill the cancer cells [32]. The use of the oligonucleotide allows us to study possible interactions within the DNA since we know the composition of the oligonucleotide.

Titrimetric analyses were performed adding aliquots of the complex solution to the biological molecules solution, and the UV-Vis spectra were recorded after each addition.

Figure 4-1 show the UV-Vis differential spectra of DNA titration with $Ti(maltolato)_2(OH)_2$. The spectrum does not show an isosbestic point which means that the free and bound species do not coexist in equilibrium and it is an evidence of no Ti-DNA interaction. This behavior of $Ti(maltolato)_2(OH)_2$ suggests that the theory of coordination of Ti(IV) to DNA bases is not the principal target within the cancer cells. Similar result was obtained for the titration of the oligonucleotide with the titanium complex.

Human serum transferrin has been extensively implicated in the transport of the metal other than iron (III) to the cancer cells [33]. The interaction assays using transferrin was accomplished and the absorbance vs. [Ti/TR] ratio at 321 nm was plot. Band at 321 nm is attributed to ligand to metal charge transfer (LMCT). **Figure 4-2** show the plot of the titration of transferrin with $Ti(maltolato)_2(OH)_2$ complex. The behavior of the plot suggest that there is no interaction of the $Ti(maltolato)_2(OH)_2$ with the protein, at least at the conditions tested. The linearity of the plot shows that the complex does not load the two lobes of the protein. In addition, bands at 240 and 295 nm that are attributed to the coordination of the metal ion to the tyrosine residue were not observed. All of these factors suggest that the transferrin as carrier protein theory is not a possible route of $Ti(maltolato)_2(OH)_2$ to reach the cell.

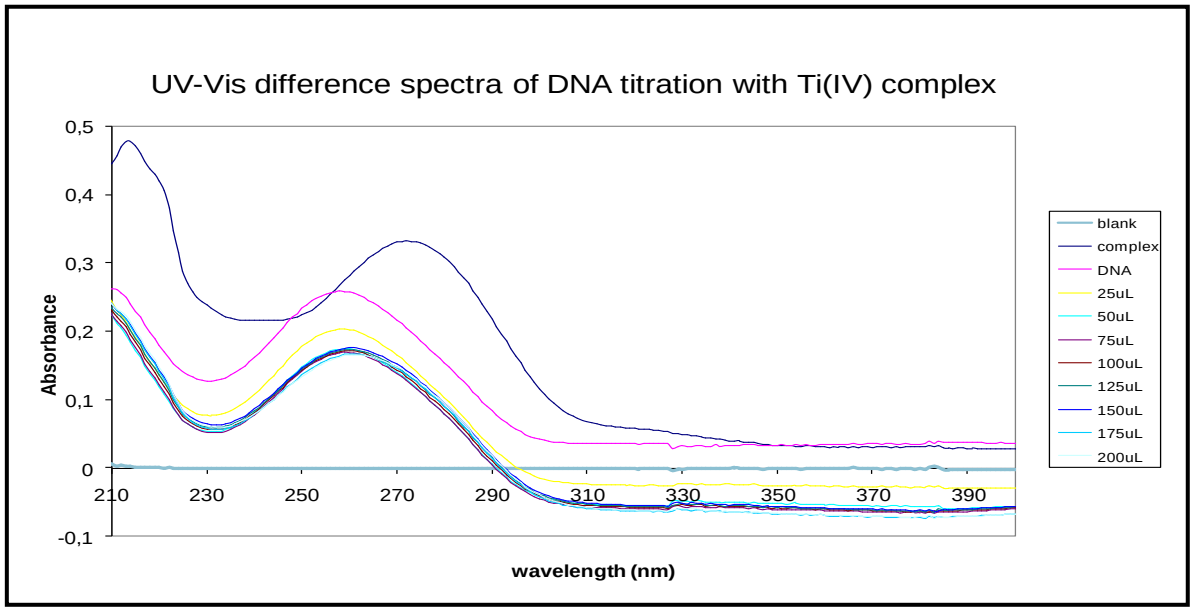


Figure 4-1: UV-Vis difference spectra of DNA titration with Ti(IV) complex in 0.1 M Tris-HCl / 10 mM NaCl / 100 mM NaHCO buffer solution

