## Title

Investigations of the biological effects of natural products on metal-mediated metabolic disease states



## Author

Lee, Vanessa Janice

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Investigations of the biological effects of natural products on metal-mediated metabolic disease states

By

VANESSA JANICE LEE

DISSERTATION

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Approved:

Marie Heffern, Chair

Matthew Augustine

R. David Britt

Dylan Murray

Committee in Charge

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*To my parents, Diana and Ken, who have shown me the best the world has to offer.*

# Contents

# Acknowledgements

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# Abstract

Metabolic disease states like diabetes, Wilson disease, and Menkes disease are linked to dysregulation of d-block metals in the body, and current therapeutic approaches aim to alter these metal populations. Natural products often serve as inspiration for the development of therapeutics due to their inherent bioactivity. This thesis describes investigations of the interactions between natural products and metal ions in the context of affecting metabolic disease states. Chapter 1 introduces the importance of micronutrients in human biology, highlights disease states associated with metal dysregulation, and finally outlines analytical tools and methods used to study bioinorganic systems. Chapter 2 provides a deeper analysis of the connections between plant-derived molecules, metal ions, and metabolic disease states while proposing further study for potential therapeutic uses. Chapters 3 and 4 outline investigations of the interactions of copper with specific plant-derived molecules, flavonoids and rice bran protein hydrolysates, respectively. Studies in controlled solutions provide a basis for further analyses of the downstream biological effects in model systems. Chapter 5 highlights NMR method and application development towards studying the cobalt center of vitamin B12 with different binding partners. Finally, chapter 6 presents a copper-dependent inhibition of bioluminescence in marine systems due to an oxidation of the luciferin substrates.

# Chapter 1

## Natural products in relation to metal trafficking and disease pathologies

## 1.1 The roles of vitamins and minerals in homeostasis

Human life requires essential nutrients obtained through diet. Macronutrients such as proteins, carbohydrates, and lipids are sources of energy and make up the bulk of our diets.[1] In contrast, micronutrients are required in much smaller amounts but still play vital roles in maintaining homeostatic processes. Vitamins and minerals comprise the term micronutrients; vitamins are produced by plants and animals and are metabolized in the human body whereas minerals are inorganic in nature and cannot be broken down.[2] Due to their lower concentrations, slight changes in micronutrient levels can result in dyshomeostasis and disease.

### 1.1.1 Vitamins

There are thirteen vitamin classes which are composed of molecules that must be obtained through diet.[2] Vitamins perform a range of biological activities including serving as cofactors, regulating cell growth, and imparting antioxidant activity.

The vitamin A class includes retinoids and carotenoids.[2] Retinoids are synthetic derivatives of naturally occurring vitamin A which exists as retinol or retinyl esters. Retinoids contain four isoprenoid units, and Vitamin A is the all-*trans* parent molecule.[1] Vitamin A is a fat-soluble vitamin that is found in milk, eggs, and beef liver.[3] Making up the remaining part of the vitamin A class are carotenoids which consist of eight isoprenoid units with an inversion center and derive from acyclic $C_{40}H_{56}$. Carotenoids are mainly found in photosynthetic plants and algae as opposed to their animal-derived retinol counterparts.[4] The class of vitamin A molecules are required for maintenance of many tissues in the body, but their most well-known function is in the eye.[5] In the retina, rhodopsin, a G-protein coupled receptor, binds vitamin A in the form of 11-*cis*-retinal. Upon light absorption, 11-*cis*-retinal isomerizes to all-*trans*-retinal which triggers a signal cascade corresponding to visualization in the cortex. Because of the integral role of vitamin A in biological processes, a deficiency in the vitamin corresponds to a plethora of

diseases.[6] Notable, xerophthalmia is a group of pathologies deriving from the inability to regenerate rhodopsin and which is treated with vitamin A supplementation.[7]

The class of vitamin B compounds includes $B_1$ (thiamine), $B_2$ (riboflavin), $B_3$ (niacin), $B_5$ (pantothenic acid), $B_6$, $B_7$ (biotin), $B_9$ (folate), and $B_{12}$ (cobalamin).[2] Vitamin B compounds are water-soluble, serve as enzyme cofactors, and are required for the proper functioning of most cell types in human biology. Thiamine acts as a coenzyme and is involved in catalytic reactions in the form of thiamine diphosphate (ThDP).[8] Importantly, ThDP serves as a coenzyme for transketolase which is required for fatty acid and nucleic acid synthesis.[9] Thiamine also serves protective roles against glutamate in neurons, and as such, thiamine deficiency can present as peripheral neuritis.[10] Riboflavin is produced by plants, yeast, and prokaryotic cells from which humans can obtain the isoalloxazine-based compound.[11] Derivatives of riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), serve as coenzymes for flavoproteins that participate in redox reactions.[12] Such redox reactions are required for the metabolism of energy, carbohydrates, lipids, and amino acids. Niacin, a group of compounds associated with nicotinamide, was identified in response to the pellagra outbreak in which patients experienced vitamin $B_3$ deficiency resulting in dermatitis, psychological changes, and muscle weakness.[13] The spread of this disease led to in depth research on the role of niacin compounds and their roles as NAD and NADP coenzymes. Like riboflavin, niacin coenzymes are involved in over 400 redox reactions and are famously involved in energy production as various forms of nicotinamide adenine dinucleotide (NAD, NADP, and NADPH). As such, sufficient levels of niacin are required for maintenance of a healthy metabolism. Pantothenic acid is ubiquitous in foods and is consumed through meats (e.g. chicken, beef), grains (e.g. oats), and vegetables (e.g. tomatoes, broccoli). The widespread nature of pantothenic acid results in low instances of deficiency.[2] Pantothenic acid plays roles in lipid metabolism, Coenzyme A synthesis, and fatty acid synthesis.[14] As such, the rare deficiency in pantothenic

acid is linked to symptoms of numbness and burning sensations of the hands and feet. Vitamin B6 is required for metabolism of proteins, lipids, and carbohydrates.[15] Vitamin B6 is refers to a group of pyridine derived compounds that share physiological functions. Along with their service as coenzymes, vitamin $B_6$ also exhibits antioxidant activity through superoxide quenching and protection against lipid oxidation. Vitamin $B_6$ deficiency from diet insufficiency is rare but can results from diseases that restrict its bioavailability. Due to its diverse utility, deficiency of vitamin $B_6$ results in neurological symptoms like seizures, cardiovascular dysfunction, and dermatitis. Biotin, vitamin $B_7$, has one bioactive form which is a bicyclic compound containing a ureido group and a tetrahydrothiophene ring.[2] Widely known for its tight binding to streptavidin, biotin is used extensively in biochemical research.[16] Biotin serves as a coenzyme for five carboxylases involved in energy and fatty acid metabolism. Common sources of biotin include egg yolk, legumes, and nuts, and biotin deficiency results in buildup of fatty acids and organic acids due to the disrupted metabolic pathways. Folate denotes a group of compounds that participate as coenzymes in the transfer of one-carbon units.[17] Commonly known for its importance during pregnancy, folic acid reduces the risk of neural tube defects and can be consumed in leafy vegetables, fruits, and legumes.[18] Methyl transfers are essential in maintaining proper homeostasis in reactions involving DNA, RNA, lipids, proteins, and histones. Thus, folate deficiency is linked many pathologies states including cancer, vascular disease, and developmental abnormalities. The last B vitamin, $B_{12}$, or cobalamin, is the only vitamin that contains a metal center.[19] The importance of this vitamin and further discussion will be detailed in Chapter 5 of this dissertation.

Vitamin C, or ascorbic acid, is historically famous for its curative activity towards scurvy.[7] Possessing both redox activity and catalytic activity, vitamin C's most famous biological function is its antioxidant activity.[20] Oxidative damage affects important biomolecules such as lipids, DNA, and proteins.[21] Vitamin C exhibits antioxidant activity through radical scavenging and can

ameliorate oxidative stress. Behaving as a reducing agent, vitamin C activates mono- and dioxygenases required for cellular function.[20] Changes in vitamin C status are correlated to several disease states including, but not limited to, cancer, stroke, and atherosclerosis.

Vitamin D is a group of molecules that affect calcium and phosphate concentrations in the serum.[22] The vitamin D family are steroid hormones and are derivatives of a cyclopentanoperhydrophenanthrene ring system. Technically a misnomer, humans can produce vitamin D precursors, so they are not micronutrients.[23] Vitamin $D_2$, or ergocalciferol, and vitamin $D_3$, or cholecalciferol, are the most vital forms for human health. The endogenously produced forms of vitamin D are inert and require ultraviolet light to undergo hydroxylation to their active forms.[24] As calcium is ubiquitous biological functions, calcium modulation by vitamin D is required for insulin production, cell growth, and muscle function. Vitamin D deficiency is historically known as the cause of rickets in children.[25] Rickets describes a condition in which bones are not properly mineralized which leads to skeletal deformities and weak bones. The anti-rachitic properties of vitamin D compounds can be affected by age, fat malabsorption, and drug interactions.

Vitamin E, most commonly in the form of α-tocopherol, is best known for its lipid-soluble antioxidant activity.[26] Serving unique roles in different tissues, the vitamin E compounds share a chromanol core structure. α-tocopherol is the only active form of vitamin E in human biology and scavenges peroxyl radicals to prevent oxidation of lipids.[27] Deficiency in vitamin E can be caused by genetic disorders such as ataxia with vitamin E deficiency (AVED) which exhibits as neurological abnormalities.[26] Because of vitamin E's lipid solubility, genetic disorders that affect lipoproteins (e.g. hypobetalipoproteinemia) or fat absorption (e.g. cystic fibrosis) can result in vitamin E deficiency. Similar to most vitamin supplements, research on the long-term effects of vitamin E produce conflicting results, but a healthy diet must contain sufficient levels of α-tocopherol in order to maintain low levels of oxidative damage.

The vitamin K class of compounds includes 2-methyl-1,4-naphthoquinone and its derivatives.[28] Vitamin K compounds can be consumed through legumes, leafy vegetables, and root vegetables, and are usually consumed in excess of biological requirements. Biochemical reactions that require vitamin K for proper enzymes including carboxylases and reductases highlight the integral role of the compounds in human health.[2] In particular, vitamin K is involved in the production of prothrombin which is required for blood coagulation.[29] In the rare instances of vitamin K deficiency, patients experience hypoprothrombinemia which results in poor blood clotting.

Each vitamin serves an integral part in maintaining proper function of the body. Treatment for diseases in which patients experience dysregulation of vitamins often includes supplementation of the required micronutrient, but the efficacy of supplementation on bioavailability still must be elucidated. Proper understanding of formulation and vitamin-protein interactions provides insight into optimized treatments and nutrition recommendations.

### 1.1.2 Minerals

Unlike their vitamin counterparts, mineral micronutrients cannot be metabolized in the body.[1] Mineral micronutrients include main group elements (e.g. boron, silicon, iodine), alkaline earth metals (e.g. magnesium, calcium), alkali metals (e.g. sodium, potassium), and d-block metals (e.g. copper, iron, cobalt, zinc, manganese, nickel). Such micronutrients are found in a variety of foods spanning dairy products, meat, fruits, and vegetables.

Calcium is one of the most commonly touted mineral micronutrients that is associated with bone health.[1] Indeed, calcium is stored in bones and teeth for its use through blood transport elsewhere in the body. Calcium participates in the named calcium channels which regulate potential changes related to cell signaling.[30] Notably, proper calcium channel maintenance is required for cardiovascular function and muscle activity. The most well-known disease linked to

calcium deficiency is osteoporosis which is characterized by loss of bone mass.[31] While genetic factors do play a role in osteoporosis, intake of calcium can mitigate risks of disease onset. Calcium is consumed through dairy products, leafy greens, legumes, and nuts.

Similar to calcium, potassium channels traffic potassium ions thereby inducing electrochemical gradients which are used for biochemical processes including cell signaling.[32] Potassium is famously consumed through bananas though also present in other fruits such as oranges and avocados.[1] Due to the functions of potassium channels, potassium deficiency is linked to muscle cramps and cardiovascular disease relating to improperly regulated electrochemical gradients.

Another s-block element cation, sodium, has ion channels that are responsible for forming action potentials in excitable cells.[33] Participating in neuronal and cardiovascular pathways, sodium is required for maintenance of human life. Sodium is sufficiently obtained through diet, perhaps in excess with the addition of table salt, sodium chloride. Though rare, sodium deficiency (hyponatremia) can occur in instances of excessive sweating, diarrhea, vomiting, and in kidney disease.[34] Hyponatremia presents as symptoms of muscle weakness, cramps, nausea, and coma.

The counterion to sodium in table salt, chloride, is an anionic micronutrient. Chloride channels which traffic chloride across cell membranes are found in tracheal, airway, and nasal epithelial cells.[35] Chloride also plays a key role in cell volume through hydration mechanisms, and its dysregulation is linked to cystic fibrosis.[36] Fluoride, an anion famously linked to dental health, is required for the mineralization of teeth and bones.[37] Fluoride is often obtained through drinking water and can be supplemented to prevent fluoride-deficiency-associated tooth decay.

Main group micronutrients are promoted for their beneficial health effects, while their d-block metal equivalents are commonly regarded as toxic. While redox-active metal

micronutrients may exhibit toxic effects when dysregulated, they play many integral roles in maintaining proper function of human biology.

## 1.2 Metals in Biology

Metal micronutrients including copper, iron, and zinc are minerals that are unable to be broken down in the body.[38] They function as structural cofactors, serve as redox centers, and participate in signaling. The most common metal micronutrients are iron, copper, and zinc, but humans also require manganese, nickel, and cobalt.

While most metals are consumed in mineral form, the notable exception is cobalt which is consumed in the form of vitamin $B_{12}$.[39] Vitamin $B_{12}$, or cobalamin, serves as a cofactor for enzymes methionine synthase and methylmalonyl-CoA mutase which participate in methylation processes including DNA synthesis and nerve maintenance.[19] Like with its mineral counterparts, dysregulation of vitamin $B_{12}$ is related to health issues including pernicious anemia and Chron's disease.

The most commonly recognized d-block metal micronutrient is iron. Iron binds to hemoglobin and is essential for respiration and oxygen transport within the body.[40] Iron is also involved in DNA synthesis and participates in controlled redox reactions in cells and blood. When dysregulation occurs in iron metabolism, redox-active iron ions can participate in Fenton chemistry causing oxidative stress. Iron is consumed through diet often in meat, legumes, fruits, and vegetables.[41] When an insufficient amount of iron is consumed, the body may experience iron deficiency in which there is no stored iron accessible.[42] Iron deficiency often leads to anemia which affects cognitive function and the immune system. Anemia is treated through an increase in iron consumption either through diet or supplements.[41] While iron deficiency can cause anemia and related health concerns, iron overload is often linked to metabolic disease states including type 2 diabetes and non-alcoholic fatty liver disease.[43] The known mechanisms

of iron metabolism under pathological conditions continues to be investigated by the Heffern lab amongst others.

Unlike iron, zinc is not redox active due to its full d-orbital electron count. Zinc is obtained through diet in high levels from oysters, red meat, and poultry. Zinc participates in human biology as a metal cofactor and transcriptional regulator affecting functions ranging from immune response to bone growth.[38] Zinc metalloenzymes continue to be discovered, but the most widely-recognized zinc-containing proteins are called zinc finger proteins.[44] Zinc finger proteins were first discovered as nuclear transcriptional factors and can bind DNA. Such zinc finger transcriptional factors regulate processes including stem cell maintenance, cell proliferation, gluconeogenesis, and adipogenesis. Zinc is also employed in insulin storage where zinc stabilizes a trimer of insulin dimers.[45,46] Due to its link to insulin, it is no surprise that zinc dysregulation is associated with metabolic diseases, most notably diabetes. Zinc deficiency is linked to high glucose levels, though the mechanisms of zinc regulation under diabetic states remain under study.

Copper, like the aforementioned d-block metals, serves as a cofactor for a host of enzymes in the body.[47] Roles as static cofactors in oxygenases, hydrolases, and transferases take advantage of the redox-active nature of copper. Notably, copper serves as a cofactor to cytochrome c oxidase which is involved in respiration through electron transfer.[48] Copper is tightly regulated because, like iron, aberrant copper species can produce reactive oxygen species via Fenton-like chemistry and in turn can cause oxidative stress. Copper is obtained from consumption of foods like oysters, spinach, and dark chocolate and is trafficked through the body by chaperones in the blood and cytosol and transmembrane transporters.[49] While tightly bound copper is required for proper function of cuproenzymes, labile, or more loosely bound, copper is importantly modulated in metabolic disease states.[50]

### 1.2.1 Copper dysregulation and metabolic disease states

While necessary to maintain proper function, copper can exhibit toxic effects if is not tightly regulated. As such, copper dysregulation is related to a host of disease states including metabolic disorders and cancer. Copper is increasingly being recognized for its role in signaling and functions deriving from labile copper pools.[51] Studying the relationships of these labile pools with disease states and understanding mechanisms to modulate these populations is a focus of research in the Heffern lab.[52–59]

Genetic diseases affecting metal transport include Menkes and Wilson diseases where patients have mutations in copper exporters ATP7A and ATP7B, respectively.[60,61] Patients with Menkes disease have low copper levels in serum, liver, and the brain which presents as symptoms including hypopigmented hair, respiratory failure, and vascular complications.[62] Conversely, patients with Wilson disease have high serum copper levels and can experience steatosis or cirrhosis without timely treatment.[63] Chronic metabolic diseases like non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes (T2D) do not have direct relationships to metal metabolism but patients often exhibit altered clinical metal levels.[46,64]

NAFLD incidences are fast rising across the world with the disease characterized by fat deposits within the liver.[65] If allowed to progress without intervention, NAFLD can lead to irreversible liver damage from non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma. Patients with NAFLD clinically present with lower serum copper levels.[64] *In vivo* studies demonstrate that copper deficiency is linked to changes in metabolism and mitochondrial function.[56] As such, there is a need to understand the underlying causal relationships between copper dysregulation and NAFLD in order to develop better treatment plans. The Heffern lab investigated the effects of fatty acids on copper status in the liver.[57] HepG2 cells treated with palmitic acid, a saturated fatty acid, experienced changes in copper trafficking that ultimately led to copper deficiency in the cell. Information like this can be

harnessed to develop therapeutics targeting the involved proteins or the improperly localized copper.

Copper trafficking molecules are currently employed for treatment in diseases including Wilson and Menkes diseases.[66] Specifically, copper chelators are used which bind copper for excretion from the body thereby preventing copper overload. Chelators such as tetrathiomolybdate, trientine, and D-penicillamine are clinically prescribed with promising results. Further investigation is warranted for copper trafficking molecules that reorganize copper within the body rather than excrete the ions given the mislocalization of copper associated with disease.[61] Towards this end, copper-binding molecules inspired by plant natural products are being investigated for their potential nutraceutical effects.[67–69]

## 1.3 Therapeutics and natural products

Plants have been historically used for medicinal purposes predating modern science.[70] As such, plant metabolites have been extensively studied for their potential biochemical activity, and many drug candidates resemble compounds found in nature.[71] Thus, identification of compounds in plants that possess therapeutic activity provides a basis for rational design of drugs. Many plant-derived compounds have therapeutic effects and will be discussed in the review in Chapter 2. Here, I will highlight two important classes of plant-derived compounds that exhibit therapeutic effects towards diseases states associated with metal dysregulation.

### 1.3.1 Flavonoids as therapeutics and metal ligands

Plant-derived food products are often identified by their color or distinctive smell and taste. These characteristics are largely attributed to secondary plant metabolites, flavonoids.[72] Flavonoids are polyphenolic molecules that have a three-ring core consisting of the A and B phenyl rings joined by the central heterocyclic pyran C ring. Substitutions on this core structure divide flavonoids into subclasses: flavanols, flavanones, flavonols, flavones, anthocyanins, and

isoflavones. Over 8000 compounds fall under flavonoid classification, with more being discovered every year.[73] Flavonoids have been implicated for therapeutic effects towards diseases states including cancers, cardiovascular complications, neurological disorders, and metabolic pathologies.[74–76]

While flavonoids serve multiple biological roles, they are often touted for their antioxidant activity.[77,78] The phenolic nature of flavonoids lends itself to antioxidant behavior through radical scavenging and prevention of ROS generation. Furthermore, flavonoids can bind metal ions potentially combatting deleterious biological effects.[79–81] Redox active metals are known to participate in Fenton or Fenton-like chemistries resulting in the production of ROS and oxidative stress in biological systems. The ability of flavonoids to bind reactive metal ions with varying binding affinities allows for modulation of dynamic metal populations in disease states.

### 1.3.2 Plant protein hydrolysates towards therapeutic applications

Peptides from plant and animal sources display therapeutic effects against human pathologies.[82–84] Providing different advantages to their protein and small molecule therapeutic contenders, peptides are better able to interact with protein interfaces and cross cell membranes.[85–87] Proteins isolated from natural sources and subsequently hydrolyzed in the lab using commercially available proteases exhibit a range of therapeutic activities. Protein hydrolysates exhibit therapeutic effects towards cardiovascular, neurodegenerative, and metabolic diseases, amongst others.[88–91]

Plant protein hydrolysates have been isolated from a wide range of consumed plants including soy, oat, and rice plants. The identity of peptides resulting from protein digestion depends on the employed hydrolysis method. Techniques include solvent and enzyme hydrolysis as well as microbial fermentation.[92] The most common digestion technique is

enzymatic hydrolysis using proteases like trypsin, pepsin, and pancreatin. Each enzyme cleaves at specific residues on a protein resulting in different peptide sequences.

Plant protein hydrolysates exhibit a wide range of therapeutic activity. Like their small molecule counterparts, hydrolysates have antioxidant, anti-cancer, and anti-inflammatory activity. Due to their natural sources, plant protein hydrolysates are ideal candidates for nutraceutical applications with lower risk for side effects compared to their synthetic analogs. The potential therapeutic efficacy can be tuned by adjusting or selecting for physical properties such as water solubility, hydrophobicity, or pH stability.

## 1.4 Methods of Study

When studying complex systems, considerations must be made regarding both the questions and tools at hand. Heterogeneous biological systems often require targeted analysis while reducing unwanted background signaling. When adding metal ions into the equation, further consideration must be given to their oxidation states and coordination environments. Thus, bioinorganic chemistry employs a wide variety of techniques to study all aspects of biological systems and their interactions with metal ions. Here, I will provide a brief overview of techniques used for the work within this dissertation, with an emphasis on the uniquely suited properties of each technique for its applied study.

### 1.4.1 Spectroscopy

Metal-small molecule interactions are often probed with spectroscopic methods. Due to the unique spectroscopic properties of different metal centers, detailed information about the state of a metal and its environment can be garnered. The oxidation states of metals affect the method of study due to the changing electron counts. For example, paramagnetic copper(II) is better suited for electron paramagnetic resonance spectroscopy (EPR) whereas its reduced counterpart, diamagnetic copper(I), is EPR silent. Likewise, zinc(II) cannot be directly probed via

electronic absorption spectroscopy due to its $d^{10}$ electron count which prevents electronic transitions. Understanding the physical properties of the systems being studied is integral to effective experimental design and execution and subsequent data analysis.

#### 1.4.1.1 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique that can access molecular information as well as macromolecular characterization. To understand NMR, we first must understand the concept of spin. Spin is an intrinsic property that all fundamental particles possess and is quantized, as first shown by the Stern-Gerlach experiment in 1922.[93] The term "spin" is a misnomer in the sense that spin-possessing particles are not actually rotating on or around an axis. Instead, spin is dimensionless and is inferred through deflection in a magnetic field. While theorized for many years by the likes of Bohr, it was not until 1922 that the Stern-Gerlach experiment confirmed quantization of spin. The Stern-Gerlach experiment employed a gradient magnetic field through which a beam of silver atoms was fired. The silver atoms were deposited onto a detector screen in two distinct bands demonstrating deflection of particles due to spin. While this experiment predated the naissance of the field, it beautifully visualizes many important concepts in quantum mechanics.

While all fundamental particles possess spin, NMR specifically probes nuclear spins. Here, a classical approach to NMR will be described for the purpose of probing bioinorganic systems.[94] NMR employs a static magnetic field, $B_0$, with which nuclear spins align. When in an applied magnetic field, nuclear spins align with (parallel or spin up) or against (anti-parallel or spin down) the external magnetic field. The discrete energy levels associated with the parallel and anti-parallel states are observed in the Stern-Gerlach experiment and the energy difference between the states is referred to as the Zeeman splitting.[95] Zeeman splitting is positively correlated to the strength of the external magnetic field and is described as

$$\Delta E = \gamma \hbar B_0$$

where ħ is Planck's constant, $B_0$ is the magnitude of the applied field, and γ is the gyromagnetic ratio (Fig. 1).

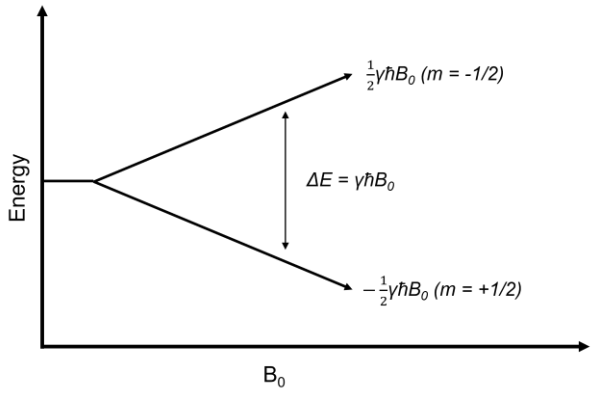



**Figure 1.1:** Zeeman splitting is dependent on the strength of the external applied magnetic field and the gyromagnetic ratio of the nucleus of interest.

When aligned with an external magnetic field, nuclear spins precess at a rate termed the Larmor frequency. Larmor frequency is described mathematically as,

$$\omega = -\gamma B_0$$

where γ is the gyromagnetic ratio, and $B_0$ is the magnitude of the applied magnetic field. The gyromagnetic ratio is described as the ratio of the charge, *q*, to the mass, *m* of the particle, and has units MHz/T:

$$\gamma = \frac{q}{2m}$$

In the presence of an external magnetic field, an ensemble of nuclei will align with the external field and have bulk magnetization in the direction of the applied field, or the +*z*-axis. A second magnetic field, $B_1$, which is perpendicular to $B_0$ is generated using a radiofrequency (RF) coil in the *xy*-plane.[95] Application of $B_1$ RF pulses tips the bulk magnetization away from $B_0$. Modern NMR experiments consist of series of RF pulses of varying lengths and frequencies to probe nuclei. The applied frequencies depend on strength of the external magnetic field as well as the Larmor frequency of the nuclei of interest.

The detection in an NMR experiment is current generated from the precessing nuclei in the magnetic field. After deflection of the bulk magnetization away from the *z*-axis by an applied RF pulse, the nuclei precess within the RF coil, generating a current which is recorded. Nuclear spins which are perturbed will relax back to alignment with the external magnetic field through two relaxation mechanisms. The first is spin-lattice, $T_1$ or longitudinal, relaxation which describes relaxation of the bulk magnetization to its parallel alignment with $B_0$. This relaxation occurs through energy transfer from the nuclear spin to the "lattice" or surrounding environment.[95] In Fourier Transform NMR (FT-NMR), the $T_1$ relaxation rate is related to the peak height. The second relaxation is spin-spin, $T_2$ or transverse, relaxation. $T_2$ relaxation describes the loss of coherence of the transverse components of magnetization.[95] $T_2$ relaxation rates are related to peak widths in FT-NMR.

