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The Synthesis and Biological Activity of Iron Binding Motifs

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The Synthesis and Biological Activity of Iron Binding Motifs

An Honors Thesis submitted in partial fulfillment of the requirements for Honors in Chemistry.

By Rachel Burke

Under the mentorship of Dr. Michele McGibony

Abstract

Iron is an essential mineral that plays a key role in the oxygen transporting molecule hemoglobin. Iron also helps muscles store and use oxygen. Excess iron in the body can attract electrons, creating harmful oxygen radicals. Iron overload can be caused by several factors such as genetics, age, gender, and having received multiple red blood cell transfusions. Excess iron can lead to many diseases, such as arthritis, liver cirrhosis, heart disease, and several forms of cancer. Iron-chelating therapies work by binding free iron in the bloodstream and by reducing the amount of iron bound in transferrin, the iron transporting molecule. Current iron therapies are time-consuming, painful, and costly. New, more effective chelation therapies are being researched.

The first step in this research is to synthetically produce iron binding motifs modeled after the naturally occurring iron binding protein, adenochrome. The goals in the synthesis are to produce a chelator with increased water solubility and a higher affinity for chelation of iron. The next step is to test the effectiveness of the synthesized chelators to bind free iron, as well as other first row transition elements, such as chromium, copper, nickel, and aluminum. Evidence has shown that accumulation of aluminum in the body may be involved in the formation of senile plaques, which occur in the brains of patients with Alzheimer's disease, and is therefore a suspect in the initial cause of the disease. The binding of aluminum could be beneficial in the therapy of early Alzheimer's patients.

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Introduction

Iron is an essential metal ion for animals, plants, and bacteria, and it is one of the most common metals in the environment. While iron is important in many metabolic processes in the body, the most commonly known is the role of iron in the transport of O_2 and other molecules in the bloodstream. The heme group embedded in myoglobin and the subunits of hemoglobin binds these molecules for transport in the bloodstream. Additionally, iron is involved in many other biological processes such as the transfer of electrons in cytochrome P-450, cytochrome proteins in the electron transport chain, and methane monooxygenase, an oxidoreductase enzyme found in methanotrophic bacteria.

Iron can serve a wide variety of purposes mainly because it is so versatile. This is due to the fact that iron is stable at two different oxidation states, Ferric iron at 3+, and Ferrous iron at 2+. Iron possesses unfilled *d* orbitals, allowing it to undergo Fenton chemistry, reactions involving the change in oxidation states[1]. These reactions will generate several reactive species including the superoxide radical, the hydroxyl radical, and the hydroperoxyl radical as demonstrated in the reactions below. While iron is very

important in many metabolic processes, too much can do more harm than good when these reactive species are in high concentrations.

Reaction #1: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^-$

Reaction #2 $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO\bullet + H^+$

In the United States, diseases arising from the body's mismanagement of iron levels in the blood stream affect over 3 million people. Some examples of these health conditions include rheumatoid arthritis, certain cancers, and, the target of this research, hemochromatosis. Hemochromatosis is the most common genetic disease in the U.S.A. according to the U.S. Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia. Patients with hemochromatosis may experience arthritis-like pain, cirrhosis or cancer of the liver, diabetes, chronic fatigue, infertility, and sometimes stroke. Approximately 1.5 million Americans are carriers of the double gene responsible for fully developed hemochromatosis[5]. Iron overload as a condition can accelerate neurodegenerative diseases, such as Alzheimer', early-onset Parkinson's, Huntington's disease, epilepsy, and multiple sclerosis[1].

The treatment options for hemochromatosis are limited and include phlebotomy and iron chelation therapies. Phlebotomy is the regular removal of blood, and can only be done if patients' hemoglobin levels are sufficient. Currently, the only viable drug for iron chelation is Desferrioxamine B (DFO, Desferal®)(Figure 1). This drug works by chelating (binding) iron and enhancing its elimination by the body via the urine. This molecule is naturally occurring as it is a bacterial siderophore produced by the Actinobacteria *Streptomyces pilosus* and contains one of the most stable ferric iron binding motifs ever identified.

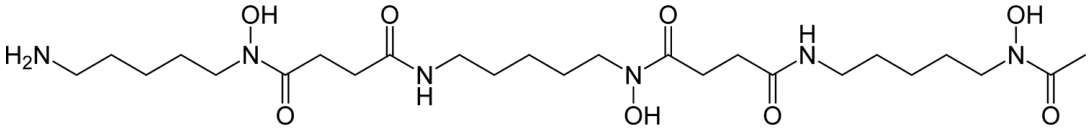


Figure 1: Desferrioxamine B

While this drug works well for iron chelation, the therapy is very costly and painful, because treatment is administered intravenously, in a dialysis-like fashion[1]. Recent research in the area of oral iron-chelation therapy centers on the synthesis of chelation agents which contain hydroxamate and/or catechol ligands, such as myxochelines, which mimic natural siderophores (Figure 2). Desferrioximane E is one such hydroxamate ligane molecule (Figure 3). These efforts have produced effective iron chelators but none that have better binding affinity than Desferal[11].