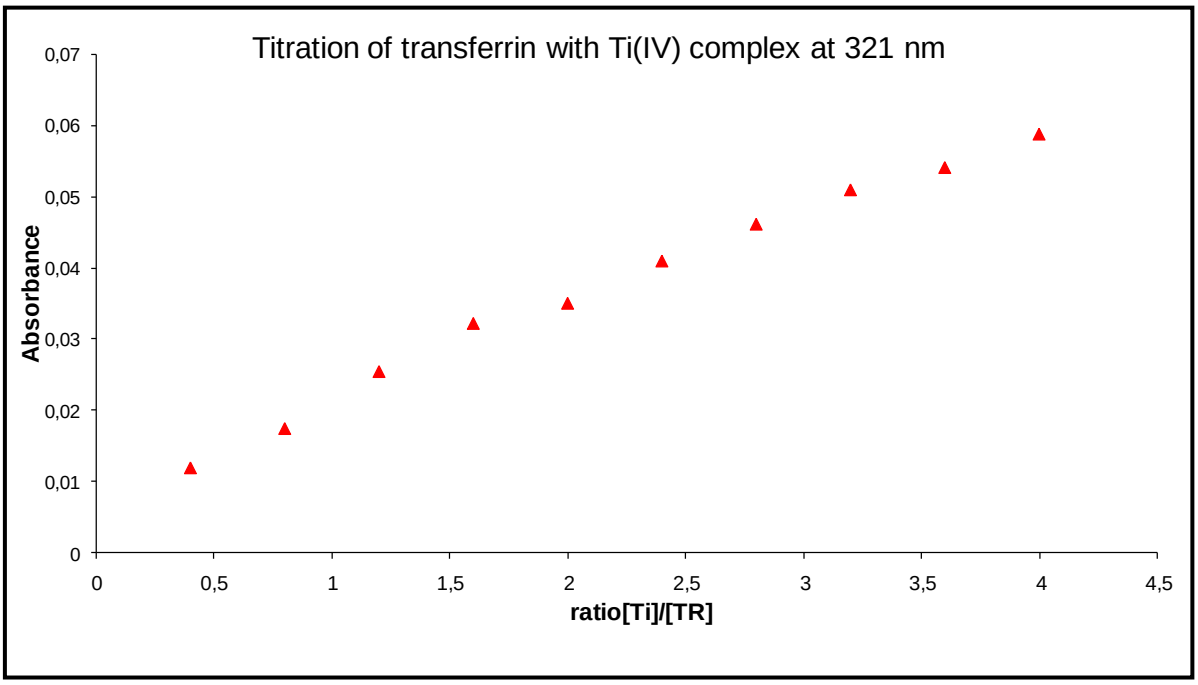


Figure 4-2: Titration of transferrin with Ti(maltolato)$_2$(OH)$_2$ complex in 0.1 M Tris-HCl / 10 mM NaCl / 100 mM NaHCO buffer solution

4.2 Citotoxic studies

4.2.1 HT-29 cells line

The cytototoxic studies were performed using MTT assay in which the reagent yellow MTT was reduced into the mitochondria living cells to formazan (purple in color) [1]. This conversion was directly related to the living cells. The assay was performed at the Ponce School of Medicine by former graduate student R. Hernández. HT-29 colon cancer cells line was used and the [Ti$_4$(maltolato)$_8$(μ-O)$_4$] complex was compared to other eight complexes including titanocene dichloride [1]. **Table 4-1** shows the IC$_{50}$ for the tested complexes. This comprehensive studied also included cytotoxic activity in

tranferrin enriched environment (in the medium) to investigate the role of transferrin in the cytotoxic pattern of the complexes.

The $Ti_4(maltolato)_8(\mu\text{-}O)_4$ complex shows better values of IC_{50} than the other eight titanium complex for all the period of times tested; its higher activity was at 96 hours, showing an IC_{50} value of 0.15 mM, followed by 0.24 mM at 120 hours and 0.28 mM at 72 hours. It is important to note that $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$ is the only titanium complex that retain activity at 120 hours, perhaps as result of its greater hydrolytic stability in aqueous solution. As mentioned previously, $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$ complex is stable at pH of 8.4 for unlimited time, an important property in the design of the anticancer drugs. The presence of transferrin does not alter the cytotoxic activity of $Ti_4(maltolato)_8(\mu\text{-}O)_4$ complex, which means that transferrin does not take part of the mechanism of transport to reach the cancer cells. That is an expected characteristic because the complex was not being able to donate the titanium to the tranferrin as shown the UV-Vis transferrin titration.

