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Coordination Chemistry of the Antitumor Metallocene Molybdocene Dichloride with Biological Ligands

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The relative affinity of molybdocene dichloride (Cp_2MoCl_2) for the thiol, amino, carboxylate, phosphate(O) and heterocyclic(N) donor ligands present in amino acids and nucleotides, has been studied in aqueous solutions at pH 2–7, using ^1H, ^{13}C and ^{31}P NMR spectroscopy. Molybdocene dichloride forms the highly water soluble, air-stable complexes $Cp_2Mo(Cys)_2$ and $Cp_2Mo(GS)_2$ with cysteine and glutathione respectively, via coordination of the deprotonated thiol groups. While coordination to the imidazole nitrogen in histidine was observed, no evidence for coordination of the amino or carboxylate groups in the amino acids cysteine, histidine, alanine or lysine to Cp_2MoCl_2 was detected. Competition experiments with dAMP, ribose monophosphate and histidine showed preferential coordination to the cysteine thiol over the phosphate(O) and heterocyclic(N) groups. $Cp_2Mo(Cys)_2$ is stable in the presence of excess dAMP or ribose monophosphate and Cys displaces coordinated histidine, dAMP or ribose monophosphate to give $Cp_2Mo(Cys)_2$. These results provide further evidence against interaction with DNA as the key interaction that is related to the antitumor activity of molybdocene dichloride. The implications of these results for the biological activity of the antitumor metallocene and the likely species formed in vivo are discussed.

Introduction

The biological chemistry of the metallocene dihalides, Cp_2MCl_2 (M = Ti, Mo, V, Nb), has attracted significant interest since the antitumor activity of these organometallic complexes was first reported by Köpf and Köpf-Maier.[1−4] Titanocene dichloride (Cp_2TiCl_2) is the first non-platinum metal complex to enter clinical trials.[5−9] The lack of cross-reactivity against cisplatin resistant cells, and the different pattern of side-effects compared with most clinically established cytostatic drugs has confirmed that non-platinum metal complexes may offer significant benefits in chemotherapy. DNA has been implicated as the principal cellular target of Cp_2TiCl_2 in vivo, as the complex induces significant and pronounced inhibition of nucleic acid synthesis,[3,10] and titanium is found in the nucleic acid rich regions of solid Ehrlich Ascites tumor cells that have been treated with the complex.[11,12] Extensive structure−activity studies[13] and model studies with nucleic acid constituents and bulk DNA support formation of stable DNA adducts.[14,15] However, full structural characterization of the mode of interaction with DNA has not been possible, mainly due to the hydrolytic instability of Cp_2TiCl_2 at physiological pH.[16,17]

In contrast to Cp_2TiCl_2, there is insufficient biological and structure−activity data on the other metallocene dihalides, Cp_2MCl_2 (M = Mo, V, Nb), to propose mechanisms of action.[13] The vastly different chemical stabilities of these

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complexes at physiological pH[16,18,19] and the different coordination chemistries of each complex point to significantly different mechanisms of antitumor action for each drug. While there is evidence that vanadocene dichloride (Cp_2VCl_2) interacts with DNA in vivo,[10] there are no detailed data on the uptake and cellular distribution of the other metallocene dihalides in tumor cells.

While little is known about the mechanism of antitumor action of molybdocene dichloride (Cp_2MoCl_2), the hydrolytic stability of the cyclopentadienyl ligands at pH 7[20] has allowed numerous model studies with nucleic acid constituents to be performed.[20−23] The metallocene coordinates to both the nucleobase nitrogens and the phosphate oxygens of nucleotides forming discrete complexes.[20−22] However, simultaneous coordination to phosphate(O) and nucleic base(N) sites, which can occur with nucleotides, is not possible in DNA as the steric accessibility of phosphate(O) coordination sites in a DNA duplex is significantly different from these sites in isolated nucleotides. Independent studies with oligonucleotides showed that while stable adducts between Cp_2MoCl_2 and DNA strands may be formed at pH < 4.0, the formation of stable metallocene−DNA adducts in vivo at pH > 6.0 is unlikely.[24] However, the possibility that plasma constituents stabilize complexes with DNA could not be ruled out.

Given the lack of interaction with oligonucleotides, the interactions of Cp_2MoCl_2 with other cellular targets, including proteins and small molecules in blood plasma as well as DNA-processing enzymes, have been considered.[22,25,26] The metal complex inhibits the function of both protein kinase C[22] and topoisomerase II,[25] and coordinates to the thiol containing tripeptide glutathione.[26]

In this paper we report a comparative study of the interaction of Cp_2MoCl_2 with nucleic acid constituents, glutathione and amino acids, including thiol-containing amino acids. These experiments were undertaken to establish the nature of the species that are transported into the cell, possible mechanisms of detoxification and transport in the cell, as well as further information on the likely cellular target that may be related to the antitumor activity of Cp_2MoCl_2. The results show that Cp_2MoCl_2 preferentially coordinates to thiols over phosphate(O), heterocyclic(N), amino, and carboxylate groups, and forms stable adducts that may be isolated and fully characterized. In addition, the preparation of stable amino acid derivatives provides highly water soluble analogues of Cp_2MoCl_2 that may offer advantages for clinical studies.

