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Synthesis and Characterization of a Biometric Model of the Tricopper Binding Site of Multicopper Oxidases

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Synthesis and Characterization of A Biomimetic Model of the Tricopper Binding Site of Multicopper Oxidases

AN HONORS THESIS

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by

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PROJECT TITLE: The Synthesis and Characterization of Biomimetic Model of Multi-Copper Active Sites
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Introduction

Multicopper Oxidases

Since this project is to mimic multicopper oxidases, it is worthwhile to briefly explain what they are and what they do. Multi-copper oxidases are a class of copper containing enzymes in organisms that catalyze the oxidation of substrates and the four-electron reduction of oxygen molecule to water. Some examples include laccase, ceruloplasmin, and ascorbate oxidase. These proteins usually contain multiple copper ligands, hence the name multicopper. Of the copper centers in the multicopper oxidases, three types could be found: type 1(T1Cu), type 2(T2Cu), and type 3(T3Cu). One T2 and one T3 copper centers form a trinuclear center that can bind dioxygen molecule, and the T1 copper center near the tricopper center transfers electron to dioxygen, reducing it to water (4).

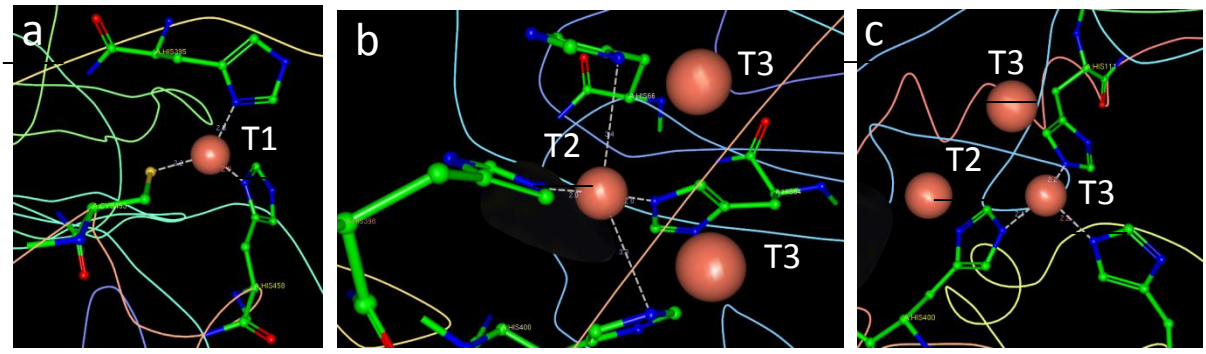

Figure 1. The three types of copper centers found in laccase. a). T1Cu is a mono-copper site, with two histidine and one cysteine residue attached to the copper ion. b). T2Cu is a mono-copper site, with four histidine residues attached. c). T3Cu is a bi-copper site. Each copper is attached to three histidine residues. T3Cu and T2Cu always show up together in multicopper oxidases as tricopper cluster. (Pictures taken from the RCSB Protein Data Bank, reference 10).

The oxygen reducing activities of the enzymes are largely due to the copper centers. T1Cu is also called blue copper because it exhibits an intense absorption at ~600nm as a result of Ligand-to-Metal-Charge-Transfer (sulfur atom on the cysteine to Cu). T1Cu is usually found in mononuclear copper proteins, responsible for intermolecular electron transfer, or found in multinuclear copper proteins and functions in intra-molecular electron transfer. T2Cu is also called normal copper, with a square planar geometry comparable to simple Cu (II) complexes. T3Cu site is a binuclear cluster, in some cases (such as oxidized ascorbate oxidase)(11), coupled through a hydroxide bridge. The copper ions themselves have unpaired electrons, and should be paramagnetic;

however, coupling with the hydroxide bridge made them antiferromagnetic. The T2 and T3 centers forms the trinuclear oxygen binding unit and the T1 center about 13 angstroms away oxidizes the substrate and transfers the electron to the trinuclear center where dioxygen can be reduced, in a series of steps, to water. These four copper centers form the minimum functional unit of a multicopper oxidase. In fact, laccase only has these four fundamental copper centers. Other multicopper oxidases may have more than four copper ions. Ceruloplasmin, for instance, has eight copper centers, four copper in the functional unit and other four T1Cu's(4).

Figure 1 below shows the four copper functional unit of laccase. The large spheres are copper ions. The T1 copper (far left) is bound to two histidine (His) residues and one cysteine (Cys) residue, and is connected to the trinuclear cluster by a bridge of His and Cys residues. The T2 copper (bottom right) is bound to four His residues, assuming a near-square-planar geometry. The binuclear T3 coppers are both bound to three His residues. When the oxygen-reducing reaction takes place, the electron donated to the T1 copper will transfer to T2/T3 cluster through the His/Cys bridge.

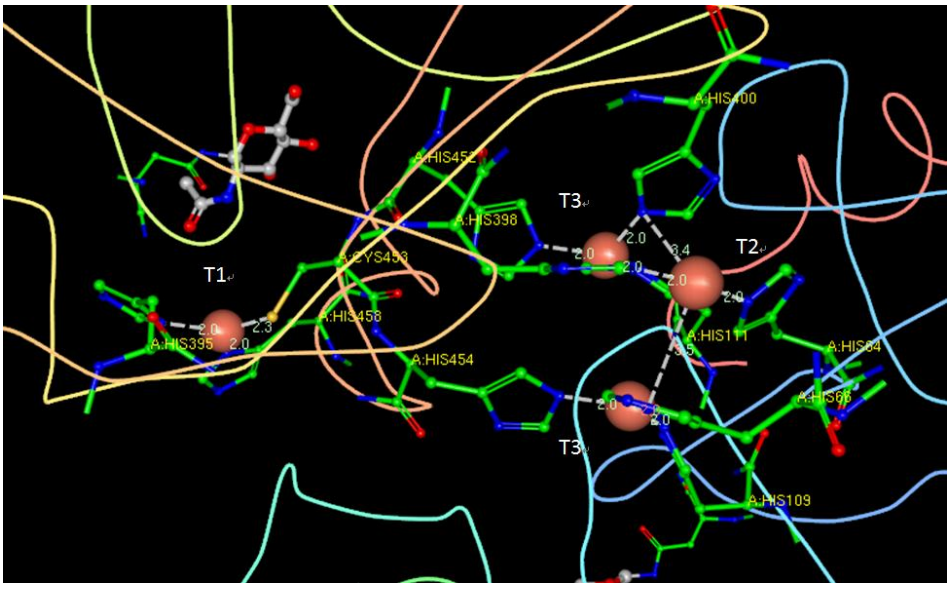

Figure 2. The four copper centers in laccase. (Figure taken from RCSB Protein Data Bank, reference 10.)