Complex	**IC_{50} (x 10^{-4}M)**			
	72h	**96h (w/o Tr)**	**96h (w/Tr)**	**120h**
$Ti_4(maltolato)_8(\mu\text{-}O)_4$	2.8(1)	1.5(1)	1.7(1)	2.4(1)
Cp_2TiCl_2	4.5(3)	4.2(3)	4.1(5)	--
$(Cp\text{-}COOEt)_2TiCl_2$	5.8(4)	5.3(3)	--	--
$(Cp)(Cp\text{-}COOEt)TiCl_2$	6.3(2)	3.0(3)	--	--
$(Cp\text{-}COOMe)_2TiCl_2$	Na	Na	Na	--
$(Cp)(Cp\text{-}COOMe)_2TiCl_2$	Na	Na	Na	--
$[Cp_2Ti(L\text{-}cysteine)_2]Cl_2$	Na	Na	Na	--
$[Cp_2Ti(L\text{-}methionine)_2]Cl_2$	Na	Na	Na	--
$[Cp_2Ti(D\text{-}penicillamine)_2]Cl_2$	Na	Na	Na	--

Table 4-1: Cytotoxicities of the complexes on HT-29 colon cancer cell line

4.2.2 Caco-2 cells line

Similar to the assay with the HT-29 cells lines, the $Ti_4(maltolato)_8(\mu\text{-}O)_4$ complex has been tested with another cell line. The cytotoxic assays were performed in collaboration with the Department of Chemical Engineering of the University of Puerto Rico at Mayaguez. **Table 4-2** shows the IC_{50} values for the tested complexes. Caco-2 cells were used to perform the analysis. These cells are human colon carcinomas cells that are able to express differentiation features of matures intestinal cells.

$Ti_4(maltolato)_8(\mu\text{-}O)_4$, titanocene dichloride and three titanium-aminoacid complex were tested. The cytotoxicity studies were performed at 24 and 72 hour of time. At first sight, it is clear that all the complexes showed an enhanced cytotoxic activity at 72 hrs as compared to 24 hrs. Titanocene dichloride and $Ti_4(maltolato)_8(\mu\text{-}O)_4$ showed one order of magnitude more citotoxic than titanium-aminoacid complexes.

Complex	**IC_{50} (mM)**	
	24h	**72h**
Cp_2TiCl_2	6.9(6)	0.109(8)
$[Cp_2Ti(L\text{-cysteine})_2]Cl_2$	2.9(6)	1.7(3)
$[Cp_2Ti(L\text{-methionine})_2]Cl_2$	3.16(1)	1.2(8)
$[Cp_2Ti(D\text{-penicillamine})_2]Cl_2$	3.29(0)	1.193(1)
$Ti_4(maltolato)_8(\mu\text{-}O)_4$	2.50(5)	0.214(5)

Table 4-2: IC_{50} values for the complexes on Caco-2 colon cancer cell line

The Inductive Couple Plasma (ICP) assay performed to determine the amount of cellular titanium uptake was designed to investigate the possible relationship between Ti uptake and cellular toxicity. The three representative complexes used were Cp_2TiCl_2, $[Cp_2Ti(L\text{-cysteine})_2]Cl_2$ and $Ti_4(maltolato)_8(\mu\text{-}O)_4$. **Table 4-3** shows the concentration values obtained for the study and **Figure 4-3** depicts the amount of the Ti(IV) complexes as function of drug exposure time.

The study demonstrated that the Ti uptake quantified for the three titanium complexes do not have correlation to their cytotoxic activities. Interestingly, the complex with the less cyyotoxic activity, $[Cp_2Ti(L\text{-cysteine})_2]Cl_2$, has the highest concentration of titanium in the cells.

Exposure Time	5 hrs	24 hrs	48 hrs	72 hrs
Compound	[pgTi/cell]	[pgTi/cell]	[pgTi/cell]	[pgTi/cell]
$Ti_4(maltolato)_8(\mu\text{-}O)_4$	0.144(2)	0.3(1)	0.11(1)	0.17(1)
Cp_2TiCl_2	0.016(2)	0.032(5)	0.049(2)	0.0667(7)
$[Cp_2Ti(L\text{-cysteine})_2]Cl_2$	0.116(2)	0.203(2)	0.27(2)	0.31(2)

Table 4-3: Concentration values for the Titanium uptake by Caco-2 cells

$Ti_4(maltolato)_8(\mu\text{-}O)_4$ complex behavior in the Ti uptake by Caco-2 cancer cells is one that deserve special attention. Complexes like Cp_2TiCl_2 and $[Cp_2Ti(L\text{-cysteine})_2]Cl_2$ have an increase in the amount of the uptaken Ti to the Caco-2 cells as a function of time. However, $Ti_4(maltolato)_8(\mu\text{-}O)_4$ complex showed an increase at shorter period of time,

from 5 to 24 hrs but a decrease at longer exposure times. This suggests that the interaction of the complex into the cells is weak and the titanium was removed from the cell as a result of the metabolic process.