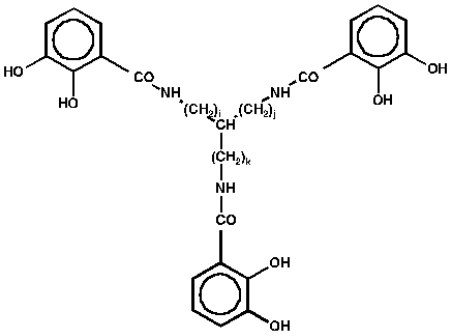


Figure 2: Myxochelines, a catechol ligand molecule

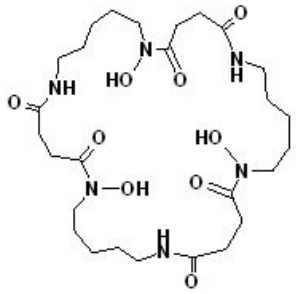


Figure 3: Desferrioximane E, a hydroxamate ligand molecule

The most recent work has focused on the TRENSOX drugs. Both N-TRENSOX and O-TRENSOX (Figure 4.) have shown promising results in iron binding (both ferric and ferrous iron) and water solubility studies, but, they only work in systems with very low pH. With its current design, TRENSOX would be completely ineffective in a system

with physiological pH. O-Trensox has been shown in vitro to remove iron from ferritin at pH of 4 and mobilize hepatic iron in rats in similar amounts to Desferal. O-Trensox was found to be more effective the Desferal in quenching hydroxyl radical in an acellular system[6].

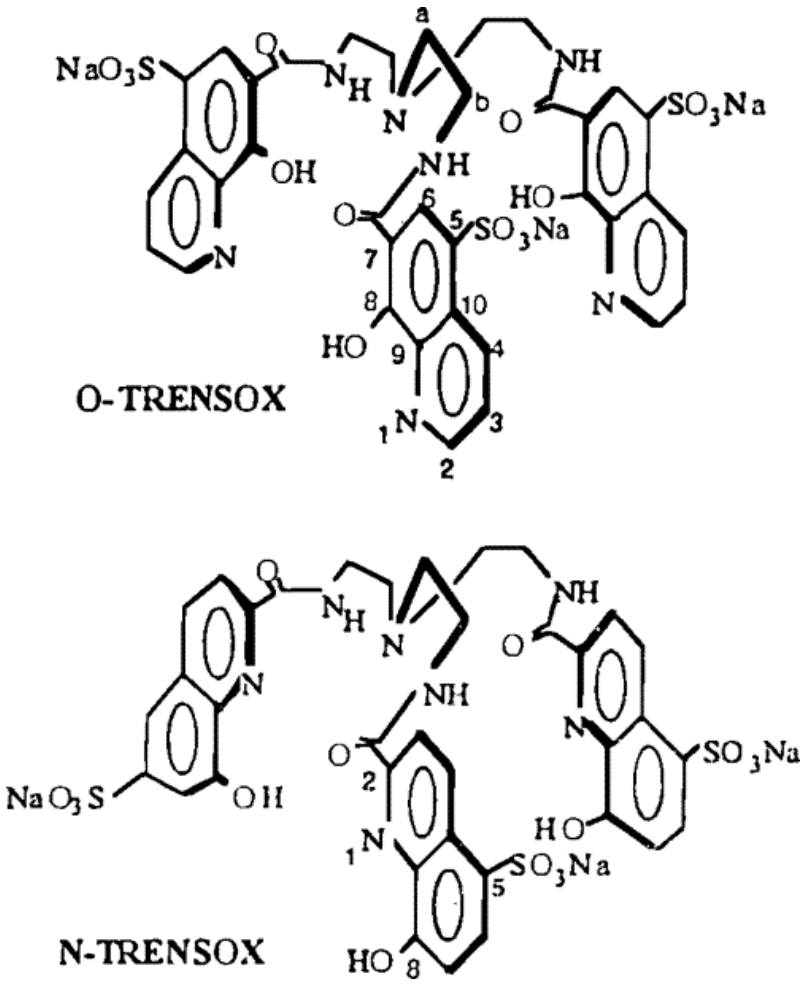


Figure 4. The structure of O-TRENSOX and N-Trensox[7].

The goal of this research is to create new binding motifs that will be stable, water soluble, and effective at biological pH. To accomplish these tasks, the quinoline units (Figure 5) of TRENSOX amines were replaced with electron-rich indole units (Figure 6) in order to reduce total carbon amount and increase solubility.