The oxygen binding and reducing pattern has been studied by many groups, but the actual mechanism still deludes researchers. One possible oxygen binding mechanism is illustrated below. When molecular oxygen binds to the trinuclear site, it is inserted between the two T3Cu's as a peroxide ion, with both atoms bound to these two copper ions. The oxygen atom close to the T2Cu is also bound to the copper. The T3Cu's assume distorted tetrahedral geometry while the T2Cu is almost square planar. The binding of oxygen results in significant increase in distance between the two T3Cu

but not much increase between the T2 and T3 copper ions, as illustrated in Figure 3. The pictures are cartoon renderings of the actual X-ray crystal structures of the tricopper site in laccase (11).

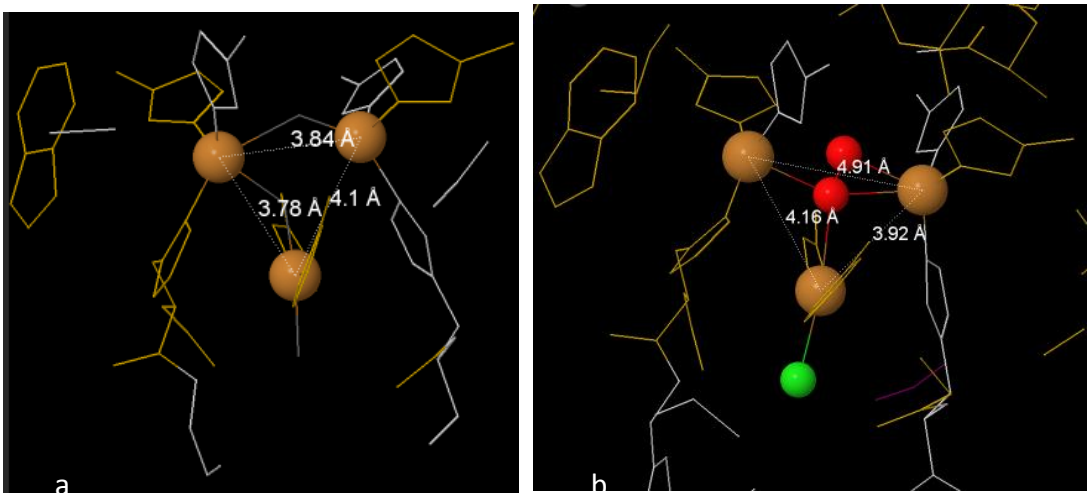

Figure 3.a) shows the trinuclear binding site of laccase without oxygen bound. b). shows the site with a bound oxygen molecule and a chloride ion. (Picture taken from RCSB Protein Data Bank reference 11).

There are many oxygen-reducing mechanisms proposed by various groups. One possible mechanism (12) that is consistent with the previous oxygen binding pattern is illustrated in Figure 4 below.

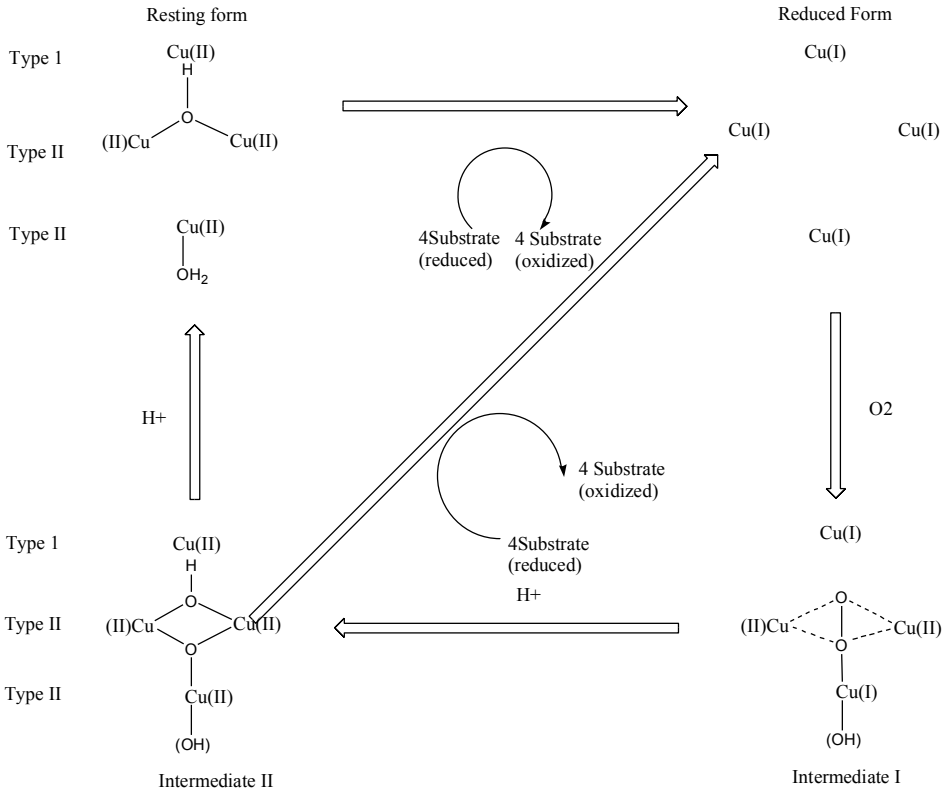

Figure 4: one mechanism for the four-electron reduction of dioxygen into water (Adapted from 12, Figure 6). The amino acid residues that the coppers are attached to are omitted in this diagram.

In the reduced form, all copper ions are Cu(I). Oxygen binds to the tricopper cluster as a peroxide ion between the two T3Cu's, oxidizing them to Cu(II) and giving rise to Intermediate I. The peroxide ion in Intermediate I then accepts two more electrons from the T2Cu and the T1Cu and a proton, breaking the O-O single bond, and Intermediate converts into Intermediate II. Intermediate II can either accept a proton and convert into the resting form, which oxidizes the substrates and decays back to the reduced form, or decays directly to the reduced form, oxidizing four substrate molecules in the process. In both cases, the bound oxygen atoms are released as water molecules (12). This reduction process occurs in two two-electron steps.

Another set of mechanisms are proposed by a different group, which is shown in Figure 5 below.

