Synthesis, Characterization, and pH-Dependent
Relaxivity of the Mn(II) Complex of a Novel Cyclen-Based Ligand

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“How lucky I am to have something that makes saying goodbye so hard.” – A.A. Milne
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Abstract

Previously, the Mn(II) complexes of macrocyclic ligands H$_4$DOTA and DOTAM have been studied to better understand their solution dynamics and investigate their potential use as MRI contrast agents. It has been shown that MnDOTA has a strong pH dependence to its relaxivity and a low stability under acidic conditions. MnDOTAM has been shown to be more stable, but its relaxivity is low and shows no pH dependence. As pH variation in the body is a potential target application of MRI contrast agents, some pH dependence of relaxivity is desirable.

This work focuses on the synthesis of a novel macrocyclic cyclen-based ligand, 1,4,7,10-tetraazacyclododecane-1,7-bis(acetate)-4,10-bis(acetamide), containing both acetate and amide functional groups. The ligand and its Mn(II) complex are characterized in solution and the solid state. The Mn(II) complex is believed to be at least six-coordinate over a large pH range, and is stable at relatively low pH. Its relaxivity is pH dependent, and at low pH can be attributed to both inner-sphere water exchange as well as prototropic exchange. This new ligand and Mn(II) complex provide insight into the roles different functional groups play in the structure of these types of complexes which can aid in the future design of pH-sensitive, stable MRI contrast agents.
Chapter 1: Introduction

1.1 MRI

Magnetic Resonance Imaging (MRI) is a useful and commonly used diagnostic technique in modern medicine. It is noninvasive and unlike CT scans or x-rays, does not require harmful external radiation, making it relatively safe for clinical use. The technology for MRI was first developed as an extension of NMR (nuclear magnetic resonance) spectroscopy in the 1970s by Paul Lauterbur. He received a Nobel Prize in 2003 for his discoveries related to magnetic resonance imaging. Lauterbur’s initial work - and all of MRI imaging - is based on the behavior of the spins of hydrogen nuclei, or protons, of water molecules in the body. These protons have an inherent spin, which can align with an applied magnetic field. These nuclei also undergo precession around the net magnetization vector. The frequency of the precession is proportional to the magnetic field strength and is known as the Larmor frequency (ω₀), given by Equation 1.1, where γ is the gyromagnetic ratio, and B₀ is the applied field. The gyromagnetic ratio is different for different atoms; for the hydrogen nucleus, it is 42.58 MHz/T. The Larmor frequency for protons in a 1.5 T field, the highest field commonly used in diagnostic imaging, is therefore 63.9 MHz.

\[ \omega_0 = \gamma B_0 \]  \hspace{1cm} (1.1)

The lowest energy state for a proton is for the spin to be aligned with the magnetic field. The energy difference between this ground state and the excited state where the spin is against the field is quite small and can be calculated using the Larmor frequency and the relationship \( E = h \nu \). At 1.5T, the difference in energy between the two states is \( 6.739 \times 10^{-27} \) J. Calculating a Boltzmann distribution based on this energy difference at 298K gives a ratio of excited to ground state spins of 0.9999983. This is to say, that for every mole of protons, \( 3.0100 \times 10^{23} \) are in the ground state, while \( 3.0099 \times 10^{23} \) are in the excited state. This marginal
excess of protons whose spins are aligned with the magnetic field provides the measurable net magnetization seen in an MRI sample.

Once the spins have reached this equilibrium state, a radiofrequency (RF) pulse, at the Larmor frequency, is applied. For example, one can imagine an RF pulse strong enough to flip the spins of the nuclei by 90°. If the applied magnetic field is assumed to be in the z-axis, then the RF pulse would flip the spin vectors of the nuclei into the x,y plane. This causes the net magnetization of the protons in the z direction to be zero.

Once the RF pulse is turned off, the spins realign with the applied magnetic field. This process is called relaxation. Rates of relaxation are determined by the chemical environment of the proton. An isolated proton would take $10^{25}$ s or $3.2 \times 10^{17}$ years to relax from an excited state in a magnetic field. The presence of nearby protons and other nuclei allow protons to relax on a timescale that is practical for NMR and MRI imaging. For example, protons in water generally have relaxation times on the order of a few seconds. Because different body tissues create different chemical environments, protons will relax at different rates depending on where they are in the body. The amount of time it takes for the proton spins to realign with the applied magnetic field along the z-axis is characterized by the longitudinal relaxation time, or $T_1$.

In addition, the relaxation in the xy plane can be measured. After a 90° RF pulse, the spin vectors are all oriented in the xy plane. Again, a small majority of the spins become aligned to produce a net magnetization in the xy plane. As the nuclei precess around the z-axis, the spin vectors of the individual nuclei gradually become out of phase with each other, and the net magnetization in the xy plane returns to zero as the system returns to equilibrium. The amount of time that characterizes this process is called the transverse relaxation time, or $T_2$. MRI images can be generated from either $T_1$ or $T_2$ values. $T_2$ weighted MRI tends to
cause image darkening, while T$_1$ weighted MRI is associated with image brightening. T$_1$ weighted MRI is more common and produces the brightened images with which most people are familiar.

To generate an MRI image, the T$_1$ of small areas of tissue are measured and matched up to their locations in the body. This is done by varying the field strength in a gradient over different parts of the body, allowing the location of the protons to be encoded by their Larmor frequencies. The computer can then convert the data into a 3d image using Fourier transform. The differences in T$_1$ are plotted on a grayscale, with lighter areas corresponding to shorter relaxation times and darker areas corresponding to longer relaxation times. Different body tissues create different chemical environments, greatly varying the relaxation time of protons and providing very good spatial resolution in MRI images.

1.2 Contrast Agents

Contrast is the difference in brightness between the light and dark areas of an image. In MRI images the contrast comes from the difference in relaxation times of various protons. The greater the difference in the relaxation times, the greater the contrast. Natural variations in the composition of different body tissues create differences in the relaxation times of water protons. Factors affecting the relaxation time of a tissue include concentration of water protons, density of tissue, rigidity of tissue, and temperature. Instrument field strength can also play a role in determining the contrast seen in an image as T$_1$ shows a dependence on the magnetic field.

While naturally occurring contrast is sufficient to generate an MRI image, diagnostic accuracy and efficacy for certain body tissues can be improved by enhancing the contrast through external means. This is achieved through the use of contrast agents. Contrast agents are small molecules containing a metal ion, which interact with water protons, causing them
to relax faster. This increase in relaxation rate enhances the contrast of an MRI image by differentially affecting the $T_1$ of some protons and magnifying natural differences in relaxation time. The most commonly used metal in current clinical MRI contrast agents is gadolinium. As shown in Figure 1.1, gadolinium contrast agents are very effective in creating contrast and elucidating details in body tissues. Image A is without contrast and image B is with gadolinium contrast. Details of the tumor structure can be seen in much greater detail once the contrast agent is used.

![MRI images showing a metastatic bone deposit in a patient’s skull both without (photo A) and with (photo B) a gadolinium based contrast agent.](image)

There are currently eleven FDA approved contrast agents in clinical use. Of these, nine are gadolinium based, and only two are non-gadolinium based. The first contrast agent developed was Magnevist (Gd-DTPA), which is still the most commonly used today. The two contrast agents not based on gadolinium(III) are complexes of iron(III) and manganese(II) ions. These ions are chosen for use as contrast agents because of their unpaired electrons and high water exchange rates. The unpaired electrons on the metal center shorten the relaxation time of the surrounding water protons due to their large magnetic moment. Ions with more unpaired electrons have more paramagnetic character and are more effective as contrast agents. Gd$^{3+}$ has a spin of $7/2$ which makes it an attractive candidate for MRI contrast agents. Mn$^{2+}$ and Fe$^{3+}$ (high spin) have a spin of $5/2$ which is somewhat less
desirable, but still has a large enough effect to be useful. Other qualities that must be considered when choosing a metal center for a contrast agent include its thermodynamic and kinetic stability with the ligand, water exchange rate, and toxicity.