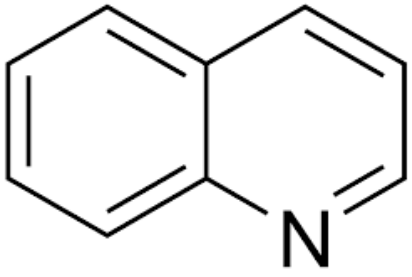


Figure 5. Structure of Quinoline[8].

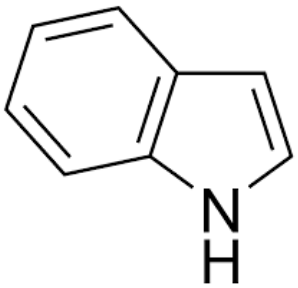


Figure 6. Structure of Indole[9].

The proposed synthetic mimics are Tris-Indole and Tris-Indole 3-Carboxylic Acid.

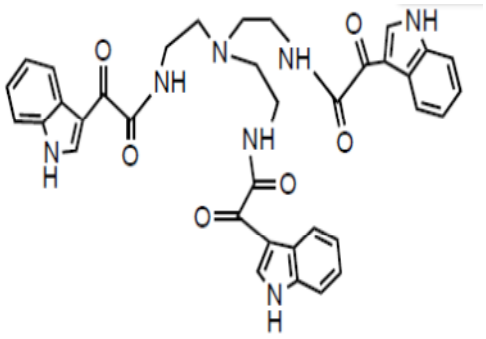


Figure 7: Tris-Indole

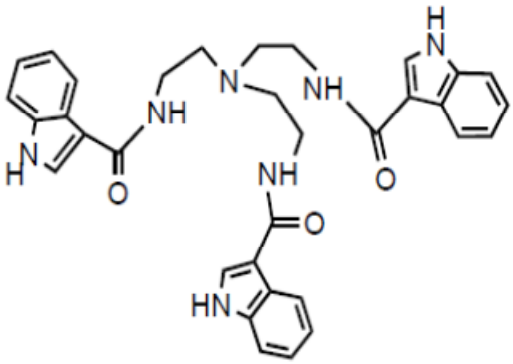


Figure 8: Tris-Indole 3-Carboxylic Acid

Both compounds present in a claw shape (Figure 9) very similar to N and O-Trensox. This tripodal shape puts the oxygens in the optimal position for iron coordination. The proposed synthetic biomimics will first be synthesized in our collaborator Dr. Christine Whitlock's laboratory, then thermodynamic and kinetic studies which will determine the binding strength and stability of the metal-protein complex with iron and other relevant first-row transition elements such as aluminum, cobalt, copper.

The studies will determine the possibility of iron binding motifs among first row transition metals by comparing complexed iron. After synthesis, NMR and Mass spectrometry will be utilized to confirm the identity of the proposed drugs.

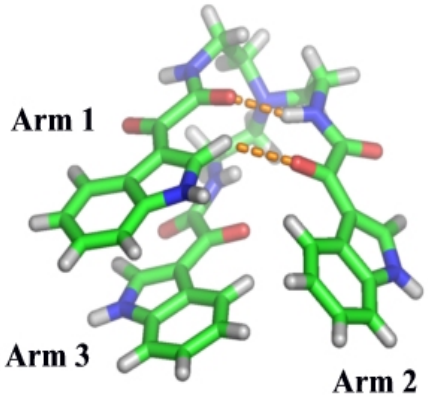


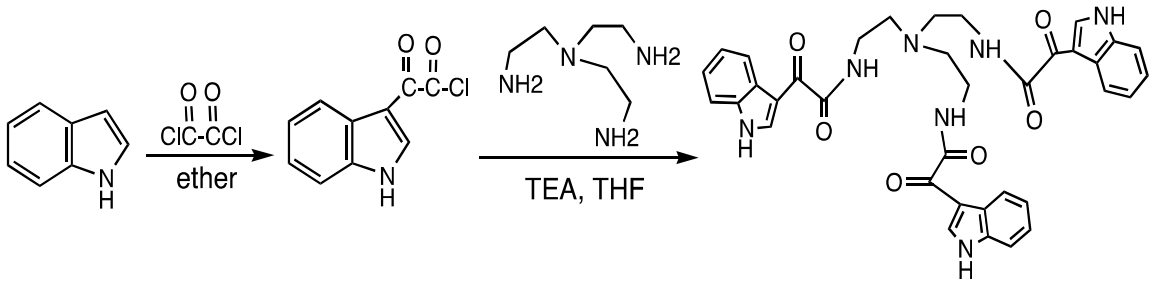
Figure 9. Claw shape of tripodal compound.