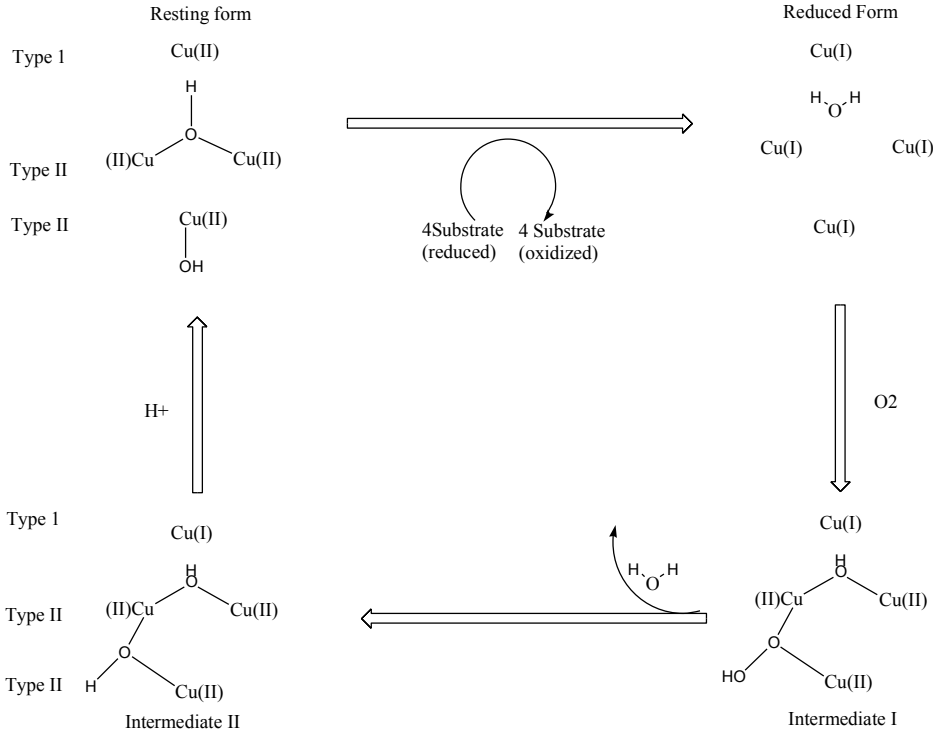

Figure 5. another proposed oxygen binding and reducing mechanism(adapted from reference 4, figure 33)

The reduced form of the binding site reacts with an oxygen molecule, reducing it to a hydro-peroxide bridge connecting a T2Cu and a T3Cu. This step activates the peroxide in intermediate I, which is further reduced to a hydroxide bridged species, intermediate II. Therefore, this mechanism is also a two two-electron-step process (reference 4). However, this mechanism is fundamentally different from the first one because it proposes a completely different oxygen binding pattern.

As there is not a consensus on the oxygen binding and reducing mechanism, more research is required. For this reason, this research projects intends to create a simplified model of the tricopper site that will shed some light on the oxygen binding mechanism.

Biomimetic modeling

Due to the immense size and complicated structures of the multicopper oxidases, to study the properties and functions of the proteins, it is easier to work with simplified models that reduce the complexity of the proteins but still retain the fundamental structures and properties of the protein active sites. These models can shed light on possible binding patterns, reaction mechanisms, reaction intermediates, and the like (9). Our project focuses on mimicking the structure and the behavior of the tricopper binding site of multicopper oxidases.

To mimic the tricopper site, a ligand that can simultaneously bind three Cu ions is required. Also, the bound coppers need to be close to each other in space and their binding environment needs to be similar to that of coppers in the multicopper oxidases. To mimic the geometry of the tricopper site, a 1,3,5-triethylbenzene scaffold is applied. The scaffold has three ethyl groups attached to 1,3,5-positions and three binding arms to 2,4,6-positions. According to theoretical calculations, the alternating conformation (shown in Figure 6), with three binding arms on one side of the benzene ring and the ethyl groups on the other side is the global minimum of for 1,3,5-triethylbenzene based scaffolds with moderately large binding arms. Most scaffolding systems researchers tested on show statistical preference for the alternating conformation (13).

Different binding arms have been tested before this project, and none of them thus far had been able to accurately mimic the structure and the behavior of the real tricopper site (1). A new ligand, 1,3,5-tri(N,N-bis(2-pyridylmethyl)-N-triazole)-2,4,6-triethylbenzene(also known as L_{tapma}), was proposed for this project.

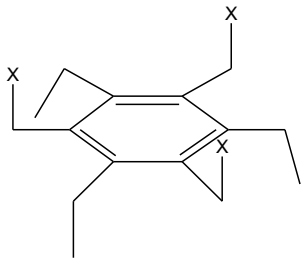

Figure 6: The alternating conformation of 1,3,5-triethylbenzene based scaffolds(adapted from Wang, X).

L_{tapma} is a promising model because 1) the three copper binding sites are likely to be on the same side of the benzene ring; 2) the copper binding side arms have two pyridyl rings that are similar to the histidine residues found in the actual binding site of the proteins. As discussed above, the copper ions in the T2/T3 trinuclear cluster were each attached to three histidine groups, assuming a trigonal

pyramidal geometry; the binding side arm of L_{tapma} can also potentially bind to a copper ion as a tridentate or tetradentate ligand, thus exhibiting similar binding patterns. Molecular simulation with Spartan was performed on this model, and the lowest energy conformation is shown to be the one with three binding arms on the same side of the benzene ring (see Figure 15).

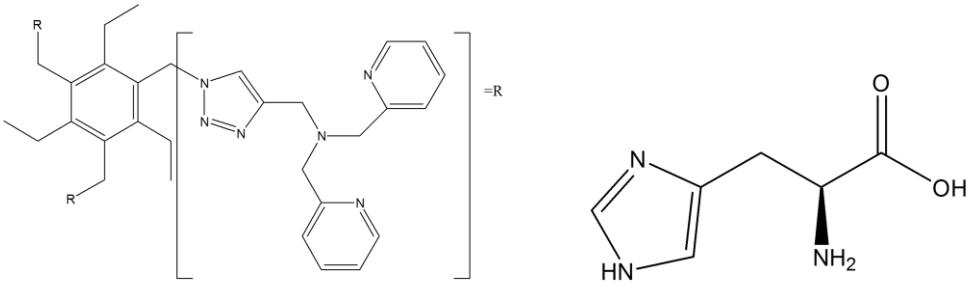

Figure 7: L_{tapma} (left) and Histidine(right)

The synthesis of L_{tapma} employs the Cu(I)-catalyzed alkyne-azide cycloaddition, which is considered an example of "click chemistry" requiring only mild reaction conditions and easy work up processes. The cycloaddition of alkyne and azide can also occur without Cu(I) catalyst; however, the product will be a 1:1 mixture of two compounds:

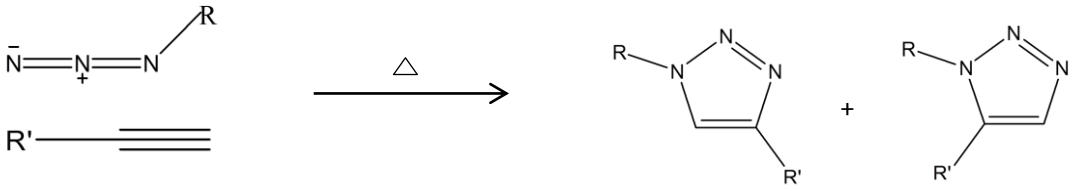

Figure 8: general scheme of thermal 1,3 cycloaddition(uncatalyzed)

With Cu(I) catalyst, only the desired product on the left would be formed. This regioselectivity is due to a di-copper intermediate, which well explained in the mechanism below (14).