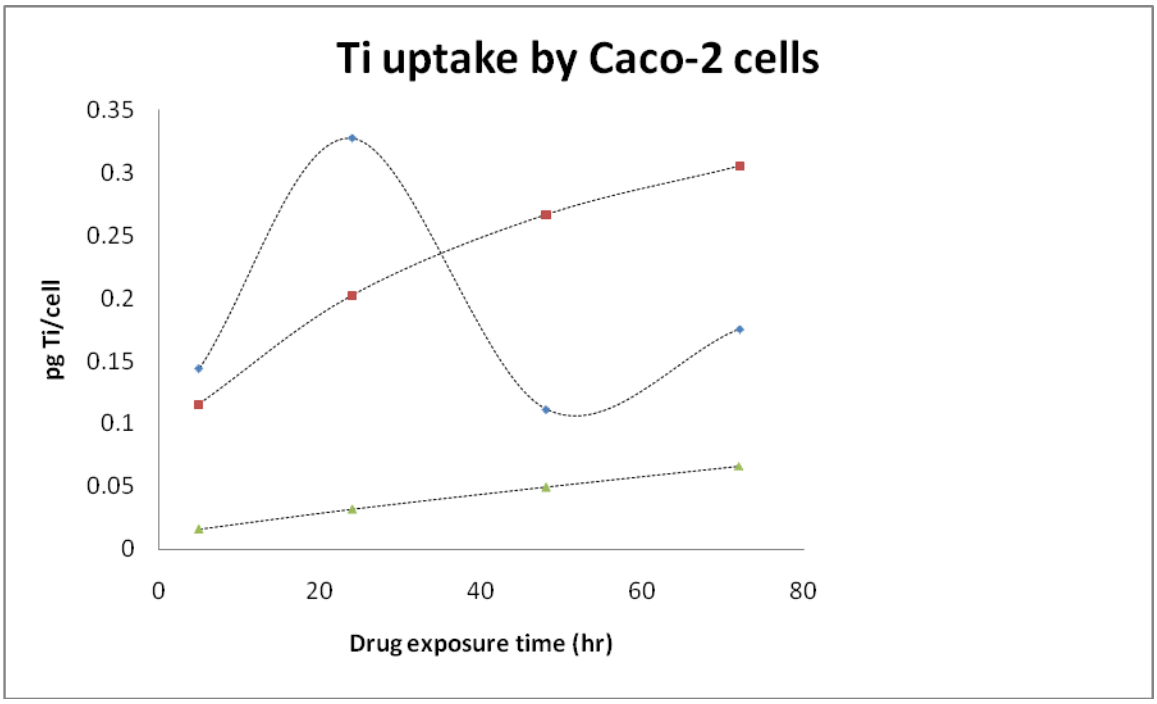


Figure 4-3: Comparison of intracellular titanium uptake by Caco-2 cells. Cp_2TiCl_2 (triangles), $[Cp_2Ti(cysteine)_2]Cl_2$ (squares) and $[Ti_4(maltolato)_8(\mu\text{-}O_4)]$ (diamonds).

The behavior of this complex into the cells would be explained using some knowledge about the structural conformation of the complex. This complex is a tetranuclear species, water stable and is not uptaken by transferrin as other titanium complexes do. The complex is supposed to enter into the cells as tetranuclear species and since its coordination sphere is saturated, it can partially intercalate between DNA bases rather than undergoing DNA coordination. An experiment using ^1H NMR spectroscopy provides evidence about DNA intercalation. In the experiment the addition of the calf thymus DNA to a mix of monomer and tetramer of the complex was performed and the signals of the H-5 and H-6 of the complex were monitored. It was observed that upon the

addition of the calf thymus DNA into the complex solution the maltol signals H-5 and H-6 of the tetrameric species broadened and then collapsed into the baseline, while the monomeric species remained intact. This behavior suggest that the $Ti_4(maltolato)_8(\mu\text{-}O)_4$ complex is intercalating between the DNA base [34].

5. Vanadium complex results

5.1 Solubility properties

In contrast with titanium-maltolato monomeric complex, the vanadium complex solubility was more encouraging. In the final step of the synthesis, the use of rotovapor to remove water as solvent, to obtain the complex, permit us to make an unequivocal prediction about water solubility of the complex, see **Table 5-1.** Furthermore, other solvents were tested in order to obtain additional information. The complex was soluble in water and dichloromethane, solvent like acetone and hexane do not dissolve the vanadium complex and methanol and ethanol partially dissolve the complex. This information could be crucial in the design of the subsequent experiments.