Materials and Methods

Synthesis of Iron Binding Compounds

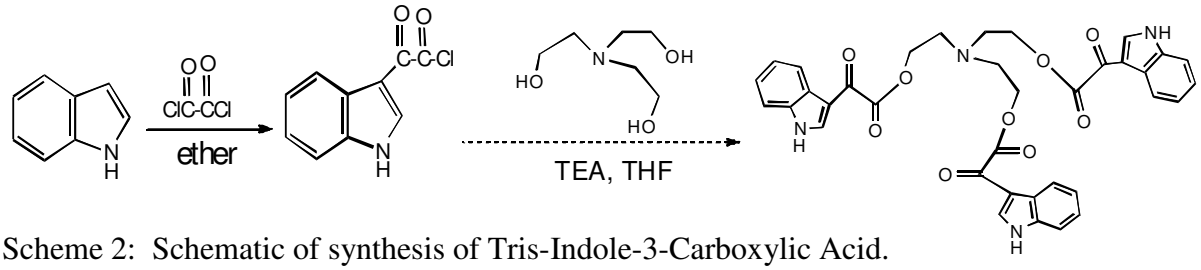
To prepare the first derivative, Tris(2-[indole-3-glyoxylamido]ethyl)amine. To indole (10.07 g, 86.1 mmol) in ether (200 mL) was added dropwise oxalyl choride (14.6 g, 114.6 mmol) over 15 mins. After stirring for 30 min, the solution was filtered to yield glyoxalyl chloride as a yellow solid, which was rapidly dissolved in Tetrahydrofuran (THF) (50 mL). To the glyoxalyl in THF at 0°C was added a solution of tris(2-aminoethyl)amine (TREN) (6.14 g, 42.0 mmol) and triethylamine (42.3 mmol) in THF (10 mL). After stirring at 0°C for 1 hour, the solution was filtered and the filtrate was evaporated to yield the Tris-Indole as a beige solid which crystallized from MeOH

(Scheme 1). Yield 5.93 g (31.4%); mp > 250°C. 1 H-NMR (250 MHz, DMSO-d6) 8.75 (s, 3H, H2), 8.65 (t, J=5.29 Hz, 3H, H1), 8.14 (d, J=6.9 Hz, 3H, H4), 7.47 (d, J=7.1 Hz, 3H, H7), 7.22 (t, J=7.0 Hz, 3H, H5 or H6), 7.16 (t, J=7.0 Hz, 3H, H6 or H5), 3.31 (m, 6H, Ha), 2.71 (bt, 6H, Hb); MS (M+1)+ = 660 for C36H33N7O6[10].



Scheme 1: Schematic of synthesis of Tris-Indole.

Having established success in the preparation of the first derivative, a related tripodal amine with one carbonyl unit per arm was also prepared by a modification of the previously stated procedure, Tris(2-[indole-3-amido]ethyl)amine. Indole-3-carboxylic acid (0.56 g, 3.4 mmol) dissolved in SOCl2 (6 mL) was stirred at 0°C. After 1.5 hours, the solution was rotary evaporated, and to the resulting oil was added TREN (0.62 g, 4.2 mmol) and triethanolamine (0.46 g, 4.6 mmol) in THF (25 mL). After stirring at 0°C for 30 min, the solution was vacuum filtered to yield the Tris-Indole-3-Carboxylic Acid as a beige solid which was recrystallized from MeOH (Scheme 2). Yield 0.28 g (43.0%); mp > 250°C. 1 H-NMR (250 MHz, DMSO-d6) 7.98 (s, 3H, H2), 7.44 (m, 3H, H1), 7.17 (m, 3H, H4), 7.15 (d, 3H, H7), 7.14 (m, 3H, H5 or H6), 7.12 (m, 3H, H6 or H5), 3.40 (m, 12H, Ha and Hb); MS (M+1)+ = 576 for C33H33N7O3[10].



Scheme 2: Schematic of synthesis of Tris-Indole-3-Carboxylic Acid.

Biological Activity in Solution

Tris-Indole or Tris-Indole 3-Carboxylic acid was added to stock iron nitrate solution (50 mg/mL) $Fe(NO_3)_3$ in a 1:1 and 1:2 ratio. Similarly, the synthetic mimics were combined with stock metal chloride solutions (100 mg/mL) each of cobalt chloride and chromium chloride. Each sample was incubated for 2 hours and analyzed every 30 minutes via UV-Visible spectrophotometry (UV-1601 Shimadzu).

Biological Activity with partially loaded Human Transferrin

Tris-Indole or Tris-Indole 3-Carboxylic acid was added to human transferrin (partially loaded from Sigma). The synthetic compounds were also added to transferrin and stock (50 mg/mL) $Fe(NO_3)_3$ in a 1:1 and 1:2 ratio. Each sample was incubated for 2 hours and analyzed every 30 minutes via UV-Visible spectrophotometry (UV-1601 Shimadzu). Each sample was chromatographed (bed volume ~15 mL) using Sephadex G-10, and fractions were analyzed for both the drug and iron. The iron-containing fractions were pooled and concentrated via lyophilization. During this process the samples were dissolved in dH_2O in order to ensure removal all of salt ions.