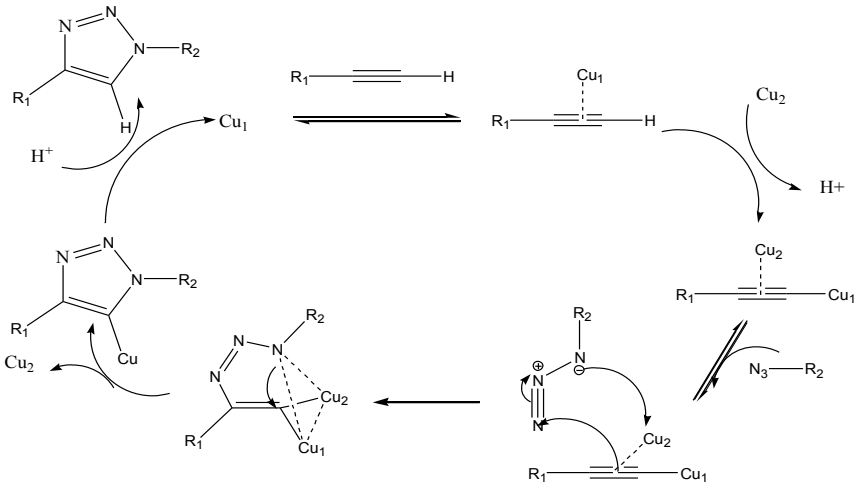

Figure 8. Cu-catalyzed azide-alkyne cycloaddition mechanism. (Figure adapted from 14, Figure 4.)

Experimental

General Methods. All chemicals and instruments are provided by the chemistry department of the College of Saint Benedict and by my advisor, Dr. Johnson, and Mardi Billman, who worked on this project with Dr. Johnson before me. All reactions involving Copper (I) ions were performed in the dry glove-box. All H^1NMR spectra were collected using JEOL-ECA 400MHz NMR. The packing material used for column chromatography is flash chromatography basic alumina, purchased from Sorbent Tech.

Synthesis of N,N-bis(2-pyridylmethyl)-N-propargylamine(alkyne 13) method 1. Dipicolylamine (0.9mL, 5.0mmol) was dissolved in 10mL of DMF in a 50mL round bottom flask (RBF). Anhydrous potassium carbonate (2.76g 20.0mmol) was added to the solution in one portion. Propargyl bromide (0.8mL, ~7.0mmol) was added drop-wise to the suspension by a syringe in about 2 minutes. No air-sensitive or water-sensitive conditions are required (if heat is applied, these conditions are recommended). The yellow suspension was then stirred for ~48 hours at room temperature and extracted with 50mL of dichloromethane three times. Dichloromethane (DCM) and tetrahydrofuran (THF) were evaporated with a rotary evaporator and the resulting gel was purified with a column of 1 inch (length) of basic alumina by eluting with ~5% methanol in dichloromethane. The resulting oil was dark yellowish orange (0.893g, 74% yield). ^{1}H NMR (400MHz, $CDCl_3$) σ ppm 8.56(2H,d), 7.65(2H, t), 7.50(2H, d), 7.25(2H, t), 3.90(4H, s), 3.40(2H,s), 2.3(1H, s).

Synthesis of N,N-bis(2-pyridylmethyl)-N-propargylamine(alkyne 13) method 2. Dipicolylamine (0.9mL, 5.0mmol) was dissolved in 10mL of DMF in a 50mL round bottom flask. Anhydrous potassium carbonate (2.76g 20.0mmol) was added to the solution in one portion. Propargyl bromide (0.8mL, ~7.0mmol) was added drop-wise to the suspension by a syringe in about 2 minutes. No air-sensitive or water-sensitive conditions are required. The yellow suspension was then stirred for 48 hours at room temperature and extracted with 50mL of dichloromethane three times. Dichloromethane was evaporated with rotary evaporator, DMF solvent was removed via Kugelrohr at 75^o C for 1.5 hours and the resulting gel was purified with a column of about 1 inch (length) of basic alumina by

eluting with ~5% MeOH in DCM. The resulting oil was dark yellowish orange (1.068g, 89% yield). ^{1}H NMR (400MHz, $CDCl_3$) σ ppm 8.56(2H,d), 7.65(2H, t), 7.50(2H, d), 7.25(2H, t), 3.90(4H, s), 3.40(2H,s), 2.3(1H, s).

Synthesis of 1,3,5-tri(N,N-bis(2-pyridylmethyl)-N-triazole)-2,4,6-triethylbenzene(L_{tapma}). Solid 1,3,5-tribromomethyl-2,4,6-triethylbenzene (0.145g 0.330mmol), sodium azide (0.076g, 1.17mmol), sodium ascorbate (0.065g 0.330mmol) and copper sulfate pentahydrate (0.050g 0.200mmol) were dissolved in 5mL water in a 50mL round bottom flask. N,N-bis(2-pyridylmethyl)-N-propargylamine (0.340g 1.25mmol) was dissolved in ~1mL dimethylformamide(DMF) and transferred into the RBF. Then appropriate amount of DMF was added into the RBF such that the total amount of DMF is 15mL. The reaction solution was stirred for 72 hours at room temperature without air-sensitive conditions. Then saturated ammonium bicarbonate (10 mL) and concentrated ammonia (10mL) were added to the RBF to quench the reaction. The mixed solutions were extracted with 3X30mL dichloromethane, and the combined organic layer was washed with saturated sodium chloride solution and dried with magnesium sulfate. DCM was evaporated with rotary evaporator and DMF was evaporated with a Kugelrohr for 1.5 hours at 75^{o}C. The dark brown gum left in the flask was then dissolved in DCM and loaded on to a 2-3-inch (length) basic alumina column and eluted with 5% methanol in DCM to yield a thick dark brown oil (~0.25g, ~75%).^{1}H NMR (400MHz, $CDCl_3$) σ ppm 8.45(3H,d), 7.58(3H, d), 7.49(3H, t), 7.41(3H,s), 7.08(3H,t), 5.60(6H,s), 3.73(18H,s), 2.76(6H, q), 0.90(9H, t).

Synthesis of $[Cu_3L_{tapma}]^{3+}$ complex. Solid tetrakis acetonitrile copper(I) tetrafluoroborate (0.066mg 0.21 mmol) and gel L_{tapma} (0.073g, 0.070mmol) were dissolved in 1.5mL acetonitrile in a vial. The solution was left to react overnight. The acetonitrile solvent was then evaporated, and the remaining gel-like mixture was washed with DCM and then with methanol. Minimum amount of acetonitrile was added to the vial to dissolve the remaining compound and appropriate amount of diethyl ether was added to the solution (add until the precipitates could barely dissolve back into the solution). The mixed solution was left in the freezer (-33^{o}C) for recrystallization.

Results and Discussion

All reaction schemes and spectra can be found in the Supplemental Materials section.