Gadolinium performs well in many of these categories; however, it is not an ideal contrast agent. The use of gadolinium based contrast agents has been linked to nephrogenic systemic fibrosis in patients with renal insufficiencies. Nephrogenic systemic fibrosis is a disease that causes hardening of the connective tissue in the body, including the skin and around the organs. Though it is relatively rare, it is very painful and can be fatal. It impacts those with impaired kidney function because contrast agents are excreted through the kidneys. In patients without renal disease, the time for half of the contrast agent dose to be excreted is usually around 1.5 hours. In patients with kidney disease, this time can be lengthened to over 5 hours. This extra time allows for more of the gadolinium to dissociate from the ligand complex and incorporate into body tissues such as the liver, kidneys, and bones. Gadolinium has been thought to undergo transmetallation with calcium and interfere with normal calcium channels and signaling in the body. Because of these problems, gadolinium-based contrast agents are no longer used for patients with renal insufficiency. Since the majority of contrast agents contain gadolinium, this leaves few options for these patients, highlighting the need for alternatives.

1.3 Relaxation

As described previously, MRI images are generated by plotting the differences in the relaxation times, or $T_1$ values, of water protons. This relaxation will happen naturally in an applied magnetic field. Contrast agents work by providing extra pathways for relaxation, thus decreasing the $T_1$ of the sample. There are a few pathways through which this process can occur. Metal centers have both an inner hydration sphere of water molecules that directly
contact the ion and an outer hydration sphere of water molecules that are influenced by the metal center to a lesser degree. A contrast agent or free metal ion in solution can influence the relaxation of protons in both of these hydration spheres.

The first mechanism of relaxation is the outer sphere relaxation mechanism that relies on through space interactions between the metal ion and the water proton. The water molecule is not directly bound to the metal center, but the electronic interaction is still strong enough to influence relaxation.

The next mechanism is inner sphere water exchange. This mechanism allows inner-sphere water molecules that are directly bound to the metal to exchange with bulk water molecules. When the water is bound to the metal, its protons relax quickly. The water molecule can then exchange with another unrelaxed molecule and the cycle can continue. This mechanism is especially effective if the metal center or complex has a high water exchange rate. Similar to this mechanism is transient water binding, where a metal temporarily increases its coordination number, to interact with a water molecule, but does not generally have a water molecule bound to it.

The final mechanism of relaxation comes from prototropic exchange. If the ligand (including any bound water molecules) has exchangeable protons, these protons are relaxed by the metal center and can then exchange with unrelaxed protons from the bulk water. This provides a pathway for relaxation to occur in the absence of any bound water molecules, or with a metal center that is highly isolated from the solution.

### 1.4 Relaxivity

The relaxivity of a contrast agent is a measure of its ability to enhance the relaxation rate of water protons or other nuclei, and is therefore the most important property of a contrast agent. The equation for the relaxivity of a complex is given by Equation 1.2.\textsuperscript{11}
\[ [M] r_i = \frac{1}{T_i} - \frac{1}{T_i^0} \quad i = 1 \text{ or } 2 \]  

Relaxivity is a measure of the difference in relaxation time, either \( T_1 \) or \( T_2 \), between a solution containing a contrast agent and the solution without a contrast agent. The relaxivity of a substance is proportional to its concentration and the units of relaxivity are mM\(^{-1}\)s\(^{-1}\). The observed relaxivity is the sum of the relaxivity contributions of the various mechanisms of relaxation described above.

The relaxivity of a contrast agent can be enhanced by choosing metals with a high spin and fast water exchange rate. Because Gd(III) has a very high spin of 7/2, it is an attractive candidate for MRI contrast agents. The somewhat lower spin of Mn(II), 5/2, is less favorable, but the lower inherent toxicity of the manganese metal helps make up for this decrease in spin. The water exchange rates of Mn(II) and Gd(III) ions are both relatively high (\( k_{\text{Mn(II)}} = 2.7 \times 10^7 \text{ s}^{-1}, k_{\text{Gd(III)}} = 8.3 \times 10^8 \text{ s}^{-1} \))\(^{12}\) which make them attractive candidates for contrast agents.

### 1.5 Ligand Design

Aside from the properties inherent to the metal, the relaxivity and stability of contrast agents can be greatly affected by the design of the ligand. Currently, among commercially available contrast agents, ligands fall into two major categories: linear and cyclic. The structures are shown below in Figure 1.2. The top two rows of contrast agents are examples of a linear chelate structure. These ligands are acyclic but contain various nitrogen and oxygen donor groups to complex with the metal. The bottom row of contrast agents are macrocyclic contrast agents, usually consisting of a polyazamacrocyclic ring with other oxygen and nitrogen-containing functional groups for binding.\(^{13}\)
Though the macrocyclic ring-based contrast agents are more stable, they often have lower relaxivities making them slightly less effective and necessitating larger doses for patients. This decrease in relaxivity is due to the increased isolation of the metal from the bulk water that comes from having a cyclic structure, as well as the absence of free metal ions in solution. The decomplexation that occurs more readily with linear chelating agents helps increase their relaxivity and is the source of the relaxivity properties of Mn(DPDP)$^{2-}$, the only current Mn(II)-based contrast agent.

For macrocycle-based compounds, the base of the ring structure is often 1,4,7,10-tetraazacyclododecane, commonly called cyclen. The four nitrogen atoms in the ring can be functionalized to provide coordination sites for the central metal ion. Because the metals used are generally hard acidic cations, the ligands employ hard basic functional groups for binding. These include negatively charge oxygen and nitrogen based functional groups. In addition,
ligands with exchangeable protons are desirable because they can influence relaxation through the mechanism of prototropic exchange. H₄DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) is a common cyclen-based ligand, with an acetic acid group attached to each of the four nitrogen atoms in the ring. DOTA and some of its derivatives are used in Gd(III) based contrast agents as shown above in Figure 1.2.

1.6 DOTA and DOTAM

In previous work, the Mn(II) complexes of H₂DOTA²⁻ and DOTAM have been synthesized and characterized.¹⁵ The Mn(II)H₂DOTA complex is known to be six-coordinate in the solid state as shown in the crystal structure in Figure 1.3. The Mn²⁺ ion is bound to the four nitrogen atoms in the ring and two of the deprotonated acetate side arms.

![Diagram of Mn²⁺ bound to DOTA](image)

**Figure 1.3. Simplified structure and crystal structure of MnH₂DOTA.¹⁵c**

The pH dependent relaxivity of [MnHₓDOTA]ₓ⁻² has been measured¹⁵a,¹⁶ and the speciation diagram has been determined by potentiometric titration.¹⁵b Figure 1.4 shows an overlay of the two plots. At low pH, the complex has a very high relaxivity, which decreases with increasing pH. At low pH however, the relaxivity of the complex is the same as that of free Mn(II), which exists in solution as Mn(II)•6H₂O. The speciation diagram shows that at low pH the complex dissociates and that the subsequent decrease in relaxivity as the pH
increases is caused by complex formation. While this is not a huge problem at physiological pH, greater complex stability is desirable in contrast agents.

![Figure 1.4: Relaxivity and speciation diagram of MnDOTA.](image)

To determine the effects of functional group variation on the properties of the complex, the ligand DOTAM has also been complexed with manganese, among other metals. Mn(II)DOTAM is 8-coordinate in the solid state as evidenced by the crystal structure shown in Figure 1.5.
DOTAM coordinates with the metal center through the oxygen atoms of the amide groups. It has no ionizable protons and therefore, there is only one species present in solution over the pH range of 1-10. The Mn(II) does not dissociate at low pH, and the amide groups are weakly basic and do not accept protons. A potentiometric titration of the complex yielded no useful data and a speciation diagram and therefore the stability constant of the MnDOTAM complex was not able to be determined. Because the structure of the complex does not change and there is no open coordination site for a water molecule, the relaxivity does not show any pH dependence.\textsuperscript{15a} It is also lower than the relaxivity at high pH of the MnDOTA complex, with a value around 1.97 mM\textsuperscript{-1}s\textsuperscript{-1}. The fact that the relaxivity does not change combined with titration data suggests that the Mn(II) remains bound to the ligand at all measurable pH values and that the DOTAM complex has a much higher stability than the DOTA complex does.