The field of NMR relaxometry uses relaxation times to acquire physical and chemical information about samples such as the fat content, viscosity, or chemical makeup of known components. As opposed to traditional Fourier Transform NMR which is often used for structural elucidation, NMR relaxometry can provide important information at lower magnetic field strengths and unique sample geometries allowing for flexibility in experimental design. The instruments used for NMR relaxometry can vary from single-sided, low-field permanent magnets to high-field cryomagnets. Regardless of magnet set-up, relaxation rates can be obtained using the common pulse sequences.

For $T_1$ measurements, either an inversion recovery or a saturation recovery pulse sequence is employed. An inversion recovery pulse sequence starts with a π pulse to invert $M_0$ to $-M_0$ after which a π/2 pulse is used to tip the recovered magnetization into the *xy*-plane. Inversion recovery data is fit using the following equation, and the desired value is $T_1$ (Fig 2).

$$M_z(t) = A\left(1 - 2e^{-\frac{\tau}{T_1}}\right)$$

where A is a constant describing the initial amplitude and τ is the varied delay time between pulses. Inversion recovery experiments are advantageous in providing consistent and robust results. However, inversion recovery sequences require repetition times of at least 5 times $T_1$ which leads to long experiment time.

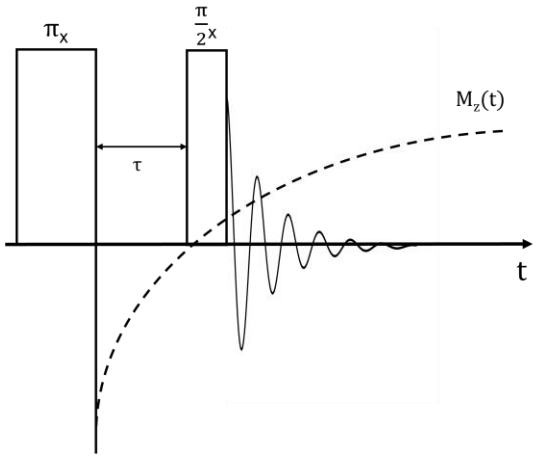



**Figure 1.2:** An inversion recovery pulse consists of a π pulse followed by a π/2 pulse. Varying the time between pulses results in a series of intensities which can be plotted and fit to determine $T_1$ values.

To circumvent this required long repetition time, saturation recovery pulse sequences can be used. Saturation recovery pulse sequences start by saturating the $M_z$ signal with a series of π/2 pulses such that $M_z = 0$ (Fig. 3). Similar to inversion recovery sequences, saturation recovery data can be fit with the following equation and solved for $T_1$ where the variables are as described above.

$$M_z(t) = A\left(1 - e^{-\frac{\tau}{T_1}}\right)$$

Though the stated advantage is shorter experiment time, saturation recovery pulse sequences have lower signal-to-noise ratios and therefore more inconsistent results.

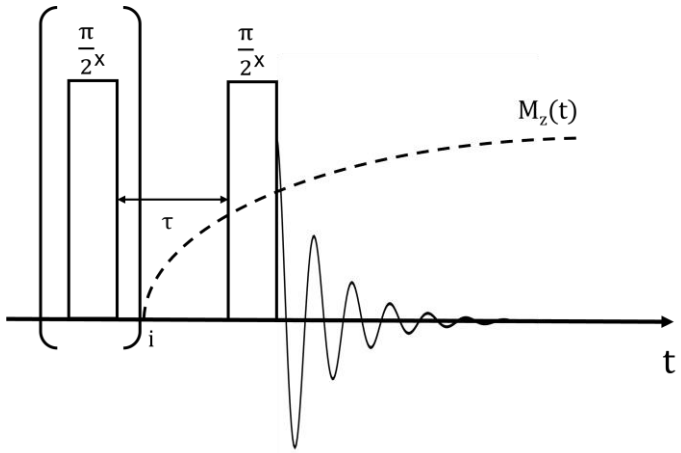



**Figure 1.3:** A saturation recovery sequences begins with a series of π/2 pulses to saturate the spins followed by a π/2 after a varying delay time. The data is collected and fit similarly to an inversion recovery experiment.

$T_2$ relaxation times are most commonly measured using the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. A π/2 pulse is applied followed by series of π pulses which produces a train of echoes. The intensity of the echoes decays overtime as the π pulses refocus less bulk magnetization due to $T_2$ relaxation. CPMG data is fit using an exponential with the desired value of $T_2$ in the exponent.

$$M_z(t) = Ae^{-\frac{t_e}{T_2}}$$

The variables are as described previously where A is the initial amplitude, and $t_e$ is the echo time, or the time between echoes.

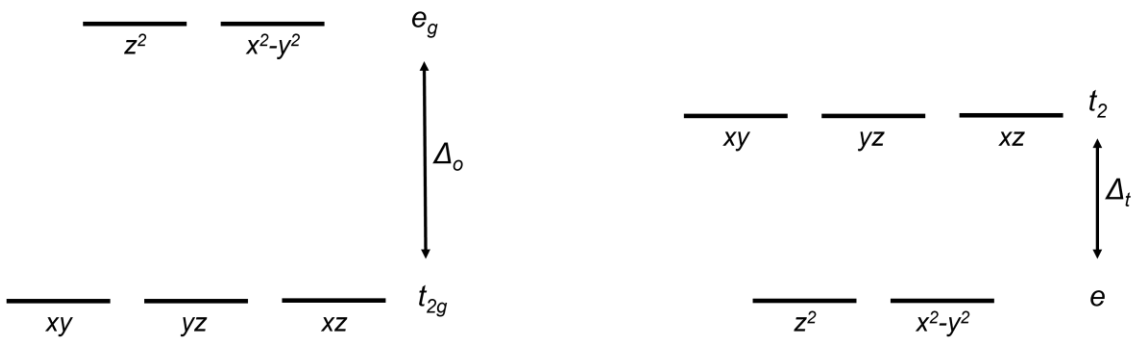
While NMR is often used in chemistry and biology for structural elucidation, relaxation rates can provide insight into properties of complex, heterogeneous solutions. Of importance to the field of bioinorganic chemistry is the sensitivity of NMR signals to the presence of paramagnetic species in solution.[95] Unpaired electrons interact with nuclear spins allowing fast relaxation to occur. This is due to the relatively large (three orders of magnitude) gyromagnetic ratio of electron spins compared to nuclear spins. Many biologically relevant metal ions are paramagnetic including $Fe^{2+}$, $Fe^{3+}$, and $Cu^{2+}$. The paramagnetic nature of these ions can be

exploited when studying a system via NMR. For example, increased relaxation rates caused by interactions with paramagnetic ions lead to wider peak widths and shorter peak heights in FT-NMR. Isolated instances of peak broadening allow for identification of molecular sites involved in metal-binding.

#### 1.4.1.2 Absorption spectroscopy

D-block metal-ligand interactions lend themselves to study by ultraviolet-visible (UV-Vis) absorption spectroscopy due to electronic transitions of the d-orbital electrons. UV-vis spectroscopy uses wavelengths between approximately 100-900 nm to probe electronic transitions of valance electrons between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO).[96]

Often, metal ion solutions are colorful due to the energy of electronic transitions that correspond to the visible region. Transitions between d-orbitals of a given metal ion produce relatively weak absorption profiles and vary depending on the geometry of the surrounding ligands. Tetrahedral complex d-d transitions are more intense compared to their octahedral complex counterparts.[97] The reason for the decreased intensity of octahedral complex d-d transitions is selection rules. The Laporte rule states that transitions between states that are both symmetric or antisymmetric with respect to an inversion center are forbidden. Because of the gerade symmetry of octahedral complex d-orbitals, the transition is forbidden. Tetrahedral complexes have no inversion center, and therefore the transition between d-orbitals in a tetrahedral complex do not violate the Laporte rule (Fig. 4). Regardless, d-d transitions occur at relatively low energy levels, absorbing longer wavelengths.




Octahedral Complex Tetrahedral Complex

**Figure 1.4:** Octahedral metal complexes have Laporte forbidden transitions and therefore have less intense UV-Vis signatures than tetrahedral metal complexes.

While d-d transitions within a metal ion can be probed, more often, charge transfer transitions are studied. There are two types of charge transfer transitions that can occur. The first is metal-to-ligand charge transfer (MLCT) in which an electron from the metal ion is excited to an unoccupied orbital of the ligand. Conversely, in a ligand-to-metal charge transfer (LMCT), an electron from the HOMO of the ligand is excited to the LUMO of the metal ion. Charge transfer bands are usually very intense and are often responsible for the vibrant colors of metal ion solutions.

In this dissertation, UV-Vis spectroscopy is used to probe interactions between metal ions and organic ligands. Commonly, metal status is determined using colorimetric chelators.[98,99] Colorimetric chelators describe a group of organic molecules that have unique absorption profiles when bound to specific metal ions. The absorbance of the chelator-metal complex can be monitored to understand competitive binding by molecules of interest providing information about the metal ions in solution. Exploiting the high intensity of charge transfer transitions and employing the Beer-Lambert law allows for calculation of concentrations of bound metal ions in solution.[100] The Beer-Lambert law,

$$A = \varepsilon l c$$

states the relationship between absorbance (A), the pathlength through which light travels (l), and concentration (c), where ε is the molar extinction coefficient which describes the probability of a given transition in L $mol^{-1}$ $cm^{-1}$.

Colorimetric chelators are specifically useful to study interactions of $Zn^{2+}$ in solution. $Zn^{2+}$ is spectroscopically silent due to its $d^{10}$ electron count meaning there are no electronic transitions occurring involving the d-electrons of $Zn^{2+}$. Instead, a colorimetric chelator, zincon, is used to track chelatable $Zn^{2+}$ in solution.[98] Generally, calculation of the concentration of metal ions in solution allows for interpretation of information about binding affinities, binding ratios, and oxidation state. For example, the Heffern Lab used phenanthroline to approximate the binding affinity of C-peptide and copper by monitoring phenanthroline-$Cu^{2+}$ absorbance.[101] Similarly, redox reactions involving $Cu^{2+}$ can be monitored using bathocuproine disulfonic acid (BCS) which is a $Cu^{+}$-specific chelator.[102]

UV-Vis spectroscopy is a versatile tool to study bioinorganic systems. Additional advantages of the technique are the ability to perform high-throughput, colorimetric assays using a plate reader.

### 1.4.2 Biological settings

Assessing metal-binding in controlled, buffered solutions provides a basis for understanding the more complex interactions in biological systems. Isolating interactions within biological settings remains a challenge, but there are techniques that prove very handy in understanding the effects of metal-ligand interactions on downstream biological effects. Here, techniques that were used to elucidate such effects are described as they pertain the data contained in this dissertation.

#### 1.4.2.1 In vitro and in vivo experiments

Cell-based studies performed outside of a living organism are considered to be *in vitro* assays. Such assays allow researchers to perform more targeted analysis by removing some of the whole-organism complexities. Growth and metabolism vary by cell line. Therefore, it is imperative to select specific cell lines based on the question at hand. Once a cell line is chosen, there are a vast number of cell-based assays that comprise the area of *in vitro* assays. Cell-based assays allow researchers to probe the effects of target molecules, study cell signaling events, and monitor organelle function, among much else. Of particular importance to this dissertation are cell viability and transport assays.

Regardless of the cell-based assay performed, it is important to understand how different treatments affect cell viability. There are different methods by which cell viability can be measured including tetrazolium reduction and ATP detection.[103] ATP detection uses firefly luciferase to generate photons as the output signal. The luciferin substrate in the presence of ATP reacts with its luciferase enzyme pair to generate light output. ATP detection assays are the most sensitive cell viability assays partly due to the bioluminescent output which affords greater signal-to-noise ratios with no background signal. An additional advantage of ATP detection assays is that there is no incubation step preventing potential disruption to viable cells.

Tetrazolium reduction assays rely on the reduction of tetrazolium compounds by the mitochondria of viable cells to generate a formazan product which can be monitored at a specific wavelength. In this dissertation, the MTS tetrazolium compound is used to assess cell viability of HepG2 cells.[59] The generated formazan product absorbs at a wavelength of 490 nm which is monitored to track cell viability.

Depending on the experimental set up, cell viability assays can also assess cell proliferation and cytotoxicity. Though cell proliferation and cell viability are two distinct properties, they can often be assessed using the same assay. Tracking cell viability over time

can provide information about cell proliferation under different conditions. Further, treatment of cells with a target compound followed by cell viability assays allows for assessment of potential cytotoxic effects of said compounds.

Transport assays allow researchers to better understand how compounds move within biological systems.[104] *In vitro* transport assays often employ permeable cell culture inserts which compartmentalize the well into apical and basolateral environments. Treatment of the cells on either side followed by analysis of the two environments provides insight into the passage of compounds across cell monolayers.

Beyond cell-based assays, cell models can be used to elucidate mechanisms of action of varied treatment conditions. In this dissertation, Western blot analysis is used to study expression of specific proteins involved in metal trafficking and metabolic processes. This data is further supported by gene expression analyzed using qPCR analysis.

While *in vitro* studies provide a basis for understanding the biological effects of various treatments, whole organism studies give a fuller picture. Whole organism, or *in vivo* studies, offer meaningful information about biological effects of various treatments. While *in vivo* tests often refer to clinical evaluations in animal models, *in vivo* assays can be performed routinely in smaller organisms such as yeast. Such assays will be discussed here for the analysis of metal trafficking within a whole organism.

In addition to having a whole organism platform, one benefit to using a yeast model is the commercially available knockout strains. A pioneering genome project led to a collection of tens of thousands of yeast knockout strains.[105] Knockout strains allow researchers to probe mechanisms by which compounds impart biological effects. For the work described in this dissertation, proteins involved in metal transport and metal metabolism can be removed from yeast models to better understand the transport mechanisms of target compounds.[106]

## 1.5 Conclusion and Overview

Complex questions require multidisciplinary approaches to provide sufficient solutions. The interplay between nutrition, disease, and metal metabolism is indeed a complex field with room for researchers from vastly different disciplines to contribute. This dissertation describes my attempts to understand even a small fraction of this area or to at least provide a foundation off which future Heffern lab members can build.

Chapter 2 describes in more detail the potential role plant-derived products can play in metal trafficking and therapies for metal-dysregulation-related disorders. Researchers draw much inspiration from nature, and it is imperative to reflect what nature can produce to better inform future studies.

Chapter 3 presents a more focused study of the interactions between plant-derived flavonoids and copper. Solution-state studies construct a basic understanding of metal-ligand interactions from which biological studies are used to probe therapeutic effects of flavonoid-metal complexes.

In Chapter 4, another group of plant-derived compounds, rice bran protein hydrolysates, are investigated for their copper-related biological effects. Building off previous understanding of the copper-binding abilities and anti-diabetic effects of plant protein hydrolysates, we describe a collection of peptides that exhibit beneficial health effects.

Chapter 5 provides a brief overview of an NMR approach to understanding the binding environment of vitamin $B_{12}$, or cobalamin. Both high-field Fourier Transform NMR and low-field NMR relaxometry were used to probe the cobalt metal center in cobalamin.

Finally, Chapter 6 highlights work performed in collaboration with Justin O'Sullivan which elucidates the mechanism by which copper inhibits bioluminescent output in marine luciferases. The information presented here should be considered when employing bioluminescent strategies in the presence of copper ions.

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# Chapter 2

## Transition Metal Chelators and Ionophores as Potential Therapeutics for Metabolic Diseases*

*This chapter is adapted from a manuscript prepared for publication entitled: Transition Metal Chelators and Ionophores and Potential Therapeutics for Metabolic Diseases. This work was a collaboration with Samuel Janisse. Sam wrote the section on metabolic diseases; Vanessa wrote the remaining sections.

## 2.1 Abstract

Transition metal dysregulation is associated with a host of pathologies, many of which are therapeutically targeted using chelators and ionophores. Chelators and ionophores are used as therapeutic metal-binding compounds which impart biological effects by sequestering or trafficking endogenous metal ions in an effort to restore homeostasis. Many current therapies take inspiration or derive directly from small molecules and peptides found in plants. This review focuses on plant-derived small molecule and peptide chelators and ionophores that can affect metabolic disease states. Understanding the coordination chemistry, bioavailability, and bioactivity of such molecules provides the tools to further research applications of plant-based chelators and ionophores.

## 2.2 Introduction

Transition metals are pervasive in biology and are increasingly recognized for their essential biological activity beyond their traditional role as tightly bound structural cofactors. It has long been established that redox-active transition metals serve as static cofactors for an estimated one-third of all proteins as well as DNA and RNA.[1] Conversely, the main group alkali earth metals that are identified for participation in signaling pathways use labile metal pools.[2] Progressively however, transition metals (most notably copper, iron, zinc, and manganese) are being studied for their involvement in signaling pathways through more loosely bound pools.[3] The makeup of these populations remains elusive, but observed differences in labile transition metal pools is associated with a host of disease states.[4–6]

Due to their potential toxicity, transition metals are tightly regulated in biology. Dysregulation of transition metals is correlated with a wide range of pathologies including cancer, cardiovascular disease, neurodegenerative diseases, and metabolic diseases.[7–12] Metabolic diseases include inherited disorders, such as Wilson and Menkes diseases, and

chronic conditions including diabetes mellitus and non-alcoholic fatty liver disease (NAFLD). Some metabolic diseases, like Wilson and Menkes diseases, have clear connections to metal metabolism through mutations in metal trafficking proteins. Others, including NAFLD and metabolic syndrome, have no direct relationship to metal metabolism, but have been correlated with transition metal dysregulation.[7,12] As such, treatments for these diseases include attempts to restore metal homeostasis through employment of chelators and ionophores.

Chelation therapy is well-established in the treatment of Wilson disease and has gone through clinical trials for the treatment of cancer and Alzheimer's disease.[7,13,14] These treatments use chelators which are small molecules that selectively bind, sequester, and evacuate metal ions from the cell. Conversely, ionophores are small molecules that bind and import metal ions into the cell circumventing standard metal ion importers. While chelators and ionophores serve different purposes, they share necessary properties for metal trafficking. These properties include a low molecular weight, hydrophobicity that is sufficient for crossing cell membranes, and the ability to specifically bind metal ions.[15]

Many of the properties that are ideal for chelators and ionophores can be found in small molecules derived from plants. Medicinal chemistry has long taken inspiration from nature, thus many drug candidates emulate small molecules and peptides found in plants.[16] Due to the association between many chronic metabolic diseases and diet, it is natural to look towards food sources in treatments of such diseases. One of the common health benefits of plant products is antioxidant activity.[17] Classes of small molecules such as polyphenols and carotenoids have long been studied for radical scavenging activity and protection against oxidative stress. These small molecules often possess antioxidant activity through interactions with metal ions. Metal ions such as iron and copper perform Fenton or Fenton-like chemistry which is a source of reactive oxygen species (ROS).[18,19] ROS production is linked to metabolic regulation, and as such, perturbation in ROS production is associated with metabolic diseases. For detailed

information on the pathways affected by ROS production in metabolic regulation, see the review by Forrester et al.[20] Oxidative damage induced by redox-active metals can be combatted by reduction or chelation of said metal ions. Thus, small molecules that can bind metal ions may reduce their reactivity and thereby reduce oxidative stress.

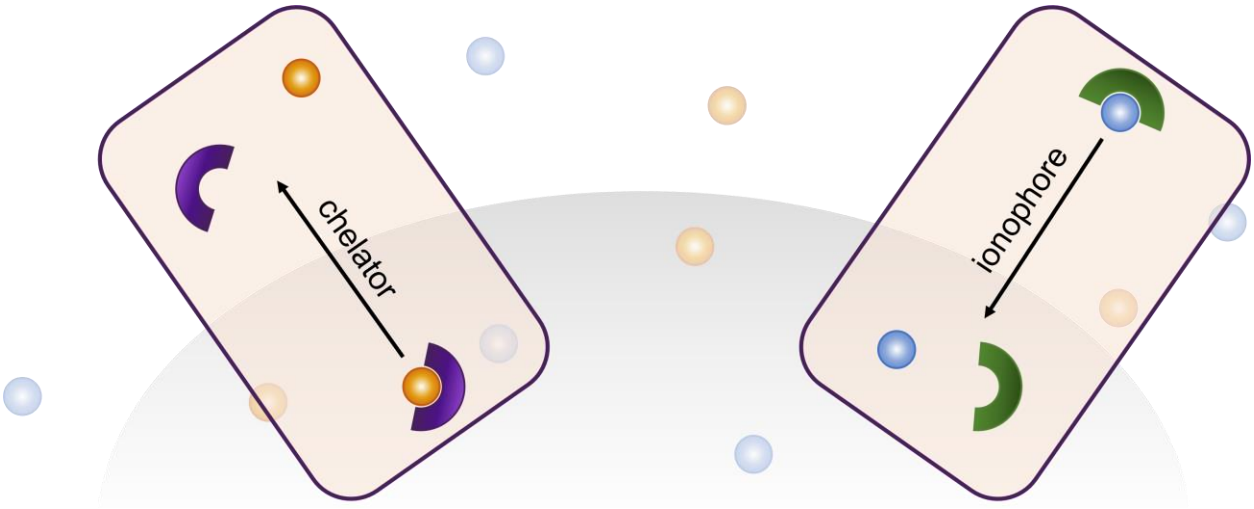


**Figure 2.1:** Chelators are small molecules that can cross cell membranes, bind metal ions, and subsequently evacuate bound ions from the cell. Conversely, ionophores are small molecules that extracellularly bind metal ions and import them into the cell passing through the cell membrane.

This review will focus on plant-derived small molecules and peptides towards therapy for metabolic diseases.

## 2.3 Metabolic diseases related to metal dysregulation and metal-binders used in treatment

### 2.3.1 Hereditary Diseases Related to Metal Dysregulation

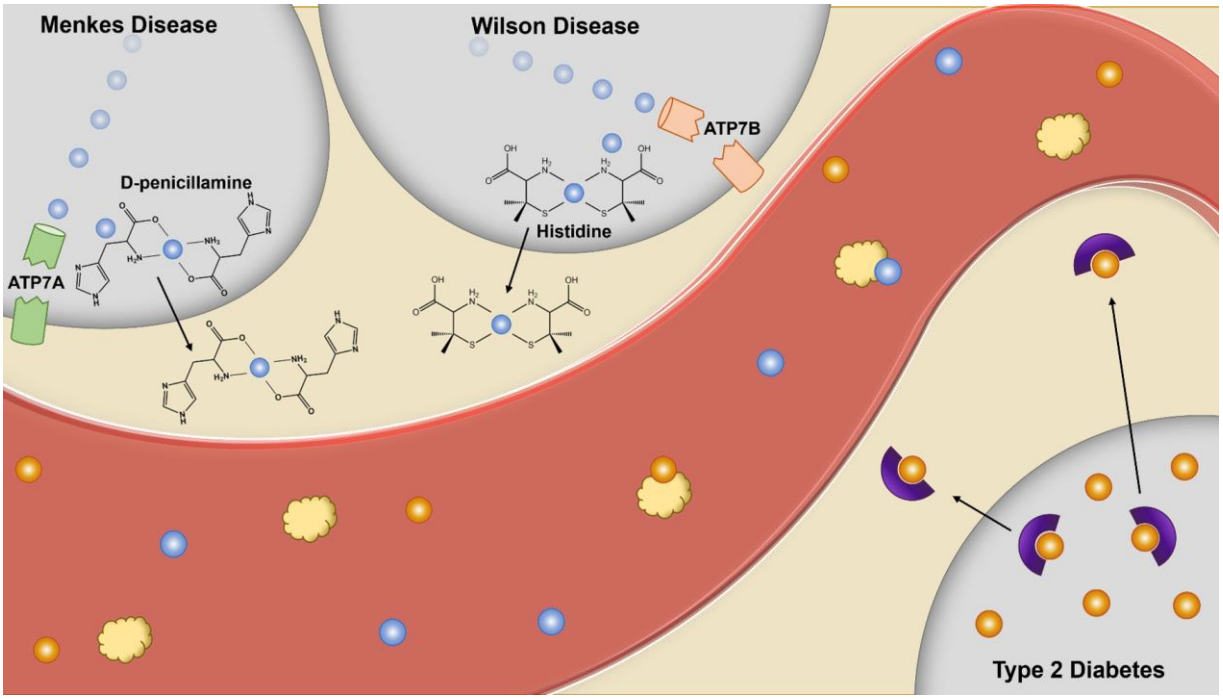


**Figure 2.2:** Hereditary diseases including Menkes and Wilson diseases are linked to mutations in copper-trafficking proteins. These mutations result in dysregulation of copper populations and subsequent detrimental symptoms.

#### 2.3.1.1 Wilson Disease

Wilson's disease (WD) is a hereditary disease effecting an estimated 1 in 30,000 [21] people worldwide. WD involves a mutation in the ATPase copper transporting beta protein (ATP7B) resulting in the obliteration of the copper transporter's ability to export copper via the biliary excretion pathway and uncontrolled copper accumulation in several organs[22]. The copper overload in patients with WD leads to deleterious neurological and hepatic outcomes, such as steatosis and cirrhosis [23] [24] [25]. While the exact mechanisms of liver damage in WD is unclear, the generation of excess oxidative stress due to the buildup of copper culminating in an increase in lipid peroxidation and hepatic dysfunction[26,27]. Moreover, the reduction in

intracellular copper transport from the mutation in ATP7B prevents adequate copper loading into the ferroxidase ceruloplasmin as it matures through the trans-Golgi network[28] . This results in a drastic increase of apoceruloplasmin, or non-copper binding ceruloplasmin, relative to holoceruloplasmin which contains copper. Currently, the diagnostic criteria for WD involves screening patients relies on serum ceruloplasmin and copper levels, which are often significantly lower and higher, respectively. The increase in non-ceruloplasmin bound copper is believed to be a direct result of the release of copper from degrading hepatocytes. Although damage can occur if copper imbalance in not remedied, if diagnosed early, WD can be managed.

Current treatments include zinc supplementation and copper chelator therapy. Zinc supplementation is believed to inhibit copper uptake in the gastrointestinal tract by inducing metallothionine production [29], which in turn results in increased metallothionein bound copper and subsequent excretion by intestinal sloughing[30]. While zinc supplementation has consistently been effective in WD maintenance, it has been suggested that it is not as effective as chelating therapies in preventing liver damage [31]. Chelation therapy for WD was first suggested in the early 1950's when researchers found that administering 2,3-dimercaptopropalol (also called BAL) lead to a significant decrease in neurological symptoms observed in patients [32]. However, despite BAL showing a profound impact increasing quality and length of life for patients with WD, it required regular invasive intramuscular injection leading to the development more accessible treatments [33].

Currently, the most common chelators used in the treatment of WD are D-penicillamine (DPA) and trientine (TETA), which are taken orally. DPA, first used as a therapeutic 1956 [34], contains three main functional groups: thiol, amine, and carboxylic acid. D-penicillamine is reported to be a bidentate chelator binding copper(I) with the amine and thiol functional group and a tridentate chelator for copper(II) where the carboxylate is thought to participate [35]. Once

binding to copper, the D-penicillamine-copper complex is excreted via the urine. While an improvement to BAL, DPA has been associated with various disadvantageous side effects, such as gastrointestinal irritation, cytopenia, proteinuria, myasthenic syndrome, and degenerative dermopathy [36,37]. Triene is the other commonly administered copper chelator used to treat WD. TETA is a polyamine containing a total of four nitrogen groups with two primary amines and two additional secondary amines separated by two aliphatic carbons ($-CH_2CH_2-$). The four nitrogen atoms coordinate copper resulting in a square planar complex. Similar to DPA, once copper is bound by TETA it is excreted via the kidneys. Moreover, it has been shown to have efficacy in reducing copper absorption is taken prior to eating. However, despite TEBA being distributed throughout various tissues post administration, the copper pool that TETA chelates is believed to be primarily in the blood whereas DPA can extract copper from tissues [38].