N,N-bis(2-pyridylmethyl)-N-propargylamine(alkyne 13)

The synthetic scheme is shown in Figure 8. The synthesis of N,N-bis(2-pyridylmethyl) -N-propargylamine, also called alkyne 13, can be performed under a variety of mild conditions and can still give reasonably high yield (~90%) and purity. This compound will serve as the side arm to bind to copper ions. Even though the reaction was initially believed to require oxygen free and moisture free conditions, as long as no intense heat is applied to the system, having air in the system has negligible impacts on purity and yield. One possible explanation is that this reduced temperature prevents oxidation of amine to imine and enamine, which could polymerize to form a dark brown by-product, and thus increases the yield and purity of the desired product. To compensate for the reduced activity that accompanies low temperature, more reaction time is required. Generally speaking, this reaction is rather robust, and can take place under various temperatures ranging from room temperature to ~45^{o}C, and requires no strictly oxygen-free conditions (oxygen free conditions would produce better results, but not significantly better). No optimal reaction time was obtained, but it is known that more than 24 hours is necessary for the reaction to reach a reasonable completion percentage. Method 1 and method 2 mainly differs in the solvent used. Overall, DMF solvent gives better yield and purity than THF solvent. However, DMF has to be removed with a Kugelrohr, and this process is extremely time-consuming. The purification of the product is relatively simple as the product was about 90% pure. The column used for chromatography was about 2cm in diameter, and only 1 inch tall of packing material was used. This pseudo-flash chromatography is essentially a filtration process, as most of the impurities were left on the column after the desired product was eluted with the chosen elution solution. Note that the impurities would move down to the bottom of the column but for some reason would not elute out.

The product was characterized by ^{1}H NMR (see Figure 9) and compared to literature. The doublets at 8.56ppm and 7.50ppm, and the triplets at 7.65ppm and 7.25ppm correspond to the H's attached to the carbons on the pyridyl rings. The singlet at 3.90ppm corresponds to the methylene groups connecting the central nitrogen atom and the pyridyl rings. The singlet at 3.40ppm corresponds to the methylene group connecting the central nitrogen atom and the alkyl group. The singlet at

2.3ppm corresponds to the H of the alkyne group. The integrals of these peaks match the theoretical H ratios of the actual structure.

1,3,5-tri(N,N-bis(2-pyridylmethyl)-N-triazole)-2,4,6-triethylbenzene(L_{tapma})

The synthetic scheme is shown in Figure 10. This synthesis is considered an example of "click chemistry" of Cu(I) catalyzed alkyne and azide coupling. The first step of the synthesis is a SN reaction of the bromomethyl group on the benzene ring and the azide group. Due to the instability of organic azide, sodium azide was added in to the solution to substitute for the bromide on 1,3,5-tribromomethyl-2,4,6-triethylbenzene. Then, the freshly made azide was coupled with the alkyne group of alkyne 13 to form triazole. This coupling is catalyzed by Cu(I), which is formed in situ by reducing CuSO4 with sodium ascorbate.

The proper ratio of reactants and catalysts was determined by Mardi Billman, who worked on this project before me. The ratios of all chemicals used in this reaction are listed in the table below.

Ltapma Synthesis			
Chemicals	grams	mmole	eq
tribromoarene	0.137	0.310	1
Sodium Azide	0.074	1.136	3.66
Sodium ascorbate	0.062	0.310	1
copper sulfate pentahydrate	0.047	0.186	0.6
alkyne 13	0.280	1.025	3.3

Table 1. L_{tapma} synthesis reagent amounts in grams, mmol and equivalents.

L_{tapma} is characterized by ^{1}H NMR (See Figure 11). The doublets around 8.45ppm and 7.49ppm, and the triplets around 7.58ppm and 7. 08ppm correspond to the aromatic CH's on the pyridyl rings. The singlet at 7.41ppm corresponds to the CH on the triazole ring. The singlet at 5.60ppm corresponds to methylene group connecting the triazole ring and the benzene ring. The singlet at 3.73ppm corresponds to the methylene groups connecting the nitrogen atom and the pyridyl rings. The quartet around 2.76ppm corresponds to the methylene groups on the ethyl groups on the benzene ring. The triplet around 0.90ppm corresponds to the methyl groups on the ethyl groups attached to the benzene ring. The ^{1}H NMR also shows alkyne 13 peaks, 1,3,5-triethylbenzene scaffold peaks, DMF

peaks (8.0ppm, 2.96ppm and 2.88ppm), water peak (1.61ppm) and some unknown impurity peaks. However, these impurities can be relatively easily removed in the third step of the synthesis (because these impurities are soluble in DCM but the product in the next step is not).

One concern with L_{tapma} is whether the three binding arms would end up on the same side of the benzene ring. Even though calculations show that the alternating conformation is the global minimum of 1,3,5-triethylbenezene scaffold, the gigantic binding arms of L_{tapma} might create too much steric stress on the same side of the benzene ring, and cause one of the arms to flip to the other side. It is suggested that if the three binding arms of this scaffold compound were not on the same side of the benzene ring, the peak that correspond to the methyl (0.90ppm) groups would show up in more than one position on the ^{1}H NMR spectrum, for example 1.1ppm. The fact that the 0.9ppm peak is much more intense than the 1.1ppm peak on the spectrum implies that for the majority of the L_{tapma} molecules, the three binding arms are likely to be on the same side.

$[Cu_3L_{tapma}]^{3+}$ complex

The synthesis of the $[Cu_3Ltapma]^{3+}$ complex is performed in the dry glove box and due to technical difficulties with chemical weighing and transfer process, more than 3 equivalent amounts of Cu(I) compound were added to the reaction solution every time. The reaction was initially performed in DCM, but for some reason Cu(I) was oxidized to Cu(II), indicated by a color change from whisky orange to dark green. It was speculated that chlorinated solvent played a role in the oxidation, and acetonitrile was then used for subsequent trials. After the solvent change, the Cu(I) complex could remain orange(color of L_{tapma}) for an extended period of time.

There had been great difficulties with recrystallization from unpurified reaction mixtures, and thus a simple pre-purification method is needed to be developed. It was discovered that the product is insoluble in methanol and DCM, but the starting copper complex was soluble in methanol and L_{tapma} was soluble in DCM. Hence the pre-purification method (evaporating the acetonitrile, washing first with DCM and then with methanol) in the experimental section was applied. However, the recrystallization results have yet to be seen.

The ^{1}H NMR of the $[Cu_3Ltapma]^{3+}$ showed broadened peaks (compared to L_{tapma}). There might be two possibilities: 1. Not all three binding arms were bound to Cu(I), but the Cu(I) can rapidly transfer between the binding sites, and the NMR spectrum is in effect the average spectrum of these

different isomers and hence the broadened peaks (this hypothesis could be tested by lowering the temperature of the sample and getting multiple sets of narrow peaks, or by increasing the temperature and observing narrower peaks); 2. Cu(I) can intrinsically broaden the peaks. There might also be other unknown explanations. However, regardless of the explanations, based on ^{1}H NMR data alone, little could be said for sure about the composition and structure of the complex.

Future work

A more efficient way of purifying the tricopper complex needs to be developed in order to obtain more quality crystals of the complex. An X-Ray crystallography needs to be obtained for the tricopper complex in order to ascertain its structure. If the structure is in agreement with the proposed structure, its binding behavior with dioxygen could be studied. If this complex fails to mimic the structure and behavior of the actual tricopper site in multicopper oxidases, another ligand scaffold would need to be proposed, synthesized, and characterized for further research.