1.7 Amide vs. Acetate Groups

Because the coordination between the metal and amide groups is not affected by changes in pH, the complex is very stable over a variety of conditions. Carboxylic acid groups however, are more susceptible to changes in protonation state, impacting the charge
on the oxygen atoms and subsequently their coordination to the metal center. Regardless of the functional group attached to them, the ring nitrogen atoms of both DOTA and DOTAM can become protonated. The first two protonations of the DOTA ligand occur at pH 12.09 and 9.68, and happen at the ring nitrogens, as these are the most basic sites on the ligand.\cite{17} The next protonations occur at a pH of 4.5 and 4.1 involving two of the acetate side arms.\cite{17} The other two acetate arms are deprotonated at all measurable pH values. In DOTAM, two of the ring nitrogens are protonated below a pH of 6.21.\cite{18} The pK\textsubscript{a} values for both DOTA and DOTAM are shown below in Table 1.1.

<table>
<thead>
<tr>
<th>Species</th>
<th>pK\textsubscript{a}</th>
<th>Species</th>
<th>pK\textsubscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>H\textsubscript{D}OTA\textsuperscript{-}</td>
<td>12.09</td>
<td>H\textsubscript{D}OTAM\textsuperscript{+}</td>
<td>7.70</td>
</tr>
<tr>
<td>H\textsubscript{2}DOTA</td>
<td>9.68</td>
<td>H\textsubscript{2}DOTAM\textsuperscript{2+}</td>
<td>6.21</td>
</tr>
<tr>
<td>H\textsubscript{3}DOTA\textsuperscript{+}</td>
<td>4.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H\textsubscript{4}DOTA\textsuperscript{2+}</td>
<td>4.13</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1. pK\textsubscript{a} values of DOTA and DOTAM.\cite{17-18}

The two highest pK\textsubscript{a} values for DOTA represent protonation of the nitrogen atoms in the ring. While the first protonation occurs at relatively high pH, the second occurs at much lower pH due to the stabilization of the nitrogen lone pairs through resonance and hydrogen bonding with the first proton bound to the ring as shown in Figure 1.6.
Similarly, hydrogen bonding can be used to explain the difference in pKa of the two acetate groups. The first acetate group becomes protonated at pH 4.55. The second group requires a slightly more acidic environment for protonation to occur. This is because, when the acetate group becomes protonated, it becomes more electron withdrawing. Because the remaining deprotonated acetate group is more electron donating, the two protons on the ring will hydrogen bond preferentially with the nitrogen attached to the deprotonated acetate as shown in Figure 1.7. This hydrogen bonding draws electron density towards the ring through induction and makes the acetate less basic.

One way to compare the Mn(II)DOTA and Mn(II)DOTAM complexes is by looking at the stability constants for each species. The stability constant, or formation constant, of a
complex is generally reported as the logβ value. This value represents the affinity of a compound for either a proton or a metal ion. The β value for a species is the equilibrium constant for its formation as shown below. For the free ligand, β values can be determined for each protonation. In this case, the logβ value is called a protonation constant.

For the metal complex, the initial logβ is the formation or stability constant, arising from the metal interacting with a fully deprotonated ligand. Subsequent β values arising from the protonation of the metal-ligand complex take into account the concentration of both the metal and the protons in solution as shown in Equation 1.3b below. Because logβ values arise from equilibrium constants of protonation, they are directly related to pKₐ. The difference between two successive logβ values gives the pKₐ for that protonation. The equations for the determination of the β values for both the protonation of the ligand and formation/protonation of the metal complexes are shown below.

$$ \beta_{HxL} = \frac{[H_xL]}{[H]^x[L]} \quad (1.3a) $$

$$ \beta_{MLH} = \frac{[M_{mL}H_h]}{[M]^m[L]^h[H]^h} \quad (1.3b) $$

The stability constant for MnDOTA has been determined to be 22.202±0.008. This is the constant for the formation of the complex between fully deprotonated DOTA⁴⁻ and the Mn(II) ion. The monoprotonated complex has a stability constant of 23.94 and the diprotonated complex has a stability constant of 26.97. Stability constants for MnDOTAM have not been reported in the literature. Experimental data however, suggests that MnDOTAM forms a very stable complex and does not dissociate even at low pH, making the determination of an accurate stability constant very challenging by standard titration methods. Since the complex is colorless and paramagnetic, studies by UV-Vis or NMR spectroscopy are very difficult, if not impossible, to conduct.
A lower bound for the stability constant of MnDOTAM can be estimated from the limiting equilibrium concentrations from experimental observation. At a pH below 7, where DOTAM is fully protonated, the equilibrium expression is shown below, along with the expression for its equilibrium constant in Equation 1.4.

\[
\beta = \frac{[\text{MnDOTAM}][H^+]^2}{[\text{Mn}^{2+}][\text{H}_2\text{DO}2\text{A}2\text{AM}^{2+}]} \tag{1.4b}
\]

Because the complex does not appear to dissociate at low pH, it can be assumed that at most, a dissociation of 1% of the complex could have occurred within experimental error. If the original concentrations of the metal and ligand were 10mM, then there is assumed to be no more than \(10^{-4}\text{M}\) Mn\(^{2+}\) now left in solution. At a pH of 1, the proton concentration is 0.1M. This gives the K for the expression as \(\geq 9900\). Taking the logK, which is comparable to log\(\beta\), gives an overall stability constant of 9.9. Since this assumes fully protonated DOTAM, the stability constant for the formation of the protonated DOTAM must be taken into account. Adding these log\(\beta\) values together gives a value \(\geq 23.1\).

To determine which of these species is more stable at a given pH, a theoretical competition experiment can be done. The HySS \(^2\) program uses stability constants to generate a speciation diagram for the components of a mixture. This program can be used to model the species present in a mixture of Mn\(^{2+}\), DOTA, and DOTAM over a range of pH values. Using the known protonation constants for H\(_4\)DOTA, DOTAM, and MnDOTA as well as the lower limit for the stability constant for MnDOTAM, the speciation diagram shown below can be produced.
As shown by the speciation diagram, at all physiologically relevant pH values below pH 10, [MnDOTAM]$^{2+}$ is more stable and comprises 100% of the Mn containing species in solution. This model is consistent with the experimental data showing [MnDOTAM]$^{2+}$ maintaining its stability even at very low pH. On the basis of this data, it appears that amide groups bind more strongly to the metal center than do acetate groups at physiological pH.

The higher stability of MnDOTA at high pH can be explained by its solution structures and pK$_a$s. At pH lower than 10, the two ring nitrogen atoms of H$_2$DOTA are protonated. These protons must be displaced by the Mn$^{2+}$ in order for binding to occur. Above pH 10, the nitrogens are deprotonated and there is no competition for Mn$^{2+}$ binding. This accounts for the increase in complex stability at high pH. The pK$_a$ of the ring nitrogens of DOTAM is lower at 7.70 and 6.21. These protons are more acidic and are more easily displaced by the Mn$^{2+}$ even at low pH. The neutral amide groups are also able to coordinate at any pH and provide extra stability to the complex below the pK$_a$ of the ring nitrogens.
1.8 DO2A2AM

This work focuses on the synthesis and characterization of the novel ligand, 1,7-
bisacetate-4,10-bisamide-1,4,7,10-tetraazacyclododecane (DO2A2AM), and its complex with manganese. This ligand, shown in Figure 1.9 below is a hybrid of DOTA and DOTAM containing two acetate and two amide functional groups. It was designed to combine the higher stability of DOTAM complexes with the pH-dependent relaxivity of DOTA complexes. Placing both amide and acetate groups on the same ligand also allows for a direct comparison of the affinity of each functional group for the metal center over a range of pH values. The synthesis of the ligand is based on work by Kovacs and Sherry, detailing a synthetic method to produce trans-disubstituted cyclen derivatives.\textsuperscript{21}

![Figure 1.9. Structure of the ligand DO2A2AM.](image-url)

It is expected that the complex will be more stable than the DOTA complex over a range of pH values, especially at low pH. In solution, it is expected that the complex will be six-coordinate with the amide side arms bound to the metal center and the acetates unbound. This prediction comes from the assertion that amides bind more strongly than acetate groups do, as well as the fact that at most physiologically relevant pH values, the acetate groups are
likely to be protonated as they are in the case of DOTA. This provides the possibility for prototropic exchange between the acetate groups and the bulk water as well as the possibility of expanded coordination to the ligand at higher pH where the acetate groups are deprotonated. The change in protonation states of the acetate groups is expected to provide a pH dependence to the solution structure and therefore relaxivity, while the amide groups will prevent the Mn$^{2+}$ from dissociating at low pH.