#### 2.3.1.2 Menkes disease

Menkes disease (MD), and the less severe occipital horn syndrome (OHS), is another hereditary disease involving the dysregulation of copper[39]. The X-linked genetic disorder involves the mutation of the copper transporter ATP7A, which regulates copper by utilizing ATP to transport copper across cell membranes. Classical MD is often lethal with an average life expectancy of less than three years [40] while those with OHS exhibit a longer lifespan. ATP7A is heavily involved in the transport of copper from the intestine after import by CTR1 and DMT1 into the blood where it is transported to the liver and other organs for utilization in various proteins. Mutations in ATP7A results in the aberrant transport of copper through the intestine resulting is low copper levels in serum, liver, and brain [41]. Diagnosis does not usually occur until the age of 3-6 months due to the appearance of hypopigmented hair that is prone to fraying, failure to thrive, vomiting, diarrhea, and loss consistent seizures. Later symptoms often include blindness, respiratory failure, and vascular complications which ultimately lead to death.

Currently, the only treatment of MD and OHS is through the subcutaneous injection of copper histidine (CuHis), which is comprised of copper coordinated by histidine in a 1:2 stochometric ratio. Early and sustained intervention with CuHis leads to an increase in life expectancy and been shown to increase serum copper, CSF copper, and ceruloplasmin levels[42–44]. The mechanism of action has not been fully elucidated, however, the injection of CuHis complex into the subcutaneous tissues bypasses the gastrointestinal track and is introduced in the bloodstream. Once in the bloodstream the copper can be chelated by the various copper chelating proteins, such as albumin, or exist as the CuHis.

### 2.3.2 Diseases Related to Metal Dysregulation Linked to Hereditary and Environmental Causes

#### 2.3.2.1 Type 2 Diabetes, Obesity, and Metabolic Syndrome

Type 2 diabetes mellitus (T2D) is a rapidly expanding disease affecting millions of people worldwide. It is a metabolic disorder involving the dysregulation of lipid and glucose metabolism. The dysregulation has been directly linked to impaired insulin secretion by the pancreas and insulin resistance in peripherical tissues such as the liver and adipose [45,46]. Various factors have been associated with the onset of T2D including the dysregulation of metal micronutrients, such as iron, copper, and zinc [47,48]. In patients with T2D, there is a positive association between serum copper to zinc ratios T2D as well as glycated hemoglobin [49].

The association with iron and T2D has been observed in patients with hereditary hemostasis (HH) which is an iron disorder leading to iron accumulation in various tissues and increased serum ferritin[50,51]. However, there is a growing interest on the role of dietary iron and non-hereditary iron overload with T2D disease progression [52]. The use of iron chelators to treat iron overload has been well documented with various animal studies illustrating their utility.

Early studies showed that obese (ob/ob lep-/-) mice were protected from deleterious effects of diabetes onset such as glucose intolerance and insulin resistance by the iron chelator FBS0701 administration [53]. Moreover, it was found that 15 day interparental administration of deferoxamine (DFO) for 15 days led to decreased insulin resistance in adipose tissues of ob/ob mice [54]. The exact mechanism of these preventative outcomes have yet to be fully elucidated. One explanation is there is a reduction of oxidative stress associated with dysregulated iron levels that produce reactive oxygen species (ROS). The increase of ROS can lead to lipid peroxidation and advanced glycation end products [54–56]. There seems to be a link between iron chelation and preventing excess weight gain, which has been linked to a decrease in systemic oxidative stress. A recent study showed that mice who were fed a high fat diet supplemented with the iron chelator deferasirox (DFS) weighed less than non-chelator high fat diet (HFD) control and obese mice on a HFD supplemented with DFS also led to a reduction in weight gained compared to HFD ob/ob mice [57].

Flavonoids have been linked to a decrease in T2D prevalence, obesity, and involved in glucose metabolism. A cross-sectional study showed a strong negative association strong negative association between daily quercetin intake and the prevalence of T2D [58] while another study showed that daily flavonoid intake lead to a lower prevalence of diabetes and the inflammation marker – C-reactive protein [59]. Moreover, the daily intake of flavonoids was found inversely related to the prevalence of obesity. Administration of quercetin to Sprague-Dawley rats with streptozocin induced diabetes showed improvements in hepatic glucose and lipid metabolism through increased Akt activity [60]. Studies conducted in skeletal muscle L6 myotubes showed that quercetin acts through the AMPK pathway in a manner similar to metformin [61], as well as GLUT4 translocation to the membrane in mouse skeletal muscle [62]. A further link between the beneficial aspects of dietary quercetin and the reduction of ferroptosis, a mechanism in which lipid peroxidation catalyzed by iron leads to programmed cell death, in

mouse pancreatic islets [63]. Interestingly, the authors also found that administration of DFO resulted in similar protective outcomes for ferroptosis induced by high glucose, potentially suggesting a mechanism where quercetin directly interacts with iron to prevent the generation of ROS, protecting the cell from the onset of ferroptosis.

#### 2.3.2.2 Cancer

Cancer is defined by a dysregulation of biochemical processes that govern proper cell homeostasis, leading to uncontrolled cell proliferation and resistance to cell death [64]. Aberrant metal micronutrients levels, such as copper, have been linked to various types of cancers[65–68]. Copper levels in the tumor microenvironment have been directly related to cancer cell proliferation and angiogenesis [69]. The mechanism of how metals such as copper influence tumor progression and metastasis is relatively unexplored. However, recent research has elucidated copper trafficking through ATP7A, ATOX1, and LOX as a key pathway in breast cancer migration [70]. Furthermore, recent studies have elucidated copper as a key regulator of the autophagic kinases ULK1/2 through direct metal binding in lung adenocarcinoma [71].

Due to the role of copper in cancer progression, there is growing interest in the application of copper depletion therapies for cancer treatments. Application of the copper chelator tetrathiomolybdate (TM) decreases the metastases of triple negative breast cancer to the lungs [72]. The exact mechanism of TM reducing cancer metastasis remains elusive, but recent research illustrates a link between the tumor microenvironment and collagen processing through the lysyl oxidase axis.[73] Additional research has revealed that TM mediates the inhibition of the mitochondrial Complex IV, which is involved in mitochondria energy production, via copper depletion. [65]

Beyond copper, the application the zinc chelator N,N,N,N-Tetrakis(2-pyridylmethyl)-ehtlyenediamine (TPEN) to pancreatic cancer results in increased cell apoptosis and autophagy

*in vitro.* [74] Iron chelation by deferasirox (DFX) inhibited the migration and reduced invasiveness of pancreatic cancer by reducing the activity of Rac1 and Cdc42, which are involved in a plethora of pro-cancer mechanisms such as tumor growth, migration, and angiogenesis.[75] This finding was significant as DFX can be given orally in contrast to DFO, which has been shown to decrease in tumor size in patients with hepatocellular carcinoma but requires intravenous application. [76]

Anti-cancer properties of quercetin have been explored and show promise in reducing the severity of cancer. Mice given quercetin via oral gavage post tumor induction had a five-fold increase in life span compared to the vehicle [77]. The authors illustrated that quercetin intercalates with the DNA in cancer cells leading to S phase cell cycle arrest and subsequent apoptosis. Quercetin can also act by repressing expression of the receptor to advanced glycation end products (RAGE) leading to an increase in apoptosis and autophagy in pancreatic cells [78].

## 2.4 Plant-derived molecules with bioactivity related to transition metals

Plants have been historically used for medicinal purposes predating modern science. As such, plant metabolites have been extensively studied for their potential biochemical activity, and many drug candidates resemble compounds found in nature. There are two major groups of plant-derived compounds that are identified for their bioactivity: phenolic compounds and peptides.

### 2.4.1 Phenolic compounds

Plant phenolic compounds, often referred to as polyphenols, are a class of small molecules that include molecules such as flavonoids, coumarins, and lignans. By definition, polyphenols are compounds that are composed of multiple phenolic rings. However, the term

has been colloquially used to describe phenolic compounds including monophenols like catechol. To read more about the history and definition of the term polyphenols, you can read a review by Quideau et al.[79]

Polyphenols are secondary metabolites from fruits and vegetables and serve a variety of purposes in plants including aroma and color. Polyphenols are found in all plant products we consume and are often the source of the health benefits advertised for various herbs, fruits, and vegetables. Most plant polyphenols exist as conjugated forms (glycosides, esters, and amides) rather than in their free forms. The seemingly endless identification of novel plant phenols broaches the variety of roles they play in plant biology. Plant phenols are involved in activities ranging from protective effects (against predators or radiation) to reproduction to signaling.[79] The vast range of plant phenols necessitates categorization. Each subgroup of plant polyphenols shares a core structure and has a wide range of substitutions on the ring structure. Of the subgroups of plant polyphenols, flavonoids are the most prevalently studied.

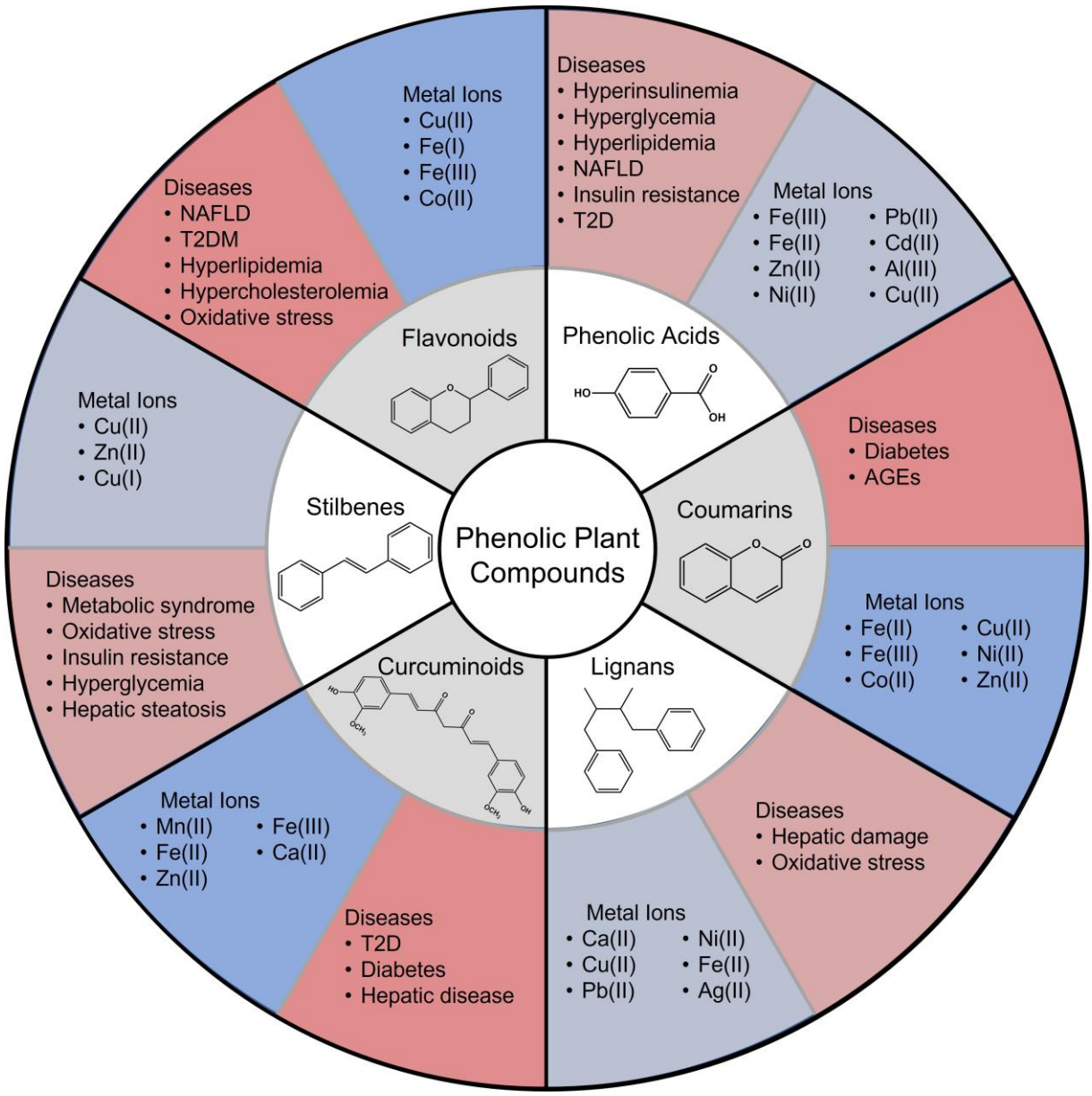


**Figure 2.3:** Phenolic plant compounds are known to affect biological function under metabolic disease states. Additionally these phenolic compounds interact with d-block metal ions with little known about the intersection between effects on metabolic disease states.

### 2.4.1.1 Flavonoids

Over 8000 molecules comprise the largest group of plant polyphenols, flavonoids. Flavonoids all share a core structure consisting of three rings: two phenyl rings (A and B) joined by a heterocyclic pyran ring (C). Subclasses of flavonoids are defined by substitution on and

oxidation of the heterocyclic C-ring. There are subclasses of flavonoids: flavanols, flavanones, flavonols, flavones, anthocyanins, isoflavones, and chalcones. Flavonoids, which are found in all parts of plants, are most often isolated via extraction from their natural sources.[79] Extraction is most commonly performed via a mixture of organic and aqueous solvents, though more current methods are continually being optimized.[80] Flavonoids have historic medicinal purposes, and modern techniques have been used to elucidate the bioactivity of flavonoids in diseases ranging from cancer to cardiovascular disease to metabolic diseases.[81–84]

Flavonoids have been used in the treatment of diabetes, non-alcoholic fatty liver disease (NAFLD), and hyperlipidemia.[82,85,86] Yi et al. highlight the advances of the flavonol quercetin in clinical trials for treatment of metabolic diseases.[85] Quercetin has entered clinical trials in the treatment of type 2 diabetes mellitus (T2DM), hyperlipidemia, hypercholesterolemia, and NAFLD. The results from these clinical trials support the use of quercetin for increasing insulin secretion and improving insulin resistance, regulating glucose homeostasis, and reducing oxidative stress. Like quercetin, the flavanol (-)-epicatechin, shows beneficial effects in the treatment of NAFLD-related symptoms.[87] In all of these applications, flavonoids are known to exhibit antioxidant and anti-inflammatory activity.[83,88]

The exact mechanisms of flavonoid antioxidant activity continue to be explored. It is understood that one path by which flavonoids prevent oxidative damage is by interacting with reactive metal species including iron and copper ions.[89–92] Flavonoid-metal complexes exhibit different behaviors than flavonoids alone.[93–95] Flavonoids have experimentally been shown to bind and reduce metal ions.[92,96,97] Samsonowicz et al. identify three main interaction sites on the flavonoid core structure at the B-ring 3’,4’-dihydroxy group, the C-ring 3-hydroxy or 5-hydroxy group, and the C-ring 4-carbonyl group.[98] These interaction sites have are supported both experimentally and computationally.[90,99] Karlíčková et al. found that isoflavones containing a 5-hydroxy-4-keto substitution pattern were able to chelate ferric, ferrous, and cupric ions. The

presence of a free 4'-hydroxyl group and the absence of a 5-hydroxyl group corresponded to redox activity in reducing Cu(II) ions.[100] However, many studies of flavonoid-metal complexes are contradictory in their characterization.[98] Binding affinities, binding ratios, and binding locations are all dynamic under varying experimental conditions. Further investigation of the effects of experimental conditions is warranted, but it is clear that flavonoid-metal interactions contribute to their antioxidant activity.[101]

Conversely, interactions between flavonoids and metal ions have also been implicated in pro-oxidant activity which contributes to observed anticancer and apoptogenic activity.[102] Similar to chelation ability, pro-oxidant interactions of flavonoids with metal ions are structure-dependent. The number of adjacent hydroxy groups and conjugation throughout the molecule affects prooxidant activity.[83] The distinction between anti- and pro-oxidant interactions between flavonoids and metal ions is slight and must be considered when thinking about these complexes in therapeutic contexts.

#### 2.4.1.2 Phenolic acids

Phenolic acids contain a carboxylic acid and are the most produced phenolic compounds by plants. Plant phenolic acids are most abundant in the seeds, leaves, and skins of fruits.[103] There are two main groups that comprise plant phenolic acids: hydroxybenzoic and hydroxycinnamic acids.[104] Some of the more abundant hydroxybenzoic acids including syringic, vanillic, and protocatechuic acids exhibit many of the beneficial health effects previously discussed.

Hydroxybenzoic acids have been demonstrated to possess protective effects against a host of diseases including cancer, cardiovascular disease, and diabetes.[105–108] Of particular interest to metabolic diseases, Chang et al. found that vanillic acid has protective effects against hyperinsulinemia, hyperglycemia, and hyperlipidemia in a study with HFD fed rats.[109] Treating

HFD rats with vanillic acid decreased blood glucose levels and increased expression of proteins associated with insulin signaling and lipid metabolism. Sreelekshmi et al. identify activation of glucokinase and reduction of lipid peroxidation by vanillic acid under hyperinsulinemic conditions in HepG2 cells.[110,111] Similarly, syringic acid protects against fat accumulation in the liver of albino rats treated with acetaminophen as reported by Ramachandran et al.[112] Protocatechuic acid can also affect lipid and glucose metabolism in NAFLD conditions and ameliorate insulin resistance associated with diabetes.[113,114] The biological pathways affected by hydroxybenzoic acids continue to be investigated, but acids such as protocatechuic acid is known to activate mitogen-activated protein kinases (MAPKs) which are involved in inflammatory responses.[106]

Commonly encountered hydroxycinnamic acids including chlorogenic, ferulic, caffeic, and sinapic acids share biological properties to their hydroxybenzoic counterparts. Notably, ferulic acid derived from cereals demonstrates anti-hypertensive effects which may be attributed in part to its antioxidant activity.[115,116] Additionally, in obese mice and high-fat fed rats, caffeic acid and sinapic acid, respectively, modulate the gut microbiome to produce fewer microbiota associated with disease and inflammation.[117,118] Associated with metabolic disease, chlorogenic acid has been extensively studied *in vivo* and clinical studies for its role as a nutraceutical against metabolic syndrome and related diseases including obesity, diabetes, and hypertension.[119] Shi et al. found that treatment of NAFLD mice with chlorogenic acid leads to decreased activation of inflammatory cytokines (TNF-α and IL-6), reduced fasting blood glucose levels and blood lipids, and reduced insulin resistance.[120] This work is supported by observations that the improved conditions of HFD mice treated with chlorogenic acid was related to changes in mRNA levels of genes involved in glucose metabolism like GYS2, PCK, GK, and PFKL.[121] The same effects of chlorogenic acid were observed in human patients with NAFLD or T2D and exhibited similar results with improved metabolic readings.[122]

Unsurprisingly, phenolic acids are known to interact with transition metal ions through their carboxylic acid and phenol moieties.[123–126] Truong et al. used a density functional theory (DFT) approach to study the antioxidant versus pro-oxidant effects of ferulic acid interactions with iron ions at the carboxyl group.[124] Antioxidant activities of ferulic acid are more prominent than pro-oxidative reduction of Fe(III) except under specific conditions such as high concentrations of ferulic acid. Mazzone also employed DFT to study the interactions of Fe(II) with caffeic acid.[127] Using DFT coupled with experimental UV-Vis data, the binding site of caffeic acid with Fe(II) was identified as the carboxyl group, and caffeic acid-Fe(II) complex formation was found to be more energetically favorable than the production of $H_2O_2$ through Fenton chemistry. Oke et al. studied the activity of a vanillic acid-Zn(II) complex under hyperglycemic conditions.[128] Similar to previous studies, the anti-oxidant activity was highlighted as a key mechanism of bioactivity. Another plant-derived carboxylic acid, nicotianamine (NA), was shown to aid in Fe(II) import facilitated by the proton-coupled amino acid transporter SLC36A1 (PAT1).[129] Nicotianamine is a small organic molecule that can be obtained through consumption of fruits, vegetables, and legumes. Murata et al. use $^{59}$Fe(II) to track iron import in Caco-2 cells by NA. Intracellular Fe(II) levels track with the concentration of NA-Fe(II) treatment, and the complex should be explored for use in iron deficiency treatments. While these studies explain a mechanism of antioxidant activity, there remains room to explore the interplay between metal chelation and protective effects against metabolic diseases of plant phenolic acids.

#### 2.4.1.3 Coumarins

Coumarins have a benzopyrone core and are found in all parts of plants though they are concentrated in fruits.[130] Like the other phenolic compounds previously mentioned, coumarins are used to treat a range of pathologies including cancer, depression, and Alzheimer's Disease.[131–134] A thorough review by Hussain et al. discusses the biological and pharmaceutical

properties of coumarins and their derivatives.[130] Some highlights pertinent to our topic include a study by Ali et al. where methanol extracts of *Angelica decrusiva* exhibited inhibitory activity of protein tyrosine phosphatase 1B (PTP1B) and α-glucosidase.[135] Correspondingly, Islam et al. also found PTP1B and α-glucosidase inhibitory activity of coumarins extracted from *Artemisia capillaris*.[136] As their involvement in diabetes is understood, PTP1B and α-glucosidase are targets for the treatment of diabetes, and thus coumarins which inhibit the activity of these enzymes possess therapeutic potential.[137,138] In animal models, coumarins and their derivates exhibit protective effects against diabetes and associated renal damage.[139,140] Kang et al. administered esculin, a coumarin derivative, to streptozotocin-induced diabetic mice and found that esculin combatted diabetes-associated symptoms including elevated blood glucose levels and increased hepatic glucose-6-phosphotase expression.[139] Non-obese diabetic mice were administered total coumarins extracted from *Urtica dentata*, and Wang et al. found that the treated mice showed decreased expression of the TLR4 gene which is involved in inflammation in type 1 diabetes.[140]

Coumarins are shown to interact with transition metal ions such as iron and copper.[141–143] García-Beltrán et al. synthesized a fluorescent probe sensitive to Cu(II) based on 3-amino-7-hydroxycoumarin.[142] While the proposed mechanism of the probe is through hydrolysis of an imine bond, Mergu et al. also designed a Cu(II)-sensitive probe which employs a coumarin moiety through which the copper ion is chelated.[141] Mladěnka et al. investigated the interactions of coumarins with iron ions *in vitro*.[143] At neutral pH, *ortho*-dihydroxy derivatives of coumarins, specifically, 7,8-dihydroxy-4-methylcoumarin, were able to tightly bind ferrous ions. However, at acidic pH, the same *ortho*-dihydroxycoumarins demonstrated potential pro-oxidant activity through reduction of ferric ions. While the groundwork for coumarin-metal interactions exists, there remains room to investigate the relationship between coumarins, metals, and the protective effects of coumarins against metabolic diseases.

#### 2.4.1.4 Stilbenes

With a core of 1,2-diphenylethylene, stilbenes are found as either *trans*- or *cis*-isomers.[144] The most known stilbene is resveratrol which is found in edible fruits and seeds such as grapes, pistachios, and berries. Over 250 clinical trials have indicated health benefits of the *trans*- form of resveratrol in addressing cardiovascular diseases, neurological diseases, and metabolic diseases like diabetes.[145] Singh et al. present a summary of clinical trial data of resveratrol in their review article.[145] Some other notable bioactive stilbenes include oxyresveratrol, piceatannol, and pterostilbene.[146] These stilbenes too possess bioactivities such as anticancer and anti-hypertensive effects.[147–149] In the context of metabolic disease, Choi et al. reported that oxyresveratrol combatted metabolic dysregulation in high-fat diet-fed mice by increasing the expression of proteins including AMP-activated protein kinase α, insulin receptor substrate 1, and insulin-dependent glucose transporter type 4 which are involved in lipid and glucose homeostasis.[150] Two studies by Choi et al. and Pan et al. note the increase of energy expenditure in high-fat diet-fed mice administered oxyresveratrol.[151,152] Both groups identify increasing expression of uncoupling protein 1 (UCP1), a mitochondrial membrane protein in brown adipose tissue, as a mechanism of induced thermogenesis by oxyresveratrol. Piceatannol exhibits anti-inflammatory and antioxidant activity in a variety of cell types and *in vivo* studies.[153] Kitada et al. studied the effects of piceatannol from *Passiflora edulis* on metabolic health in humans.[154] The preliminary results presented indicate that piceatannol increases insulin sensitivity and decreases blood pressure and heart rate. Similarly, pterostilbene reduces adiposity in white adipose tissue at a higher efficacy than resveratrol.[155] Resveratrol, though being the most-studied stilbene, has low oral bioavailability which supports the study of other stilbenes as potential therapeutics.[156]

Stilbenes interact with metal ions, and their complexes have demonstrated biological activity. Stilbene-copper complexes have been studied for their antitumor activity.[157,158] Resveratrol-Cu(II) and piceatannol-Cu(II) complexes induce apoptosis through production of ROS and DNA damage.[158–160] Tamboli et al. use electrospray ionization mass spectrometry (ESI-MS) paired with DFT calculations to understand the mechanisms by which resveratrol interacts with copper.[161] While the previously discussed phenolic compounds interact with metal ions mainly through oxygen-containing groups, resveratrol interacts with copper through its aromatic carbon atoms and alkenyl group. Though resveratrol does exhibit some copper chelating activity, Granzotto et al. suggest that resveratrol poses more of a risk of producing ROS than chelating copper.[162] Metal-interactions with resveratrol have been studied computationally[163] *in vitro*, but the exact mechanisms of interaction *in vivo* remain elusive. Majewski et al. studied the effects of resveratrol on copper deficient Wistar rats and found resveratrol to increase copper and zinc levels as well as superoxide dismutase (SOD) and ferric reducing antioxidant power (FRAP) which are related to antioxidant activity. While the clinical relevance of stilbenes is well-established in diseases associated with metal dyshomeostasis, the direct effects of stilbene-metal interactions on pathological states remains largely unexplored.

#### 2.4.1.5 Lignans

In plants, lignans serve as structural compounds in the formation of lignin in the cell wall.[164] Lignans have a 2,3-dibenzylbutane structure and are consumed in fibrous foods like grains and legumes.[165] Though relatively low-abundant, lignans, as with other plant phenolic compounds, exhibit a range of biological activity from anti-cancer activity[165] to gut microbiota modulation[166] to cholesterol reduction.[167] Lignans are consumed largely through cereals in western diets and are known to affect metabolic systems through nuclear receptors (NRs),

particularly estrogen receptors (ERs).[168] Zanella et al. highlight the relationship between plant lignans and metabolic syndrome (MetS). Epidemiological studies show an inverse correlation between lignan intake and incidence of T2D, dyslipidemia, and fasting insulin serum levels. Additionally, the structural similarity of lignans to steroid hormones such as estrogen can play a role in modulation of hormone-related tumors by lignans.[169] Lignans including pinoresinol, sauchinone, sesamin, and honokiol can combat hepatic oxidative stress which is often associated with metabolic diseases.[170–175] Mice with liver injury induced by $CCl_4$ or *tert*-butyl hydroperoxide were treated with lignans which activated pathways including AMPK, JNK, SIRT3, and Nrf2/ARE. Due to their potent antioxidant activity, it is no surprise that lignans can bind metal ions.

Lignans have known interactions with metal ions, particularly iron. Donoso-Fierro et al. extracted, isolated, and studied the iron-binding abilities of lignans from *F. cupressoides* and *A. chilensis*.[176] Five lignans with iron-binding capacity of over 87% were identified as isolariciresinol, isotaxiresinol, matairesinol, methylmatairesinol, secoisolariciresinol, and didemethylmatairesinol. This work was followed up by Fucassi et al. who focused on secoisolariciresinol digucoside (SDG) and found that SDG was able to bind calcium, copper, lead, nickel, iron, and silver ions.[177] While some of these metals are implicated in metabolic diseases affected by lignans, the direct connections between metal chelation, lignan intake, and instance of metabolic disease remain largely unexplored.