References

1. Billman, M. M. Molecular Modeling of Multicopper Oxidases: the Binding of O_2 to a Tri-copper Active Site, May 3, 2011
2. Fulmer, G. R. et al. *Organometallics*, 2010, *29* (9), pp 2176–2179
3. Mirica, L. M., et al. *Chem. Rev.*, 2004, *104* (2), pp 1013–1046
4. Solomon, E I. et al. *Chem.Rev*. 1996, 96,2563-2605
5. Huang, S., Clark, R. J, *Org. Lett.*, 2007, *9* (24), pp 4999–5002
6. Zhou. et al. *Org. Lett.*, 2011, *13* (6), pp 1290–1293
7. Rosenthal, J, Lippard, S. J. *J. Am. Chem. Soc.*, 2010, *132* (16), pp 5536–5537
8. Rajakumar, P., et al. *Tetrahedron Letters*,2010, 51, 5167–5172
9. Holm, R. H. and E. I. Solomon, *Chem. Rev.* 2004. 104(2): 347-348
10. Bento,I, Peixoto, C., Zaitsev, V.N., and Lindley, P.F. *Acta Crystallogr*, 2007, Sect. D 63:240
11. Hakulinen, N., et al. *Nat Struct Biol* 2002, 9(8): 601-605.
12. Kataoka, K., et al. J Biol Chem 2009, 284(21): 14405-14413.
13. Wang, X. and F. Hof. *Beilstein Journal of Organic Chemistry*,2012, 8: 1-10.
14. B. T. Worrell, J. A. Malik, and V. V. Fokin *Science* 26 April 2013: 340 (6131), 457-460