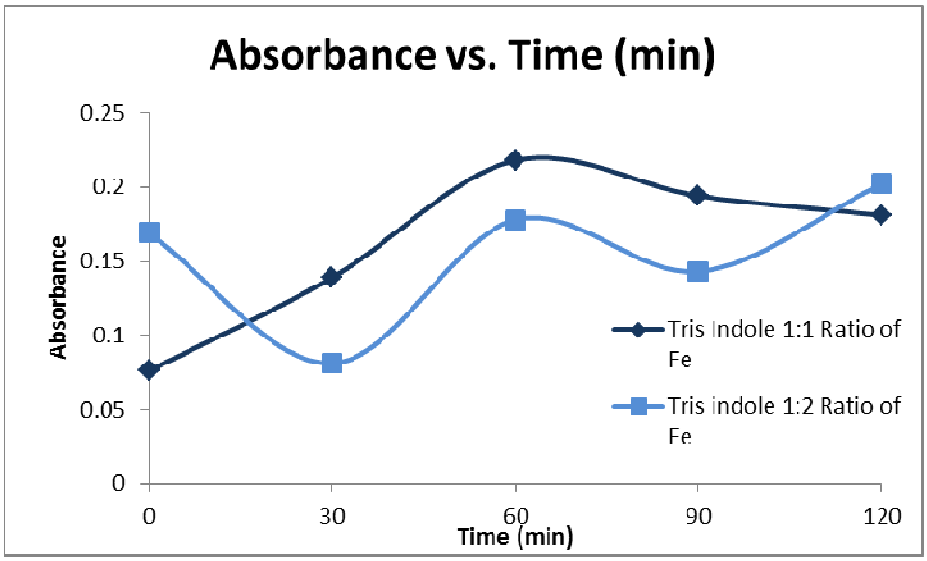
Miscellaneous

H-NMR spectra were obtained using a Bruker 250 MHz multi- nuclear spectrometer. MS were measured using a Shimadzu QP 5050A instrument. Melting points are uncorrected. All concentrated samples were analyzed via UV-Visible spectroscopy (UV-1601 Shimadzu) and MALDI-ToF (Brucker Microflex) in order to determine extent of iron binding. For all experiments either Trizma/Tris (50 mM, pH = 7.42) or citrate (50 mM, pH = 7.47) buffers were utilized. Synthetic compounds were dissolved in a minimum of DMSO.

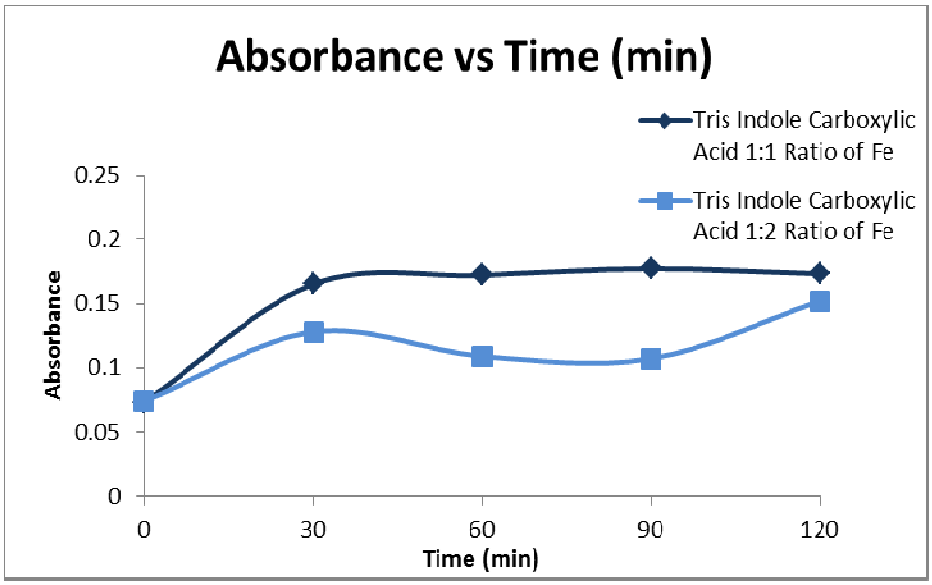
Results and Discussion

Table 1. Percent yields of the synthetic mimics.