#### 2.4.1.6 Curcuminoids

Curcuminoids are found in the rhizome of turmeric and have gained attention for their bioactivity.[178] The most common curcuminoid, curcumin, is a yellow polyphenolic pigment that contains two ferulic acid residues bridged by a seven-carbon methylene group. Clinical trials implicate curcumin in treatment for a range of disease states from rheumatoid arthritis to

inflammatory bowel disease to Alzheimer's disease. Reviews by Pivari et al. and Zheng et al. highlight current understanding of treatment of diabetes with curcumin.[178,179] Yuan et al. performed meta-analysis of the effects of curcuminoids on blood lipids in adults with metabolic diseases.[180] While the results are preliminary, consumption of curcuminoids correlated with decreased levels of triglycerides, total cholesterol, and LDL and an increase in HDL. Newer work by Ibrahim et al. demonstrates hepatoprotective effects of curcuminoids. Hepatic damage was induced in Wistar rats by administration of $CCl_4$, and curcuminoids were administered in doses of 75, 150, and 300 mg. Liver enzyme levels (alanine transaminase, aspartate transaminase, and alkaline phosphatase) increase with liver damage caused by $CCl_4$ but are restored upon treatment with curcuminoids.

Expectedly, curcuminoids exhibit antioxidant activity and interact with metal ions. Pitchumani Violet Mary et al. used DFT in gas and DMSO solvent phases to study interactions of curcumin with Mn(II), Fe(II), and Zn(II). Curcumin-Zn(II) complexes are the most stable of the three, though DMSO solvent interactions destabilize the complex. The binding site is identified as the diketone moiety, and metal complexes show increased antioxidant activity as compared to free curcumin. These calculations are supported by experimental results by Hieu et al.[181] Curcumin complexes with Fe(III), Ca(II), and Zn(II) were assessed for their solubility and antioxidant activity. Increased solubility of the metal complexes as compared to free curcumin correlated with increased antioxidant activity as assessed by the DPPH assay. A review by Prasad et al. summarizes the increased pharmacological activity of curcumin when complexed with metal ions.[182] Curcumin-metal complexes modulate a host of biomarkers involved in metabolic diseases including inflammatory cytokines IL-6, TNF-α, and NF-κB. Yuan et al. exploit the anti-inflammatory effects of curcumin-metal complexes in their Fe-Curcumin nanozyme employed for ROS scavenging and anti-inflammatory activity.[183] The strong chelating behavior

of curcumin paired with its therapeutic effects requires further investigation for modulation of metal populations in metabolic disease states.

### 2.4.2 Carotenoids

Over 700 compounds comprise the group of natural pigments called carotenoids which impart yellow, red, and orange colors.[202] In plants, carotenoids serve roles in photosynthesis and in protection against oxidative damage.[203] Carotenoids, like the previously discussed compounds found in plants, have been studied for their potential use in therapeutics for pathologies including cardiovascular disease and various cancers.[204] Another key role of carotenoids in human health is as a precursor for vitamin A and antioxidants which implicates their protective activity against oxidative damage.[203]

A study by Christensen et al. of 2003–2014 National Health and Nutrition Examination Survey (NHANES) data showed that increased intake and serum levels of carotenoids correlates with decreased instance of NAFLD.[205] Specifically, α-carotene, β-carotene, β-cryptoxanthin, and lutein/zeaxanthin show strong associations with decreased risk of NAFLD which was assessed using ultrasonography.[206] While a healthy diet may largely affect the risk of disease onset, Christensen et al. show that including the healthy eating index of 2015 in their analysis did not eliminate the inverse relationship between increased serum carotenoid levels and risk of fatty liver disease.[205] As such, carotenoids exhibit therapeutic effects towards fatty liver diseases through an unclear mechanism of action. Elvira-Torales et al. highlight some mechanisms by which carotenoids impart their protective effects against liver damage through reduction of oxidative damage and modulation of genes associated with lipid metabolism.[207] Researchers note that levels of inflammatory cytokines including *TNF-α*, *IL-6*, and *MCP-1* are repressed upon oral administration of carotenoids, specifically β-cryptoxanthin.[207,208] These

cytokines likewise play a role in diabetes mellitus which is a chronic inflammatory disease. Expectedly, carotenoids present anti-diabetic properties as presented in a review by Roohbakhsh et al.[209] Researchers highlight that carotenoids reduce insulin resistance by affecting JNK, IKKβ, and PPARγ. JNK and IKKβ regulate phosphorylation of insulin receptor substrates, specifically IRS-1; PPARγ assists in metabolism of carbohydrates and decreases inflammation in the cell.

Unlike many of the previously mentioned plant-derived compounds, carotenoids have not been studied for chelation-based interactions with metal ions. Due to their lipophilic nature, the context under which carotenoid-metal ion interactions are studied is in reference to lipid oxidation.[210] Interactions between carotenoids and redox-active metals may contribute to their pro-oxidant activity by producing carotenoid radical cations through electron-transfer.

### 2.4.3 Peptides

Another group of plant-derived compounds that is of interest to human health are peptides. Peptide sequences within plant proteins are increasingly being recognized for their potential bioactivity and use as nutraceuticals.[184]

Many bioactive peptides are hydrolysis products of plant proteins where the proteins themselves do not present the same bioactivity. The hydrolysis products naturally occur through consumption by digestive enzymes such as trypsin and pepsin.[185] A typical workflow for preparation of plant-protein derived bioactive peptides involves hydrolysis of proteins through one of three methods: gastrointestinal digestion, enzymatic hydrolysis, or fermentation.[186] Enzymatic hydrolysis is the most common method and has been performed with a wide variety of enzymes derived from plants and microbes.[187] The proteolytic enzyme selected for digestion affects the potential bioactivity of the resulting peptides because of the varied cleavage sites.[187] Hydrolysates from a range of foods consumed through diet have been studied for their

bioactivity. Similar to their phenolic compound counterparts, plant protein hydrolysates are known to possess biological properties including anti-cancer, anti-inflammatory, and cardiovascular effects.[188–195] Within the realm of metabolic disease, peptides from plant protein hydrolysates have exhibited anti-diabetic, anti-obesity, and anti-oxidant activity.[184,196] Jakubczyk et al. highlight specific peptide sequences that demonstrate bioactivity towards ameliorating metabolic syndrome in their review.[184] Other recent reviews highlight therapeutic effects of plant-derived peptides towards diabetes and related complications.[196,197]

There are known metal-binding amino acid residues, thus it is expected that plant protein-derived peptides have metal-binding capacity.[198] Esfandi et al. hydrolyzed oat bran proteins using four proteases, Alcalase, Flavourzyme, papain, and Protamex.[199] Antioxidant assays and iron-chelating assays support the varied bioactivity of peptides produced by different proteases, with papain-hydrolyzed peptides having the highest iron-chelating activity. Hu et al. further investigated iron chelation by oat bran protein hydrolysates prepared with papain, ficin, and bromelian, separating peptides by size.[200] Larger peptides (> 10 kDa) hydrolyzed by papain have higher iron-chelating capacity than those produced by ficin and bromelian whereas small peptides (< 1 kDa) hydrolyzed by ficin have higher iron-chelating capacity than those produced by papain and bromelian. Kubgloḿsong et al. studied rice bran albumin hydrolysates from papain hydrolysis for their copper-chelating activity.[201] Using gradient elution by HPLC, the more hydrophilic peptides demonstrated the highest copper-chelating activity. Identification of the peptides from the strongest chelating fraction showed characteristic moieties such as sulfur-containing amino acids, repeating serine residues, tryptophan, and arginine. Similar to the plant phenolic compounds, the interplay between plant peptides, metal ions, and metabolic disease has much room to be explored.

## 2.5 Metal complexes of plant-based molecules as potential therapeutics for metabolic diseases

Current therapeutic design targeting metal dysregulation focuses largely on metal-trafficking small molecules.[14,15] Such molecules can act as metal chelators or metal ionophores. Chelators sequester metal ions from the intracellular space and evacuate them out of the cell; ionophores bind metal ions in the extracellular space and traffic them into the cell across the cell membrane. Physiochemical requirements of small molecule chelators and ionophores include a moderate binding affinity to specific metal ions, sufficient lipophilicity to penetrate the cell membrane, and adequate complex stability.[14] Plant-derived compounds deserve to be considered for their therapeutic potential as metal chelators and ionophores. With known metal ion interactions, a range of binding affinities and lipophilicities, and varying complex stabilities, plant-derived small molecule-metal complexes possess the chemical properties to traffic metal ions. Indeed, small molecules like flavonoids have been studied in these contexts.

Flavonoids are known to interact with redox active metals such as copper and iron which are implicated in metabolic disease states such as diabetes, Wilson and Menkes disease, and metabolic syndrome. To date, much of the research regarding flavonoid-metal interactions focus on their antioxidant activity. A review by Selvaraj et al. highlights the potential of flavonoid-metal complexes as therapeutics mainly for antioxidant and anti-inflammatory activity.[211] However, flavonoids are good candidates to study for their potential chelator or ionophore activity. Dai et al. present their study on flavones as Cu(II) ionophores.[212] Researchers highlight 3-hydroxyflavone as being the most effective copper ionophore. Using human hepatocytes, HepG2 cells, as a model system, 3-hydroxyflavone is shown to import copper into the cell at up to a 150-fold change. While the experimental conditions induce cell death due to cuproptosis[213], the ionophore activity of 3-hydroxyflavone can be harnessed to address diseases under which

intracellular copper levels are decreased. Further studies of flavonoid-metal interactions by our lab, show that flavonoids can modulate expression of proteins involved in copper trafficking.[101] Copper chaperone for superoxide dismutase (CCS), which is used as a marker for intracellular copper, shows decreased expression upon treatment with 3-hydroxyflavone and Cu(II) and increased expression when treated with quercetin and Cu(II). Compared to the other molecules studied, 3-hydroxyflavone is the one of the more lipophilic compounds. The lipophilicity paired with the binding ability of 3-hydroxyflavone maintains the important chemical properties of an ideal ionophore and should serve as inspiration for future investigations.

The other groups of plant-derived compounds remain largely unexplored for chelator and ionophore activity. Based on their structures, characterized interactions with metal ions, and known health benefits, it behooves researchers to pursue studies in these areas.

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# Chapter 3

## Structure-activity assessment of flavonoids as modulators of copper transport*

*This chapter is a modified version of the following published article: Lee, V.J., Heffern, M.C. Structure-activity assessment of flavonoids as modulators of copper transport. *Front. Chem.* 23 August 2022.

## 3.1 Abstract

Flavonoids are polyphenolic small molecules that are abundant in plant products and are largely recognized for their beneficial health effects. Possessing both antioxidant and prooxidant properties, flavonoids have complex behavior in biological systems. The presented work investigates the intersection between the biological activity of flavonoids and their interactions with copper ions. Copper is required for the proper functioning of biological systems. As such, dysregulation of copper is associated with metabolic disease states such as diabetes and Wilson's disease. There is evidence that flavonoids bind copper ions, but the biological implications of their interactions remain unclear. Better understanding these interactions will provide insight into the mechanisms of flavonoids' biological behavior and can inform potential therapeutic targets. We employed a variety of spectroscopic techniques to study flavonoid-Cu(II) binding and radical scavenging activities. We identified structural moieties important in flavonoid-copper interactions which relate to ring substitution but not the traditional structural subclassifications. The biological effects of the investigated flavonoids specifically on copper trafficking were assessed in knockout yeast models as well as in human hepatocytes. The copper modulating abilities of strong copper-binding flavonoids were largely influenced by the relative hydrophobicities. Combined, these spectroscopic and biological data help elucidate the intricate nature of flavonoids in affecting copper transport and open avenues to inform dietary recommendations and therapeutic development.

## 3.2 Introduction

Flavonoids are a class of small phenolic secondary plant metabolites. Ubiquitous in the plant kingdom, flavonoids are recognized as micronutrients that are biologically active in mammalian systems.[1] Flavonoids are widely studied for their health benefits which include anti-inflammatory

and anti-cancer properties.[2] However, the mechanisms of their biological activity remain unclear due to flavonoids' complex activity as both anti- and pro-oxidants. Flavonoids share a core structure containing two benzene rings connected by a heterocyclic pyran ring (Figure 1). Derivatization of this core structure differentiates flavonoids into subclasses based on oxidation and substitution of the heterocyclic C-ring (Table 1). Modest structural differences correspond to changes in biological effects with variations in hydroxyl group positions relating to differing antioxidant activity.[3] Often, flavonoids are studied by subclasses with the assumption that molecules within subclasses behave similarly.

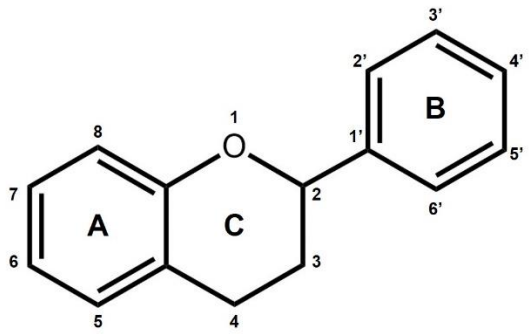
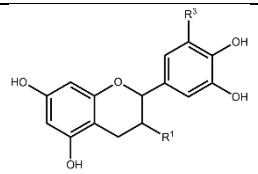
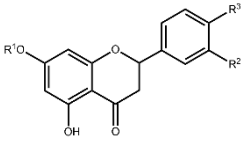
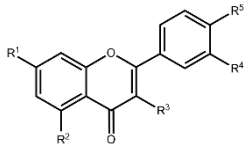
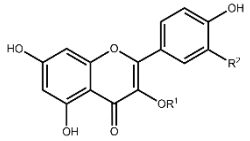
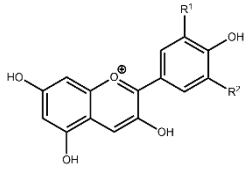
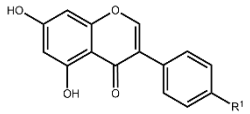



**Figure 3.1:** The core structure shared by flavonoids is composed of two benzene rings (rings A and B) connected by a heterocyclic pyran ring (ring C).

**Table 3.1:** Structures and subclasses of flavonoids.

| Subclass | Structure | Flavonoids | Substitutions |
|---|---|---|---|
| Flavan-3-ols |  | Epicatechin (EC)<br>Catechin (Cat)<br>Epigallocatechin (EGC)<br>Epicatechin gallate (ECG)<br>Epigallocatechin gallate (EGCG) | $R^1$ = OH, $R^2$ = H<br>$R^1$ = OH, $R^2$ = H<br>$R^1$ = OH, $R^2$ = OH<br>$R^1$ = gallate, $R^2$ = H<br>$R^1$ = gallate, $R^2$ = OH |
| Flavanones |  | Hesperetin (Htin)<br>Hesperidin (Hdin)<br>Naringenin (Ngenin)<br>Naringin (Ngin) | $R^1$ = H, $R^2$ = OH, $R^3$ = $OCH_3$<br>$R^1$ = glucoside, $R^2$ = OH, $R^3$ = $OCH_3$<br>$R^1$ = H, $R^2$ = H, $R^3$ = OH<br>$R^1$ = glycoside, $R^2$ = H, $R^3$ = OH |
| Flavones |  | Luteolin (Lut)<br>Chrysin (Chrys)<br>3-hydroxyflavone (3-HF) | $R^1$ = OH, $R^2$ = OH, $R^3$ = H, $R^4$ = OH, $R^5$ = OH<br>$R^1$ = OH, $R^2$ = OH, $R^3$ = H, $R^4$ = H, $R^5$ = H<br>$R^1$ = H, $R^2$ = H, $R^3$ = OH, $R^4$ = H, $R^5$ = H |
| Flavonols |  | Quercetin (QT)<br>Kaempferol (Kae)<br>Rutin (Rut) | $R^1$ = H, $R^2$ = OH<br>$R^1$ = H, $R^2$ = H<br>$R^1$ = rutinoside, $R^2$ = OH |
| Anthocyanins |  | Cyanidin (Cyan) | $R^1$ = H, $R^2$ = OH |
| Isoflavones |  | Biochanin A (Bio A)<br>Genistein (Gen) | $R^1$ = $OCH_3$<br>$R^1$ = OH |

Interactions of flavonoids with metal ions are a source of both anti- and pro-oxidant activity.[4] Flavonoids have been shown to perform Fenton-type reactions in the presence of Fe(III) and Cu(II) ions.[5] Furthermore, coordination to metal ions affects the oxidative activity of flavonoids. There is evidence that flavonoids interact with metal ions including Fe(III), Zn(II), and Cu(II) with varying reports on the potential biological effects.[6–13]

While copper is an essential trace metal micronutrient, its dysregulation and mislocalization is correlated with a host of pathologies including Wilson's disease, neurodegeneration, and some cancers.[14] The causal relationship between these pathologies and copper dysregulation is still undetermined, but redirecting copper trafficking, either by removal or altering the localization of the metal ion, is emerging as a viable therapeutic strategy.[15] For instance, copper chelation therapies have shown encouraging effects in reducing morbidity in Wilson's disease patients and restricting tumor angiogenesis.[14] By in large, the focus on copper-modulating agents have been on synthetic chelators and ionophores, with less attention paid to natural products such as flavonoids. Investigating the copper-modulating properties of flavonoids may not only facilitate rational design but may also inform on nutrition-based effects of such plant-derived products. As flavonoids display metal chelating properties, their health benefits may in part be due to their ability to affect copper trafficking.[11]

Indeed, some flavonoids reportedly protect against symptoms of copper-dysregulation associated pathologies,[16] but the structure/function relationship between how their chelating abilities relate to their potential therapeutic actions in these scenarios requires further elucidation. To this end, this work sought to elucidate the relationship between flavonoid-copper interactions and their ability to affect copper trafficking in cell-based models. A library of 18 flavonoids was compiled composed of bioavailable flavonoids that are available through food consumption and recognized for their health benefits. The Cu(II)-binding properties of the 18 flavonoids spanning the subclasses (Table 1) were directly compared spectroscopically, and

their impact on Cu(II)-associated redox activity was assessed with *in vitro* assays. Flavonoids with appreciable Cu(II)-binding abilities were assessed for their ability to alter copper trafficking in cell-based eukaryotic models. Taken together, this study offers a deeper understanding of how specific structural features of flavonoids may relate to their potential therapeutic activity in copper-associated disorders.

## 3.3 Materials and Methods

All chemicals were used as purchased without further purification. (-)-Epicatechin (EC), (-)-Epicatechin gallate (ECG), quercetin (QT), 3-hydroxyflavone (3-HF), hesperetin, ACES, tricine, and bathocuproine disulfonic acid disodium salt (BCS) were obtained from Sigma Aldrich (St. Louis, MO). Catechin (Cat), luteolin (Lut), (-)-epigallocatechin (EGC), and biochanin A were purchased from VWR (Radnor, PA). (-)-Epigallocatechin gallate (EGCG), chrysin, naringenin, naringin, hesperidin, rutin, kaempferol, cyanidin, genistein, hematoxylin, coumarin-3-carboxylic acid (CCA), ascorbic acid, ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl-$d_3$)amino-$d_2$-methane (deuterated Tris, Tris-$d_{11}$), MOPS, Bis-Tris, and all solvents were obtained from Fisher Scientific (Waltham, MA).

I. Determination of binding ratio and binding affinity

All UV-Vis studies were performed on a Shimadzu UV-1900i at 30°C or 37°C using quartz cuvettes (Starna) with a pathlength of 1 cm. Milli-Q water was the reference for all studies, and the spectrum of the buffer was subtracted, and the spectra were normalized after data collection.

50 μM solutions of flavonoids were prepared in 50 mM buffer (pH 7.4). $CuSO_4$, dissolved in Milli-Q water, was titrated into the solution in ratios ranging from 10-250 μM.

II. Identification of binding location

a. $^{1}$H Studies

Spectra were recorded on a Bruker 400 MHz NMR spectrometer with Topspin 3.2 running ICONNMR. 50 mM solutions of flavonoids were prepared in DMSO-$d_6$ and 12.5 mM solutions of $CuSO_4$ were prepared in MeOD. Samples were prepared of flavonoids alone at a final concentration of 12.5 mM flavonoid and with 0.25 equivalents of $CuSO_4$ dissolved in 50:50 DMSO-$d_6$:MeOD.

III. DPPH radical scavenging of flavonoids

The radical scavenging abilities of flavonoids were studied using the DPPH$^{\bullet}$ assay as previously described.[17,18] In short, 50 μM 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) in ethanol was added to preincubated solutions of 50 μM flavonoid, or ascorbic acid as the positive control, with and without 50 μM $CuSO_4$ or $ZnCl_2$ in a 96-well plate. Time dependent absorbance measurements were recorded at 515 nm over the course of 60 minutes using a Spectramax i3x microplate reader (Molecular Devices, San Jose, CA). The radical scavenging ability was calculated using the following equation:

$$DPPH\ scavenging\ \% = [1-(A_{sample}-A_{flav})/A_{DPPH}] \times 100\%$$

where $A_{sample}$ is the absorbance of the flavonoid and DPPH, $A_{flav}$ is the absorbance of the flavonoid alone, and $A_{DPPH}$ is the absorbance of DPPH alone at the end of the 1-hour measurement.

IV. Measurement of $^{\bullet}$OH in solution

The amount of •OH in solution was measured as previously described.[19] A solution of 2.5 mM CCA and 500 µM ascorbic acid was prepared in 10 mM phosphate buffer, pH 7.4. 50 µL of the CCA/ascorbic acid solution was added to 200 µL of pre-incubated flavonoid/$CuSO_4$ solutions at the described concentrations. Time-dependent fluorescence intensity measurements were recorded with excitation at 388 nm and emission at 450 nm over the course of 90 minutes using a Spectramax i3x microplate reader (Molecular Devices, San Jose, CA).

V. Assessment of copper transport in yeast

Saccharomyces cerevisiae strains used in this study are listed in Table 4. Yeast cells were cultured in YPD (1% yeast extract, 2% peptone, 2% glucose) and YPGE (1% yeast extract, 2% peptone, 3% glycerol, 2% ethanol) media. Growth in liquid YPD media was monitored at 600 nm. For qualitative observations, spot plate assays were performed on YPD and YPGE plates supplemented with the indicated flavonoids and copper. 10-fold serial dilutions of overnight YPD cultures were spotted on YPGE agar plates supplemented with 25 µM flavonoids or 5 µM $CuSO_4$ and incubated at 37°C for the indicated time.

VI. HepG2 cell culture and stimulations

HepG2 cells were maintained in 4.5 g/L DMEM media supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% glutamine, 1% Penicillin-Strepomycin. Cells were incubated at 37°C with 5% $CO_2$ in complete media until 70% confluence. HepG2 cells were plated at 300,000 cells/well in 6-well plates and allowed to grow for 24 hours. Stimulations of 20 µM flavonoid with and without 50 µM CuSO4 were added to the wells and incubated for 24 hours.

VII. Cell viability

Cell viability was measured using the MTS assay (Promega, Madison, WI). HepG2 cells were plated at 10,000 cells/well in a 96-well plate and allowed to grow for 24 hours. Wells were stimulated with 20 μM flavonoid with and without 50 μM $CuSO_4$. After 24 hours, media was removed, and cells were washed with DPBS to remove excess flavonoid. 20 μL of MTS reagent was added to each well and incubated for 1 hour. Absorbance at 490 nm was measured.

VIII. Intracellular copper measurements

After stimulations, HepG2 cells were washed with cold 50 mM EDTA in HEPES buffer to remove any copper on the cell surface followed by washes with HEPES buffer. Cells were resuspended in 50 μL HEPES buffer and added to 250 mL 70% nitric acid. Samples were boiled for 1 hour at 95°C then allowed to sit at room temperature for 24 hours. Samples were diluted to 5 mL with nanopure water. Metal analysis was performed using a Perking Elmer 5300 CV optical emission ICP with auto sampler. Measured copper levels were normalized to the control sample.

IX. Western blot analysis

After stimulations, HepG2 cells were washed with DPBS and lysed with 60 μL RIPA buffer. Whole cell lysates were centrifuged at 15,000 rpm at 4°C for 1hour. The supernatant was collected and stored at -20°C. Protein quantification was determined using the BCA assay. 20 μg of protein sample was loaded into 15-well SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were incubated at 4°C overnight with primary antibodies. Primary antibodies used were anti-CCS (1:2000, sc-55561, Santa Cruz Biotechnology, anti-mouse), and anti-α-tubulin (1:10,000, MA1-80017, ThermoFisher, anti-rat). Primary antibodies were removed, and the membranes were washed with tris buffered saline with Tween (TBST). Membranes were then incubated in secondary antibodies at room

temperature for 1 hour. Secondary antibodies used were anti-mouse IgG AlexaFluor800 (A32789, 1:5000 Invitrogen) and anti-rat IgG (A21428, 1:5000 Invitrogen), AlexaFluor647, and a horseradish peroxidase-conjugated secondary antibody. Blot images were recorded on a BioRad ChemiDoc imaging system and processed in ImageLab.

## 3.4 Results and Discussion

### 3.4.1 Spectroscopic characterization of flavonoid-copper binding interactions in solution

A breadth of techniques have been applied to probe aspects of flavonoid-metal interactions including computational methods, electrochemical studies, and various spectroscopies.[20–22] However, reports have used varying experimental conditions including solvents, buffers, and pH conditions, yielding contradicting parameters for flavonoid-metal interactions.[22] Thus, to allow connections to be made between structural features and biological function, we applied a universal approach that would allow us to comparatively study our selected library of flavonoids across the subclasses. The conjugated structure of flavonoids lends itself to analysis by absorption spectroscopy. There are two major peaks in UV-Vis spectra for flavonoids. Band I corresponds to absorbance by the B ring while Band II appears at lower wavelengths due to absorbance by the A ring (Figure 2A).[23] Flavanols and flavanones do not have conjugation between the A and B rings. This lack of conjugation is reflected in the presence of a strong absorbance between 270-295 nm (Band II) and a weak Band I absorbance that appears as a shoulder at slightly longer wavelengths (Figure 2B).[3]

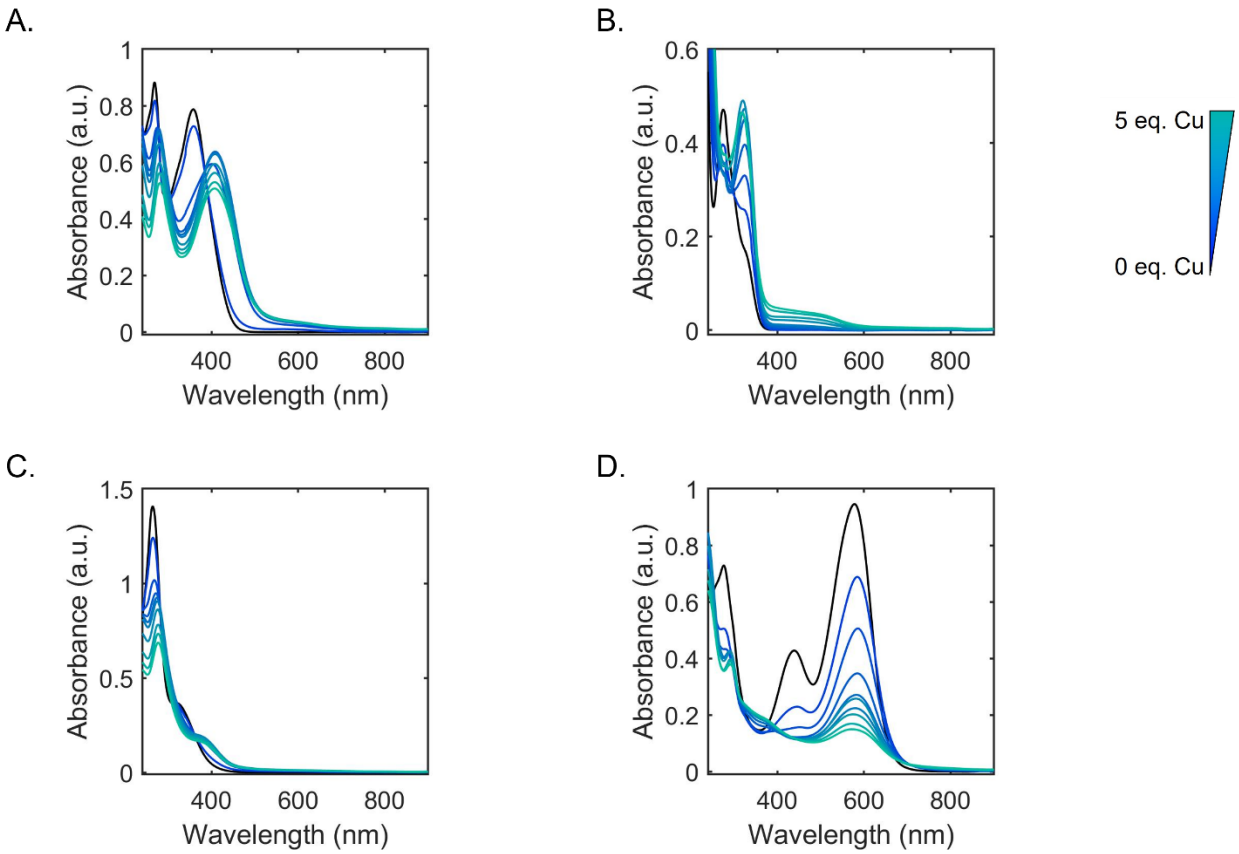



**Figure 3.2:** Electronic absorption spectra of representative flavonoids a) luteolin, b) EGCG, c) biochanin A, and d) cyanidin (black) in 50 mM MOPS buffer (pH 7.4) titrated with $CuSO_4$. Flavonoids were prepared at 50 µM and $CuSO_4$ was titrated into the solution from 0.2 to 5 molar equivalences. Flavonoids typically have two distinct absorption bands: a strong absorbance between 270-295 nm (Band II) and a weak Band I absorbance that appears as a shoulder at slightly longer wavelengths. Changes in the intensities and shifts in the lambda max wavelengths of these bands, indicate interactions of Cu(II) with the flavonoids. Electronic absorption spectra of the remaining flavonoids tested are shown in Figure 3.