References


Chapter 2: Materials and Methods

2.1 Synthesis of DO2A t-buty1 ester

The synthesis of DO2A t-buty1 ester was performed according to the procedures published by Kovacs and Sherry. Benzyl chloroformate was used to protect the cyclen nitrogen atoms in the first step of the synthesis. The reaction was allowed to stir for an extra twelve hours after the chloroformate addition to increase product yield. Careful control of pH during protection also resulted in improved yields. After the addition of the t-buty1 acetate arms, the compound was dissolved in ether and filtered through a 2 cm pad of silica gel. This method produced the desired compound in comparable purity to the silica gel column procedure described in the literature. The reaction scheme, with experimental yields is shown below in Figure 2.1.

![Reaction Scheme](image)

Figure 2.1. Synthesis of DO2A2AM

2.2 Initial Experiments

The synthesis of the ligand DO2A2AM was first attempted using a similar procedure to that used to add the t-buty1 acetate groups in the synthesis of the di-tert-buty1 ester described above, heating the di-tert-buty1 ester compound with chloroacetamide and diisopropylethylamine in MeCN at 60°C overnight. This procedure did not yield any of the
desired product and instead produced a mixture of starting material. The synthesis was also attempted by adding the amide groups directly to the benzyl protected cyclen first instead of the acetate groups. This too, was unsuccessful, with the product giving a $^1$H NMR spectrum that could not be assigned. Ultimately, a procedure by Chalmers, et al. was adapted to produce the desired compound using the addition of chloroacetamide to the ester using $\text{K}_2\text{CO}_3$ as a base.\textsuperscript{2} The reaction was also successful using bromoacetamide.

### 2.3 Synthesis of 1,4,7,10-tetraazacyclododecane-1,7-bis(\textit{tert}-butyl acetate)-4,10-bis(acetamide)

0.2058 g ($5.15\times10^{-4}$ mol) DO2A-\textit{tert}-butyl ester was dissolved in 20 mL anhydrous MeCN. To this mixture was added 0.584g ($4.23\times10^{-3}$ mol) $\text{K}_2\text{CO}_3$, 0.1.82 g ($1.16\times10^{-3}$ mol) 2-chloroacetamide, and a catalytic amount of KI. The reaction was stirred at 95°C for eighteen hours. The solvent was removed by rotary evaporation and the solid residue was partitioned between chloroform and water. The organic layer was isolated and the chloroform was removed by rotary evaporation yielding 0.2403 g ($4.68\times10^{-3}$ mol) of an off-white solid powder. Yield 90.8%

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.67 (s, 2H, NH$_2$), 3.11 (s, 4H), 3.02 (s, 4H), 2.6 (m, 16H, NCH$_2$), 1.42 (s, 18H, \textit{t}-Bu)

### 2.4 Synthesis of 1,4,7,10-tetraazacyclododecane-1,7-bis(acetate)-4,10-bis(acetamide) (DO2A2AM)

0.2403 g ($4.68\times10^{-3}$ mol) 1,4,7,10-tetraazacyclododecane-1,7-bist(\textit{tert}-butyl acetate)-4,10-bis(acetamide) was stirred with 3 mL trifluoroacetic acid and 1mL dichloromethane at room temperature for five hours. The solvent was removed by rotary evaporation to yield a viscous orange oil. This oil was dissolved in a minimum of deionized water.
Purification: A column was prepared using Dowex 50X4-400 ion-exchange resin in 0.01 M HCl. The product, dissolved in water was loaded onto the column. The column was first eluted with approximately 200 mL deionized water, until the pH of the eluate was approximately 5-6. The column was then eluted with 0.05 M NH₃ and fractions were collected. The fractions collected as the pH of the eluate increased to 10 were collected and the solvent was removed by rotary evaporation to yield 0.1745 g (4.34x10⁻⁴ mol) of a clear, pale yellow-gold solid. Overall yield: 84%.

¹H NMR (300 MHz, D₂O) δ 3.82 (s, 4H, CH₂NH₂), 3.45 (s, 12H, CH₂COOH), 3.09 (m, 4H, NCH₂), 2.97 (m, 4H, NCH₂)

¹³C NMR (75MHz, D₂O) δ 178.3, 175.3, 170.0, 169.9, 56.5, 55.3, 51.3, 48.6, 48.3

IR cm⁻¹ (KBr pellet): 1676 (amide C=O stretch), 1630 (acetate C=O stretch)

ESI MS (50:50 MeOH:H₂O, m/z +): 202.19, 403.26, 425.3

IR cm⁻¹ (solution, D₂O): 1725, 1678, 1580

2.5 Synthesis of Mn(II)DO2A2AM•2HCl

0.030g (7.46x10⁻⁵ mol) of DO2A2AM was dissolved in a minimum amount of water, with 0.0148g (7.46x10⁻⁵ mol) MnCl₂•6H₂O. The mixture was allowed to sit for 3 hours and then was added dropwise, slowly into a test tube of acetone. A white precipitate formed immediately. The solution was centrifuged and the supernatant was decanted away from the solid to yield 0.0251 g (5.5x10⁻⁵ mol) of a white powder. Yield 74%.

The ligand was mixed with MnCl₂ in a 1:1 molar ratio in six drops of deionized water. Slow diffusion of acetone into this solution yielded very small needle-like crystals.

ESI MS (50:50 MeOH:H₂O, m/z +): 456 (MnHDO2A2AM⁺), 228 (MnH2DO2A2AM²⁺)

IR cm⁻¹ (KBr pellet): 1676 (amide C=O stretch), 1618 (acetate C=O stretch)

IR cm⁻¹ (solution, D₂O): 1736, 1693, 1593

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Anal. Calcd for $\text{C}_{16}\text{H}_{38}\text{O}_{10}\text{N}_{6}\text{MnCl}_{2}$: C 32.05; H 6.34; N 14.02 Found: C 32.21; H 6.33; N 14.01

2.6 Potentiometric Titrations – Solution Preparation

All titrations were performed using water freshly distilled from KMnO$_4$ solution. Titrations were carried out in a water-jacketed cell at 37 °C under nitrogen that had been bubbled through a solution of 0.1 M KNO$_3$. A 1 M KNO$_3$ solution was made with 10.1150 g (0.100 mol) KNO$_3$ that had been recrystallized in H$_2$O from a stock sample dissolved in 100 mL of distilled water.

2.7 Standardization of KOH

0.5884 g KOH was dissolved in 100 mL distilled water. The KOH solution was then titrated against a solution of 0.2122 g (0.00103 mol) KHP in 10 mL water. The amount of base added to reach the equivalence point was 11.4 mL and the concentration of the KOH solution was determined to be 0.0911 M.

2.8 Standardization of HNO$_3$

A solution was made using 0.6 mL concentrated HNO$_3$ in 100 mL H$_2$O. 10 mL of this solution was placed in a water-jacketed cell at 37°C under nitrogen. Previously standardized 0.0911 M KOH solution was added in 0.1 mL increments via a syringe. The pH was measured and recorded 10-15 seconds after each addition after the value had stabilized. 11.8 mL of base were required for the titration. The concentration of acid was determined to be 0.1011 M.