Supplemental Materials

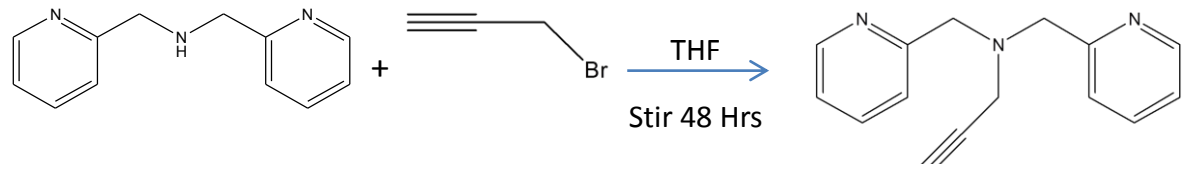

Figure 9: Synthesis of Alkyne 13

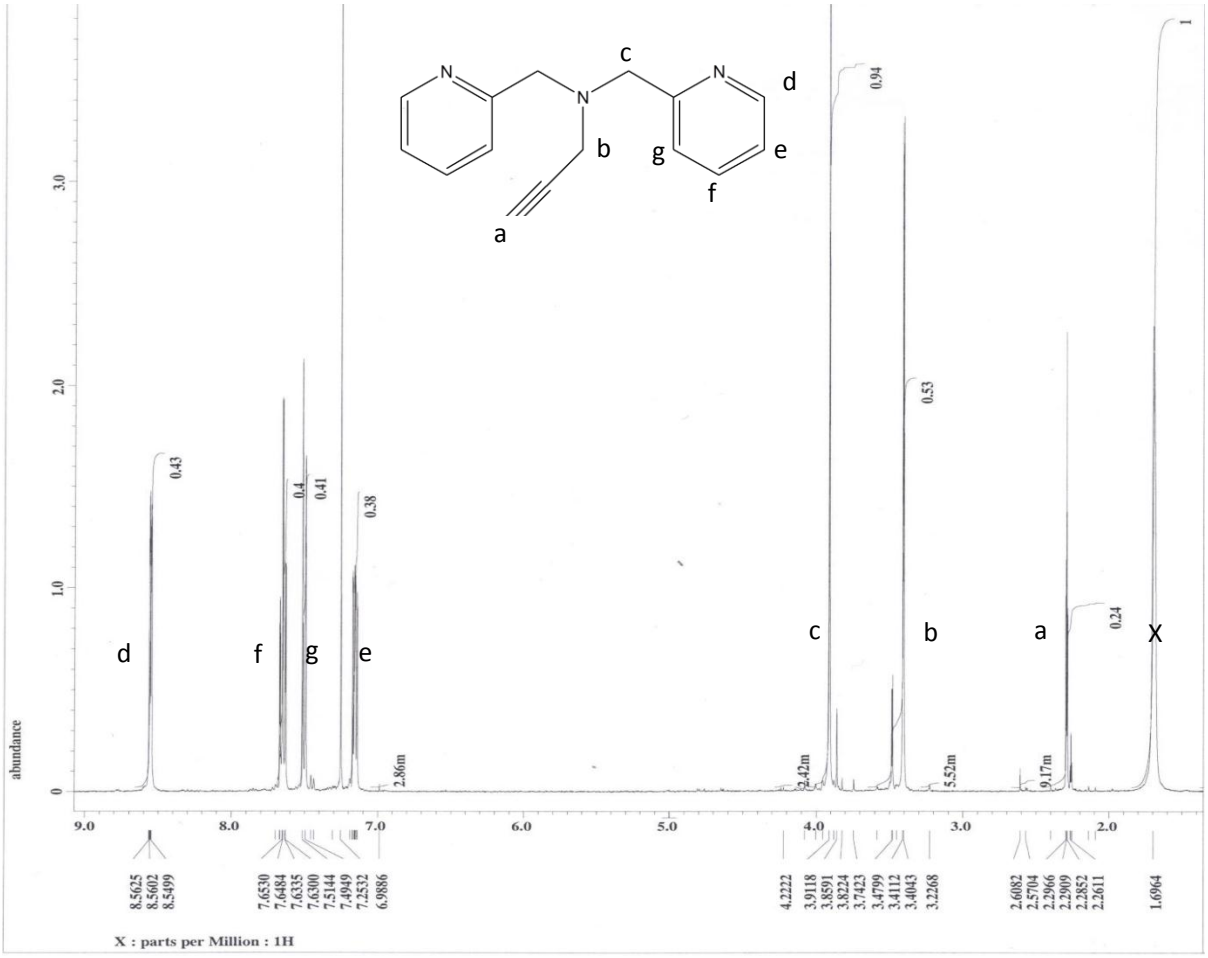

Figure 10: ^{1}H NMR of Alkyne 13

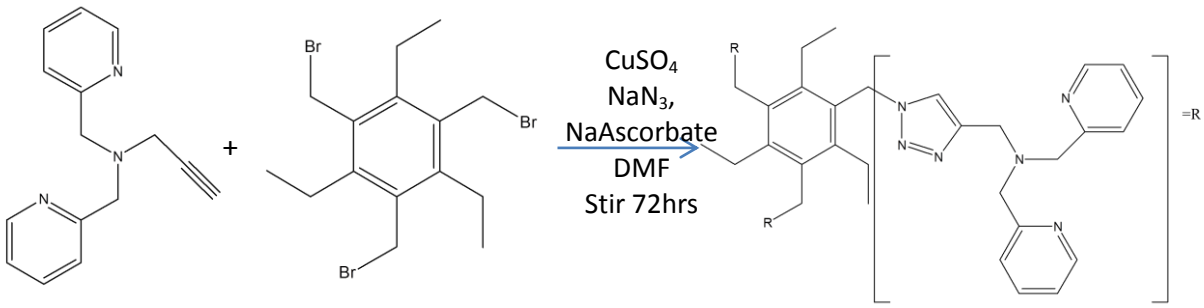

Figure 11: Synthesis of L_{tampa}

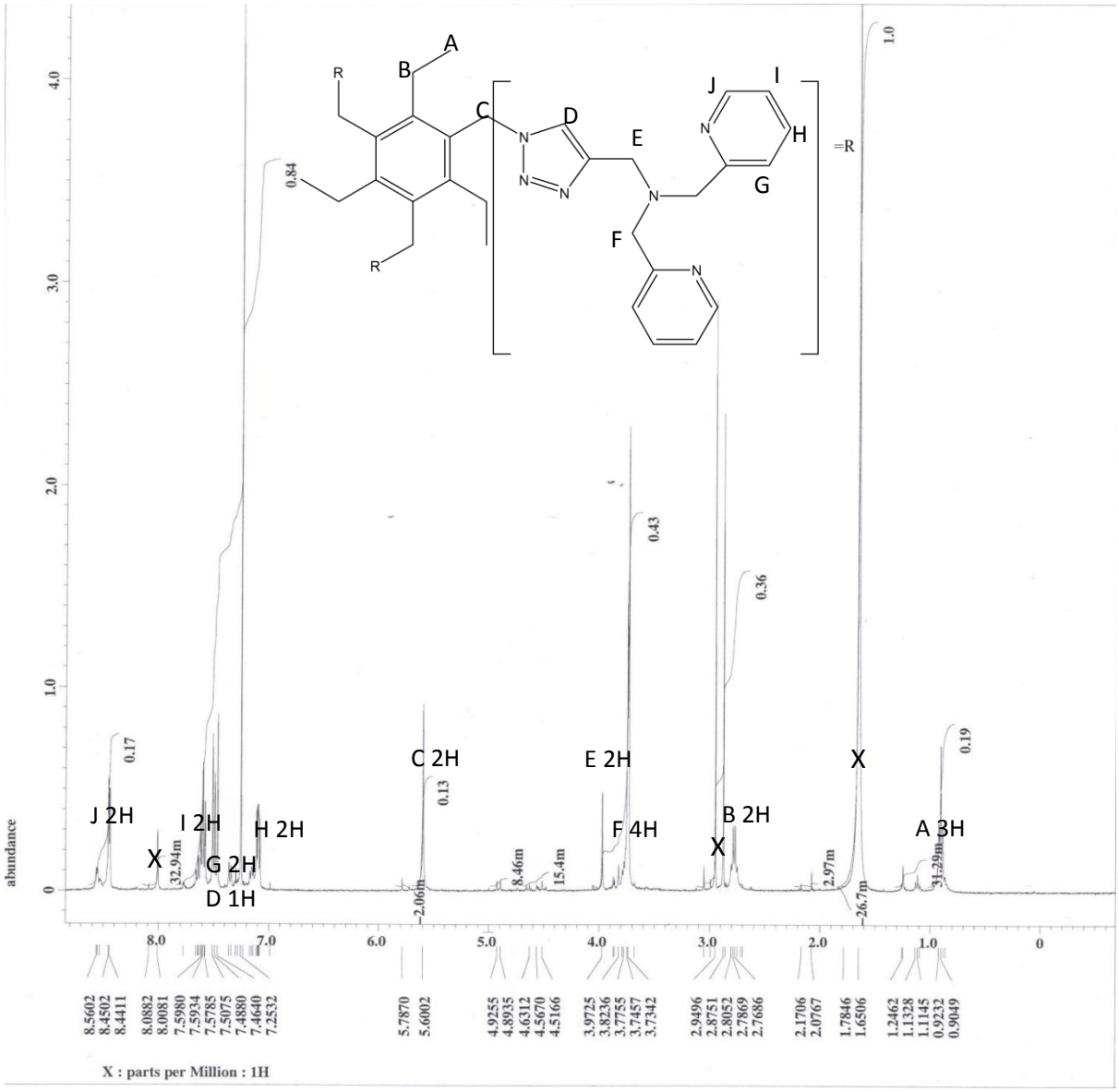

Figure 11: ^{1}H NMR of crude L_{tapma}

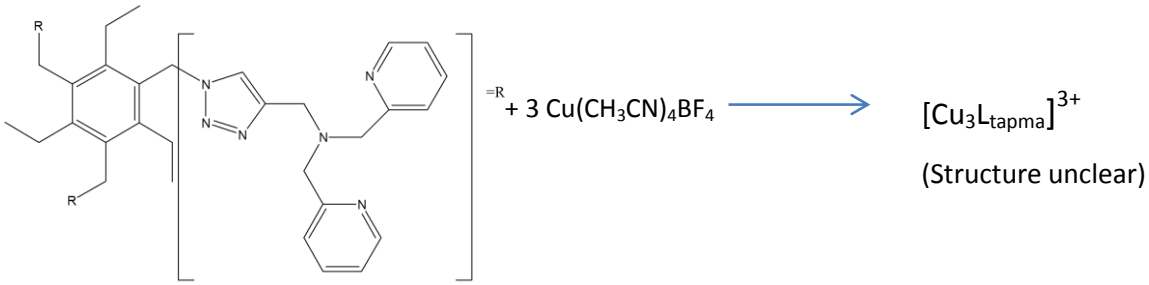

Figure 13: $[Cu_3L_{tapma}]$ synthesis scheme

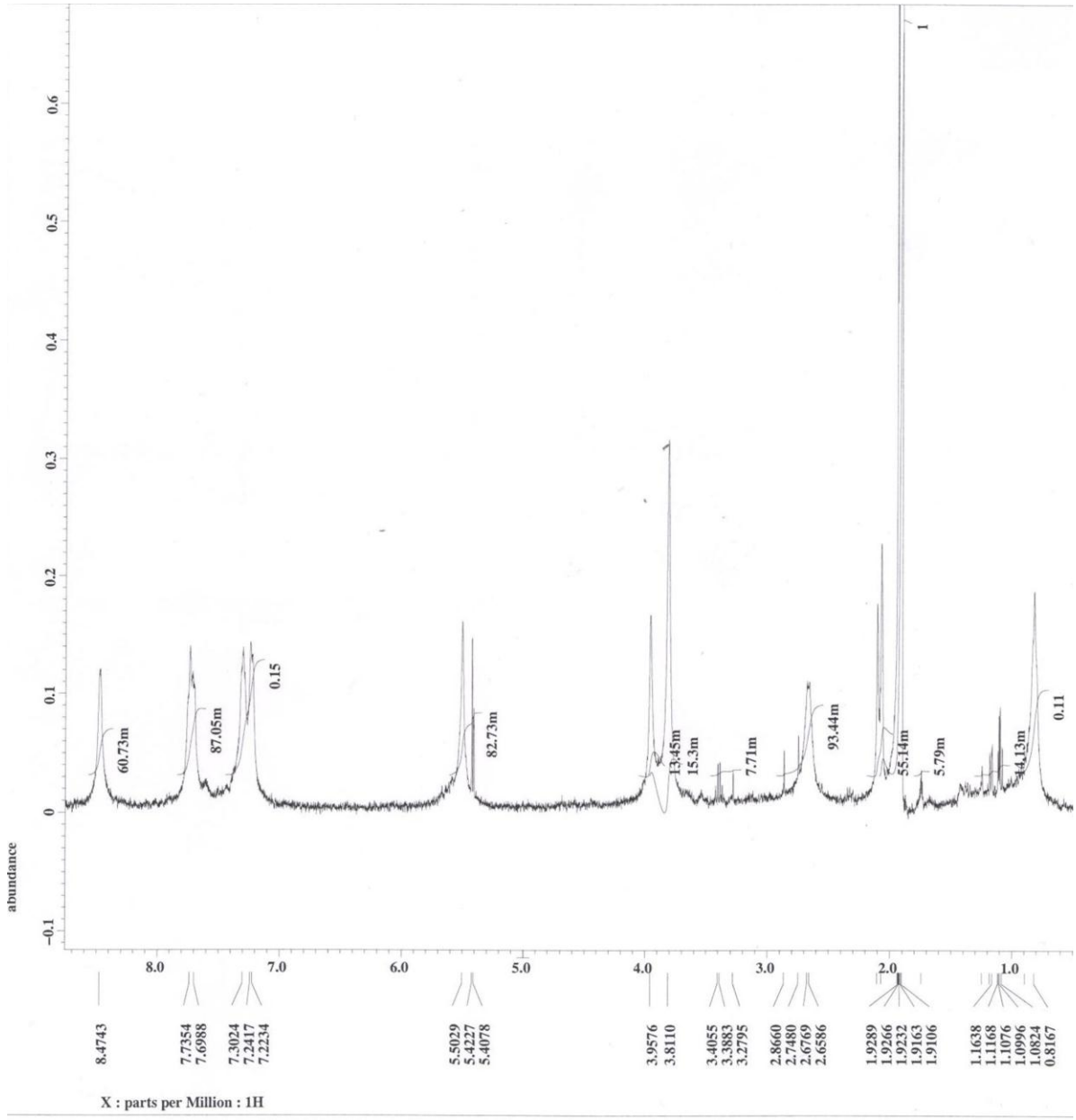