Experimental Section

General. Cp_2MoCl_2 was purchased from the Aldrich Chemical company; glutathione (GSH), deoxyadenosine monophosphate (dAMP), ribose monophosphate, and the amino acids cysteine (Cys), histidine (His), alanine (Ala), and lysine (Lys) were purchased from the Sigma Chemical company. All reagents were used as provided. UV−vis spectra were recorded on a Cary 5E UV−vis spectrophotometer at 25 °C. NMR spectra were recorded using a Bruker WM AMX 400 (400 MHz, ^{1}H; 100.6 MHz, ^{13}C; 162 MHz, ^{31}P) or a Bruker Avance 300 (300 MHz, ^{1}H; 121.5 MHz, ^{31}P) spectrometer at 300 K in D_2O referenced to TSP at δ 0.00 ppm (^{1}H), to external $CDCl_3$ at δ 77.00 ppm (^{13}C), or to external triphenyl phosphite at δ 140.85 ppm (^{31}P). Standard water presaturation was used in all ^{1}H 1D NMR experiments. Spectral assignments were made with the aid of standard 2D NMR techniques, including COSY, HSQC, and HMBC experiments. pD values were measured using a Beckman Φ11 meter and a Mettler NMR tube pH probe and are related to the pH meter reading by the formula pD = pH(meter reading) + 0.4.[27] Measured pD values are ±0.3 due to fluctuations in sample pD which occurred over time. Electrospray ionization mass spectra were recorded on a Finnigan LCQ ion trap mass spectrometer.

NMR Titrations. In a typical experiment, Cp_2MoCl_2 (3.0 mg, 0.010 mmol) was sonicated in D_2O (0.5 mL) for 2−3 h to give a deep maroon solution at pD < 2, referred to as $Cp_2MoCl_{2(aq)}$ **(1)** in the text. This sample was prepared using the same method reported in our earlier studies[17,24] without rigorous exclusion of oxygen; under these conditions, no significant oxidation of $Cp_2MoCl_{2(aq)}$ occurs. For titrations performed at pD 6, the sample pD was adjusted to 6 ± 0.5 with NaOD (0.01 M, <10 μL). The solution was transferred to a sealed NMR tube and deoxygenated thoroughly via several purge−pump cycles.

Stock solutions of Cys, GSH, His, Lys, Ala, dAMP, and ribose monophosphate were prepared in D_2O (100 mM, pD 6), the appropriate reagents were added to the NMR tube via a syringe (0−2 equiv, 0−200 μL), and the reaction was monitored via ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy, as required.

For competition experiments, Cys, dAMP, or ribose monophosphate (0−2 equiv, 0−200 μL, 100 mM) was added to the relevant solution via syringe. Some precipitation of an unknown and insoluble species occurred over time.

Preparation of $Cp_2Mo(Cys)_2$ (3). A suspension of Cp_2MoCl_2 (51 mg, 0.17 mmol) was sonicated in water (7 mL) until dissolution was complete (2−3 h), to give a deep maroon solution. This solution was degassed via several freeze−pump−thaw cycles, and then a similarly degassed solution of Cys (40.7 mg, 0.34 mmol) in water (0.5 mL) was added via syringe. The reaction mixture was stirred at room temperature for 4 days, and the solvent was removed by freeze-drying to give a red-brown solid. Recrystallization from aqueous acetone afforded $Cp_2Mo(Cys)_2$ **(3a)** (73 mg, 92%) as orange crystals. λ_{max} (H_2O)/nm 235 (log ϵ 3.27), 325 (2.62). ^{1}H NMR (400 MHz; D_2O, pD ~2) δ 2.74 (2dd, 4H, $J_{\alpha\beta}$ 3.9 Hz, $J_{\alpha\beta'}$ 7.3 Hz, $J_{\beta\beta'}$ 13.2 Hz, β-H), 4.02 (dd, 2H, $J_{\alpha\beta}$ 3.9 Hz, $J_{\alpha\beta'}$ 7.3 Hz, α-H), 5.41 (s, 10H, Cp H). ^{13}C NMR (100.6 MHz) δ 26.95 (β-C), 46.31 (α-C), 87.71 (Cp), 162.14 (−COOH). *m*/*z* (+ve ion ESI) 490.9 ($M + Na^+$, 35%), 466.9 (M^+, 10), 348.1 (M^+ −Cys, 100). HRMS (ESI): $MoC_{16}H_{22}N_2O_4S_2Na$ ($M + Na^+$) requires 490.9971; found 490.9898.

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Preparation of $Cp_2Mo(GS)_2$ (6). A suspension of Cp_2MoCl_2 (49.9 mg, 0.17 mmol) was sonicated in water (7 mL) until dissolution was complete (2−3 h), to give a deep maroon solution. The pH was adjusted to 6 with NaOH (0.1 M). A solution of GSH (103.2 mg, 0.34 mmol) in water (0.5 mL) was added which resulted in a decrease of the solution pH, which was then readjusted to 6. The reaction mixture was stirred at room temperature for 12 h and filtered, and the solvent was removed by freeze-drying to give **6** as a red solid (140 mg, 98%), which was >98% pure by integration of the ^{1}H NMR spectrum. λ_{max} (H_2O)/nm 252 (log ϵ 3.24), 322 (2.87), 477.5 (1.78). ^{1}H NMR (400 MHz; D_2O, pD ∼6) δ 2.20 (m, 4H, Glu-βH), 2.57 (m, 4H, Glu-γH), 2.67 (m, 4H, Cys-βH), 3.81 (t, 2H, Glu-αH), 3.83 (s, 4H, Gly-αH), 4.41 (m, 2H, Cys-αH), 5.40 (s, 10H, Cp H). ^{13}C NMR (100.6 MHz) δ 26.2 (Glu-βC), 31.4 (Glu-γC), 37.9 (Cys-βC), 42.9 (Gly-αC), 54.1 (Glu-αC), 56.7 (Cys-αC), 96.9 (Cp), 172.7 (Cys-CONH), 174.0 (Glu-COOH), 174.9 (Glu-CONH), 175.8 (Gly-COOH). *m*/*z* (−ve ion ESI) 839.3 (M^-, 97%), 861.3 ($M^{2-} + Na^+$, 100), 883.3 ($M^{3-} + 2Na^+$, 50), 905.4 ($M^{4-} + 3Na^+$, 60). *m*/*z* (+ve ion ESI) 929.1 ($M + 4Na^+$, 78%), 906.9 ($M + 3Na^+$, 100), 534 (M − GSH, 45). HRMS (ESI): $MoC_{30}H_{40}N_6O_{12}S_2Na_3$ ($M + 3Na^+$) requires 907.0897; found 907.1066.