Synthetic Mimic	Yield
Tris-Indole	31.4%
Tris-Indole-3-Carboxylic Acid	43.0%



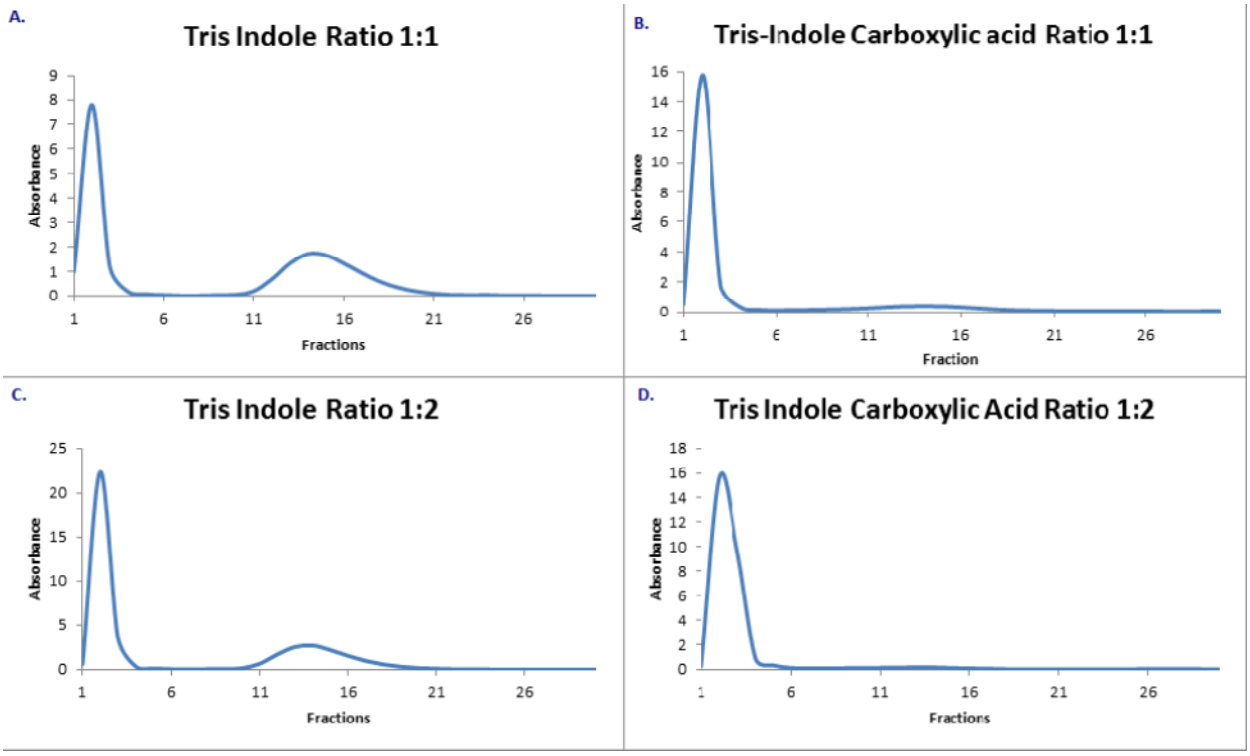
Graph 1. Tris-Indole with Iron at 475 nm[3].



Graph 2. Tris-Indole 3-Carboxylic Acid with Iron at 475 nm[3].

From the two graphs, see that Tris-Indole more successfully bound iron. Tris-Indole was incubated with $Fe(NO_3)_3$ and the absorbance at 475 nm increased reaching a maximum indicated that within 1 hour iron was bound to this molecule as indicated in

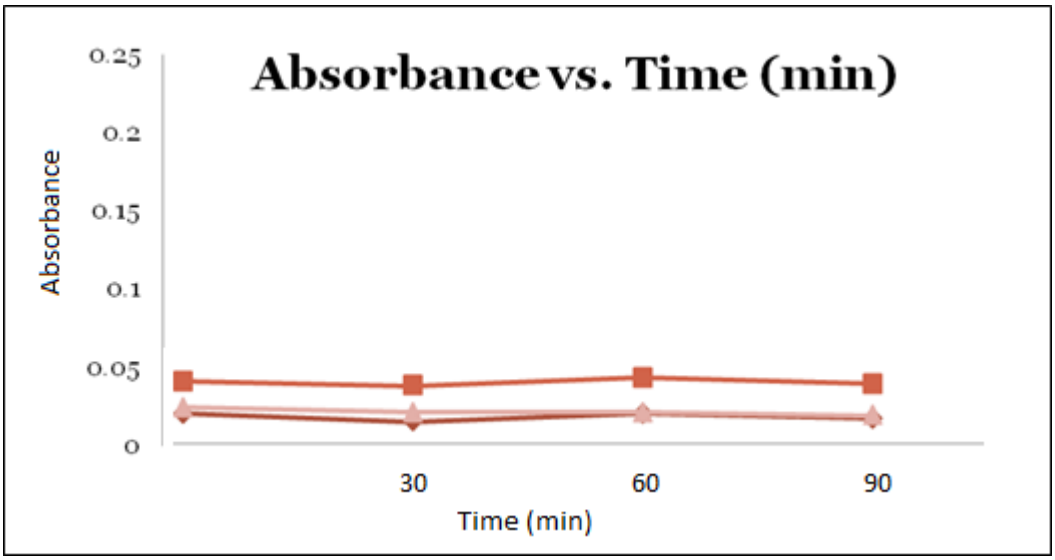
graph 1. Increasing the ratio from 1:1 to 1:2 had no bearing on the time or amount of iron bound to the complex. Tris-Indole-3-Carboxylic Acid was incubated with $Fe(NO_3)_3$ and the absorbance at 475 nm increased, reaching a maximum at 30 minutes for both a 1:1 and 1:2 ratio. Altering the ratio from 1:1 to 1:2 had no effect on the time or amount of iron bound to the complex.