Figure 14 $[Cu_3L_{tapma}]$ ^1H NMR

Spartan Simulation of L_{tapma}

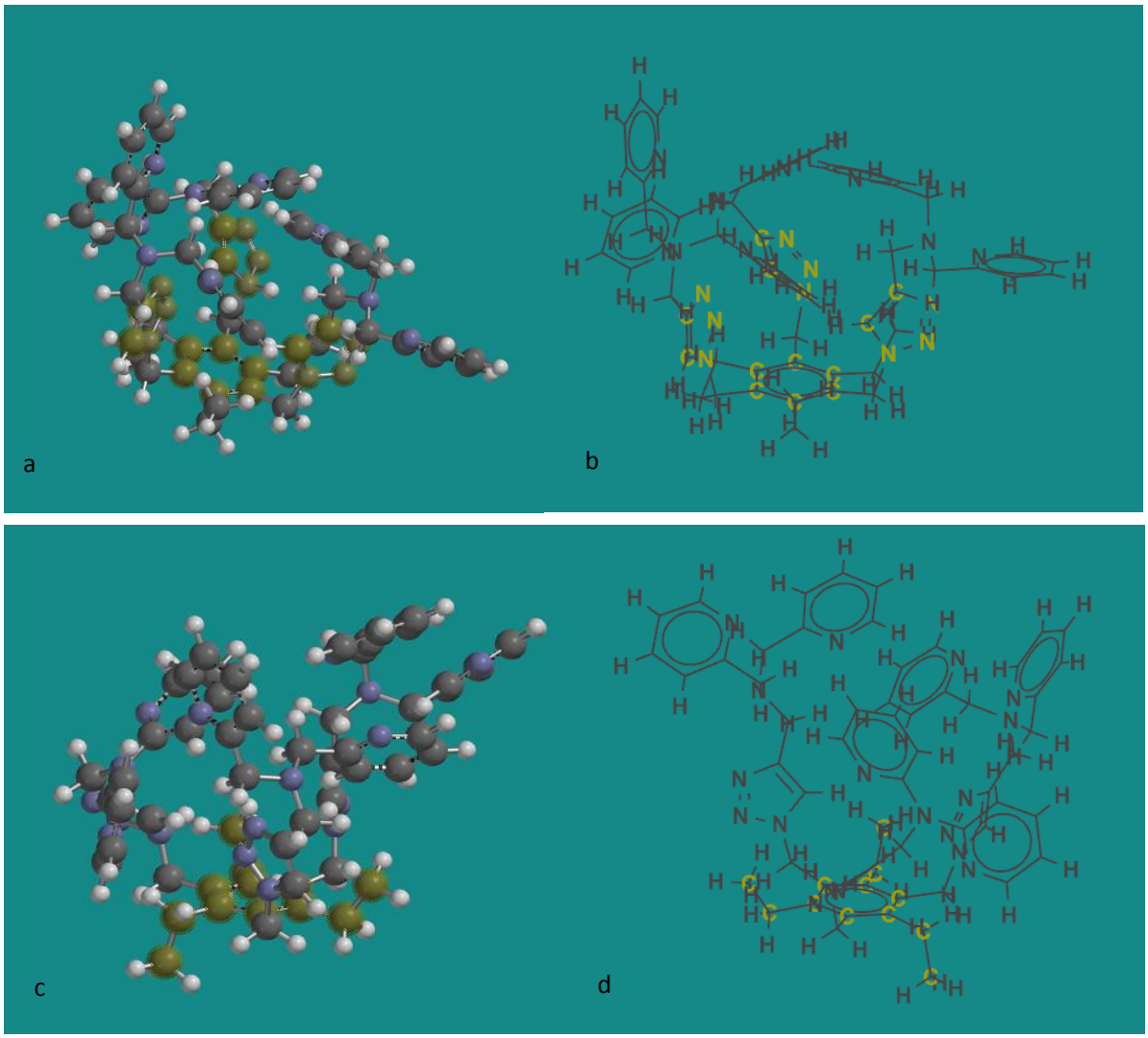

Figure 15: Lowest energy conformation predicted by Spartan 08: a) ball-and-stick model with the benzene ring and the triazole rings highlighted; b) line model with the benzene ring and the triazole rings highlighted; c) ball-and-stick model with the benzene ring and the ethyl groups highlighted; d) line model with the benzene ring and the ethyl groups highlighted;