Formation of $Cp_2Mo(Cys)$ (2). Cp_2MoCl_2 (3 mg, 0.01 mmol) was sonicated in D_2O (0.5 mL) in an NMR tube until dissolution was complete (2−3 h), to give a deep maroon solution. A solution of Cys (1.2 mg, 0.01 mmol) in D_2O (100 μL) was added, and the reaction mixture was allowed stand at room temperature for 24 h. Analysis of the solution by ^{1}H NMR spectroscopy showed that the major complex present was $Cp_2Mo(Cys)$ **(2)** (∼50%) along with the bisadduct **3** and unreacted Cys. ^{1}H NMR (**2**) (400 MHz; D_2O, pD ∼2) δ 2.34 (dd, 1H, $J_{\alpha\beta'}$ 12.0 Hz, $J_{\beta\beta'}$ 13.0 Hz, β'-H), 2.91 (dd, 1H, $J_{\alpha\beta}$ 2.8 Hz, $J_{\beta\beta'}$ 13.0 Hz, β-H), 3.87 (dd, 1H, $J_{\alpha\beta}$ 2.8 Hz, $J_{\alpha\beta'}$ 12.0 Hz, α-H), 5.70 (s, 10H, Cp H). *m*/*z* (+ve ion ESI) 348 (M^+, 100%).

Results

NMR Titration Experiments with Cys. In order to study the interaction between Cp_2MoCl_2 and Cys, a series of titration experiments in D_2O were monitored by ^{1}H and ^{13}C NMR spectroscopy. Cys contains three potential coordination sites, the thiol, carboxylate, and amino groups. The pH of the solution is clearly important in determining the exact species present (pK_a values Cys 1.96, 8.18, and 10.28), and hence, the pH was monitored closely. Oxygen was rigorously excluded in order to avoid formation of the oxidized amino acid cystine. In addition, as the chloride ligands in Cp_2MoCl_2 rapidly dissociate in aqueous solution,[20] and salt concentration has been shown to affect the amount of complexation with nucleotides,[24] titrations were also performed in 50 mM NaCl.

Figure 1 shows the ^{1}H NMR spectra obtained on titration of Cys into a solution of Cp_2MoCl_2 in D_2O. In agreement with the literature,[28] a solution of $Cp_2MoCl_{2(aq)}$ (20 mM) at pD 2 contained 2 singlets (δ 6.09, 5.90 ppm) (Figure 1a) due to the aquated dimeric (**1b**) and monomeric (**1a**) species, respectively (Scheme 1). The relative amounts of **1a** and **1b** are highly concentration and pH dependent,[28] and hence, the equilibrium mixture of these two species in a given aqueous solution is hereafter referred to as $Cp_2MoCl_{2(aq)}$ (**1**). Addition

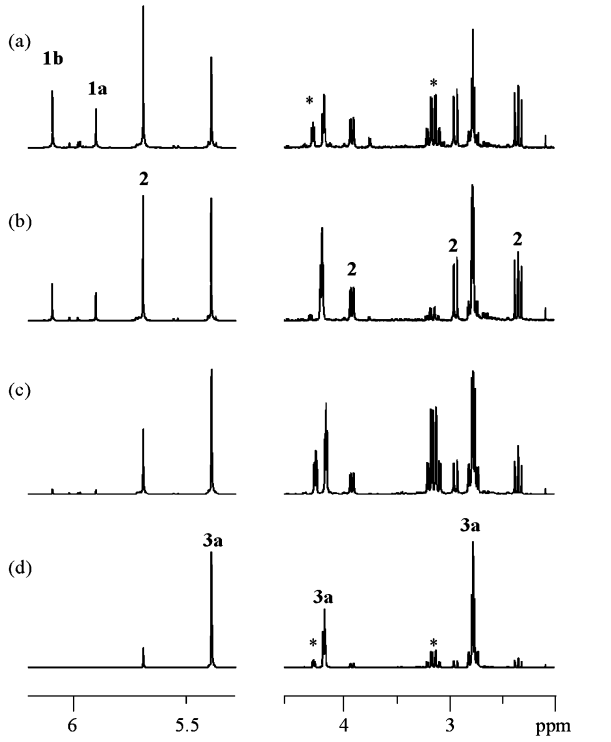
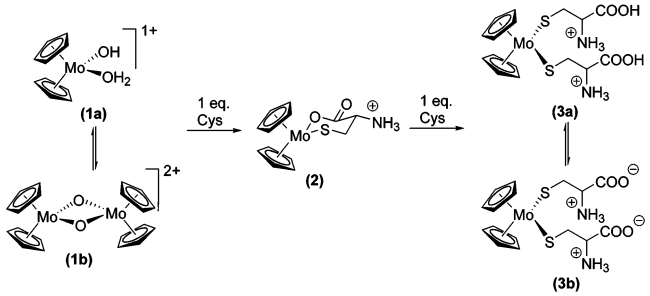


Figure 1. ^{1}H NMR spectra (400 MHz, D_2O) showing addition of a solution of Cys (100 mM, pD 6) to a solution of $Cp_2MoCl_{2(aq)}$ (20 mM, pD 2) in the absence of oxygen: 1 equiv after (a) 30 min, (b) 24 h, and after addition of a second equivalent (c) 30 min, and (d) 24 h. Asterisk (*) refers to Cys.