Solvent	Soluble	Partially soluble	Insoluble
Water	X		
Methanol		X	
Dichloromethane	X		
Ethanol		X	
Acetone			X
Hexane			X

Table 5-1: Solubility test for $V(maltolato)_2Cl_2$ complex.

5.2 Electron Paramagnetic Resonance Characterization results

The characterization of complex with paramagnetic metal center is a challenge. Due to the magnetic properties of V(IV) species, d^1 spin system, the use of NMR spectroscopy was ruled out. Electron Paramagnetic Resonance spectroscopy use the interaction of unpaired electrons in the sample with a static magnetic field produced by an electromagnet or a supraconducting magnet to gain insight into the identity, structure and dynamics of the sample under study. **Figure 5-1** shows the EPR spectrum of the V(maltolato)$_2$Cl$_2$ complex. The spectrum was performed at Bruker Co. The EPR spectrum shows 8 lines consistent with V(IV) d^1 spin system. The isotropic coupling constant A_{iso} determined was 114 Gauss. This value differs from the value of the vanadocene dichloride (78 Gauss), confirming the presence of new species.

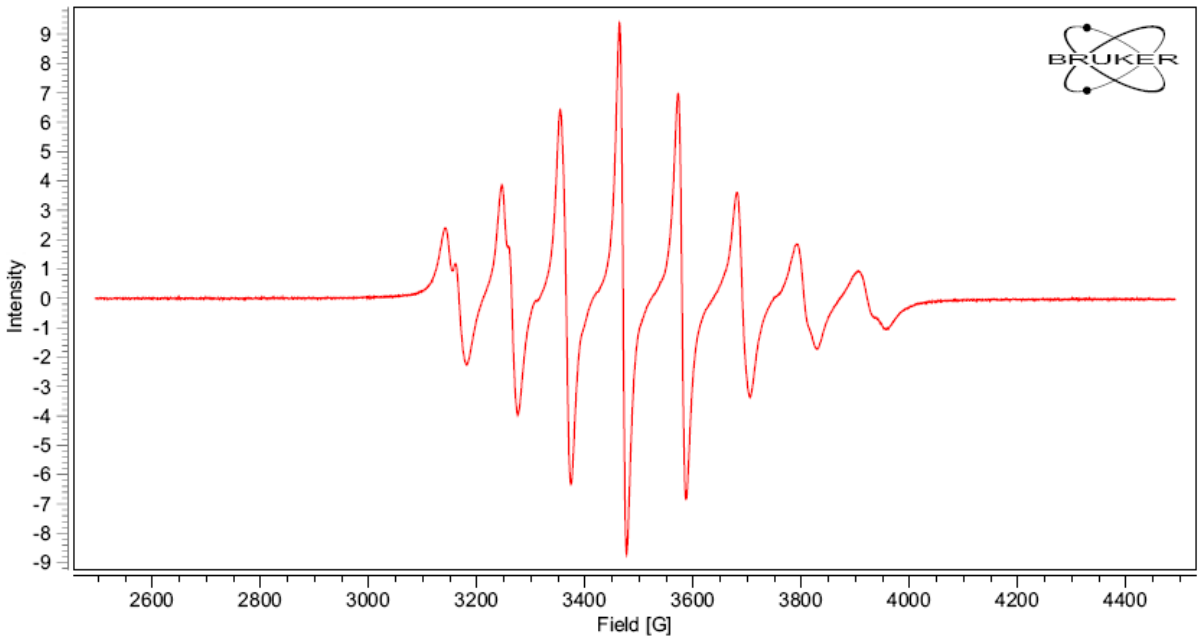


Figure 5-1: EPR spectrum for V(maltolato)$_2$Cl$_2$

5.3 IR characterization

The IR spectrum for $V(maltolato)_2(Cl)_2$ was recorded and showed three predominant bands in the region of 1400-1620 cm^{-1}. These bands are characteristic in the free maltol. The band at 1660 cm^{-1} assigned to υ(C=O) stretching vibration modes is shifted to 1600 cm^{-1} while the combination bands of the υ(C=O) and υ(C=C) vibration modes at 1626 cm^{-1} and 1565 cm^{-1} are shifted to 1551 cm^{-1} and 1474 cm^{-1} respectively. **Figure 5-2** shows the $V(maltolato)_2(Cl)_2$ complex spectra. These shifts in the vibration frequencies corroborate that maltol is coordinated by V(IV).