**Table 3.2:** Binding affinity ranges of flavonoids with Cu(II).

| **Binding affinity ranges** | | | |
|---|---|---|---|
| 100 µM | 17.4 µM | 5.3 µM | 50 nM |
| EC, Cat, EGC, Hesperetin, Hesperidin, Naringenin, Naringin, Chrysin, Biochanin A, Genistein | Rutin | | ECG, EGCG, 3-HF, Luteolin, QT, Kaempferol, Cyanidin |

Cu(II) binding near or at sites of conjugation yields spectral changes to these bands, which indicate metal-flavonoid interactions. We used these spectral changes to monitor metal binding

in the presence of Cu(II)-binding buffers to categorize the flavonoids based on approximate apparent binding affinity ranges. Flavonoids were classified into Cu(II) binding affinities based on their ability to compete for Cu(II) binding with 50 mM MOPS ($K_d$ = 100 μM), ACES ($K_d$ = 17.4 μM), BisTris ($K_d$ = 5.3 μM), and Tricine ($K_d$ = 50 nM) (Figure 4 and Table 2).[10,24–27]

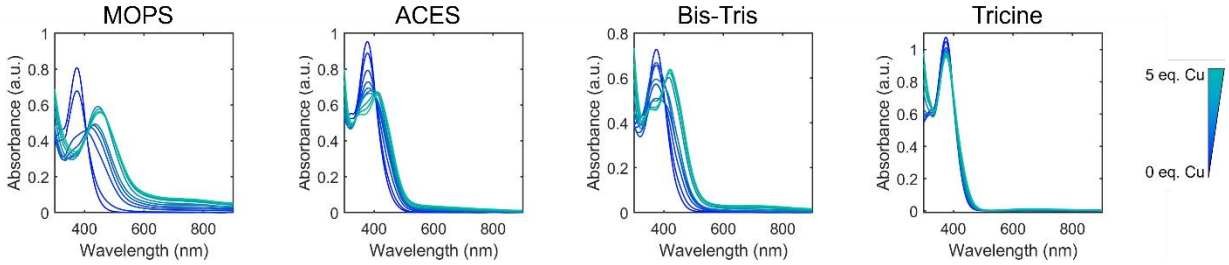



**Figure 3.3:** Representative electronic absorbance spectra of QT and $CuSO_4$ in various 50 mM buffers (pH 7.4) for determining binding affinity ranges. $CuSO_4$ was titrated into a 50 μM solution of QT from 0.2 to 5 equivalences. Spectral changes correspond to binding between QT and Cu(II) which indicates that QT has a stronger binding affinity than the corresponding buffer. Conversely, the lack of spectral changes upon Cu(II) addition in tricine buffer indicates that the binding affinity between tricine and Cu(II) is stronger than that of QT and Cu(II). These experiments allow us to estimate the binding affinity of QT and Cu(II) to be between that of BisTris-Cu(II) (5.3 μM) and tricine-Cu(II) (50 nM). Electronic absorption spectra of the remaining flavonoids tested are shown in Figure 4.

| Flavonoid | MOPS (-log K = 4) $K_d$ = 100 μM | ACES (-log K = 4.76) $K_d$ = 17.4 μM | BisTris (-log K = 5.27) $K_d$ = 5.3 μM | Tricine (-log K = 7.3) $K_d$ = 50 nM |
|---|---|---|---|---|
| EC | | | | |
| Cat | | | | |
| EGC | | | | |
| ECG | | | | |
| ECGC | | | | |
| QT | | | | |
| Hesperetin | | | | |
| 3-HF | | | | |

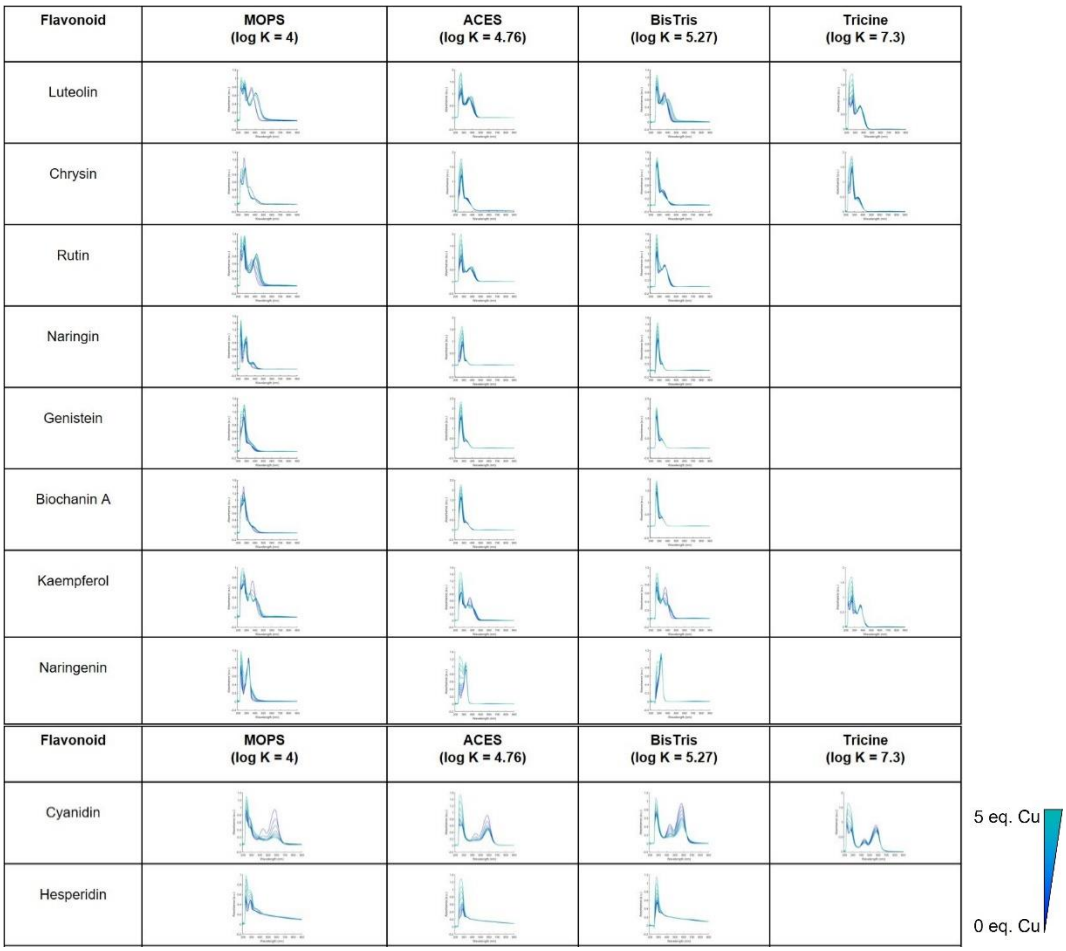



**Figure 3.4:** Electronic absorption spectra of flavonoid solutions titrated with CuSO4 in buffers MOPS, ACES, BisTris, and Tricine (pH 7.4). Flavonoids were prepared at 50 µM and CuSO4 was titrated into the solution from 0.2 to 5 molar equivalences.

Spectra were recorded for the flavonoids alone in each of the buffers as well as after copper addition. Changes in the spectra upon Cu(II) addition indicate that the flavonoids have a greater affinity for copper than the buffer and therefore interact in solution. If the buffer has a greater binding affinity for Cu(II) than the flavonoid, no spectral changes were observed. All flavonoids tested showed binding in 50 mM MOPS, indicating $K_d$ < 100 µM. While binding affinity ranges showed some correlation to flavonoid subclasses, some exceptions arise based on the type of functional group and position. The flavanones and isoflavones were all relatively weak binders with $K_d$ >17.4 µM. While flavanols with -OH modifications at the C-3 position also showed relatively weak binding affinities with $K_d$ >17.4 µM, the presence of a gallate modification at the

C-3 position in epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) increased the $K_d$ of the flavanols to 5.3 μM – 50 nM. This indicates that a gallate moiety on the C-ring can endow stronger Cu(II)-binding ability to this subclass, while, surprisingly, a catechol on the B-ring does not. Interestingly, the anthocyanin, cyanidin, which differs from epigallocatechin (EGC) by the introduction of conjugation between the A and C rings, shows a strong binding affinity, suggesting that such conjugation may contribute to the electron donating abilities of the flavonoid. Among the flavones luteolin and 3-hydroxyflavone (3-HF) were strong binders with $K_d$ between 5.3 μM and 50 nM whereas chrysin showed weak binding ($K_d$>17.4 μM). Luteolin and chrysin only differ by the presence of a catechol moiety on the B-ring of the former, suggesting that Cu(II)-binding to luteolin likely occurs at this site. While the catechol moiety is absent in 3-HF, this flavone differs from chrysin by the presence of an -OH group in the C-3 position of the C-ring, suggesting that this functional group, likely in cooperation with the ketone at the C-4 position, coordinates to Cu(II). Of the flavonols, both quercetin (QT) and kaempferol are strong binders with $K_d$ in the 5.3 μM – 50 nM range. However, the C-3 O-rutinoside analogue of QT, rutin, showed reduced binding affinity ($K_d$ in the 17.4 μM to 5.3 μM range), suggesting that this sugar modification disrupts Cu(II)-binding. The determined binding affinity ranges of QT and rutin support previously modeled binding affinities.[28]

To help further understand the contributions of specific structural features, we used the changes in absorption spectra to identify the location of Cu(II) interaction. Cu(II) interactions with the flavanols and flavanones increase the Band II absorbance, and flavanol-Cu(II) interactions also produce a bathochromic shift in Band I absorbance. These spectral shifts are attributed to changes in the conjugation on the B-ring where Cu(II) is hypothesized to interact. Flavonols and flavones likely interact with Cu(II) at the C-ring carbonyl which is supported by a bathochromic shift in Band I upon introduction of the metal ion in solution. Similar to the flavanones, the isoflavones show a large Band II absorbance and a smaller Band I absorbance

that appears as a shoulder (Figure 2C). Cu(II) interactions cause a bathochromic shift in Band I absorbance likely due to interactions with the hydroxy group on the A-ring. Finally, the spectrum of cyanidin likewise shows a bathochromic shift in Band I upon addition of Cu(II) as well as significant decreases in absorbance (Figure 2D). These interaction locations were confirmed by $^{1}$H-NMR: as Cu(II) is paramagnetic, coordination will cause faster relaxation of nuclei within electronic proximity, resulting in observed broadening in the spectra. This property can be exploited to confirm the site of interaction on the flavonoid molecules. The addition of 0.25 equivalents of Cu(II) to epicatechin (EC) results in broadening of the downfield peaks which correspond to the B-ring protons (Figure 5A).[29] The localized broadening confirms the interaction of Cu(II) ions at the diol on the B-ring. The spectra of EGCG also present localized broadening around the most downfield protons (Figure B). The peak most downfield corresponds to the protons on the gallate group indicating that rather than interacting at the B-ring, Cu(II) ions interact at the gallate moiety.[30] This confirms that it is indeed the interaction of Cu(II) with the gallate group that contributes to the stronger binding observed in the binding affinity estimation. It does appear that there is some interaction with the hydroxy groups on the B-ring evidenced by the large decrease in intensity of the peak at 5.8 ppm corresponding to the B-ring protons. Study of the flavanols is made easier by a saturated C-ring which results in no conjugation between the A- and B-rings and therefore more localized broadening. When conjugation is present throughout the molecule, introduction of paramagnetic Cu(II) to the flavonoid solutions results in universal broadening in the $^{1}$H-NMR spectra. Interpretation of spectra is more difficult with broadening across all peaks, but changes in intensity of peaks provide insight into the location of interaction. The spectrum of chrysin and Cu(II) shows broadening throughout the spectrum but has a significantly larger decrease in peak intensity of the peaks corresponding to the A- and C-ring protons (Figure 5C).[31] This confirms the expected interaction of Cu(II) with the 4-carbonyl of the C-ring and the 5-OH on the A-ring. These results

are supported by the UV-Vis interactions observed as well as previously reported crystal structures and computational studies.[7,11,32]

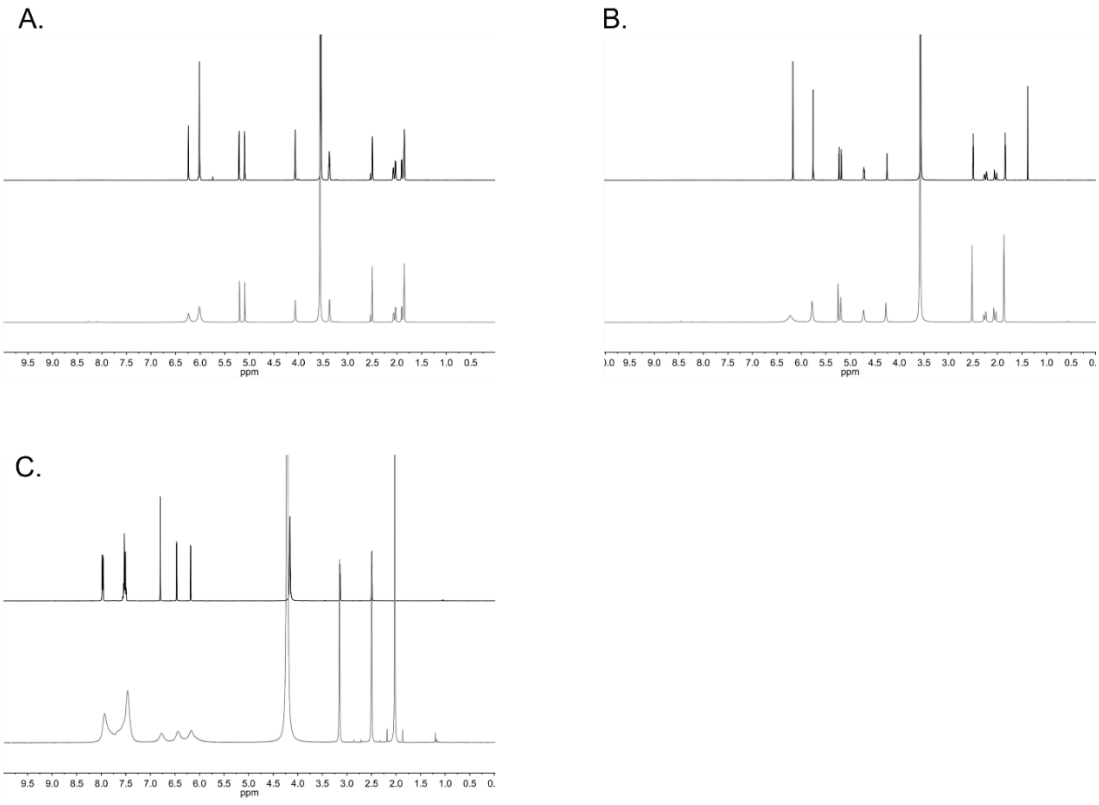



**Figure 3.5:** Representative 1H NMR spectra of 50 mM A) EC, B) EGCG, C) chrysin with (gray) and without (black) 0.25 equivalents of CuSO4 in 50:50 DMSO-d6:MeOD.

The UV-Vis titrations were also examined to determine whether binding affinity classifications were associated with ligand-to-metal stoichiometries. Although the method of continuous variation (Job's plot) has been applied to study some flavonoid/metal complexes, this method primarily works for interactions with large dissociation constants.[33] Thus, to allow for broader analysis of the flavonoid library, we opted to apply the mole ratio plot analysis using the characteristic absorbance of the Cu(II)-flavonoid complexes to approximate and compare binding stoichiometries of flavonoids to Cu(II) under buffered aqueous conditions at physiological pH. To achieve this, we monitored the equivalents of Cu(II) at which the intensities

of the absorbance of flavonoid-Cu(II) peaks plateau, indicating stable species formation (Figure 6).

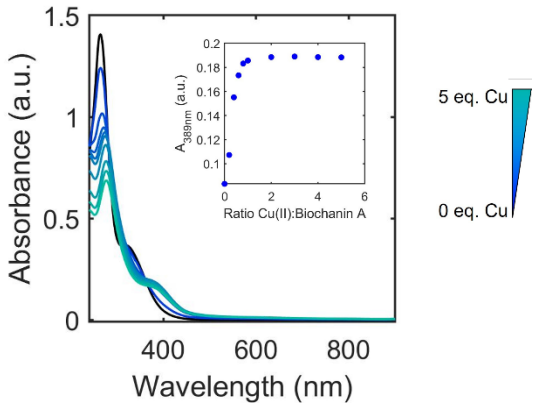



**Figure 3.6:** Representative electronic absorbance spectra of biochanin A upon addition of 0.2 to 5 equivalences of copper in 50 mM MOPS buffer (pH 7.4) for the determination of binding ratios. The inset shows absorbance at 389 nm where spectral changes occur due to Cu(II) binding to biochanin A. The changes in absorbance at 389 nm plateau at 1 equivalence of Cu(II) which indicates a 1:1 binding ratio between biochanin A and Cu(II).

Consistent with literature, the binding ratios vary between 1:2, 1:1, and 2:1 copper-to-flavonoid (Table 3). In the same way gallate modifications affects binding affinity, flavanols with gallate groups have altered binding ratios (1:1 copper-to-flavonoid) compared to their ungallated counterparts, which typically have 2:1 copper-to-flavonoid binding ratios. Additionally, as observed with the binding affinities, the O-rutinoside modification on rutin alters the binding ratio relative to its unmodified counterpart, QT, with the former having a 1:1 binding ratio in contrast to the 2:1 ratio of the latter. The 2:1 ratio of QT suggests the presence of two binding sites for Cu(II) likely occurring at the B-ring diol as well as the 4-carbonyl on the C-ring and either the 5-OH of the A-ring or the 3-OH on the C-ring. The glycone on rutin likely disrupts interactions around the 4-carbonyl allowing for binding to occur only at the B-ring. However, beyond these two observations, the binding ratios do not seem to have dependence on or correlation to structural subclass or binding affinities.

**Table 3.3: Binding ratios of flavonoids to Cu(II) ions in solution.**

| Binding ratios (Cu:flavonoid) | | |
|---|---|---|
| 1:2 | 1:1 | 2:1 |
| EC, Cat, Hesperidin, Naringin, 3-HF, Luteolin, Chrysin, Kaempferol | EGC, ECG, EGCG, Hesperetin, Naringenin, Rutin, Cyanidin, Biochanin A, Genistein | QT |

### 3.4.2 Flavonoids impart antioxidant activity through multiple mechanisms

Flavonoids are well-known to exhibit antioxidant activity, with reactive oxygen species (ROS) scavenging being one of the most common hypothesized mechanisms. The flavonoid library was classified by their antioxidant activity with the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical assay.[17,34,35] The stable DPPH free radical is purple in solution, and its reduction by an antioxidation discolors the solution, allowing loss of absorbance at its $\lambda_{max}$=515 nm to be used to assess antioxidant activity. Ascorbic acid was used as a positive control and scavenged 97% of DPPH in solution (Figure 7A). The antioxidant capacity of the flavonoids alone were tested in the absence of Cu(II) using endpoint reads of the DPPH free radical signal. The flavanols tested, all of which contain o-catechol groups in the B-ring were able to scavenge over 80% of DPPH in solution whereas the flavanones, which are similar to the flavanols but do not contain the B-ring *o*-catechol, did not exhibit DPPH radical scavenging abilities. Similarly, the flavones and isoflavones tested were unable to scavenge DPPH radical in solution except for luteolin. Of importance to note is that luteolin has an *o*-catechol group on its B-ring which the non-scavenging flavonoids lack. These results are consistent with previous studies and indicate the importance of the *o*-catechol group in antioxidant activity.[36] An exception is kaempferol, which lacks the *o*-catechol group but exhibits radical scavenging abilities, suggesting that the hydroxy group on the B-ring may participate in antioxidant activity.

Little differences were observed in the endpoint reads of the DPPH assay when Cu(II) was added. While the DPPH reads at endpoint allow for general classification of the antioxidant capacity of the flavonoids, solvent effects, particularly those that may associate with the radical, have been shown to influence endpoint quantitation.[17] A more accurate comparison on antioxidant activity can be made by monitoring scavenging kinetics. Thus, to thoroughly understand the scavenging kinetics of flavonoids in comparison to their Cu(II)-bound forms, absorbance measurements were recorded over time, beginning immediately after addition of DPPH to solution. For all flavonoids that exhibit DPPH radical scavenging properties based on endpoint reads, the presence of Cu(II) increased the kinetics of the scavenging reaction (representative plots of EC and luteolin shown in Figure 7B). It has been suggested that rather than the antioxidant activity occurring from redox reactions with the Cu(II) center, coordination of Cu(II) at the hydroxy groups on the B-ring stabilizes a semiquinone radical intermediate facilitating the DPPH radical scavenging mechanism.[32] To test this hypothesis, Zn(II) was substituted for Cu(II). Zn(II), like Cu(II), behaves as a Lewis acid, but it is redox-inactive. The scavenging activities of flavonoids in the presence of Zn(II) show similar trends as in the presence of Cu(II) (Figures 7C and 7D). The rate of scavenging was increased in the presence of Zn(II) which supports the hypothesis that the increased antioxidant activity with Cu(II) addition is due to its interaction with the B-ring hydroxy groups as a Lewis acid rather than metal-centered redox activity. The DPPH assay demonstrates that flavonoids' radical scavenging abilities are affected by Cu(II) but are not correlated with the strength of copper interaction.

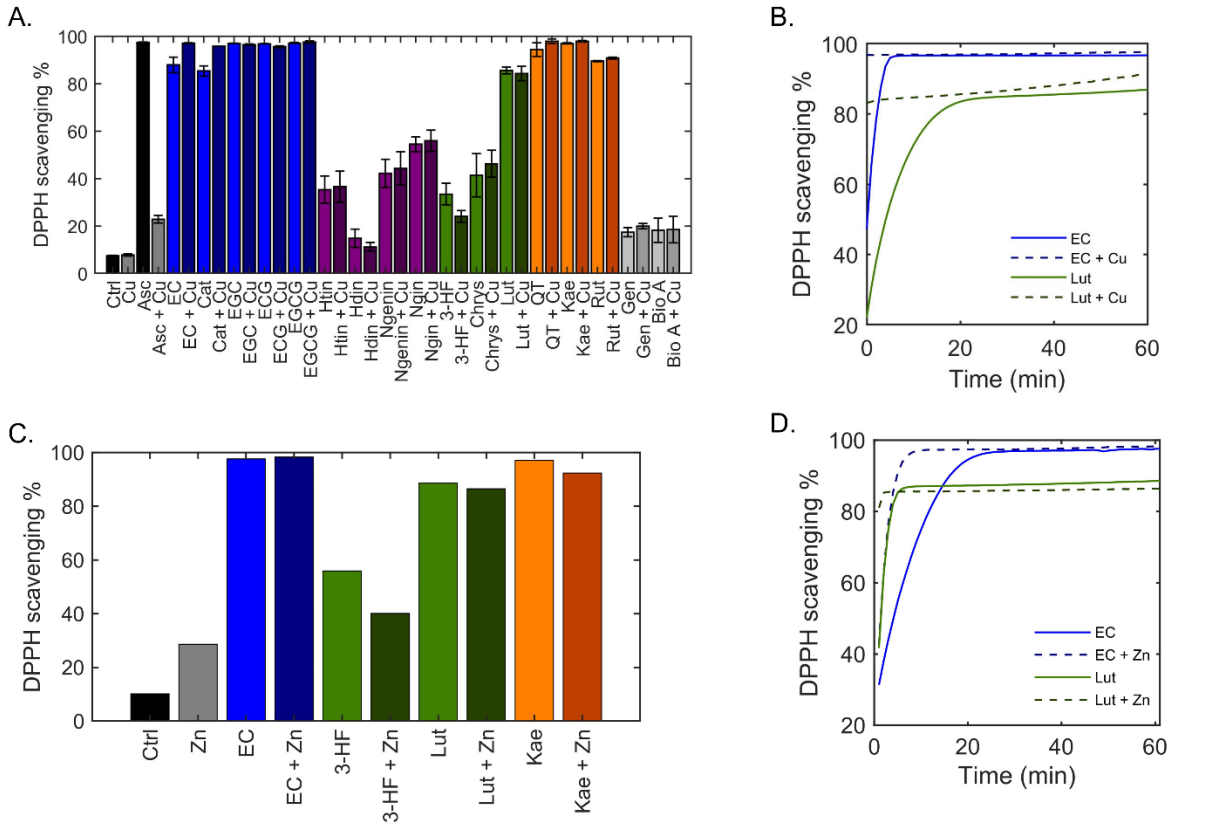



**Figure 3.7:** Determination of the ability of flavonoids to scavenge the DPPH radical, depicted as DPPH scavenging %. 50 μM flavonoids or ascorbic acid was incubated with 50 μM $CuSO_4$ before addition of 50 μM DPPH•. (A) The DPPH scavenging % was determined using the 515 nm absorbance of DPPH at the end of a one-hour reaction. (B) Time-dependent scavenging activity of representative flavonoids EC and luteolin in the presence of 50 μM $CuSO_4$ measured over the course of one hour. (C) End-point DPPH scavenging % of flavonoids in the presence of 50 μM $ZnCl_2$ after a one-hour reaction. (D) Time-dependent DPPH scavenging activity of representative flavonoids EC and luteolin in the presence of 50 μM $ZnCl_2$ measured over one hour.