Scheme 1



of 1 equiv of Cys resulted in the appearance of a new Cp resonance at δ 5.70 ppm, as well as new Cys resonances at δ 3.87 (Hα), 2.34 (Hβ'), and 2.91 (Hβ) (Figure 1a). These new signals increased over time (Figure 1b), relative to the signals for Cys and $Cp_2MoCl_{2(aq)}$, and were assigned as arising from the bidentate 1:1 complex (**2**) (Scheme 1). This assignment was based on the characteristic large axial−axial coupling of 12 Hz between Hα and Hβ'. The six-membered ring in **2** also allows hydrogen bonding between the equatorial amino group and the carbonyl oxygen and is supported by an nOe interaction between the Cp protons at δ 5.70 ppm and the Hα and Hβ' protons at δ 3.87 and 2.34 ppm, respectively. Coordination of the amino group of cysteine to **1**, rather than the carboxylate group, to give a five-membered ring has been reported under different reaction conditions.[29,30] Hence, the experiment was repeated with 1 equiv of *N*-acetylcysteine. The new Hβ protons arising

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from the 1:1 complex had similar chemical shifts and identical coupling constants to those obtained in the corresponding titration with cysteine, consistent with the formation of **2**.

Upon the addition of a second equivalent of Cys, the signals due to the 1:1 adduct **2**, as well as those due to Cys and $Cp_2MoCl_{2(aq)}$, decreased in intensity (Figure 1c), and after 24 h, the major signals present were assigned to the protonated bisadduct $Cp_2Mo(Cys)_2$ (**3a**) (Figure 1d). Minor amounts of unreacted Cys and $Cp_2MoCl_{2(aq)}$ remained after several days. In order to drive the reaction to completion and obtain only signals due to **3a**, the experiments were repeated using different mole equivalents of reagents and with different reaction times. However, in all experiments, signals arising from minor amounts of starting materials (<10% by integration) remained.

The signals present in the spectrum shown in Figure 1d at pD 2 were assigned to the 2:1 complex $Cp_2Mo(Cys)_2$ (**3a**) by analysis of the ^{1}H NMR spectrum, and ESI mass spectrometry which gave a positively charged species at *m*/*z* 466.9, with the expected Mo isotope pattern. Coordination to sulfur rather than the carboxylate or amino groups is consistent with the large upfield shift of the Cys β protons in the ^{1}H NMR spectrum, and a smaller upfield shift of the α protons, compared with free Cys. Similarly, in the ^{13}C NMR spectrum, the Cys β carbon is shifted downfield by 12 ppm, compared with only a 1 ppm downfield shift of the Cys α carbon. In support of this assignment, a similar set of titrations was performed with Cys methyl ester (data not shown). Similar changes in chemical shifts of the α and β protons were observed with both Cys and Cys methyl ester, supporting coordination of sulfur to the metal.

A similar titration experiment was performed, in which the pD was adjusted to ~6 after the addition of Cys, to give the zwitterionic complex **3b** (data not shown). The new signals appeared at chemical shift values similar to those observed at pD 2, consistent with formation of **3b** as the predominant product.

A third analogous titration was performed in 50 mM NaCl solution, in order to determine whether competitive coordination of chloride to molybdenum would reduce the amount of coordination to Cys. The ^{1}H NMR spectra obtained in the presence of salt were almost identical to those presented in Figure 1, in the absence of salt (data not shown).

Properties and Stability of $Cp_2Mo(Cys)_2$. The optimized reaction conditions identified in the NMR titration experiments were used to prepare $Cp_2Mo(Cys)_2$ (**3**)[31] on a larger scale. The complex was precipitated from an aqueous solution (pH 2) by addition of acetone and was obtained as an orange crystalline solid. As in the NMR titration experiments, this solid was estimated to be ~98% pure by integration of the ^{1}H NMR spectrum. The complex was recrystallized from aqueous acetone, but despite numerous attempts, and the use of other aqueous solvent mixtures, crystals suitable for X-ray diffraction were not obtained.

Not surprisingly, $Cp_2Mo(Cys)_2$ is highly water soluble, compared to Cp_2MoCl_2. At low pH, the complex **3a** is overall dipositively charged, while at pH 7 the complex **3b** is neutral but doubly zwitterionic. While the improvement in aqueous solubility was not quantified, as a comparison, dissolving 3 mg of Cp_2MoCl_2 in 0.5 mL of water requires 2−3 h sonication, while the same molar equivalent of $Cp_2Mo(Cys)_2$ dissolves instantaneously in the same volume of water.

The use of water as solvent for the preparation of **3** is important. Initial attempts to form the complex using methanol (i.e., the conditions reported for the synthesis of amino acid derivatives of titanocene dichloride[32,33]) were unsuccessful. In contrast to the analogous titanocene amino acid complexes, precipitation of **3** from methanol was not observed, and mixtures of **3** and unreacted starting materials were obtained.