2.9 Potentiometric Titration of DO2A2AM

0.0406 g (1.0x10$^{-4}$ mol) of DO2A2AM was added to 10 mL H$_2$O to make a 0.01 M solution. The 10 mL of solution was added to 12.5 mL distilled H$_2$O and 2.5 mL 1.0 M KNO$_3$ in a water-jacketed cell at 37°C under nitrogen that had been bubbled through a 0.1 M KNO$_3$
solution. The initial pH was 6.06. 2.6mL of 0.1011M HNO₃ was added in 0.1mL increments using a syringe to achieve a pH of 2.00. Then, 5.7 mL 0.0911 M KOH solution was added in 0.1 mL increments using a syringe to give a final pH of 11.49. The pH was recorded after each KOH addition.

2.10 Potentiometric Titration of MnDO₂A₂AM

0.0596 g (9.9x10⁻⁵ mol) of MnDO₂A₂AM was added to 10 ml H₂O to make a 0.01 M solution. The 10 mL of solution was added to 12.5mL distilled H₂O and 2.5 mL 1.0M KNO₃ in a water-jacketed cell at 37°C under nitrogen that had been bubbled through a 0.1M KNO₃ solution. The initial pH was 2.53. 3.4 mL of 0.0911M KOH solution was added in 0.1mL increments using a syringe to give a final pH of 11.38. The pH was recorded after each KOH addition.

References

Chapter 3: Characterization of H$_2$DO2A2AM and Complexes

3.1 Characterization of H$_2$DO2A2AM

$^1$H NMR

The synthesis of the H$_2$DO2A2AM ligand was confirmed first by $^1$H NMR spectroscopy. The spectrum, shown in Figure 3.1 shows two side-arm methylene peaks, one for the acetate side arm and one for the amide side arms.

![Figure 3.1. 300MHz $^1$H NMR of DO2A2AM.](image)

The spectrum was taken using D$_2$O as a solvent which has been referenced to $\delta$4.75. The peak at $\delta$3.8 corresponds to the methylene protons of the acetate side arms. The signal at $\delta$3.4 appears as a broad singlet with a shoulder. This is due to the equivalence of some of the ring methylene protons with the methylene protons of the amide side arms. The remaining ring
methylene protons appear as a broad multiplet at 3.0ppm. The ring methylene protons can be differentiated as those on the carbons to the amide substituents and those on the carbons closest to the acetate substituents. Furthermore, there are differences between the faces of the ring, giving rise to the broad multiplets and splitting seen in the spectrum.

**13C NMR**

The $^{13}$C NMR spectrum was taken in D$_2$O. The spectrum was taken at a pD of 6.84. According to the speciation diagram for the ligand, shown in section 4.1, there are multiple species present at this pH. Both HDO2A2AM and H$_2$DO2A2AM are present. This could account for some of the extra peaks seen in the methylene region of the spectrum. Somewhat more likely however, is the possible presence of impurities, such as ligand molecules with only one amide side arm. These compounds would be harder to detect in the proton NMR, but could cause both extra methylene and extra carbonyl carbon signals in the $^{13}$C NMR as shown below.
Figure 3.2. $^{13}\text{C}$ NMR of DO2A2AM

ESI-Mass Spectrometry

The ESI mass spectrum of the DO2A2AM ligand was taken in 50:50 MeOH:H$_2$O with a drop of glacial acetic acid. The spectrum is shown below.

Figure 3.3. ESI-Mass Spectrum of DO2A2AM
The mass spectrum shows a peak at m/z=403, corresponding to H₃DO₂A₂AM⁺. Two ring nitrogen atoms and one acetate group are protonated giving rise to the complex as a monocation. The peak at m/z=202 corresponds to the fully protonated ligand, H₄DO₂A₂AM which is present as a dication, giving a peak at half the molecular weight of the ligand. The peak at m/z=425 corresponds to [NaH₂DO₂A₂AM]⁺.

**Solid State IR Spectroscopy**

The IR spectrum was analyzed using a KBr pellet. The most distinctive feature of the spectrum is the presence of two carbonyl peaks, one at 1676cm⁻¹ and the other at 1629cm⁻¹. The higher frequency band is consistent with the carbonyl of an amide group. The lower frequency band is consistent with the carbonyl stretch of a protonated acetate group.¹ There is also a broad peak at 3409cm⁻¹ due to the amide N-H stretch.

![Figure 3.4. KBr IR of DO₂A₂AM.](image)
**Solution IR Spectroscopy**

The solution IR of the ligand was analyzed over a pH range of 1-9. This was done by making two 10mM solutions of the ligand in D$_2$O. D$_2$O is used instead of H$_2$O to shift a strong vibrational mode from 1630cm$^{-1}$ to lower frequency away from the carbonyl region of the spectrum where ligand peaks appear. One solution was adjusted to pH 1 using DCl and the other adjusted to pH 9 using NaOD. The solutions were mixed in different ratios to produce the intermediate pH values. In the spectra, three peaks were of major interest: 1723cm$^{-1}$, 1680cm$^{-1}$, and 1580cm$^{-1}$. The 1680cm$^{-1}$ band is due to the carbonyl stretching frequency of the amide groups. The 1723cm$^{-1}$ and 1580cm$^{-1}$ bands are consistent with the carbonyl stretching frequencies of acetate groups seen in the H$_4$DOTA ligand. The higher frequency is due to a carboxylic acid carbonyl, while the lower frequency is due to a carboxylate carbonyl. Changes in peak intensity at these frequencies should correspond to changes in the protonation state of the acetate side arms. The peak at 1630cm$^{-1}$ visible in the spectra below has been shown to appear in many IR spectra taken in D$_2$O and comes from a mode of the small amount of H$_2$O that is present in the samples.\(^2\) Below pH 3, as shown in the spectra below, DO2A2AM has two major peaks in the carbonyl region of the spectrum: 1723cm$^{-1}$ and 1680cm$^{-1}$. At higher pH, the spectrum becomes dominated by the 1630cm$^{-1}$ peak. At low pH, the 1580 peak is absent from the spectrum as all acetates are protonated. At the highest pH in this study, the 1580cm$^{-1}$ peak, corresponding to the deprotonated acetate groups, is clearly seen.
An unexpected feature of these spectra is the apparent loss of the 1680cm⁻¹ peak at high pH. As the 1630cm⁻¹ peak grows in intensity, likely due to a change in proton content from pH adjustment, it possibly overshadows and overlaps the 1678cm⁻¹ amide carbonyl peak. Another potential explanation is that the amide carbonyl stretching frequency changes as the protonation state of the ligand changes. Above a pH of 3, the ligand is present mostly as H₂DO₂A₂AM, with two ring nitrogens protonated and both acetates deprotonated. When the acetate groups are deprotonated, they are good electron donating groups and make the ring nitrogen atoms to which they are attached slightly more basic. As a result, the two protonations of the ring occur on the nitrogen atoms with the deprotonated acetate groups.
This structure is shown below in Figure 3.6a and is also the structure seen in H₄DOTA. At a pH of below 3, the ligand is present as H₄DO₂A₂AM₃⁺. In this case, two ring nitrogens and two acetate groups are protonated. When the acetate groups are protonated, they lose some of their electron donating ability. The amide groups become the more electron donating side arms, making the ring nitrogens to which they are attached more basic and creating the structure shown in Figure 3.6b. This change structure could possibly cause a shift in the frequency of the amide carbonyl peak, allowing for greater overlap with the 1630cm⁻¹ water peak.

![Figure 3.6. a) Postulated H₂DO₂A₂AM solution structure at pH 4-8 b) Postulated H₄DO₂A₂AM₃⁺ solution structure at pH < 3.](image)

**ZnDO₂A₂AM ^1H NMR**

As both confirmation of ligand structure and an attempt to elucidate more structural information about metal complexes of DO₂A₂AM, the Zn(II) complex of the ligand was made by adding excess ZnCl₂ to a solution of the ligand in D₂O. The spectrum at neutral pH is included in the Appendix. While the splitting of the ring protons becomes more complicated upon the addition of zinc, the integrations of the side arm methylene protons and the ring protons are seen as two equal singlets, each with an integration of four, the expected
values for the ligand. The spectrum of the Zn complex was also taken at high and low pH as shown in Figure 3.7.