Another mechanism by which flavonoids potentially possess antioxidant activity is by chelating redox-active Cu(II) ions thereby preventing production of ROS produced via Fenton-like chemistry.[19] To assess this, coumarin-3-carboxylic (3-CCA) was used as a probe to examine the protection of flavonoids against Cu(II)-induced production of •OH .[19] Ascorbic acid and copper undergo a redox cycling reaction which produces •OH. 3-CCA reacts with •OH to form 7-hydroxy-3-carboxycoumarinic acid which fluoresces with excitation at 388 nm and emission at 450 nm. The fluorescence intensity can be monitored to observe production of •OH in solution. As expected, the concentration of Cu(II) ions in solution was positively correlated to

the amount of •OH detected (Figure 8A). Introduction of all flavonoids to solution decreased detected •OH, with increasing amounts of flavonoids decreasing •OH production (Figure 8B, Figure 9). In contrast to the flavonoid effects on DPPH scavenging, the protective effects of the flavonoids against Cu(II)-induced production of ROS correlates to their binding affinities. For example, at 4-fold excess, EC is unable to reduce production of •OH to baseline levels (Figure 8C), whereas QT was able to stop •OH generation at 1 equivalent (Figure 8D).

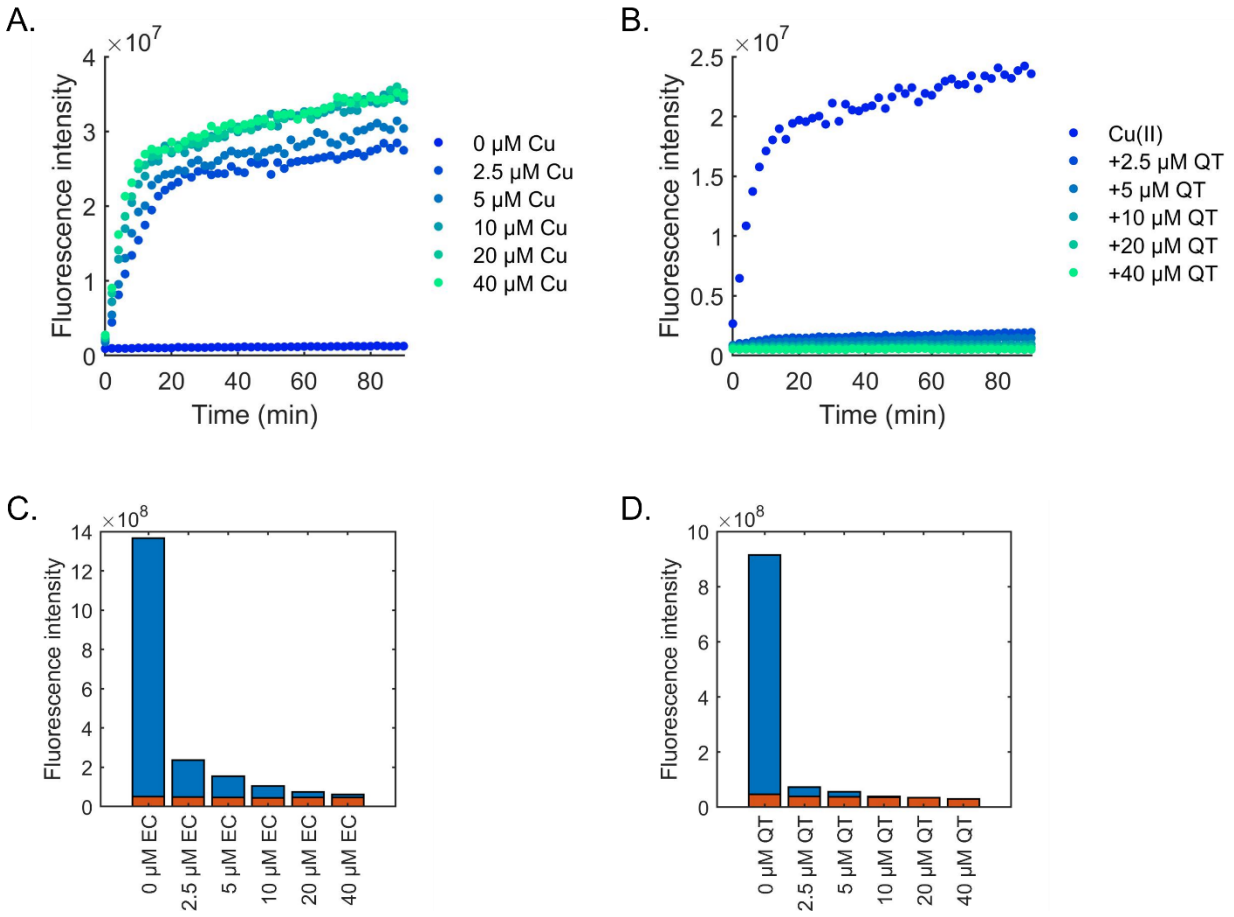



**Figure 3.8:** CCA fluorescence intensity measurements for determining the effects of flavonoids on the time-dependent generation of •OH. Flavonoid effects were compared to (A) Cu(II) addition alone, with (B) QT shown as an example. 2.5 mM CCA and 50 μM ascorbic acid were used for all experiments. The calculated area under the curve over the course of 90 minutes was measured for flavonoid solutions. (C) EC and (D) QT effects are shown as representative plots in the presence (blue) and absence(red) of Cu(II).

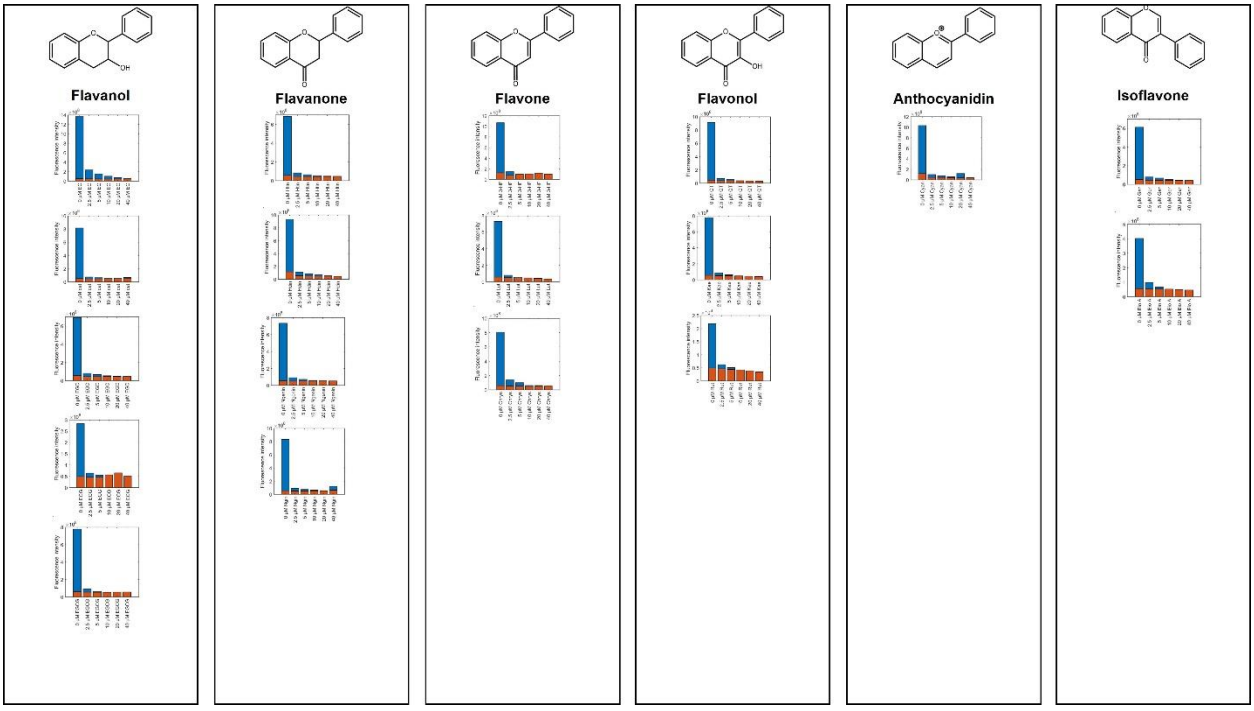



**Figure 3.9:** CCA fluorescence intensity measurements for determining the effects of flavonoids on the time-dependent generation of •OH. Flavonoid effects were compared to Cu(II) addition alone and with addition of flavonoid. 2.5 mM CCA and 50 µM ascorbic acid were used for all experiments. The calculated area under the curve over the course of 90 minutes was measured for flavonoid solution. Flavonoid effects are shown in the presence (blue) and absence (red) of Cu(II).

Flavonoids are often touted and have been extensively studied for their antioxidant effects. Due to their known interactions with copper ions, it has been hypothesized that some of their antioxidant activity is a result of those interactions.[7–10] The discrepancies between the CCA and DPPH assays indicate that while copper interactions do have an effect on the antioxidant activity of flavonoids, the mechanism by which copper has its influence varies based on the flavonoid. While copper binding affinity generally correlates with •OH levels from Cu(II)-induced Fenton-like chemistry as measured with the CCA, these trends do not apply to general free radical scavenging as measured with the DPPH assay. It is important to note that the ability to protect against •OH generation is not directly correlated to structure of the flavonoids, but rather to the binding affinities of the flavonoids to Cu(II). Stronger binders, like quercetin and kaempferol, can prevent •OH production, whereas weaker binders, like epicatechin, cannot

eliminate •OH generation. Both the CCA and DPPH assays demonstrate that antioxidant activity of flavonoids is not delineated by subclass but rather functional group location and identity.

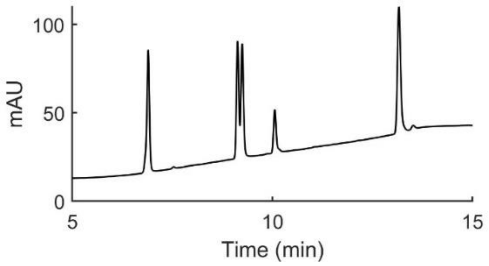
### 3.4.3 Effects of Flavonoids on Copper Trafficking in Yeast

Having gained an understanding of the structural features important to flavonoid-copper interactions in solution, we extended investigations to probe how the flavonoids' appreciable interactions with Cu(II) in buffered solutions influence copper trafficking in cell-based models. The ability of these flavonoids to transport copper was probed in *Saccharomyces cerevisiae (S. cerevisiae*) as a model eukaryotic organism.[37] Two knockout strains of *S. Cerevisiae*, ***ctr1Δ*** and ***ccc2Δ***, were treated with various flavonoids to observe differences in growth. The *ctr1* gene encodes a high-affinity copper transporter localized at the plasma membrane, and its knockdown restricts copper import from the extracellular space into the cytosol.[38] The *ccc2* gene encodes a copper-transporting ATPase hypothesized to translocate copper from the cytosol to extracytosolic compartments, and its knockdown has been shown to induce a functional copper deficiency.[39]

**Table 4: Strains of Saccharomyces cerevisiae used in this study.**

| Strain | Genotype | Source |
|---|---|---|
| BY4741 WT | MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 | Horizon |
| BY4741 *ctr1Δ* | MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, *ctr1Δ* | Horizon |
| BY4741 *ccc2Δ* | MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, *ccc2Δ* | Horizon |

Under aerobic growth conditions (e.g. YPGE media), compared to its wild-type (WT) counterpart, the knockout strains show impaired growth that is rescued with copper supplementation (Fig. 10).




| Elution time (min) | Flavonoid |
|---|---|
| 6.896 | EGCG |
| 9.129 | Luteolin |
| 9.244 | Quercetin |
| 10.058 | Kaempferol |
| 13.160 | 3-hydroxyflavone |

**Figure 3.10:** A mixture of 50 μM flavonoids was prepared in methanol. A gradient liquid chromatography experiment was performed ramping from 90:10 $H_2O$:acetonitrile to 10:90 $H_2O$:acetonitrile. The order of elution supports the relative hydrophobicities reported previously (Table 5).

**Table 3.5: Reported log P values of Cu(II)-binding flavonoids.**

| Flavonoids | Log P |
|---|---|
| EGCG | 0.46[40] |
| 3-HF | 4.17[41] |
| Luteolin | 0.7[42] |
| QT | 1.82[43] |
| Kaempferol | 1.872[44] |

We posited that copper-binding flavonoids could affect copper bioavailability to the knockdown strains, which could be monitored by differences in yeast growth. Specifically, we investigated the activity of 3-HF, luteolin, QT, and kaempferol, as these represent strong binders with a common ring conjugation structure. Growth rescue of *ctr1Δ* cells would suggest that copper is being imported by the flavonoid while rescue of *ccc2Δ* cells would indicate that the flavonoid can improve intracellular copper availability to extracytosolic compartments. To assess growth with a large dynamic range of detection, a spot assay was performed, where yeast were plated on agar in a 1:10 dilution series of four dilutions. Interestingly, despite the similarities of the flavonoids with respect to solution-based binding affinities and ring structures, they showed notable differences in effects on growth rescue of the knockouts (Figure 11).

In both knockout strains, 3-HF further restricts growth, which may indicate chelation of extracellular copper without transport into the cell. Conversely, luteolin treatment exhibits growth rescue effects of both *ctr1Δ* and *ccc2Δ* suggesting that it can transport copper into the cell and affect its intracellular availability. The differences in the behavior of these two may be attributed in their relative hydrophobicities. *S. cerevisiae* have cell walls that are hydrophilic.[45] The relatively higher hydrophobicity of 3-HF (reported log P = 4.17[41]) may explain its inability to transport copper into yeast cells in contrast to the more hydrophilic luteolin (reported log P = 0.7[42]) (Table 4). The simultaneous treatment with both 3-HF and luteolin result in a combined effect, with growth rescue that is less than luteolin but similar to copper supplementation alone suggesting that the two flavonoids may compete in their effects (Figure 11B). This may point to the possibility of modulation of copper ionophoric effects based on flavonoid content. Kaempferol and QT have reported log P values that are similar to one another but are in between those of luteolin and 3-HF (log P = 1.87[44] and 1.82[43] respectively). Interestingly, while kaempferol shows only modest to no growth rescue of the *ctr1Δ* and *ccc2Δ,* QT shows a strong growth rescue of the *ctr1Δ* strain comparable to that of luteolin. This may be in part due to the ability of QT to complex Cu(II) at a 2:1 ratio of copper-to-flavonoid, allowing for translocation of higher concentrations of copper into the cell. The growth rescue by both luteolin and QT of the *ctr1Δ* strain may also point to the importance of the catechol in the B-ring in facilitating copper import. However, in the *ccc2Δ* strain, no growth rescue is observed by QT, suggesting that while QT may facilitate copper import, it does not have a beneficial impact on extracytosolic copper availability.

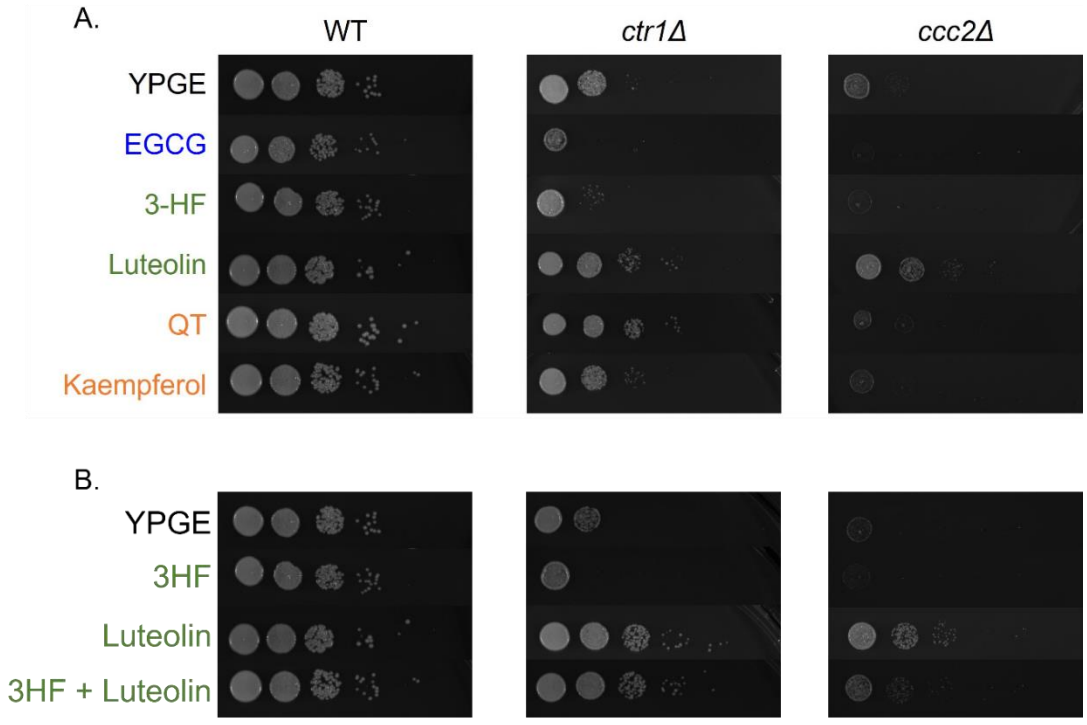



**Figure 3.11:** Serially diluted S. cerevisiae strains were spotted on the noted YPGE plates and incubated at 37°C for 4 days before imaging. (A) YPGE agar plates were prepared with 25 μM of the noted flavonoid. (B) Mixed ligand plates were prepared with 25 μM of each noted flavonoid. Treatment with mixed ligands exhibit changes suggestive of competition between ligands.

Additionally, we tested the effects of EGCG on the growth of the knockout strains, as EGCG is a well-reported copper modulator in cell-based assays. In both strains, EGCG exhibited potent inhibition on cell growth, suggesting that it may serve as an extracellular Cu(II) chelator to withhold the metal ion from the organism.

### 3.4.4 Effects of Flavonoids on Copper Trafficking in Human Hepatocytes

The inability of 3-HF to improve copper availability to the yeast knockout strains was a surprising observation, given that recent work reported that this particular flavonoid acts as a potent copper ionophore in mammalian cancer cell lines. In this context, Dai et al. posited that the delivery of copper by 3-HF could cause a redox imbalance in the cell, leading to copper-

induced cell death,[2] or "cuproptosis".[46] We thus assessed the same flavonoids in HepG2 cells, a human hepatocarcinoma cell line sensitive to cuproptosis. Cell viability was measured using the MTS assay in which an MTS tetrazolium compound is reduced by the mitochondria of viable cells to produce a colored formazan complex whose absorbance is monitored. Consistent with the previous report, our results show that in the presence of supplemented copper, 3-HF significantly reduces cell viability (Figure 12A, Table 6), and this effect correlates with increased intracellular copper levels, as measured by ICP-OES (Figure 12B).

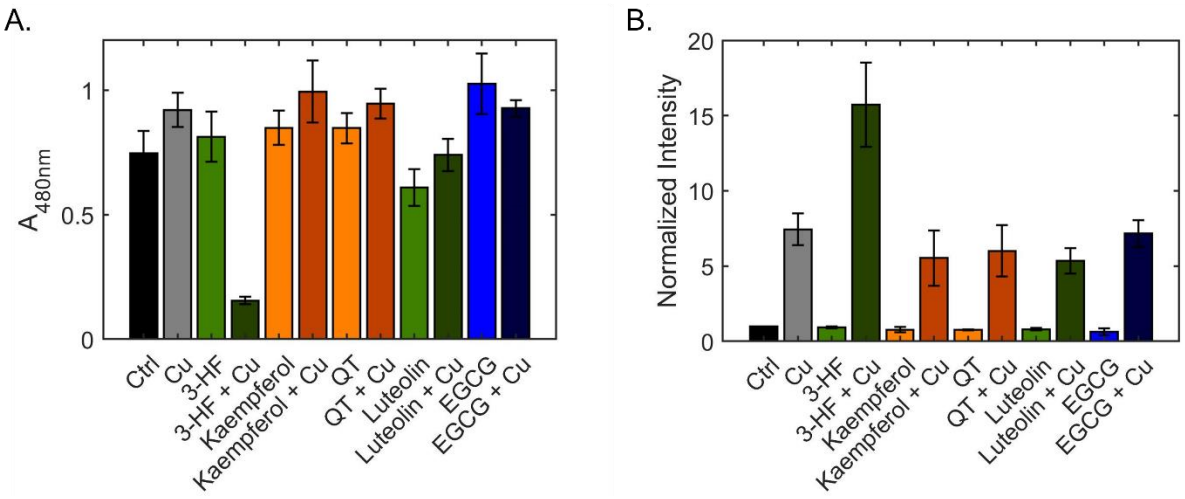



**Figure 3.12:** (A) Cell viability of HepG2 cells in the presence and absence of flavonoids and Cu(II), measured using the MTS assay. HepG2 cells were stimulated with 20 μM flavonoid with or without 50 μM $CuSO_4$. The MTS reagent was added after 24 hours. After 1 hour incubation with the reagent, absorbance was measured at 490 nm. (B) Quantification of total cellular copper of HepG2 cells in the presence and absence of flavonoids and Cu(II), measured by ICP-OES. HepG2 cells were stimulated with 20 μM flavonoid with or without 50 μM $CuSO_4$ for 24 hours. Error bars represent SD, n = 3.

**Table 3.6: p-values versus the vehicle control for the MTS assay and ICP-OES analysis.**

| | MTS assay | | | ICP-OES analysis | | |
|---|---|---|---|---|---|---|
| **Treatment** | **mean** | **SD** | **p-value** | **mean** | **SD** | **p-value** |
| Control | 0.7474 | 0.0891 | | 255.8 | 65.78 | |
| EGCG | 1.0251 | 0.1120 | 0.0104 | 159.8 | 54.75 | 0.1238 |
| 3-hydroxyflavone | 0.8130 | 0.1008 | 0.3669 | 241.1 | 77.30 | 0.8139 |
| Luteolin | 0.6096 | 0.0735 | 0.0544 | 205.6 | 50.90 | 0.3543 |
| Quercetin | 0.8473 | 0.0604 | 0.1129 | 192.8 | 39.85 | 0.2290 |
| Kaempferol | 0.8490 | 0.0680 | 0.1199 | 192.6 | 28.78 | 0.2017 |
| Cu Control | 0.9208 | 0.0681 | | $1.88\times10^{3}$ | 409.0 | |
| EGCG + Cu | 0.9270 | 0.0330 | 0.8753 | $1.86\times10^{3}$ | 617.7 | 0.9590 |
| 3-hydroxyflavone + Cu | 0.1552 | 0.0148 | $5.83\times10^{-7}$ | $3.94\times10^{3}$ | 803.3 | 0.0166 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Luteolin + Cu | 0.7399 | 0.0650 | 0.0085 | $1.39\times10^3$ | 544.9 | 0.2824 |
| Quercetin + Cu | 0.9456 | 0.0600 | 0.6057 | $1.61\times10^3$ | 824.7 | 0.6396 |
| Kaempferol + Cu | 0.9947 | 0.1237 | 0.3362 | $1.49\times10^3$ | 789.9 | 0.4970 |

Conversely to yeast cells, HepG2 cells have a hydrophobic cell membrane, which may explain the differential effects in copper import between the two systems. The effect of 3-HF on copper metabolism is further validated by changes to the expression of the copper chaperone for superoxide dismutase (CCS), a reported marker for cytosolic copper deficiency.[47] Interestingly, supplemental copper increases CCS expression, while the addition of 3-HF with supplemental copper decreases expression (Figure 13). Although CCS expression has been shown to increase in the presence of copper chelators and copper-deficient conditions, its response to acute treatment of supplemental copper remains unclear. While this complicates the interpretation of the CCS expression data, the difference between supplemented copper with and without 3-HF remain apparent, suggesting that the flavonoid modulates copper by different mechanisms than treatment with Cu(II) salt alone.

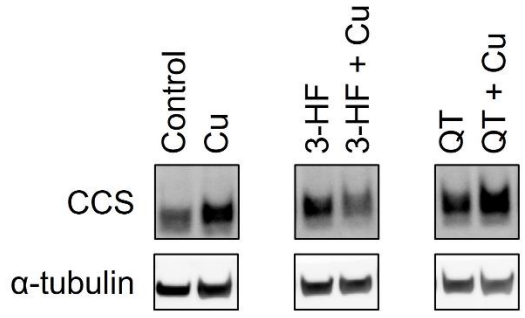



**Figure 3.13:** Western blot analysis of the CCS protein in lysates of HepG2 cells in the presence and absence of the flavonoids 3-HF or QT and Cu(II). HepG2 cells were stimulated with 20 μM flavonoid with and without 50 μM $CuSO_4$ and incubated for 24 hours. Cell lysates were collected, and Western blot analysis was performed using antibodies specific for CCS with α-tubulin as a control. Western blot images for the remaining assessed flavonoids are shown in Figure 14.

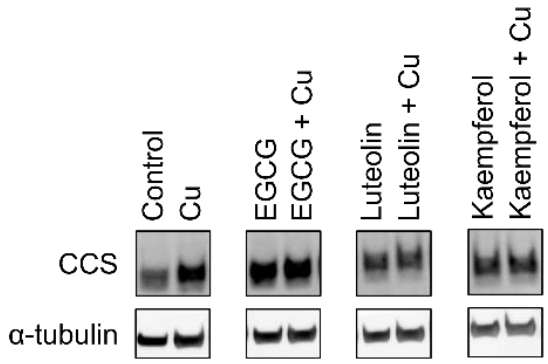



**Figure 3.14:** HepG2 cells were stimulated with 20 μM flavonoid with and without 50 μM CuSO4 and incubated for 24 hours. Cell lysates were collected and Western blot analysis was performed using antibodies specific for CCS with α-tubulin as a control.

While none of the other flavonoids tested significantly altered intracellular copper levels relative to the no-flavonoid controls, luteolin and EGCG treatments signficantly affected cell viability. Previous work has shown that while EGCG shows antioxidant properties, its copper complex conversely functions as a pro-oxidant.[48] These contrasting effects may be reflected in the cell viability data where EGCG improves cell viability over the control whereas the presence of Cu(II) reduces this effect. This may suggest the differences in cell viability with EGCG may be due to affecting the reactive oxygen species balance in the cell rather than by copper-modulating mechanisms. Luteolin, on the other hand, reduces cell viability, and this effect is attenuated by Cu(II) addition. Given the relative hydrophilicity of luteolin, it is possible that the flavonoid alters reactive oxygen species balance in the extracellular environment, but further studies are required to assess this effect. However, as both EGCG and luteolin treatments show no significant changes in either intracellular copper levels or CCS levels, their impact on cell viability may be independent of copper modulation. In contrast, while QT shows no changes in intracellular copper levels nor cell viability, the flavonoid induces a notable increase in expression in CCS in the presence of Cu(II). This may suggest that while QT does not affect copper import, it may modulate intracellular copper distribution and availability.

The cell-based assays demonstrate that despite similar behaviors of the flavonoids in solution, these are not necessarily predictive of their *biological* behavior. Moreover, even among eukaryotic systems, the structural features of the flavonoids may lend to differences in interactions in components such as membrane environments.