The biscysteine complex **3** is stable to the loss of the cysteine ligands in the presence of oxygen. Thus, while bubbling oxygen into an aqueous solution of Cys results in complete oxidation of Cys to cystine within 10 min, when oxygen was bubbled into a solution of **3a** for 1 h, there was no significant change in the ^{1}H NMR spectrum, with <5% of species due to minor byproducts observed. Thus, the Cys ligands in $Cp_2Mo(Cys)_2$ (**3**) are not labile, and this stability means that the complex can be readily handled and stored in air without decomposition. The complex is also stable in solution in the pD range 2−6 for a period of weeks.

Competition Experiments with Amino Acids. In order to test the relative affinity of Cp_2MoCl_2 for the thiol group of Cys compared to other potential coordinating ligands present in the amino acid side-chains, competition experiments were carried out between the amino acids Ala, Lys, His, and Cys. NMR titration experiments were performed in which 2 equiv of an amino acid was added to a solution of $Cp_2MoCl_{2(aq)}$ in D_2O. After 24 h, 2 equiv of Cys was added.

Addition of either Ala or Lys showed no significant changes in the ^{1}H NMR spectra, showing that under the conditions studied (pH 6) coordination of either the carboxylate or the amino groups in these amino acids to Cp_2MoCl_2 is weak. Addition of Cys to the 1:2 mixture of Ala and $Cp_2MoCl_{2(aq)}$, or Lys and $Cp_2MoCl_{2(aq)}$, resulted in the appearance of new signals previously assigned as arising from **3**.

In the case of His, clear evidence for coordination of the imidazole ring to molybdenum was observed (Figure 2). On addition of 1 equiv of His to a solution of $Cp_2MoCl_{2(aq)}$, several new aromatic signals were detected, consistent with formation of new species (Figure 2a). While the exact structures of the complex(es) are unknown, the changes in the aromatic region suggest coordination of the imidazole-(N). However, upon addition of Cys to this mixture, these new signals decreased in intensity (Figure 2b), and after 24 h, all signals were assigned to a mixture of **3** and His (Figure 2c).

Competition Experiments with Nucleic Acid Components. Given that Cp_2MoCl_2 forms nonlabile cyclic chelates

(31) The compound number **3** is used in the text to indicate either **3a** or zwitterionic **3b** depending on solution pH. Similarly complex **6** contains six ionizable functional groups, and while the major species present at pH 7 is shown in Figure 4, other species may be present depending on the solution pH.

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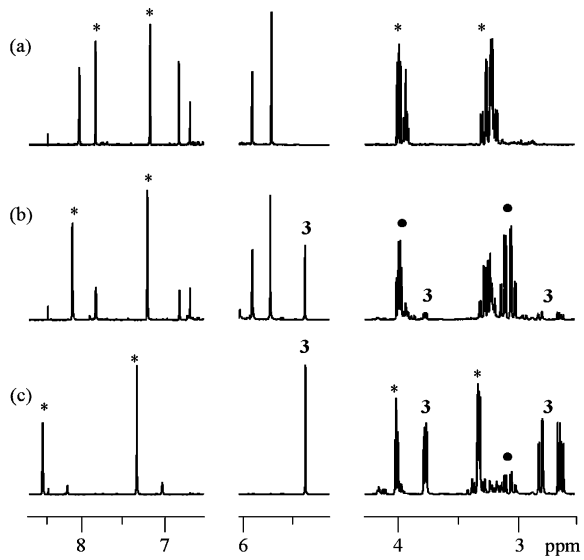
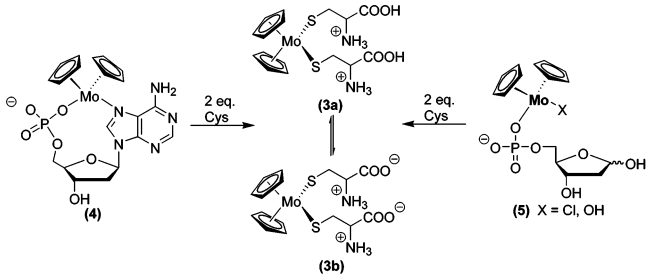


Figure 2. ^{1}H NMR spectra (400 MHz, D_2O) showing addition of a solution of His (100 mM, pD 6) to a solution of Cp_2MoCl_2 (20 mM, pD 6), followed by the addition of Cys: (a) 1 equiv of His after 1 h, (b) 2 equiv of Cys after 30 min, (c) 2 equiv of Cys after 24 h. Asterisk (*) refers to His. Dot (●) refers to Cys.

Scheme 2



with nucleotides,[20−22] the relative affinity of $Cp_2MoCl_{2(aq)}$ for the donor sites in the nucleic acid constituents, dAMP and ribose monophosphate, was compared with Cys. Previous studies have shown that that molybdocene forms Cp_2Mo-dAMP (**4**) and Cp_2Mo-ribose monophosphate (**5**) with dAMP and ribose monophosphate, respectively (Scheme 2).[20]

Three experiments, which differed in the order of addition of the reagents, were performed. First, either dAMP or ribose monophosphate was added to a solution of **3**, in order to determine whether the coordinated Cys ligands would be displaced by the phosphate(O) and/or the heterocyclic(N). Second, a mixture of Cys and either dAMP or ribose monophosphate was added to a solution of $Cp_2MoCl_{2(aq)}$, in order to determine whether a statistical mixture of **3** and **4** or **5** would be formed. Finally, Cys was added to a solution containing **4** or **5** in order to determine whether the amino acid is able to displace a coordinated nucleic acid constituent. The reactions were monitored by ^{1}H and ^{31}P NMR spectroscopy. ^{31}P NMR spectroscopy is an excellent tool for detection of phosphate(O) coordination, since coordination to molybdenum via the phosphate oxygen induces a downfield shift of the phosphate phosphorus of δ 40−50 ppm.[20,23]