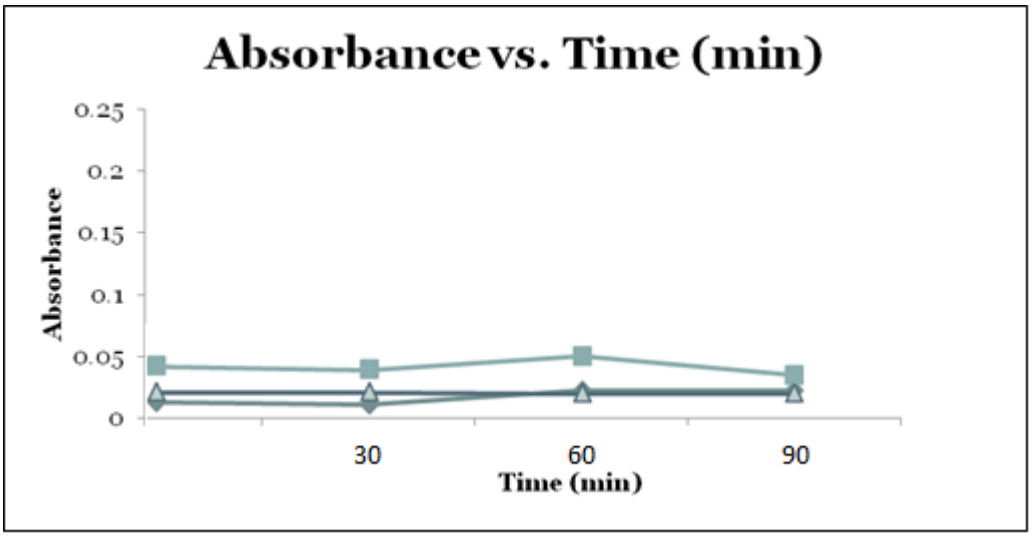
Graph 3. Elution profiles of both Tris-Indole and Tris-Indole Carboxylic Acid in iron solution[3].

The middle peak in each of the elution profiles indicated the complexed iron. The Tris Indole in both 1:1 and 1:2 ratios had visible peaks, indicating the presence of the complexed iron (Graphs A, C). The Tris-Indole Carboxylic Acid with a 1:1 ratio (Graph B) has a minor peak, while the 1:2 ratio of the Tris-Indole Carboxylic Acid has little to no

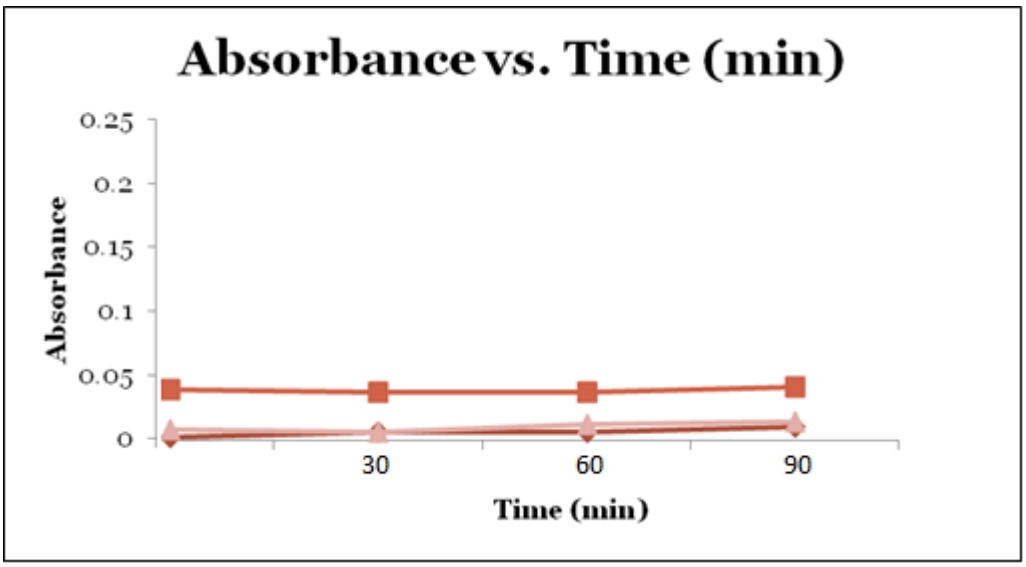
peak. The elution profiles indicate that the Tris-Indole more successfully bound the iron than did the Tris-Indole Carboxylic Acid.