The acetate methylene peak furthest downfield undergoes the greatest change in chemical shift as a result of the change in pH due to its change in protonation state. There is less of an effect on the amide methylene protons as the amide groups are less affected by pH due to their coordination to the metal center. As in the free ligand, the ring protons are differentiated by their proximity to the acetate and amide side arms, as well as their axial or equatorial position in the ring. Since the addition of zinc locks the complex into a fixed conformation, interconversion between ring geometries is slowed enough to see greater inequivalence and splitting of the ring protons. At high pH, the splitting increases, perhaps due to increased rigidity from acetate interaction with the metal center.

Figure 3.7. a) ZnDO2A2AM spectrum at pH 8 b) Zn H2DO2A2AM spectrum at pH 2.
3.2 Characterization of MnH2DO2A2AM^{2+}

ESI Mass Spectrometry

The ESI mass spectrum of the MnH2DO2A2AM^{2+} complex was performed in a 1:1 mixture of MeOH:H2O with a drop of glacial acetic acid. The complex was observed as a cation with large peaks at m/z = 456 and 228.

![Figure 3.8. ESI Mass Spectrum of MnDO2A2AM.](image)

The peak at m/z = 456 corresponds to the complex with one Mn^{2+} ion and one of the acetate groups protonated, giving the complex an overall charge of +1. The peak at m/z = 228 corresponds to the complex with one Mn^{2+} ion and both of the acetate groups on the ligand protonated, giving the complex an overall 2+ charge. The peak at m/z = 478 represents the ligand with one deprotonated acetate group and the other acetate group coordinated to a Na^+ ion. These results are consistent with the predicted molecular weights and protonation states of the complex. The peak at m/z of 228 indicating that both acetates can be protonated with
Mn$^{2+}$ still present in the complex suggests that the coordination between the ligand and the metal occurs primarily through the amide side arms.

**Elemental Analysis**

The elemental analysis was performed by Robertson Microlit Laboratories. A CHN analysis was performed. The observed CHN percentages were 32.21, 6.33, and 14.01 respectively. This analysis was consistent with a molecular formula of Mn(H$_2$DO$_2$A2AM)Cl$_2$•4H$_2$O which gives calculated CHN percentages of 32.05, 6.34, and 14.04. The ligand has both acetate groups protonated with two chloride counter ions and four waters of hydration. The presence of chloride ions was confirmed by a positive silver nitrate test. The molecular formula supports a six-coordinate structure with both acetate arms protonated and the Mn(II) coordinated through the amide side-arms. This gives the complex a molecular mass of 600.35 g/mol, which was used in all stoichiometric calculations.

**Solid State IR Spectroscopy**

The solid state IR spectrum of the complex was obtained in a KBr pellet. Similarly to that of the ligand, the spectrum of the Mn(II) complex showed two carbonyl peaks. The amide peak was observed at 1676.21 cm$^{-1}$ and the acetate peak was observed at 1618.08 cm$^{-1}$. The peak for the amide carbonyl remains at the same frequency as the corresponding peak in the free ligand spectrum. This is consistent with trends seen in DOTAM complexes where the frequency of the amide peak is relatively unchanged by metal coordination.$^1$ The frequency of the acetate peak is shifted to slightly lower frequency likely due to its interaction with bound water and chloride ions in the solid state. This data also suggests coordination through the amide carbonyl groups.
Figure 3.9. Solid state (KBr) IR spectrum of MnDO2A2AM

Solution IR Spectroscopy

The solution IR spectra of the Mn(II) complex were obtained similarly to the spectra of the free ligand. The pH of the more basic solution was kept at just above 7 because no protonation changes were expected above that pH range, and if the pH gets too high (>10), Mn(II) will precipitate as Mn(OH)₂. Similarly to the free ligand, four peaks were observed in the spectra: 1736 cm⁻¹, 1693 cm⁻¹, and 1593 cm⁻¹. Peaks at 1736 cm⁻¹ and 1593 cm⁻¹ were assigned as the protonated and deprotonated acetate carbonyl stretching bands respectively. The peak at 1693 cm⁻¹ was assigned as the amide carbonyl stretching frequency. The 1693 cm⁻¹ peak is present at all pH values and can be attributed to the carbonyl of the amide
bound to the Mn(II) causing the shift of approximately 20 cm\(^{-1}\) from the free ligand. The peak at 1636/1646 cm\(^{-1}\) is attributed to residual H\(_2\)O stretching vibrations. Spectra are shown below in Figure 3.10.

![Spectra](image)

a.pD=1.9  

b.pD = 3.4  

c. pD=7.4

Figure 3.10. pH-dependent solution IR spectra for MnDO2A2AM

As expected, the acetate peaks show a change in protonation state from low to high pH while the amide peaks do not change drastically.

References


Chapter 4: Stability Constants and Speciation Diagram

4.1 DO2A2AM

Stability constants for both the ligand and the Mn complex were determined by entering the potentiometric titration data into the Hyperquad program. To obtain the stability constant, a model including all of the species present in the pH range of the titration must be chosen. Models are varied by including only certain protonation states based on hypotheses about the solution structure of the compound of interest. In prior work, Hilary Schrieber was able to determine the stability constants and speciation diagram for [MnH₄DOTA]⁺². Multiple models were used to fit the data, each using a different protonation scheme and some including free Mn²⁺. Ultimately, the final model, which is shown in Section 1.6, was chosen based on a step-wise protonation scheme. This model was consistent with other literature values for stability constants of the complex and fit well to the pH dependent solution IR and relaxivity data. A similar approach was used to determine the stability constants and speciation diagram for Mn₃DO2A2AM.

To obtain the protonation constants for the DO2A2AM ligand, the potentiometric titration data was fit using Hyperquad. First, the titration of the ligand was performed and the data was entered into the program. The fit in Hyperquad is shown below.
The model assumes that there are four possible protonation states of the ligand within the pH range of the titration data. The possible protonation sites occur on the two acetate groups and on two ring nitrogen atoms. The cyclen ring can only be protonated on two of the nitrogens because the high concentration of positive charge that would be generated from protonating all four nitrogen atoms is electronically very unfavorable. From this data, the protonation constants of the ligand were determined and shown below as logβ values in table 4.1. For each species, in the model, the program calculates a value for logβ where β is defined in equation 1.3 as the equilibrium constant for the protonation reaction. The pKₐ value of a species corresponds to the difference between the logβ of the species and that of the next lowest protonation state.
The protonation constants for the ligand are consistent with other values seen for [H₂DOTA]²⁻ and DOTAM. The first two protonations, which are predicted to occur on two of the ring nitrogen atoms similarly to DOTA have higher pKₐ values of 10.47 and 8.28, due to the basicity of the nitrogens. In DOTA the ring nitrogens have pKₐ values of 12.09 and 9.76. In DOTAM, the nitrogens have lower pKₐ values of 7.70 and 6.21. The values for DO₂A₂AM fall between these limiting values. The first protonation of an acetate group in DO₂A₂AM occurs at a pH of 3.55, which is similar to the pKₐ for the protonations of the acetate groups in DOTA. Due to the large fit error in the protonation constant for the fully protonated ligand, the pKₐ for the protonation of the second acetate group is not able to be determined, though it must be lower than 3.55.