## 3.5 Conclusion

In this work, we focused on characterizing and assessing the ability of flavonoids to serve as modulators of copper trafficking. Structure-based characterization of flavonoid-Cu(II) interactions in buffered aqueous solutions showed wide diversity in the binding affinities and ratios that correspond more closely to specific functional group modifications and locations rather than the traditional subclassification of flavonoids by ring structures. Binding affinities correlated to protective effects against $^{\bullet}OH$ generation but not necessarily general scavenging activity. We further assessed how structural factors may affect the ability of flavonoids with appreciable Cu(II) affinity to traffic the metal in biological contexts. In yeast models, we found that hydrophobicities trend with the transport of copper into the cells. The more hydrophilic flavonoids, luteolin and QT, can cross the yeast cell wall and rescue growth of strains with copper trafficking proteins knocked out. However, a different trend was observed in mammalian HepG2 cells, which have relatively hydrophobic cell membranes, with the most pronounced copper transport effects exhibited by 3-HF. 3-HF increased intracellular copper levels and subsequently induced cuproptosis. While the exact mechanisms of 3-HF transport require further elucidation, decreased CCS protein expression suggests that 3-HF affects cellular copper trafficking mechanisms. Though 3-HF had the most marked effect on copper trafficking markers, treatment with QT also demonstrated effects on CCS expression suggesting potential modulation of intracellular localization. Taken together, our studies demonstrate an approach for linking the structure of natural products to their potential functions as copper modulators. Future

work should take into account organism-specific copper modulation as well as the heightened importance of functional group substitution and hydrophobicities rather than traditional subclassifications. The data offers insight to guiding principles for identifying such agents within the flavonoid structural family, which can both inform nutritional recommendations as well as therapeutic agent design for impacting copper-associated disorders.

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# Chapter 4

## Copper-binding peptides isolated from rice bran protein hydrolysates combat insulin resistance in HepG2 cells*

*This chapter is adapted from a manuscript in progress for publication titled: Copper-binding peptides isolated from rice bran protein hydrolysates combat insulin resistance in HepG2 cells. This work was a collaboration with Samuel Janisse, Rebeca Fernandez, and Justin O'Sullivan. Sam optimized the IMAC workflow and performed the mass spec and proteomic analysis. Rebeca performed the qPCR and ICP-MS analysis. JJ designed the pic-DTZ probe and collected the bioluminescence data. Vanessa led development of the proposed project and performed the protein extraction and hydrolysis, the IMAC enrichment experiments, and cell studies.

## 4.1 Introduction

Rice bran, a major byproduct of the rice processing industry, exhibits beneficial health effects including antioxidant activity, antidiabetic properties, and anticancer activity.[1] Rice bran is the outer section of the rice grain and contains nutrients including polyphenols, various vitamins, fats, and proteins.[1] Rice bran contains 10-16% protein which can be fractionated into albumin, globulin, glutelin, and prolamin through pH adjustments during extraction.[2,3] Isolation of such protein and further digestion yields potentially bioactive peptides. Peptides are of interest in therapeutic contexts due to their increased specificity, safety, and membrane permeability compared to their parent proteins.[4] Thus, proteolysis of rice bran proteins and identification of the resulting peptides provides insight into the mechanisms of their imparted biological effects.

One such bioactivity of rice bran albumin hydrolysates is copper chelation linked to tyrosinase inhibition.[3] Tyrosinase is a copper-dependent enzyme, and copper chelation influences tyrosinase activity.[5] Hydrophilic fractions of rice bran albumin hydrolysates exhibit both copper chelating abilities and corresponding tyrosinase inhibition. Beyond its role in tyrosinase activity, copper is an essential micronutrient that serves multiple functions in mammalian health ranging from being a static cofactor to participating in signaling pathways.[6,7] Due to its essential nature, dysregulation of copper is correlated to a host of disease states including various cancers and diabetes.[8,9] Some modern treatments of copper-related diseases focus on relocation of copper within the body to restore homeostasis.[10] For example, copper chelation therapy is a promising treatment for certain cancers and Wilson disease.[11]

Beyond copper-chelating activity, rice bran protein hydrolysates demonstrate favorable bioactivity under insulin resistant conditions by affecting the AMP-activated protein kinase (AMPK) pathway.[12] AMPK is involved in energy metabolism and is specifically responsive to glucose levels.[13] Patients with diabetes often experience high glucose levels, or hyperglycemia,

which is linked to insulin resistance and can be altered through activation of the AMPK pathway.[14,15] In insulin-resistant human hepatocyte (HepG2) cells, treatment with RBPH activated the AMPK pathway and improved glucose metabolism.[12] Curiously, treatment with copper salts also increased AMPK activity in HepG2 cells.[16] Hyperglycemia is linked to changes in copper homeostasis, and modulation of copper populations can in turn influence blood glucose levels.[17] Together, this suggests that RBPH may be able to restore AMPK activity through its copper-binding abilities. Here, we isolate copper-binding fractions from RBPH, study their effects on the AMPK pathway in insulin-resistant hepatocytes, and identify bioactive peptides.

## 4.2 Materials and Methods

*Chemicals and reagents*

Commercially available rice bran was obtained from NOW Foods. Papain, trypsin, pepsin, TNBSA, potassium sodium tartrate tetrahydrate, $CuSO_4$ were purchased from Millipore Sigma (St. Louis, MO). Dinitrosalicylic acid was purchased from Thomas Scientific (Swedesboro, NJ). Phosphate buffered saline (PBS), trifluoroacetic acid (TFA), ethylenediaminetetraacetic acid (EDTA) were obtained from Fisher Scientific (Waltham, MA). All solvents were purchased from Fisher Scientific (Waltham, MA). DSC-18 SPE tubes were purchased from Millipore Sigma (St. Louis, MO).

*Rice Bran Protein Extraction and Hydrolysate Preparation*

Rice bran was defatted with 1:3 w/v of rice bran to hexanes three times for 30 minutes each. The mixture was centrifuged at 4000 g at 25°C for 30 minutes. The supernatant was discarded, and the precipitate was dried overnight.

Dried defatted rice bran protein was stored in aluminum foil bags at -20°C. A mixture of 1:4 w/v rice bran to nanopure water was prepared. The pH was adjusted to 9.5 with 1 M NaOH. The mixture was stirred at 500 rpm for 45 minutes at room temperature. The mixture was centrifuged at 15,000 g for 30 minutes. The pH of the supernatant was adjusted to 4.5 with 1 M HCl and centrifuged at 15,000 g for 30 minutes. The precipitate was collected, freeze dried, and stored at -20°C.

Rice bran protein extracts (RBPE) were prepared in nanopure water at 8 mg/mL. For hydrolysis with papain, 1:100 w/w enzyme was added to the protein extracts and incubated at 37°C. The pH was adjusted to pH 8 with 1 M NaOH, and the solution was incubated at 37°C for 30 minutes. The enzymatic hydrolysis was stopped by placing the solution in boiling water for 5 minutes followed by an ice bath. The protein hydrolysates were freeze-dried and stored at -20°C. For hydrolysis with pepsin and trypsin, 1:100 w/w pepsin was added to the RBPE solution and the pH was adjusted to pH 1.5. The solution was shaken at 37°C for 120 minutes and then neutralized with 1 M NaOH to stop digestion. 1:100 w/w trypsin was added to the mixture and incubated at 37°C for 120 minutes. The solution was heated at 95°C for 10 minutes to stop digestion. The mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant included the rice bran protein hydrolysates (RBPH) and was freeze dried and stored at -20°C.

*Immobilized metal affinity chromatography*

1 mL Profinity IDA IMAC resin (Promega, Madison, WA) in a centrifuge tube was washed with three column volumes of nanopure water. 2 mL of 1 M $CuSO_4$ was added to the column to form IDA-$Cu^{2+}$ resin and washed with 3 column volumes of nanopure water. Three column volumes of PBS, pH 7.4, was used to equilibrate the column. 25 mg of rice bran protein hydrolysates (RBPH) in 4 mL PBS were added to the column and incubated for 1 hour after which the

unbound protein hydrolysates were collected. The column was washed with 30 column volumes of PBS before elution with 50 mM EDTA or 500 mM acetic acid.

*DSC-18 clean up*

3 mL volume DSC-18 SPE columns were flushed with 3 mL of acetonitrile followed by equilibration with 6 mL of washing solution (1% TFA and 2% acetonitrile in nanopure water). The samples were added to the column and washed with three column volumes of washing solution. The RBPH were eluted in 80:20 acetonitrile:nanopure water. The samples were freeze-dried and stored at -20°C.

*Copper chelation assessment*

Copper chelating capacity was assessed using a colorimetric $Cu^{2+}$ chelator, pyrocatechol violet. 10 µg of each sample, determined by the Pierce quantitative fluorometric peptide assay (ThermoFisher, Waltham, MA), was added to each well in a 96-well plate. Stock solutions of 2 mM pyrocatechol violet, 2 mM zincon, and 2 mM $CuSO_4$ were prepared. Final concentrations in each well were 200 µM pyrocatechol violet, 200 µM zincon, and 100 µM $CuSO_4$. Each sample in PBS, pH 7.4 was allowed to incubate with the $CuSO_4$ for 10 minutes at 37°C before addition of pyrocatechol violet. The pyrocatechol violet-copper complex absorbs at 600 nm at pH 7.4. The zincon-copper complex absorbs at 615 nm at pH 7.4. The copper chelating percent was calculated as follows:

$$\text{Copper chelating \%} = [(A_{control}-A_{sample})/A_{control}]\times 100\%$$

where $A_{sample}$ is the absorbance of the sample and copper subtracted from the absorbance of the sample, copper, and chelator.

*Degree of hydrolysis*

2,4,6-Trinitrobenzenesulfonic acid (TNBSA) was used to assess the degree of hydrolysis after enzyme digestion. 0.1% (v/v) TNBSA solution in nanopure water was prepared. 2 mg of each sample was prepared in 1 mL of 10 mg/mL sodium dodecyl sulfate (SDS). 125 µL of each sample, with water as a control, was added to 1 mL of sodium phosphate buffer (250 mM, pH 8.5) and 1 mL of TNBSA. Leucine was used as a control (0-5 mM standards). Solutions were incubated at 50°C in the dark for 1 hour. 2 mL of 100 mM HCl were added to stop the reaction. Solutions were allowed to sit for 30 minutes before absorbance measurements at 340 nm. The degree of hydrolysis was calculated as follows:

$$DH(\%) = (h/h_{tot})\times 100\%$$

where h is the hydrolysis of each sample as compared to the standard curve and $h_{tot}$ is the total molar equivalences per gram of protein.

*Cell culture*

Hep G2 cells were maintained in 1 g/L DMEM media supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% glutamine, 1% Penicllin-Strepomycin. Cells were incubated at 37°C with 5% $CO_2$ in complete media until 70% confluence. Cells were plated at 300,000 cells/well in 6-well plates and allowed to grow for 24 hours. To induce insulin resistance, complete media was replaced with high glucose (10 g/L) starvation media lacking fetal bovine serum for 18 hours. Cells were stimulated with low glucose (1 g/L glucose) complete DMEM or high glucose (10 g/L) complete DMEM and supplemented with the RBPH fractions for 24 hours.

*Western Blot Analysis*

After stimulation, cells were washed with DPBS and lysed with 60 µL RIPA buffer. Whole cell lysates were centrifuged at 15,000 g at 4°C for 1 hour. The supernatant was collected and stored at -20°C. Protein quantification was performed using the BCA assay. 10 µg of protein was loaded into SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated at 4°C overnight with primary antibodies (pAMPK, AMPK, β-actin). The membranes were washed three times with tris buffered saline with Tween (TBST) and incubated in secondary antibodies. Images were recorded using a BioRad ChemiDoc imaging system and processed in ImageLab.

*Glucose measurements*

Preliminary glucose measurements were performed using the dinitrosalicylic acid (DNS) assay. After cell stimulations, cell media was collected for each condition. DNS reagent was prepared as previously reported. In short, 1 g of DNS was dissolved in 20 mL of 2 M NaOH. A separate solution of 30 g of potassium sodium tartrate tetrahydrate in 50 mL of nanopure water was prepared. Both solutions were stirred until completely dissolved. The DNS solution was added to the potassium sodium tartrate tetrahydrate solution and the mixture was heated to homogenize. Nanopure water was added for a final volume of 100 mL. The solution was stored at 4°C away from light. For glucose measurements, 100 uL of sample was added to 100 uL of DNS reagent. The solutions were heated at 100°C for 5 minutes and allowed to cool to room temperature. Solutions were diluted to 1 mL with nanopure water and the absorbance was measured at 540 nm. Glucose consumption was calculated as follows:

$$\text{Glucose consumption (\%)} = [1-(A_{cellmedium}-A_{nocellmedium})/A_{nocellmedium}]\times 100\%$$

*Peptide Synthesis and Purification*

Peptides were synthesized at a 0.2 mmol scale. Coupling reagents, (2(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and N,N-diisopropylethylamine (DIEA), were used in 4 times molar excess for each coupling. Wang resin was shaken in 3 times the volume of N,N-dimethylformamide (DMF) to swell the resin. The resin was end-capped using 50 times the concentration of acetic anhydride and pyridine in DMF. Between each step of the synthesis, the resin was washed three times with DMF; after each coupling, the resin was washed three times with DMF, methanol, and dichloromethane. All amino acids were Fmoc-protected and were coupled in 4 times molar excess to the resin at 95°C for 20 minutes. A Kaiser test was performed after each coupling to confirm completion. Amino acids were Fmoc deprotected using 3 times volume of 25% piperidine in DMF at 95°C for 5 minutes. Peptides were cleaved in a trifluoroacetic acid cocktail containing 1.5 g phenol, 1 mL water, 0.5 mL triisopropyl silane, 1 mL thioanisole, and 16 mL trifluoroacetic acid.

*Protective effects against ROS production using 3-CCA*

Levels of generated $^{\bullet}OH$ in solution were measured as previously described.[18] A stock solution of 2.5 mM 3-coumarin carboxylic acid and 500 μM ascorbic acid was prepared in 10 mM phosphate buffer, pH 7.4. 50 μL of the CCA/ascorbic acid solution was added to 200 μL of pre-incubated peptide/$CuSO_4$ solutions at the described concentrations in 10 mM phosphate buffer, pH 7.4. Time-dependent fluorescence intensity measurements were recorded with excitation at 388 nm and emission at 450 nm over the course of 90 minutes using a Spectramax i3x microplate reader (Molecular Devices, San Jose, CA).

## 4.3 Results and Discussion

### 4.3.1 Enrichment of copper-binding peptides in RBPH using IMAC

Hydrolysis of rice bran protein was achieved using two enzyme systems: an *in vitro* digestion using pepsin and trypsin and a papaya-derived enzyme, papain. Each of these enzymatic digestions results in production of different peptides due to the unique cleavage sites of the enzymes. While pepsin has largely unspecific cleavage, it more readily cleaves at hydrophobic and aromatic residues. Trypsin cleaves at the C-terminal side of lysine and arginine residues, and papain cleaves at basic amino acids, leucine, and glycine. The efficiency of the enzyme digestions was assessed by measuring the degree of hydrolysis using TNBSA. *In vitro* digestion using pepsin and trypsin showed a slightly higher degree of hydrolysis than the papain method (Figure 1).

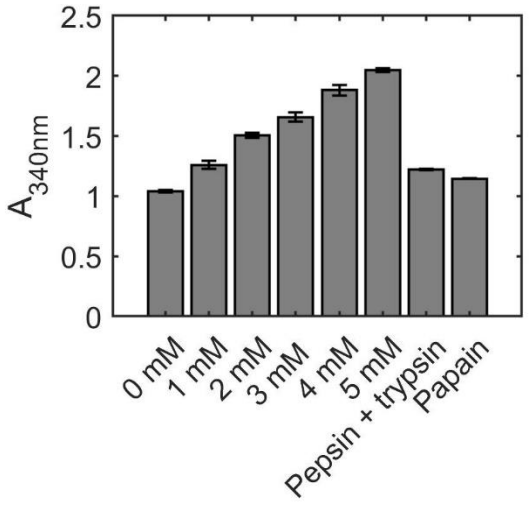


**Figure 4.1**: Pepsin and trypsin hydrolysis results in a higher degree of hydrolysis. Leucine is used as a control, and the degree of hydrolysis is calculated as: DH(%) = ($h/h_{tot}$)x100% where h is the calculated concentration of free amino groups, and $h_{tot}$ is the quantity of free amino groups per g of protein (7.4 meqv per g protein for rice bran).

Enrichment of RPBH copper-binding peptides was accomplished using an immobilized metal affinity chromatography workflow which separates the applied samples into strong

copper-binding fractions and non-copper-binding or weakly copper-binding fractions. These fractions were assessed for their copper-binding capacity using a colorimetric chelator, zincon, with a known copper binding $K_d$ of $4.68 \times 10^{-17}$ M.[19] As expected, copper-binding fractions isolated from the IMAC workflow had higher copper-binding capacity compared to their non-copper-binding counterparts (Figure 2).

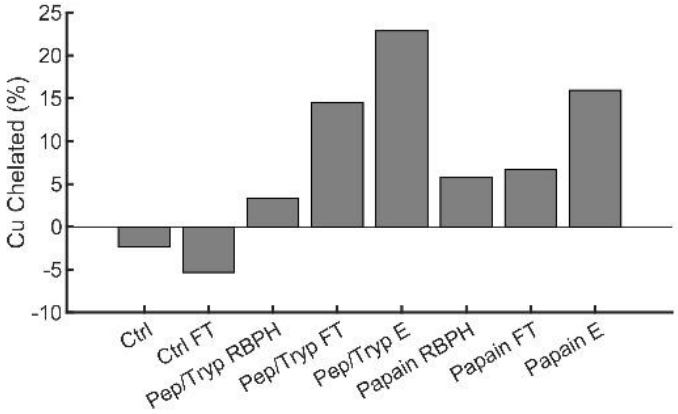


**Figure 4.2:** IMAC elution fractions (E) exhibit increased copper-binding capacity compared to their flow through (FT) counterparts. This supports the use of IMAC to enrich copper-binding populations from complex, heterogeneous peptide solutions.

The biological effects of different copper-binding populations were assessed in insulin resistant HepG2 cells through analysis of the AMPK pathway. When activated, AMPK is phosphorylated at threonine 172, and relative activation can be monitored by western blot analysis. An increase in phosphorylated AMPK correlates to an increase in AMPK activation. Preliminary results showed that the strong copper-binding elution (E) fractions increased AMPK activation more than the weak copper-binding flow through (FT) fractions (Figure 3).

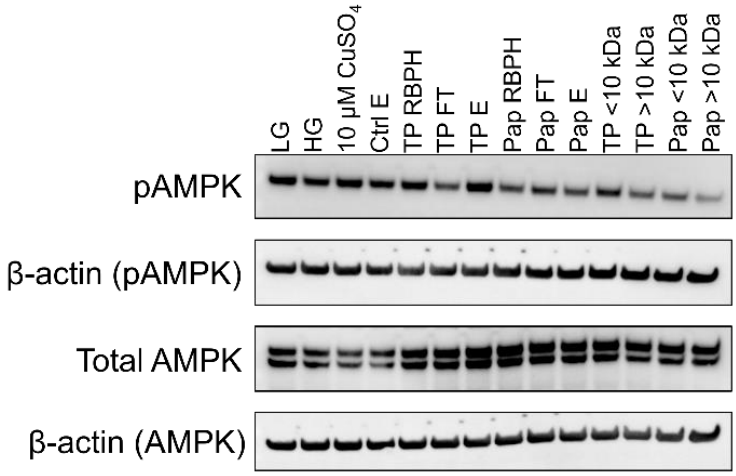


**Figure 4.3:** There is a decrease in AMPK activity under high glucose-induced insulin resistance in HepG2 cells. Treatment with rice bran protein hydrolysates show modulation of AMPK activity that may be based on their copper-binding abilities.

These results suggest that there is a relationship between the copper-binding abilities of RBPH and the observed downstream biological effects. To further investigate this relationship, peptides in the copper-binding fraction of RPBH were identified using mass spectrometry proteomics.

## 4.3.2 RBPH peptide identification and selection

Peptide sequences that were identified in both the pepsin/trypsin digestion as well as the papain digestion were selected for further analysis. Peptides with varying length and hydrophobicity were synthesized using solid phase peptide synthesis, and their sequences are listed in Table 1. The relative hydrophobicity of the peptides was assessed using reverse phase liquid chromatography mass spectrometry (RP-LCMS). Longer elution times indicate increased hydrophobicity. The 1 series of the peptides is the most hydrophilic while the 2 series are the most hydrophobic.

**Table 4.1:** Synthesized peptide sequences derived from papain and *pepsin/trypsin* digestions of rice bran protein.

| Descriptor | Peptide Sequence | Elution time (min) |
|---|---|---|
| 1A | RHASEGG | 1.98 |
| 1B | ASEGGHG | 2.26 |
| 1C | RHASEGGHG | 1.89 |
| 2A | HWPLPPF | 65.71 |
| 2B | PHWPLPPF | 65.71 |
| 2C | GPHWPLPPF | 65.71 |
| 3A | VPSGHPI | 30.20 |
| 3B | VVPSGHPI | 33.57 |
| 3C | FVVPSGHPI | 55.99 |

### 4.3.3 Copper-binding assessment of RBPH peptides

Copper binding capacities were assessed for each of the synthesized peptides using the zincon assay. While all peptides exhibited some level of copper-binding abilities, peptides 1A, 1C, and 2B bind the largest amount of copper in solution (Figure 4A). This copper-binding data was supported using a bioluminescent copper probe, pic-DTZ.[20] Pic-DTZ reports the amount of coordinatively accessible copper in solution. Peptides 1A, 1C, and 2B have the lowest available copper in solution corresponding to their increased copper-binding (Figure 4B). The sequences of peptides 1A, 1C, and 2B all have a histidine residue in the second position on the N-terminal side. This motif is reminiscent of the well-known copper-binding peptide, GHK, which binds copper in a tridentate manner with the N-terminus and the histidine residue.[21]

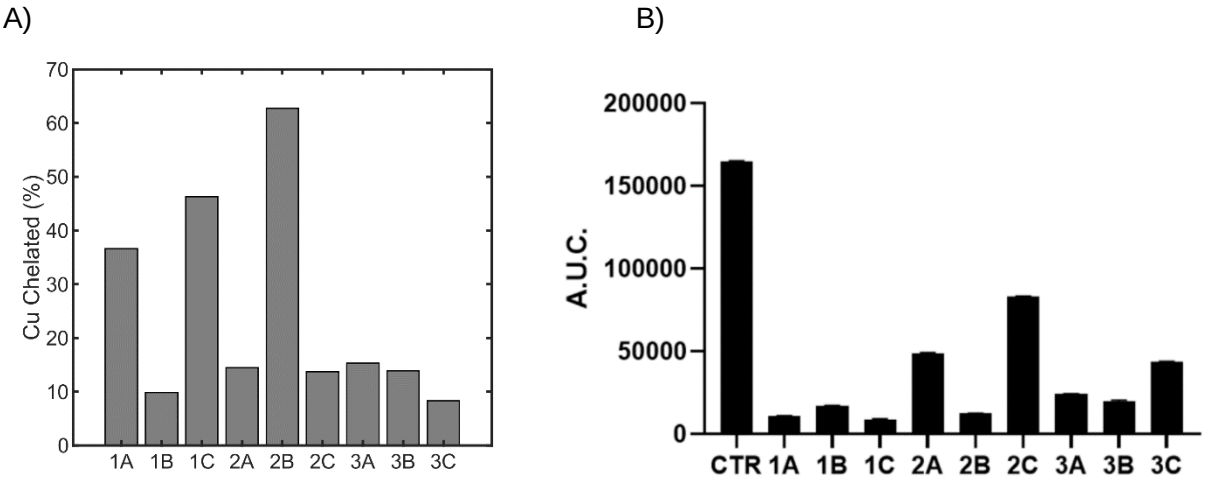


**Figure 4.4:** Using the previously described zincon assay, binding capacities of the synthesized peptides were studied. A) Peptides 1A, 1C, and 2B have increased binding affinities which are attributed to a histidine residue in the 2nd position allowing for copper binding to occur between the histidine and the N-terminus. B) Use of pic-DTZ, a bioluminescent copper probe, supports the decreased availability of copper in the presence of stronger binding peptides 1A, 1C, and 2B.

## 4.3.4 Antioxidant properties of RBPH linked to copper-binding

Copper is redox-active and therefore can produce reactive oxygen species (ROS) by participating in Fenton-like chemistry.[22] Thus abberant copper biology is linked to deleterious biological effects, and it is important that copper is tightly regulated.[7] To assess the ability of rice bran peptides to protect against ROS production, the 3-CCA assay was employed.[18] Protective effects against •OH production correlates to copper-binding ability of the RBPH peptides (Figure 4). Strong binding peptides 1A, 1C, and 2B prevent production of •OH whereas weaker binders

with accessible copper cannot fully inhibit •OH production.

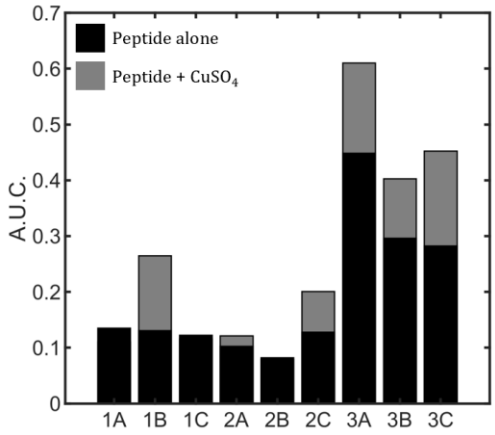


**Figure 4.5**: The CCA assay measure levels of •OH in solution. Production of •OH in solution occurs through a reduction of Cu(II) by ascorbic acid. The generated •OH then reacts with 3-CCA to produce a fluorescent derivative, 7-OH-CCA. When Cu(II) is bound by stronger binding peptides (1A, 1C, and 2B), •OH production is prevented supporting the potential of these peptides to exhibit antioxidant activity in biological systems.

## 4.3.5 Biological effects of RBPH peptides

Insulin-resistant HepG2 cells treated with the synthesized peptides show varying AMPK activation (Figure 6). All peptide treatments increased AMPK activity compared to the high glucose control treatment. Interestingly, the more hydrophobic 2 series of peptides increased AMPK activation the most. Likely, the increased hydrophobicity allows the peptides to behave like ionophores where they bind copper in the extracellular environment and import the ions through the hydrophobic membrane.[10]

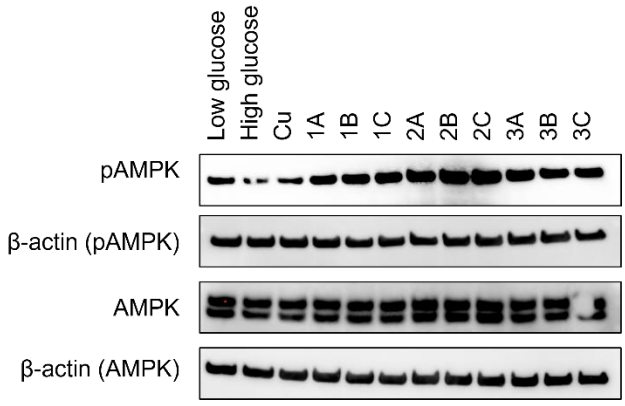


**Figure 4.6:** Activation of the AMPK pathway involves phosphorylation of AMPK at Thr-172. Insulin-resistant HepG2 cells treated with synthesized peptides show differing levels of AMPK activation. High glucose conditions inhibit AMPK phosphorylation, but treatment with copper and all copper-binding peptides show increases in phosphorylation. The more hydrophobic peptides, the 2 series, are better able to cross the cell membrane, and therefore may be able to transport copper into the cell to affect the AMPK pathway.

## 4.4 Conclusion

In this work, we present potential therapeutic inspiration in copper-binding peptides derived from rice bran protein. Isolation and identification of peptides hydrolyzed from rice bran proteins allows for an understanding of the importance of amino acid sequence, physiochemical properties, and biological activity. Strong copper-binding peptides often contain a histidine residue, and positioning the histidine residue at the second position on the N-terminal side further increases binding abilities. Increased hydrophobicity increases mobility across the cell membrane which allows access to greater bioactivity. Copper-binding populations of RBPH warrant further research to better understand how to harness beneficial biological actions.