Figure 3 shows a typical set of ^{1}H and ^{31}P NMR spectra that were obtained on addition of a solution of Cys (pD 2) to a solution containing **4** (pD 6). In the ^{1}H spectra (Figure 3A), the aromatic resonances of the nucleic base allow the presence or absence of **4** to be readily monitored. Compared to dAMP (Figure 3b), addition of 1 equiv of $Cp_2MoCl_{2(aq)}$ (Figure 3c) resulted in the appearance of a second set of aromatic peaks, arising from **4**. In addition, two new Cp signals were observed (δ 5.86 and 5.53 ppm), upfield of the signals due to $Cp_2MoCl_{2(aq)}$ (**1**) (Figure 3a). After the addition of 2 equiv of Cys (Figure 3d), the aromatic signals arising from **4** (δ 8.36 and 8.24 ppm) decreased in intensity, and new signals due to **3** appeared. Over time, the signals previously assigned to **3** increased, and after 4 days (Figure 3(e) all signals were assigned to **3**, plus dAMP and a minor amount of unreacted Cys.

Formation of **3** by displacement of dAMP from **4** is also readily detected in the ^{31}P NMR spectra (Figure 3B). Phosphate coordination of dAMP to $Cp_2MoCl_{2(aq)}$ to form **4** is clearly indicated by the downfield shift of the ^{31}P resonance to δ 33 ppm (Figure 3c), compared with the signal at δ 0 ppm, due to dAMP (Figure 3b). Upon addition of 2 equiv of Cys to this solution, the signal at δ 33 ppm immediately decreased relative to the dAMP signal at δ 0 ppm (Figure 3d), and after 24 h there was no signal remaining due to **4** (Figure 3e). Identical results were also obtained when the experiments were performed with ribose monophosphate, in the place of dAMP (data not shown). The results of these experiments are summarized in Scheme 2.

Similar results were obtained in the titration experiments in which the order of addition of Cys and dAMP or ribose monophosphate was varied. In each case, $Cp_2MoCl_{2(aq)}$ coordinated preferentially to Cys rather than dAMP or ribose monophosphate, to form **3** (data not shown).

Studies with GSH. We have previously reported that GSH coordinates to Cp_2MoCl_2 in aqueous solutions.[26] However, the study focused on the addition of 1 equiv of GSH to Cp_2MoCl_2 compared with other metallocenes, and the complexes formed were not isolated or characterized. Given the high stability of **3**, the synthesis of the corresponding GSH complex was investigated.

An NMR titration experiment similar to those carried out with Cys was performed. GSH (2 equiv) was added to a solution of $Cp_2MoCl_{2(aq)}$ in D_2O, and the reaction was monitored by ^{1}H and ^{13}C NMR spectroscopy. A single set of signals was present after 24 h, and was assigned to $Cp_2Mo(GS)_2$ (**6**) (Figure 4) on the basis of the significant upfield shift of the cysteinyl α and β protons (from δ 4.58 and 2.93 ppm, to δ 4.38 and 2.62 ppm, respectively). Similarly, the ^{13}C NMR spectrum (data not shown) shows a downfield shift of the cysteinyl β carbon from δ 22.2 to δ 37.9 ppm, while the other carbon signals remain largely unchanged, compared to GSH. This reaction was also performed on a larger scale and routinely gave material >98% purity by integration of the ^{1}H NMR spectrum of the crude reaction mixture. The high water solubility of the complex did not allow successful recrystallization from a range of different solvents. Purification via reverse phase HPLC was attempted, but while the complex was stable to analytical HPLC, purification of **6** by preparative HPLC resulted in almost complete product degradation.