Once these stability constants were determined, they were entered into the Hyss program to produce a speciation diagram for the DO₂A₂AM free ligand shown below in Figure 4.2. Around neutral pH, H₂DO₂A₂AM is the dominant species. Two ring nitrogen atoms are protonated which are attached to the deprotonated acetate groups. This creates a neutral ligand, with positively charged nitrogens and negatively charged acetates, similar to the structure seen in H₂DOTA at similar pH.
4.2 MnH₄DO₂A₂AM

The stability constants for the MnDO₂A₂AM complex were determined using Hyperquad to fit the titration data. Again, a stepwise protonation scheme was employed for the model similar to the MnDOTA complex. A relatively good fit was able to be obtained, producing low standard deviation in the logβ values. The stability constants, the model, and the fit are shown below.
Figure 4.3. Hyperquad fit of the MnDO2A2AM titration data. The diamonds are titration data points and the red dotted line is the fit. Blue points were included in the fit, while red points above pH of 9 were excluded. There is one very dominant species at high pH and the program cannot accurately fit the data.\textsuperscript{1, 4} The other colored lines represent the abundance of various species in the model.

<table>
<thead>
<tr>
<th>Species</th>
<th>Log$\beta$</th>
<th>pK$_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnDO2A2AM</td>
<td>18.65±0.0153</td>
<td></td>
</tr>
<tr>
<td>MnHDO2A2AM$^+$</td>
<td>21.86±0.0298</td>
<td>3.21</td>
</tr>
<tr>
<td>MnH$_2$DO2A2AM$^{2+}$</td>
<td>24.02±0.0272</td>
<td>2.16</td>
</tr>
<tr>
<td>HDO2A2AM</td>
<td>10.47</td>
<td></td>
</tr>
<tr>
<td>H$_2$DO2A2AM</td>
<td>18.75</td>
<td></td>
</tr>
<tr>
<td>H$_3$DO2A2AM</td>
<td>22.2</td>
<td></td>
</tr>
<tr>
<td>H$_4$DO2A2AM</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2. Stability constants and pK$_a$ values for the MnDO2A2AM complex. In the fits, the values of the free ligand protonation constants were fixed.
This model was chosen based on what is known about the structure of the MnDO2A2AM complex. It is assumed that when Mn$^{2+}$ is bound in the complex, the only possible protonation sites will be on the acetate groups since the amide groups are not easily protonated. The free ligand and all of its protonation states were included in the model to account for the possibility of protons out-competing Mn$^{2+}$ and causing the dissociation of the metal as seen in the MnDOTA complex at low pH.$^{4,5}$ The addition of a third proton to the MnDO2A2AM complex would have to occur on one of the ring nitrogens, thereby displacing the Mn$^{2+}$. This can only happen if the binding of the protons to these sites is stronger than the coordination of Mn$^{2+}$ and is accounted for by the inclusion of all the protonation states of the free ligand in the model.

Though the fit does not appear to be very good around pH 4-5, and could possibly be improved by including more species in the model, the model chosen included only the most chemically reasonable species. This model fit the data relatively well, giving minimal error in the log$\beta$ values. A model omitting the mono-protonated species of the complex was tried, but a good fit was not able to be obtained. The pK$_a$ values of the acetate groups are similar to those seen in the DOTA complexes and are slightly lower than those seen in the free ligand.

The speciation diagram generated using the Hyss program shown below in Figure 4.4 shows results that are consistent with the hypothesis that amides bind more strongly than acetates because at low pH, when the acetate groups are protonated, there is little free Mn$^{2+}$ in solution.
Above pH 5, the compound is fully deprotonated and no further changes in protonation state are observed. The pKₐ values for the acetate arms are both around 3-4 and those are the only sites that can accept a proton. At low pH, there is very little free Mn, which is due to the fact that the stability constants for all the protonation states of the metal-ligand complex are higher than the stability constants for the various protonation states of the free ligand. Even at low pH, the protonated metal complex is more stable than the protonated ligand. This is not true in the DOTA case, where the stability constants for the protonation of the free ligand are higher than the stability constants for the protonation of the metal-ligand complex. The amide groups contribute considerable stability and allow the complex to remain intact even at very low pH as seen in the case of MnDOTAM^{2+}. This is in contrast to the DOTA case, where the Mn^{2+} ion dissociates from the complex at low pH. Since the
coordination of the acetate groups is not necessary for complex formation, their protonation does not have a great effect on the stability of the complex.

Similarly to the free ligand, the solution IR data can also be used to confirm the validity of the speciation diagram. In the solution IR data, the peak for the protonated acetate groups decreases as the peak for the deprotonated acetate groups increases. This inflection happens between a pD of 2 and 3, which corresponds to the pK\textsubscript{a} values for the acetate groups and the change in speciation over this pH range seen in the speciation diagram.

![Figure 4.5. pH-dependent IR peak data for MnDO2A2AM.](image)

The speciation diagrams for both DO2A2AM and the Mn\textsuperscript{2+} complex are consistent with the pH-dependent solution IR spectra and follow the inferences made from the DOTA and DOTAM complexes.
Chapter 5: pH Dependent Relaxivity

5.1 $^1$H Longitudinal Relaxivity

The longitudinal proton relaxivity ($R_1$) of the MnDO2A2AM complex was determined using pH dependent $T_1$ measurements taken on a Bruker Minispec at 20 MHz and 37°C. The relaxivity was determined using equation 5.1 with 3.58 s as the measured value for the $T_1^0$ of water.

$$R_1 = \frac{1}{T_1^0} - \frac{1}{T_1} \frac{1}{[\text{MnDO2A2AM}]}$$  \hspace{1cm} (5.1)

When plotted as a function of pH, the $^1$H relaxivity shows a pH dependence as seen in Figure 5.1.

![Figure 5.1. pH-dependent $^1$H relaxivity of MnDO2A2AM](image)

Similarly to complexes of [MnH$_2$DOTA]$^{x-2}$, the relaxivity is highest below a pH of 3 and levels off at a value just below 3 mM$^{-1}$s$^{-1}$ at higher pH values. At a pH of just below 2,
the relaxivity increases to a maximum of 7.04 mM\(^{-1}\)s\(^{-1}\) which is above that of free Mn\(^{2+}\) in solution. To obtain a relaxivity value higher than what is possible with inner sphere water exchange of Mn\(^{2+}\), there must be an additional pathway of relaxation provided by the ligand. Since the speciation diagram shows that the Mn\(^{2+}\) is still bound to the ligand at low pH, the possibility exists for prototropic exchange. At low pH, the protons on protonated acetate groups can freely exchange with water.

### 5.2 \(^{17}\)O Transverse Relaxivity

To test the hypothesis of potential prototropic exchange, the pH dependent \(^{17}\)O transverse relaxivity, \(R_2\), of MnDO2A2AM was measured. The \(R_2\) of \(^{17}\)O must be measured since the \(^{17}\)O nucleus has a spin of 5/2 and therefore has a quadrupolar moment. The \(T_1\) of the \(^{17}\)O nucleus is so dominated by the quadrupolar relaxation pathways that it is unaffected by the concentration of Mn(II). The \(T_2\) however, is influenced by paramagnetic ions and produces a measureable \(R_2\) value for Mn(II) complexes.

Since \(^{17}\)O relaxation is solely dependent on water interactions with the metal, any differences between the \(^{17}\)O relaxivity and the \(^1\)H relaxivity arise from an alternative pathway for proton relaxation, namely prototropic exchange between exchangeable protons on the ligand and protons in the bulk water. The \(^{17}\)O measurements were taken with 0.1mM solutions of the MnDO2A2AM complex in D\(_2\)O. The pH was adjusted using NaOD and DCl solutions. Measurements were taken with a 400 MHz varian NMR spectrometer at room temperature. The data below is presented as ratios of the \(^1\)H and \(^{17}\)O relaxivity of MnDO2A2AM to the \(^1\)H and \(^{17}\)O relaxivity, respectively, of free Mn\(^{2+}\). The data is reported as a ratio to negate the difference in scale between \(R_1\) and \(R_2\) measurements. \(R_2\) relaxivities are three orders of magnitude larger than \(R_1\) values, so normalizing the data to free Mn\(^{2+}\) allows for a more direct comparison of the data.
The plot of $^{17}$O relaxivity shows a similar trend to the $^1$H relaxivity in that it is higher at low pH and lower at high pH. The crucial difference however, comes when the data is compared to the value for free Mn$^{2+}$. The $^1$H relaxivity is higher than that of free Mn$^{2+}$ (ratio >1) at pH values near 2 while the $^{17}$O relaxivity never exceeds that of free Mn$^{2+}$. This data suggests that the increase in proton relaxivity above that of the free metal ion is due to prototropic exchange from the ligand and is not from mechanisms involving the exchange of water.