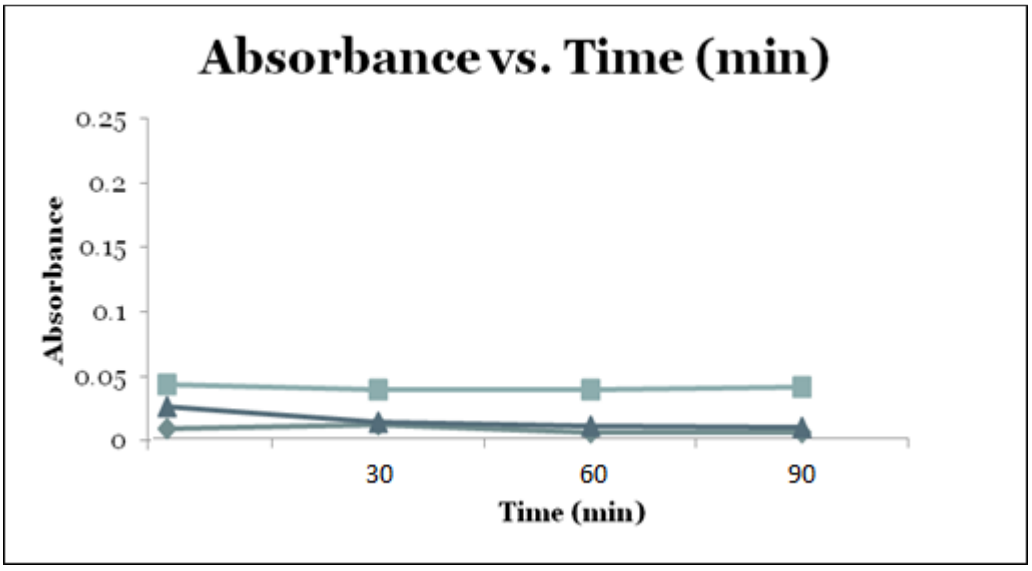
Graph 4. Tris-Indole with Cobalt Chloride at 475 nm.



Graph 5. Tris-Indole 3-Carboxylic Acid with Cobalt Chloride at 475 nm.



Graph 6. Tris-Indole with Chromium Chloride at 580 nm.



Graph 7. Tris-Indole 3-Carboxylic Acid with Chromium Chloride at 580 nm.

The graphs of the absorbance at the specific wavelengths for Cobalt and Chromium show that the drugs had little to no binding of the free iron. Over the 90 minute period, none of the samples had more than a 0.05 absorbance at the corresponding wavelengths for the bound metal ions (Cobalt and Chromium).

Conclusion

The synthesis of Tris-Indole and Tris-Indole-3-Carboxylic Acid was successful, with yields of 31.4% and 43.0%, respectively. There structures were confirmed via melting point, NMR, and MALDI-ToF. From the absorbance graphs, it can be seen that that Tris-Indole derivative more successfully bound the free iron than did the Tris-Indole-3-Carboxylic Acid. Maldi-Tof mass spec confirmed the total weight of the complex with the bound iron, indicating complexation of the complex with iron in the ferric oxidation state. While the absorbance graphs of the drugs with the cobalt and chromium no binding, alterations to the experimental procedure will be made in an attempt to increase binding.

Future Work

Aluminum is ubiquitous in the environment. About ninety-five percent of aluminum is bound to transferrin and albumin intravascularly and is eliminated renally, but it can also exist as a free ion in the bloodstream, in small quantities[13]. Aluminum is a widely recognized neurotoxin that inhibits more than 200 biologically important functions and causes various adverse effects in plants, animals, and humans, when it occurs in toxic levels. Such effects include encephalopathy, osteomalacia, aplastic bone disease, proximal myopathy, increased risk of infection, and decreased myocardial function[12].

Like iron, aluminum is another metal ion associated with the acceleration of neurodegenerative diseases. Studies have shown increased levels of aluminum in the brains of patients with Alzheimer's disease. One possible connection between aluminum and the progression of Alzheimer's disease is the presence of concentrated aluminum in the extracellular senile plaques[12]. It has been hypothesized and debated that elevated

levels of aluminum in brain tissue could be linked to the progression of Alzheimer's disease.

Though aluminum is not a transition metal, we postulated that it may fall under the same binding motif as iron. Due to the fact that aluminum is not a transition metal, the binding cannot be observed using the same methods as the other compounds tested. Atomic absorption spectrometry will be used to determine the binding ability of the synthetic mimics toward aluminum.

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