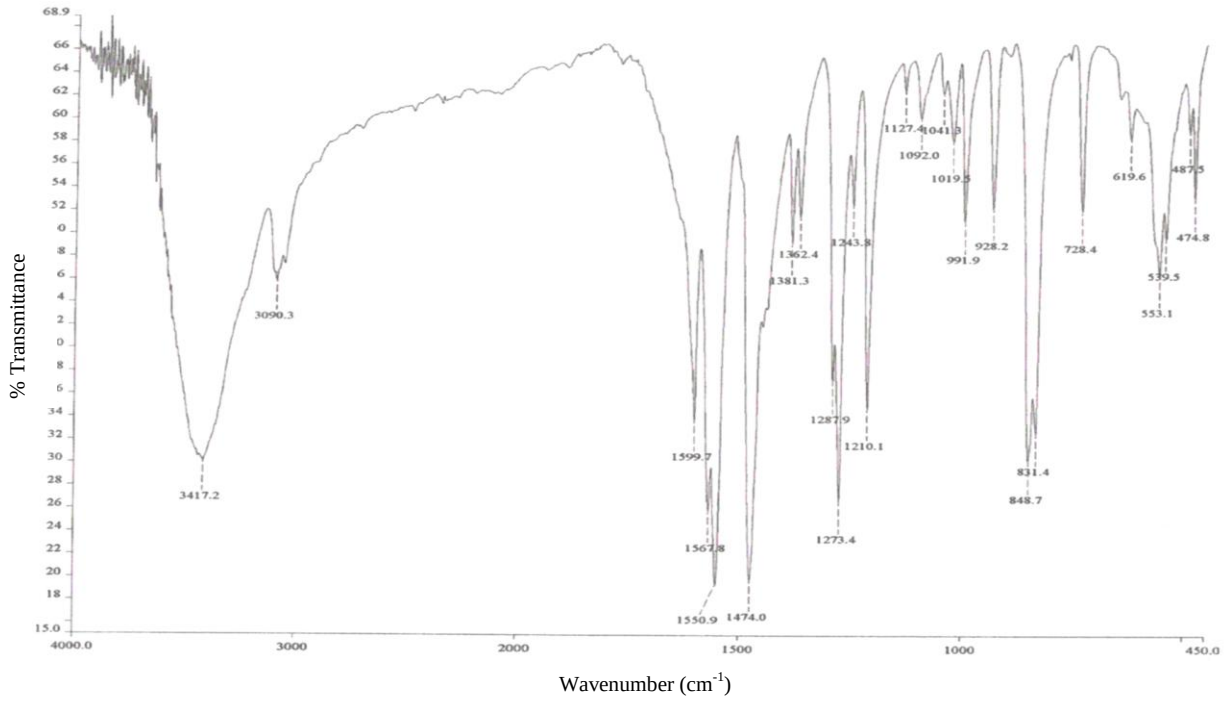


Figure 5-2: Infrared spectra for $V(maltolato)_2(Cl)_2$ complex

5.4 Elemental analysis results

Analytically pure sample of V(maltolato)$_2$(Cl)$_2$ was sent to Atlantic Microlab in order to determine the composition. The calculated values for the complex were 33.83% for the C atoms, and 3.78% for H. The values found were 33.97% and 33.93% for C, 3.43% and 3.45 for H. These values indicate that the complex was a hydrate that contains three molecules of water.

Compound	Element	Theory (%)	Found (%)	
V(maltolato)$_2$(Cl)$_2$·(H$_2$O)$_3$	C	33.83	33.97	33.93
	H	3.78	3.43	3.45

Table 5-2: Percent of composition of V(maltolato)$_2$(Cl)$_2$

5.5 MS characterization results

The Mass Spectroscopy characterization was performed using electrospray positive ion mode. The V(maltolato)$_2$Cl$_2$ complex was dissolved in formic acid. The experimental data shows parent peak at 371.0 *m/z*. This signal corresponds to the formation of the molecular ion [M$^+$] for [V(maltolato)$_2$Cl$_2$]$^+$, that appear when a simple electron was removed from the original molecule. This data corroborates the proposed formula for the complex to be V(maltolato)$_2$(Cl)$_2$. Evidently, the water molecules of the hydrate cannot be determined in this medium. The spectra also shows dominant signals

at 301.0 and 336.0 *m/z*, corresponding to $[V(C_6H_5O_3)_2]^+$ and $[V(C_6H_5O_3)_2Cl]^+$ species respectively.

5.6 UV-Vis interaction results

The UV-Vis titration with transferrin was achieved and the absorbance vs ratio metal/protein was plot. **Figure 5-3** shows the plot of the titration. In contrast with the titanium complex, V(maltolato)$_2$(Cl)$_2$ complex shows a non linear behavior suggesting the existence of an interaction with the protein. The behavior of the plot clearly shows a non progressive increase starting at two equivalent of the metal. This means that the two lobes of the transferrin were loaded with V(IV) species, an important step in the biological characterization because transferrin could be implicated in the transport of the vanadium complex to the cancer cells.