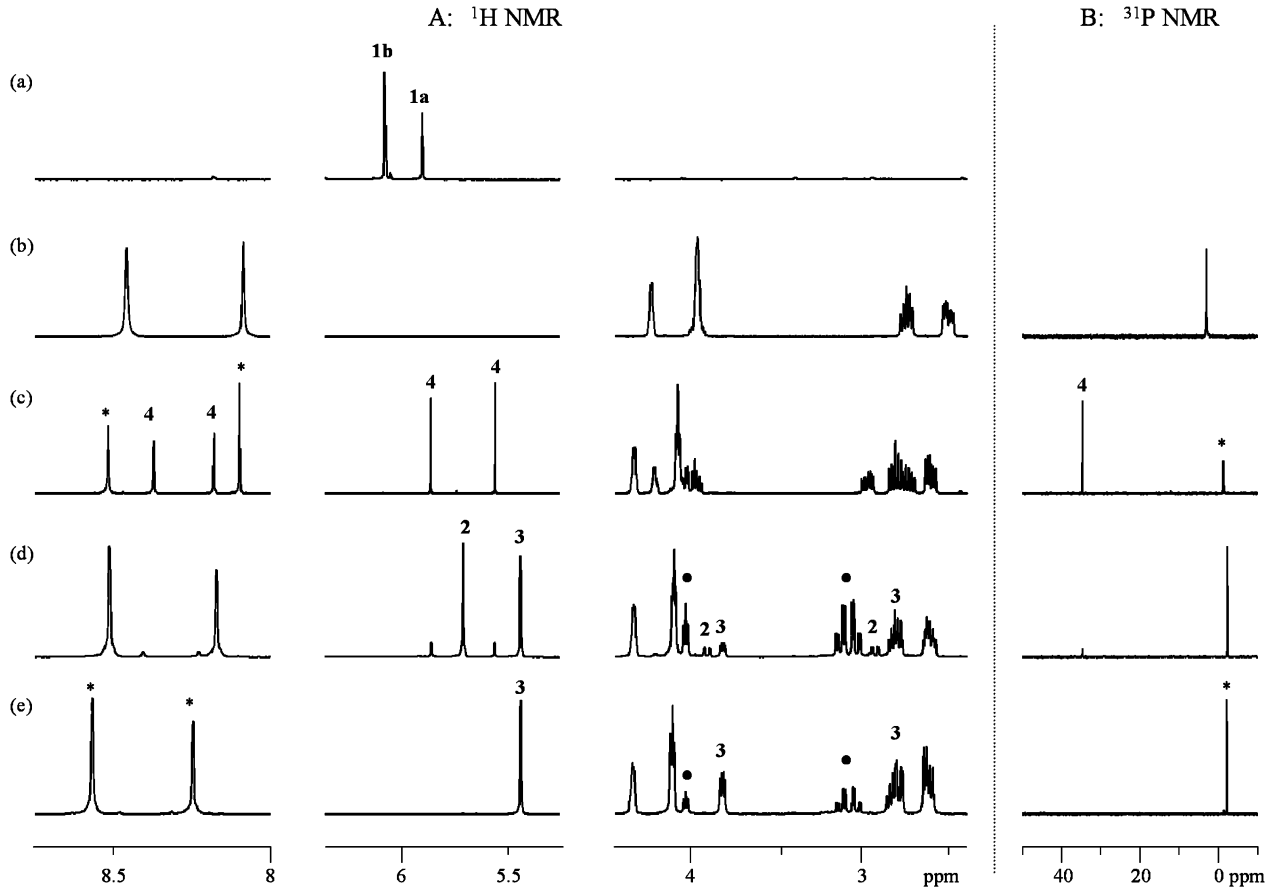


Figure 3. (A) ^{1}H NMR spectra (400 MHz, D_2O) and (B) ^{31}P NMR spectra (121.5 MHz, D_2O) showing addition of a solution of Cys (100 mM, pD 6) to a solution of Cp_2Mo-dAMP (20 mM, pD 6): (a) Cp_2MoCl_2 (20 mM, pD 6), (b) dAMP (pD 6), (c) 1:1 mixture of $Cp_2MoCl_{2(aq)}$ and dAMP after 1 h, (d) addition of 2 equiv of Cys, after 30 min, (e) 2 equiv of Cys, after 24 h. Asterisk (*) refers to dAMP. Dot (●) refers to Cys.

The reaction of Cp_2MoCl_2 with GSH to form **6** is significantly faster than the corresponding reaction with Cys to form **3**. Thus, while **6** forms within 1−2 h of addition of GSH, coordination of Cys to $Cp_2MoCl_{2(aq)}$ takes 2−3 days. The slower rate in the case of Cys is most likely related to the initial formation of the 1:1 cysteine complex **2** which must then undergo dissociation in the presence of excess Cys to form **3**. In contrast, formation of a similar intramolecular chelate is not possible with GSH, and hence, direct coordination with the preferred donor ligands occurs.

Discussion

While many articles refer to the antitumor metallocenes as a general class of antitumor agents that have broad spectrum activity against a range of animal tumors and xenografted tumors,[4,34−36] extensive comparative studies are restricted to Cp_2TiCl_2, Cp_2VCl_2, and several titanocene derivatives.[35,36] The antitumor testing of Cp_2MoCl_2 is currently limited to a single animal tumor model. An optimum cure rate (100%) against CF1 mice bearing fluid Ehrlich Ascites tumors was achieved with an optimum dose range 75−100 mg/kg and an LD_{50} of 175 mg/kg.[37] Both the optimum dose and LD_{50} are higher than the corresponding values achieved with titanocene dichloride.

This study reports the first synthesis of bisamino acid derivatives of Cp_2MoCl_2 prepared in water at physiological pH values. These solution studies differ from previous reports of 1:1 amino acid complexes of Cp_2MoCl_2 which were prepared under basic conditions with the complexes extracted into liquid SO_2 and precipitated as hexafluorophosphate salts.[29,38,39] In the solid state, these 1:1 complexes showed coordination of the carboxylate and amino groups to Mo, and in the case of cysteine, thiol and amino coordination. However, the stability and structures of the complexes in water are unknown and the corresponding bisamino acid complexes were not prepared. The solution chemistry of molybdocene amino acid complexes is important, given that while titanocene amino acid analogues have been crystallized and show coordination of the carboxylates to Ti,[32,40−43] in

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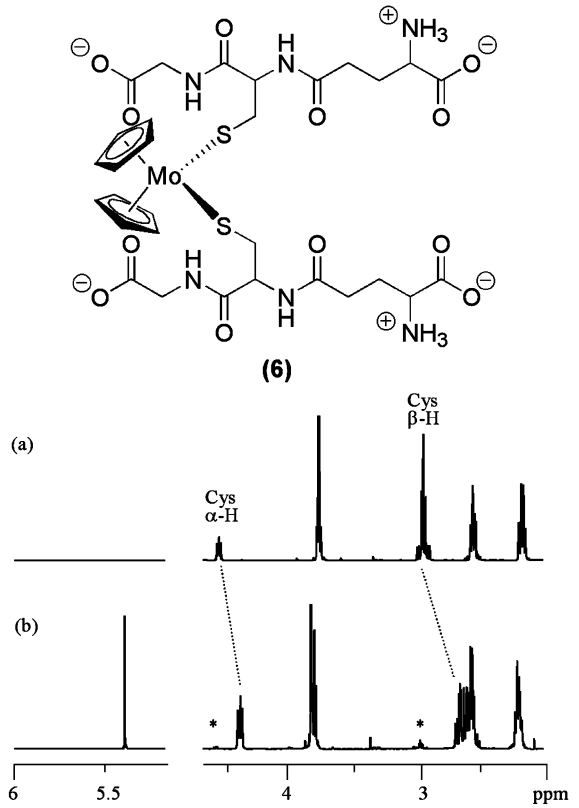