There is also evidence for prototropic exchange at high pH since the relative proton relaxivity is much greater than the relative $^{17}$O relaxivity. The relative $^{17}$O relaxivity is very low at high pH indicating that the complex may adopt a higher coordination number at high pH, with one or both of the acetates coordinating to the metal. To account for the still
relatively high proton exchange at high pH, it is also possible that the deprotonated acetates serve to attract protons towards the metal center and facilitate an increase in transient water binding or outer sphere relaxation.

5.3 Comparison to DOTA and DOTAM

**MnDOTAM**

The most obvious difference between the pH dependent relaxivities of MnDOTAM and MnDO2A2AM is that the relaxivity of the mixed ligand shows a pH dependence, while that of the DOTAM complex does not, Figure 5.3.

![Figure 5.3. Comparison of relative $^1$H and $^{17}$O values for MnDO2A2AM and MnDOTAM$^{2+}$](image)

The next difference is that the proton relaxivity of the MnDO2A2AM complex at high pH is higher than the proton relaxivity of MnDOTAM$^{2+}$ though the $^{17}$O relaxivities at high pH are similar. For DO2A2AM at high pH, the deprotonated acetates can potentially coordinate and block water access to the metal center causing the decrease in the $^{17}$O relaxivity. The $^1$H relaxivity does not decrease as drastically because of the possibility of prototropic exchange between the ligand and bulk water. The acetate groups for DO2A2AM contribute significantly to this pathway as it is not seen in the DOTAM case which only has
exchangeable amide protons. A potential mechanism of prototropic exchange is base catalyzed exchange of amide protons at high pH.

**MnDOTA**

Both the $^1$H and the $^{17}$O relaxivity profiles for MnDO2A2AM closely mirror those seen in MnDOTA.

![Graphs showing comparison of relative $^1$H and $^{17}$O values for MnDO2A2AM and MnH$_x$DOTAM$_y^{2-}$](image)

**Figure 5.4. Comparison of relative $^1$H and $^{17}$O values for MnDO2A2AM and MnH$_x$DOTAM$_y^{2-}$.

The major difference is that at low pH, the relative proton relaxivity for MnDO2A2AM is higher than the relative $^{17}$O relaxivity. This is due to prototropic exchange between the amide groups, protonated acetate groups and bulk water which does not happen in the MnDOTA complex. In fact, the mechanisms of proton relaxation for MnDOTA and MnDO2A2AM are very different at low pH.

For MnDOTA, the decrease in relaxivity from low to high pH is the result of complex formation as evidenced by the speciation diagram. Below pH 2, the ligand becomes fully protonated and is displaced from the Mn$^{2+}$. As expected, below pH 2, the relaxivity for both proton and $^{17}$O is the same as that of free Mn$^{2+}$ in solution.
The proton and $^{17}$O relaxivities of MnDO2A2AM differ at low pH, providing evidence that the compound does not fall apart like MnDOTA. This assertion is further supported by the speciation diagram.

![speciation and $^1H$ relaxivity plots for MnDO2A and MnH$_x$DOTAM$^{x-2}$](image)

**Figure 5.5.** Speciation and $^1H$ relaxivity plots for MnDO2A and MnH$_x$DOTAM$^{x-2}$.

When the relaxivity is overlaid with the speciation diagram it becomes clear that the pH dependence of the relaxivity for MnDO2A2AM can be attributed to a change in protonation state of the acetate side arms. The correlation between the decrease in relaxivity and the increase in concentration of the fully deprotonated MnDO2A2AM species supports the hypothesis that an increase in coordination number due to the binding of deprotonated acetate arms is restricting water access to the metal center and therefore decreasing the relaxivity. At lower pH, once the monoprotonated species becomes more dominant, the relaxivity begins to increase. The relaxivity increases even further as pH is lowered due to
increased prototropic exchange of the protonated acetate groups and the decreased ability of the acetate groups to bind to the metal center.

From these data, it is shown that at low pH, the metal is still bound to the ligand, and the complex is in the fully protonated form. This allows for the most direct access of water to the metal center and accounts for the high relaxivity of the complex seen at low pH. Evidence for prototropic exchange at low pH comes from the fact that the proton relaxivity is higher than the $^{17}$O relaxivity at those pH values. This prototropic exchange allows the relaxivity of the complex to equal and possibly exceed that of free Mn$^{2+}$. The protonated acetate groups can readily exchange protons with the bulk water possibly providing this extra mechanism of relaxation. In addition, water molecules that are bound to the Mn$^{2+}$ center can exchange protons with the bulk water. If this exchange happens faster than water exchange it can also account for the enhanced proton relaxivity at low pH.

The decrease in both proton and $^{17}$O relaxivity, which occurs as pH is increased, closely correlates with the increase in the prevalence of the fully deprotonated complex. Because both the relaxivities are decreasing, it is likely that this decrease is caused by a change in water access to the metal center. In the mono-protonated and fully deprotonated forms, it is possible that the complex adopts a higher coordination number. It is likely 7-coordinate because the relaxivities at high pH are still higher than they are for the 8-coordinate MnDOTAM$^{2+}$ complex. The proton relaxivity at high pH is also higher than the $^{17}$O relaxivity suggesting that the acetate groups (now deprotonated) could still be playing a role in a prototropic exchange mechanism.


Conclusion

The mixed functional group ligand, H₂DO₂A₂AM was successfully synthesized. Both proton and HSQC NMR show the expected peak signals for the free ligand, showing two separate side-arm methylene signals. Solid state IR confirmed the presence of two different carbonyl groups with two distinct peaks in the spectrum. The speciation diagram was able to be obtained from a potentiometric titration. The $pK_a$ data obtained were similar to the values seen for other compounds and correlated well with solution IR data.

The Mn(II) complex of DO₂A₂AM was also successfully synthesized and isolated in the solid form as a chloride salt. It was characterized by shifts in the IR peak frequencies from those seen in the free ligand as well as by ESI mass spectrometry and elemental analysis. The speciation diagram was obtained using potentiometric titration data. The $pK_a$ values and stability constants were comparable to those seen in the DOTA and DOTAM complexes. The speciation diagram was confirmed by its comparison to pH-dependent solution IR data, which showed protonation and deprotonation of the acetate groups over the expected pH ranges. The most notable feature was that at low pH, there was very little free Mn(II) in solution unlike in acidic solutions of MnH₂DOTA.

$^1$H and $^{17}$O pH-dependent relaxivity studies were also performed. Both the $^1$H $R_1$ and the $^{17}$O $R_2$ showed pH dependences similar to H₂DOTA, however, at low pH, the $^1$H $R_1$ appears to exceed that of free Mn(II) unlike the MnH₂DOTA case where the relaxivity at low pH is caused solely by free Mn(II). At low pH there is also a difference between the $^{17}$O and $^1$H relaxivities that suggests a prototropic exchange mechanism of relaxation in addition to water exchange. This difference, and therefore potential prototropic exchange, is also seen at high pH. The decrease in relaxivities as pH increases follows from a change in protonation
state of the ligand. It is not due simply to complex formation, but to a possible change in coordination number that can occur when the acetates become deprotonated at higher pH.

The Mn(II)DO2A2AM complex successfully combined the higher stability seen in DOTAM complexes with the pH dependent properties seen in DOTA complexes. The majority of the ligand remained bound to Mn(II) even at low pH and produced a pH-dependent relaxivity profile. This complex demonstrates the potential for rational design of MRI contrast agents with specific applications, properties, and stabilities through the integration of different functional groups into the same molecule.
Appendix: $^1$H NMR of ZnDO2A2AM in D$_2$O.