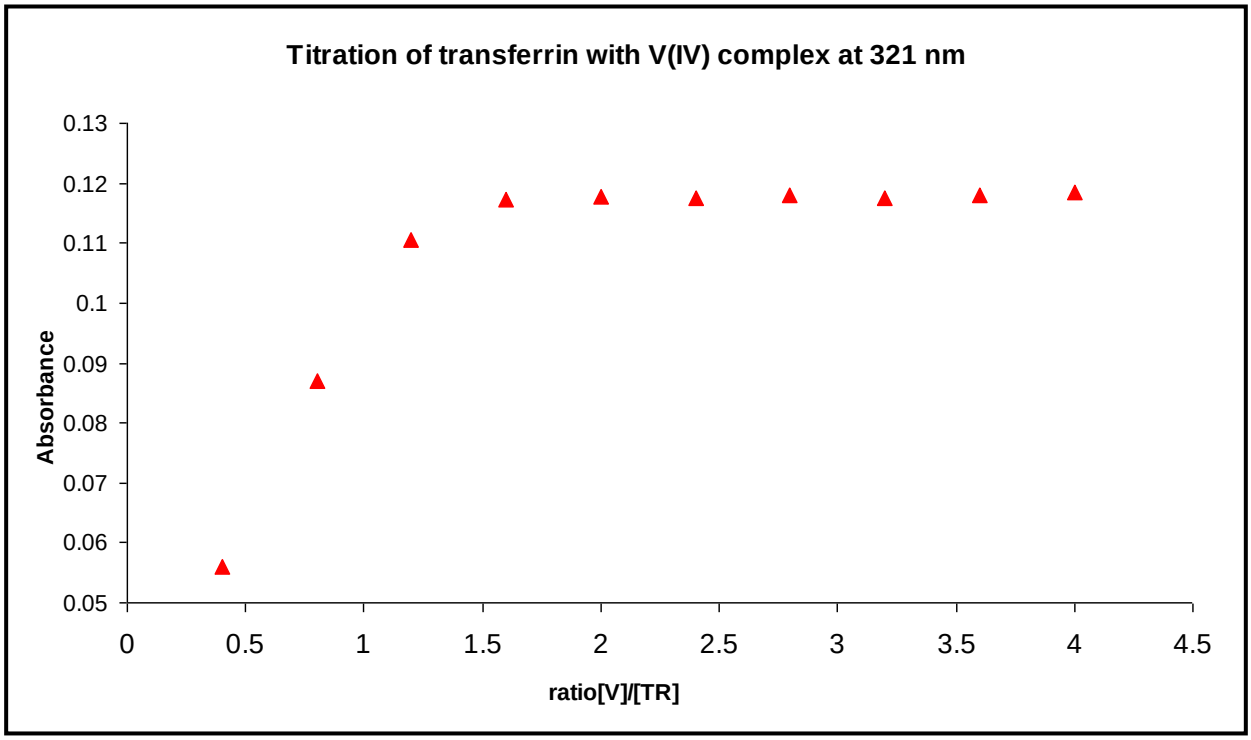


Figure 5-3: Titration of transferrin with V(maltolato)$_2$(Cl)$_2$ complex in 0.1 M Tris-HCl/ 10 mM NaCl / 100 mM NaHCO$_3$ buffer solution.

5.7 Cytotoxic studies on HT-29 colon cancer cell line

The cytototoxic studies of the $V(maltolato)_2Cl_2$ complex were performed using MTT assay. The analysis was performed at the Ponce School of Medicine by former graduate student I. Feliciano, using a period of time of 72 hours and ranges of concentration from $7.92x10^{-6}$ to $6.40x10^{-4}$. $V(maltolato)_2Cl_2$ complex exhibited less toxicity than their counterpart, vanadocene dichloride (Cp_2VCl_2). At 72 hours, $V(maltolato)_2Cl_2$ complex showed an IC_{50} of 0.14mM whereas vanadocene dichloride showed a IC_{50} of 0.0364mM. Substitution of the Cp (cyclopentadienyl) by maltol ligand does not improve the cytotoxic activity of the complex. However, the vanadium-maltol complex shows better IC_{50} values than the titanium-maltolato, a robust complex that shows to be better antitumoral agent that all the titanium complexes tested, for 72 hours of interaction [35].