Figure 4. ^{1}H NMR spectra (400 MHz, D_2O) showing addition of a solution of GSH (100 mM, pD 6) to a solution of Cp_2MoCl_2 (20 mM, pD 6) in the absence of oxygen: (a) GSH (100 mM, pD 6), (b) 2 equiv of GSH after 30 min. Asterisk (*) refers to GSH.

aqueous solution under physiological conditions, these complexes are weak and labile,[40] and hence are not likely to be formed in vivo. Smets et al. have reported the hydrolysis and coordination of glycine and glycine methyl ester to Cp_2MoCl_2 at pH 10, but limited characterization data or evidence for the complexes was presented.[44] Bidentate thiolate, phenolate, and amino complexes of Cp_2MoCl_2 formed in organic solvents,[45−47] as well as metal−sulfur bond enthalpy calculations,[48] support the possible formation of complexes with amino acids and proteins involving oxygen, nitrogen, and sulfur donor ligands. The focus of this research was to establish the relative affinity of $Cp_2MoCl_{2(aq)}$ for these ligands in water at physiological pH, and hence to predict the complexes that would most likely form in biological media, including blood plasma.

No evidence for coordination of the carboxylate or amino groups in Lys, His, Ala, or Cys to $Cp_2MoCl_{2(aq)}$ was detected using ^{1}H NMR spectroscopy in the pD range 2−7. In contrast, nonlabile thiol coordination of Cys and GSH has allowed isolation and characterization of $Cp_2Mo(Cys)_2$ (**3**) and $Cp_2Mo(GS)_2$ (**6**). These complexes are highly water soluble and are stable to oxygen and in the pH range 2−7. There is weak coordination to the imidazole group of His, but the coordinated ligands are readily displaced by the thiol in Cys.

On the basis of the strong coordination to Cys and GSH, coordination of $Cp_2MoCl_{2(aq)}$ to thiols in blood plasma will almost certainly occur and must be considered in the mechanism of action. The major thiol in blood plasma is the protein human serum albumin (HSA) which is present at approximately 0.63 mM.[49] This protein serves a number of important functions including the transport of drugs, metals, and hormones.[50] Interaction of metal complexes with the free thiol in HSA has been implicated in the mechanism of Pt(II),[51,52] Pt(IV),[53,54] and Ru(II)[55] anticancer drugs. While the single available thiol in HSA is buried below the surface of the protein[49] and hence is less accessible than the thiol in Cys and GSH, the results of this study suggest that Cp_2MoCl_2 will also interact with HSA and form stable adducts.

The tripeptide GSH was studied due to the high concentration of this thiol in most cells and as the interaction of GSH with transition metal ions is fundamental to a number of cellular processes.[56] Elevated levels of GSH render some cancer cells resistant against platinum drugs and in the case of cisplatin lead to detoxification by a rapid binding mechanism.[51,57] The formation of $Cp_2Mo(GS)_2$ reported in this study strongly suggests that in cell culture media and fluids containing GSH that $Cp_2MoCl_{2(aq)}$ will be converted to $Cp_2Mo(GS)_2$ (**6**). Further studies are required to establish whether formation of **6** leads to detoxification and excretion in a manner similar to related adducts with platinum drugs, and whether **6**, which lacks a labile Mo−X bond, retains anticancer activity.

The coordination chemistry of Cp_2MoCl_2 contrasts with Cp_2TiCl_2, which shows negligible interaction with thiols including GSH, and weak, labile coordination to carboxylate groups in amino acids, which are readily displaced by nucleotides.[26,40] Similar coordination of the carboxylate and amino groups of amino acids to Cp_2MoCl_2 occurs in the solid state,[38,39] but in aqueous solutions these ligands are also labile. This is not the case for $Cp_2Mo(Cys)_2$ (**3**) which is stable in the presence of competing donor ligands present in nucleic acids and amino acids. The complex is stable in the presence of excess dAMP or ribose monophosphate, and furthermore, Cys displaces coordinated dAMP or ribose

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monophosphate to give $Cp_2Mo(Cys)_2$ (**3**). These results show that thiol containing amino acids, and thiols on the surface of proteins including serum albumin, are attractive targets for molybdocene dichloride. The results are also consistent with independent studies that do not implicate molybdocene−DNA adducts in the mechanism of action, and reinforce the fact that each of the metallocene dihalides, Cp_2MoCl_2 (M = Ti, V, Nb, Mo), operate via independent mechanisms of action.

Conclusions

The results reported in this study strongly suggest that Cp_2MoCl_2 will preferentially coordinate to thiols in vivo. This coordination mode is distinct from the coordination chemistry of Cp_2TiCl_2 and supports suggestions that the different metallocenes operate via different mechanisms of action and that interaction of $Cp_2MoCl_{2(aq)}$ with DNA is not directly related to anticancer activity. Further studies to determine the transport mechanisms and cellular uptake of both Cp_2MoCl_2 and $Cp_2Mo(Cys)_2$ would be useful in establishing the mechanism of action of these compounds. Antitumor testing of the water soluble, stable complex $Cp_2Mo(Cys)_2$ is also required to establish whether labile Mo−X coordination is required for biological activity.

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