6. Conclusion and future works

Two new complexes were synthesized using Ti(IV) and V(IV) as a metal center and an oxygen-containing chelating ligand, maltol. $V(maltolato)_2(Cl)_2$ and $[Ti_4(maltolato)_8(\mu\text{-}O_4)]$ were synthesized and characterized chemically and biochemically using ^1H NMR, FT-IR, UV-Vis, Mass Spectrometry, cyclic voltammetry and X-ray. Interaction with biological important molecules like transferrin and Calf Thymus DNA were accomplished, in order to design a possible route of entrance to the cancer cell by the complex. Also the cytotoxic behavior of complexes was tested on HT-29 cells line and Caco-2 cancer cells.

6.1 Titanium complex

An unequivocal results of the x-Ray crystallography confirm that Titanium-maltolate, $Ti_4(maltolato)_8(\mu\text{-}O_4)$, is a tetranuclear complex. This complex is very stable at physiological pH, an important feature because this complex overcomes the extensively hydrolysis that is one of the major concern with antitumoral metal complexes. The Ti-maltol complex showed to be stable up to ten days at physiological pH. This complex at low pH (5.4) exist as monomeric species $[Ti(maltolato)_2(OH)_2]$, but an increase in pH (8.4) promotes the tetramerization. Tetramer species present a pseudo octahedral conformation with angles that were deviated from the octahedral conformation due to the strain cause by the five member ring of the maltol molecule.

Interactions studies performed using transferrin shows that the $Ti_4(maltolato)_8(\mu\text{-}O_4)$ complex do not have interaction with the protein. Bands at 240 nm and 295 nm and 321 nm were monitorated and they were not present. The 240 and 295 nm bands are attributed to the deprotonation and subsequent coordination of the tyrosine residues to the metal ion and 321 nm bands which is attributed to ligand to metal charge transfer (LMCT). The calf thymus DNA interaction shows similar result. A UV-Vis study was accomplished and the spectra obtained do not show an isosbestic point, which mean that the species does not coexist in equilibrium. The fact that the complex do not transfer the titanium to the transferrin and do not have interaction with the calf thymus DNA could be attributed to the rich oxygen environment that encloses the titanium metal in the complex. This environment produces a robust complex and could delay the stripping process, an important step for the transfer and transportation of the metal to the cells by transferrin. It is important to mention that more recent studies performed by members of our

laboratory showed that exist a weak interaction between titanium complex and the Calf Thymus DNA. In contrast to the study presented in this document in which UV-Vis was used to monitor the interaction, other members of our group have used ^{1}H NMR spectroscopy. The study suggests that the mechanism of action for this complex could be the intercalation within the DNA strain.

In addition, studies of cytotoxic activity were performed and the $Ti_4(maltolato)_8(\mu\text{-}O_4)$ complex was compared to others eight complexes. The titanium-maltol complex showed to be better antitumoral agent than the others. Values that, in most of the cases, are different from the compared complexes for one order of magnitude were obtained. This complex was an active species on HT-29 cancer cells for a period of 120 hours, a characteristic that no one had. Also shows to be active on Caco-2 cancer cells, this assay was performed in a similar manner to the HT-29 assay and the result obtained for the titanium-maltol complex was an atypical one. In contrast with the tested complexes, in which an increase of the Ti uptake by Caco-2 cells with the time was obtained, titanium-maltol complex shows an increase in the Ti uptake by Caco-2 cancer cell line at 5 and 24 hours, but it decrease with longer time exposure. An explanation to this behavior could be a metabolic process occurring into the cells that remove the titanium.

6.2 Vanadium Complex

In addition to the synthesis of the titanium-maltol complex, another complex was synthesized using vanadium as metal center. This complex contains two maltol molecules coordinate the metal center and two atoms of chlorides. This complex was

completely soluble in water and stable at physiological conditions. Also, $V(maltolato)_2(Cl)_2$ complex could donate the metal center to transferrin. Cytotoxies studies shows that the vanadium complex contains less toxicity than vanadocene dichloride even though it is more water soluble. However vanadium-maltol complex shows better IC_{50} values than the titanium-maltol complex.

6.3 Future works

Future work must be done in order to:

1- Describe the nature of the interaction of these complexes with biological important molecules.

We have presented evidence of the no interaction of the titanium-maltol complex with transferrin that rule out this protein as a carrier. However the use of more sensitive instruments and the design of new experiments by our colleagues of laboratory recently show a weak interaction with the Calf-Thymus DNA, a fact that could not rule out the possible intercalation as interaction mode with DNA bases. The interaction of transferrin with vanadium-maltol complex should be studied more extensively in order to design better antitumor metal based drugs.

2- Evaluation of the complexes in other cells lines

The evaluation of the cytotoxic activity of these complexes with different cancer cell lines could help with the design and development of more efficient antitumoral agent.

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