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# Chapter 5

## Studying Vitamin B12 with NMR Relaxometry

## 5.1 Abstract

Vitamin B12 (cobalamin) is an essential micronutrient required for the proper functioning of mammalian cells. Despite the ubiquity of cobalamin in biology, there lacks a comprehensive assay to identify and quantify different forms of B12 in biological fluids with specificity. As only certain forms of B12 are considered bioavailable, understanding the speciation of B12 and relative amounts of cobalamin-containing species in a system can provide improved insight into B12 status and associated physiological and pathological states. Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique that can provide information about both macroscopic properties and microscopic interactions in heterogeneous solutions. Here, NMR is applied to study cobalamin in various environments through 59Co NMR, 31P NMR, and 1H NMR relaxometry. The multinuclear approach presented can help elucidate information about speciation and quantification of cobalamin in biological fluids and impact strategies for diagnoses and treatments.

## 5.2 Introduction and Background

Vitamin $B_{12}$ (cobalamin, Fig. 1) is a cobalt-containing biomolecule required for the proper functioning of all human cells. Cobalamin serves as a cofactor for two essential enzymes in the body, methionine synthase and methylmalonyl-CoA mutase, which are involved in methylation processes including DNA synthesis and nerve maintenance.[1] In the blood, the majority of cobalamin is bound by two proteins, 80% to haptocorrin (HC) and 20% to transcobalamin (TCNII).[2] Cobalamin bound to HC (holoHC) is not considered bioavailable due to the lack of a receptor on the cell surface; cobalamin

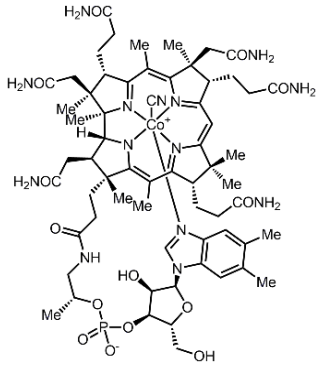


**Figure 5.1:** Structure of cobalt-containing cobalamin shown here as cyanocobalamin with a β-axial **CN**

bound to transcobalamin (holoTC) can be internalized and is considered the bioactive form of cobalamin.[3]

Vitamin $B_{12}$ deficiency is linked to disease states including pernicious anemia and neurological diseases such as Parkinson's and Alzheimer's, but the underlying mechanisms of these pathologies remain elusive. Despite its importance, few methods are available for tracking cobalamin *in vivo*, and state-of-the-art clinical assays require lengthy times and lack important information about the bioavailability of $B_{12}$ in the body.[4]

The biological pathways and interactions of cobalamin have been investigated through techniques including fluorescence, immunoassays, and radiolabeling. Nonetheless, there remains much unknown about cobalamin metabolism and function including its protein-carrier distribution within complex biological environments.[5] Nuclear magnetic resonance (NMR) is a powerful tool for analyzing complex mixtures and can provide a wealth of information about environments within a sample. NMR is most frequently applied to the structural elucidation of compounds inferred from chemical shift and J-coupling data. These applications typically use high-field magnets to probe pure solutions, but high-resolution NMR signals can be convoluted when analyzing highly complex biological systems due to the large number of nuclei with similar chemical shifts. To circumvent this issue, low-resolution NMR relaxometry can be alternatively implemented. Although low-resolution NMR lacks chemical shift information, it can provide insight into local environments and the movement of target analytes through relaxation ($T_1$ and $T_2$) and diffusion times ($D$). Additionally, NMR relaxometry can be performed using small, portable, and relatively cheap instruments expanding its broad applicability and clinical impact.[6]

## 5.3 Methods

### $^{59}$Co NMR of packed powder solid cyanocobalamin

A probe designed for a 9.4 T instrument was built in-house and tuned to a frequency of 95.18 MHz corresponding to the Larmor frequency of $^{59}$Co. Pulse widths were calibrated, and $T_2$ values were determined by acquiring a series of FIDs with varying last delay values.

### $^{31}$P NMR relaxometry of cyanocobalamin in aqueous solution

A probe designed for a 9.4 T instrument was built in-house and tuned to a frequency of 161.67 MHz corresponding to the Larmor frequency of $^{31}$P. 10 mM solutions of cyanocobalamin in nanopure $H_2O$ and glycerol were prepared. $T_2$ values were determined using a CPMG pulse sequence and the collected data was fit to a single exponential.

### $^{1}$H NMR relaxometry of cyanocobalamin in aqueous solution

To measure $T_1$ and $T_2$ values of aqueous solutions of cyanocobalamin, a pulse sequence (Figure 5) was developed. A gradient was applied across the sample of interest and a control sample to account for variation in measurements due to temperature. The resulting data was processed using a Fourier transform resulting in simultaneous $T_1$ and $T_2$ measurements for two samples.

## 5.4 Results and Discussion

Cobalamin is the main source of cobalt in the body, so it would be ideal to probe the cobalt center using $^{59}$Co NMR. Medek *et al*. reported detection of a cobalt signal from cobalamin in 1997, but suggested that high-field magnets (≥11.4 T) be used due to the extremely wide line widths and low signal-to-noise ratio.[7] Attempting to build upon this work, a probe was modified for use in a 9.4 T superconducting magnet to study cobalamin. Despite the higher field requirement stated by Medek *et*

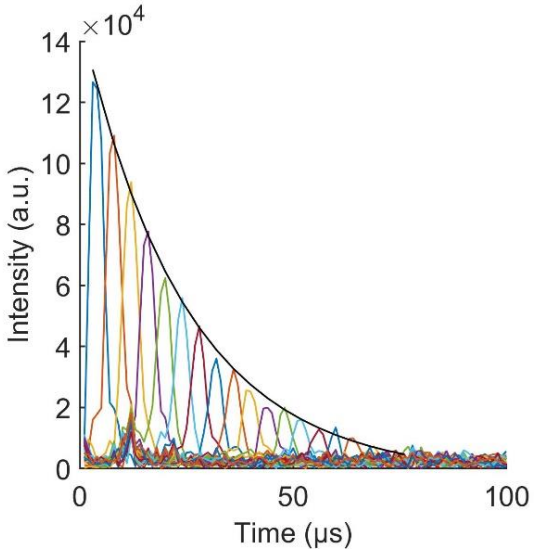


**Figure 5.2:** $^{59}$Co spin echo signal as a function of pulse spacing for a packed powder solid sample of vitamin $B_{12}$. The exponential decay curve for $T_2$ = 45 μs.

*al.*, a signal from the central quadrupole transition was detected in a packed powder solid and the transverse relaxation time was determined to be 45 μs which is supported by a recent publication (Fig. 2).[8]

Unfortunately, in aqueous environments, the extremely short transverse relaxation time of the cobalt nucleus means the signal decays faster than the ringdown from instrumentation. Although the low background signal offers a promising advantage, $^{59}$Co NMR alone is not sufficient to study cobalamin with the available instrumentation in the lab.

To validate the ability to use NMR to detect cobalamin in different environments, $^{31}$P experiments were performed on aqueous and glycerol solutions of cobalamin at 9.4 T. The changes in relaxation times support the continuing efforts to study cobalamin through multinuclear NMR approaches (Fig. 3).

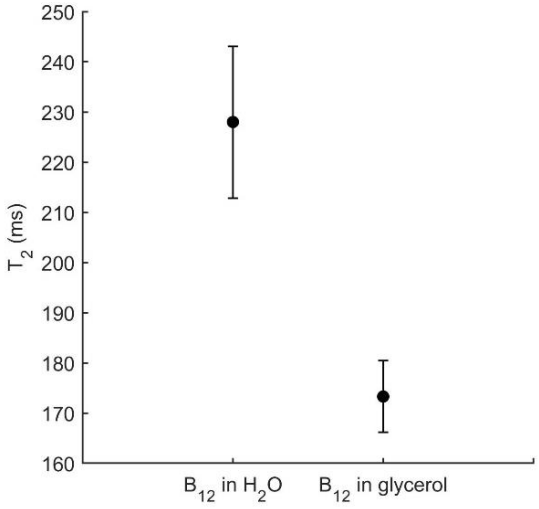


**Figure 5.3:** The $^{31}$P NMR transverse relaxation signal for the phosphate contained in the α-axial ligand of cobalamin is solvent dependent.

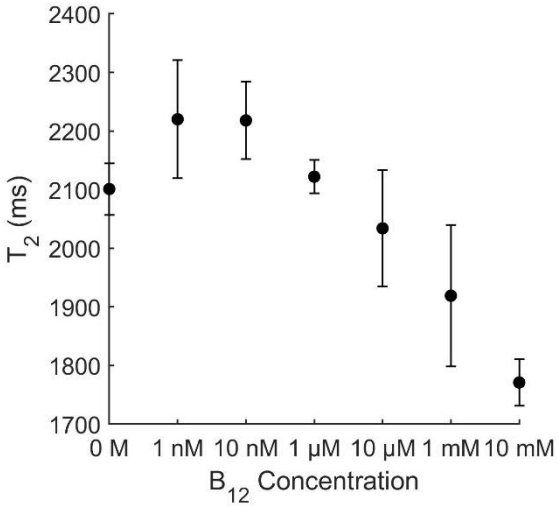


**Figure 5.4:** The $^{1}$H transverse relaxation times as a function of cobalamin concentration in aqueous

In order to circumvent the challenges of using $^{59}$Co NMR to study cobalamin, NMR relaxometry was implemented. Here, aqueous samples of cyanocobalamin were prepared at various concentrations

and studied with $^{1}$H-relaxometry at 9.4 T. The data show an inverse correlation between the resulting transverse relaxation times and cobalamin concentration. (Fig. 4).

$^{1}$H-relaxometry measurements were then performed at a lower 1 T magnetic field. Due to the small concentration differences between samples, the relaxation times of all concentrations span only a small range. Repetition of measurements revealed a temperature sensitivity of the instrument which yielded a relatively wide range of relaxation times for the same sample that confounded the concentration-dependent differences.

To address the temperature variability in the two samples and between measurements, a simultaneous two-sample pulse sequence was developed. Using a gradient to spatially encode two sample vials 2D $T_1$-$T_2$ measurements were performed using the following pulse sequence (Fig. 5).

With this measurement, a Fourier transform is used to deconvolute signals from the two

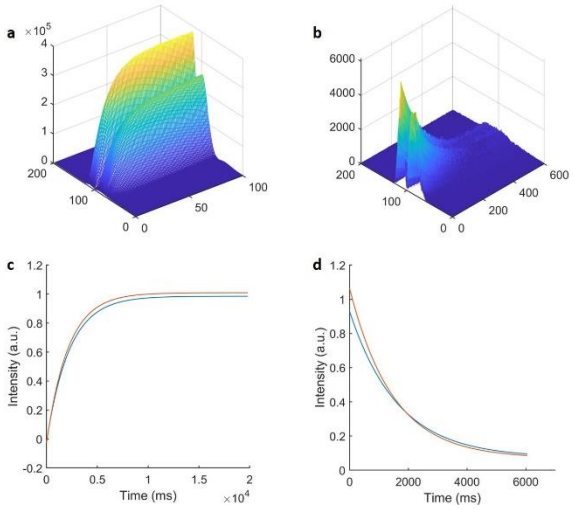


**Figure 5.6:** (a) A Fourier transform of the data in the indirect and direct dimensions yields a 3D plot of the (a) $T_1$ recovery and (b) $T_2$ decay, respectively, from the two samples. Integration of the 3D plots produces (c) recoveries and (d) decays which are fit to extract $T_1$ and $T_2$ data.

samples resulting in two recovery signals ($T_1$) and two decay signals ($T_2$) (Fig. 6).

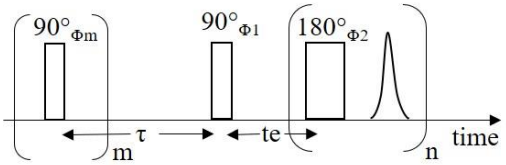


**Figure 5.5:** 2D $T_1$-$T_2$ correlation pulse sequence used to measure the relaxation times of two samples simultaneously. τ is a table of variable times to capture the saturation recovery. A gradient is applied during acquisition to spatially encode the resulting signals from the two samples.

With temperature variation corrected in the measurements, $T_1$ and $T_2$ relaxation times show an inverse correlation with cobalamin concentration, similar to what was observed a high fields.

While the concentration-dependence with relaxation times could be successfully measured, the experiments were only performed with free cobalamin. Cobalamin does not exist in its free form in the body, however, and the feasibility of the method must be considered in the protein-bound form of the molecule. The specific proteins that interact with cobalamin, TCNII and HC, have significantly different molecular weights which would be expected to manifest as different relaxation and diffusion times in solution. As a model for serum and heterogeneous media, measurements were performed on solutions of $B_{12}$ and bovine serum albumin (BSA) which is commercially available at low cost. Recent literature suggests interactions of BSA and $B_{12}$ through π-π interactions.[9] To determine whether these interactions can be probed via NMR relaxometry, solutions of 0.6 mM BSA were titrated with increasing concentrations of cobalamin and relaxation times were measured at 1 T. With increasing concentration of cobalamin, an inverse correlation with $T_2$ was observed, suggesting that BSA-cobalamin interactions may be probed with $^1$H relaxometry (Fig. 7).

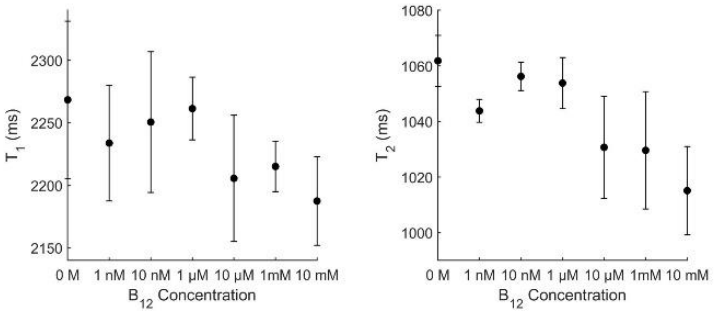


**Figure 5.7:** $T_1$ and $T_2$ relaxation times of 0.6 mM BSA titrated with various concentrations of cobalamin.

To further investigate cobalamin-protein interactions, diffusion measurements of $D_2O$ solutions of cobalamin and BSA were performed. The self-diffusion coefficient of BSA alone was longer than that of a solution of BSA with cobalamin. This indicates that BSA alone diffuses slower than when interacting with cobalamin. These findings support literature which reports a more folded tertiary structure of BSA resulting from cobalamin interactions (Table 1).[9]

**Table 5.1:** Summary of $T_1$, $T_2$, and D values for 2 mM solutions of $B_{12}$ and BSA.

| Measurement | $B_{12}$ | BSA | $B_{12}$ + BSA |
|---|---|---|---|
| $T_1$ | 82 ± 14 ms | 185 ± 6 ms | 182 ± 2 ms |
| $T_2$ | 62 ± 11 ms | 5.6 ± 0.4 ms | 5.7 ± 0.1 ms |
| $D$ | $3.1 \times 10^{-10}$ m²/s | $3.6 \times 10^{-11}$ m²/s | $5.8 \times 10^{-10}$ m²/s |

## 5.5 Conclusion

Here, various NMR methods were applied to study vitamin $B_{12}$ towards the ultimate goal of identification and quantification of different forms of $B_{12}$. Although $^{59}$Co NMR proved challenging in aqueous environments due to short relaxation times, $^{1}$H relaxometry data shows promise in elucidating differences in $B_{12}$ states.

A continued effort can be put forth to detect $^{59}$Co NMR signal from solutions of cobalamin to use as a control method to which relaxometry data can be compared. After expression of sufficient amounts

of TCNII in HEK293 cells, relaxometry measurements can be performed on cobalamin-TCNII solutions to determine if the relaxation and diffusion times can reflect differential protein binding. After establishment of a method to detect cobalamin-protein complexes, further heterogeneity will be introduced into solution to better mimic biofluids before moving onto serum and blood samples. In addition to cobalamin-protein complexes, speciation of cobalamin in heterogeneous solutions should be pursued to investigate the limit of detection of paramagnetic cobalt nuclei through $^{1}H$ relaxometry.

## 5.6 References


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# Chapter 6

## Copper Mediated Oxidation of Imidazopyrazinones Inhibits Marine Luciferase Activity

*This chapter is a modified version of the following published article: O'Sullivan, J. J., Lee, V. J., Heffern, M. C. Copper-mediated oxidation of imidazopyrazinones inhibits marine luciferase activity. *Luminescence.* **2022**, 1. JJ performed the synthesis and bioluminescence assays. Vanessa performed the absorption spectroscopy analyses.

## 6.1 Abstract

The development of bioluminescent based tools has seen steady growth in the field of chemical biology over the past few decades ranging in uses from reporter genes to assay development and targeted imaging. More recently coelenterazine-utilizing luciferases such as *Gaussia*, *Renilla*, and the engineered Nano- luciferases have been utilized due to their intense luminescence relative to firefly luciferin/luciferase. The emerging importance of these systems warrants investigations into the components that affect their light production. Previous work has reported that one marine luciferase*, Gaussia*, is potently inhibited by copper salt. The mechanism for inhibition was not elucidated but was hypothesized to occur via binding to the enzyme. In this study, we provide the first report of a group of non-homologous marine luciferases also exhibiting marked decreases in light emission in the presence of copper(II). We investigate the mechanism of action behind this inhibition and demonstrate that the observed copper inhibition does not stem from a luciferase interaction but rather the chemical oxidation of imidazopyrazinone luciferins generating inert, dehydrated luciferins.

## 6.2 Results and Discussion

Bioluminescence is a natural phenomenon where a small molecule substrate, luciferin, is oxidized by its enzyme, luciferase. This enzyme-catalyzed reaction results in the production of photons through relaxation of an excited state oxidation product. Though observed in nature for thousands of years, it was not until the second half of the 20th century that scientists began to fully characterize and elucidate the molecular mechanisms of bioluminescence.[1] Since then, bioluminescent systems have steadily gained popularity as a unique tool in biochemical research ranging in uses from reporter genes to molecular imaging probes.[2,3,4,5,6] Compared to other optical modalities such as fluorescence, bioluminescence-based platforms find a key

advantage in requiring no excitation light, affording near-zero background signal and minimizing phototoxicity.[7]

The three most well-studied classes of bioluminescent systems are insect, bacterial, and marine luciferin/luciferases.[8] The studied marine ecosystems in particular contain a vast diversity of luminescent organisms. For example, in one study about 76% of individuals in the water column were observed to have luminescent capability.[9] Currently known marine-based bioluminescent systems are representative of different taxa, are of various complexity, and are of different molecular mechanisms. Interestingly, while diverse in the structures of their enzymes, most of these systems show similarities in their substrates, utilizing luciferins with a shared imidazopyrazinone core, with coelenterazine (CTZ, Figure 1) being the most well-studied and commonly used.[10]

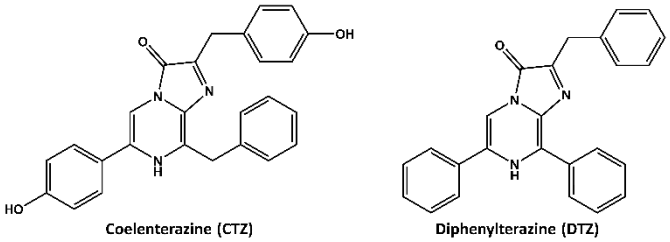



**Figure 6.1:** Chemical structures of native marine luciferin, coelenterazine, and synthetic analog, diphenylterazine.

Biochemical research on coelenterazine-utilizing organisms to date have primarily focused on the *Renilla reniformis*, *Gaussia princeps*, and *Oplophorus gracilirostis* organisms.[11,12,13] Additionally, Nanoluciferase (Nluc), a recently engineered luciferase derived from *Oplophorus* luciferase, has gained notable traction due to its small size, high thermal stability, and intense light emission in the presence of its partner imidazopyrazinone, furimazine.[14] It has previously been reported that *Gaussia* luciferase is potently inhibited by Cu(II) ions.[15,16] Inhibition was posited to occur via direct interactions with the enzyme. However, in our previous work developing a copper-responsive bioluminescent imaging probe based on the imidazopyrazinone, diphenylterazine (DTZ, Figure 1), and luciferase, Nluc, we observed

bioluminescence inhibition at high Cu(II) concentrations despite the dissimilarities between the *Gaussia* luciferase and Nluc structure.[17] This led us to the hypothesis that copper-mediated inhibition of marine luciferase bioluminescence may actually arise from transformation of the imidazopyrazinone substrate core.

We validated that Cu(II) ions could alter the activity of a series of imidazopyrazinone-based luciferases. We investigated the effects of Cu(II) on the bioluminescence of *Renilla*, Nano-, and *Gaussia* luciferases. These marine luciferases are non-homologous as they evolved independently.[18] For these studies we used CTZ for all three luciferases (Figure 2) as well as DTZ with Nanoluciferase (Figure 3) as *Renilla* and *Gaussia* luciferases do not accept DTZ.

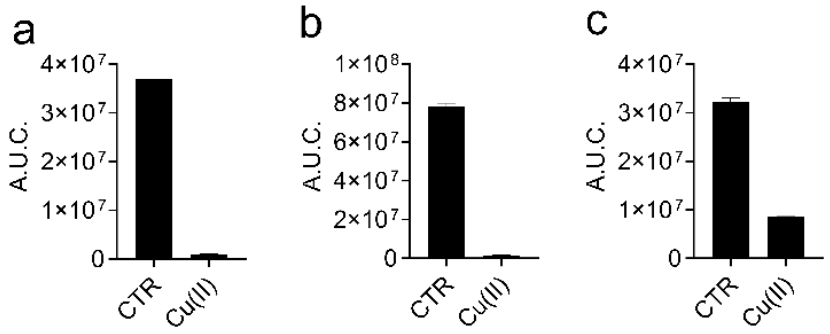



**Figure 6.2:** Calculated area under the curve (A.U.C.) over twenty minutes of kinetic luminescence measurements of CTZ (10 µM) in the absence (CTR) or presence of Cu(II) ions (100 µM) with A) Renilla, B) Nano or C) Gaussia luciferases (0.4 µg/mL).

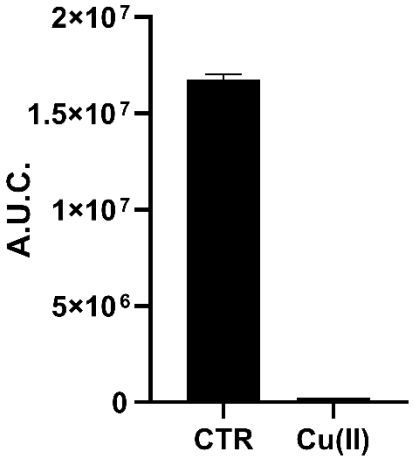



**Figure 6.3:** Calculated area under the curve over twenty minutes of kinetic luminescence measurements of DTZ (10 µM) in the absence or presence of Cu(II) ions (100 µM) with recombinant Nluc (0.4 µg/mL).

In all cases, bioluminescence was potently inhibited by Cu(II) upon immediate addition to the enzyme substrate solution. The level of inhibition shows a response to dose, suggesting a non-catalytic inhibition. Interestingly, inhibition of light output is potent, occurring even with as low as 0.1 equivalents of copper into DTZ and reaching maximum inhibition by 0.5 equivalents (Figure 4).

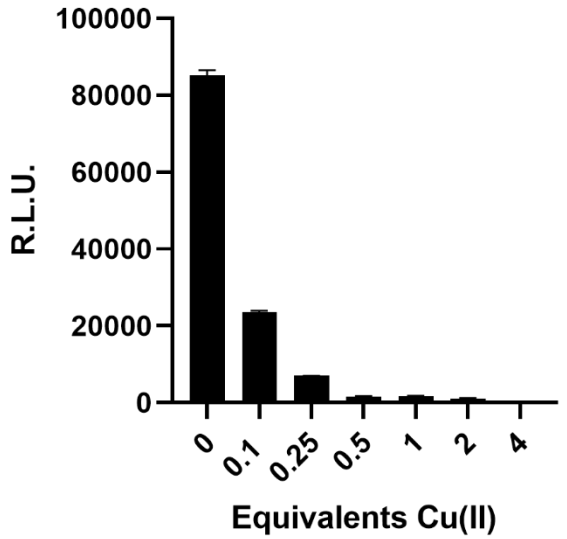



**Figure 6.4:** Calculated area under the curve over twenty minutes of kinetic luminescence measurements of DTZ (1 µM) in the absence or presence of varying stoichometric equivalents of Cu(II) ions (0 to 4 equivalents) with recombinant Nluc (0.4 µg/mL).

Furthermore, we hypothesized that the lower level of inhibition observed with Gaussia luciferase likely stemmed from faster binding kinetics of the substrate to the enzyme pocket, reducing Cu(II) access to the free substrate. To test this hypothesis, we performed an experiment where Cu(II) was preincubated with DTZ for 10 minutes prior to addition of Gaussia luciferase (Figure 5) and observed a complete loss of signal.

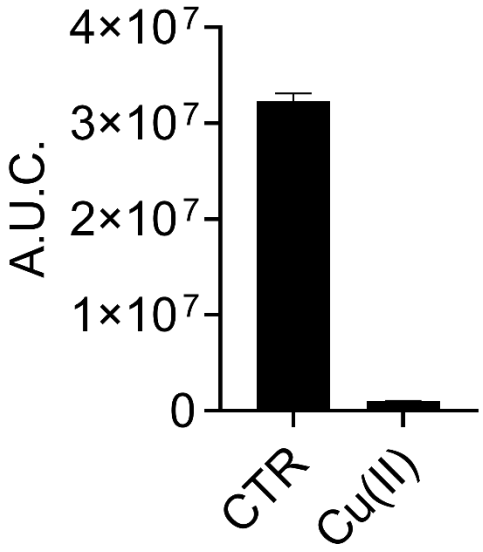



**Figure 6.5:** Calculated area under the curve (A.U.C.) over twenty minutes of kinetic luminescence measurements of DTZ (10 µM) in the presence of Gaussia luciferase (0.4 µg/mL) with or without preincubation with Cu(II) for 10 minutes.

This supports the hypothesis that the observed decrease in inhibition with *Gaussia* is likely due to kinetic competition between Cu(II) reaction and substrate-enzyme binding.

We therefore aimed to determine the structural impact of Cu(II) on the partner luciferins (DTZ

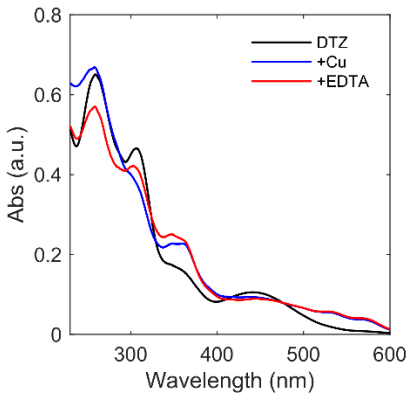



**Figure 6.6:** Electronic absorption spectrum of 50 μM DTZ with and without addition of 50 μM CuSO4 in ethanol. Spectral changes suggest oxidation of DTZ upon interaction with copper. Upon addition of 500 μM EDTA, the DTZ spectrum is not restored which confirms that the interaction is irreversible by chelation of the metal ion.

and CTZ) by Cu(II) as the driver of bioluminescence inhibition. As inhibition was observed with both substrates, we chose to use DTZ for the majority of studies due to its increased stability relative to CTZ and its facile synthesis. The conjugated nature of marine luciferins lends itself to study by electronic absorption spectroscopy. To begin understanding the interactions between the luciferins and Cu(II), we recorded absorption spectra in the absence and presence of Cu(II) ions. We noted significant spectral changes upon addition of $CuSO_4$. Furthermore, the luciferin spectrum was not restored upon addition of excess EDTA, indicating a change that is irreversible by metal ion chelation (Figure 6).

Additional experiments with CTZ show similar results (Figure 7).

**a** **b**

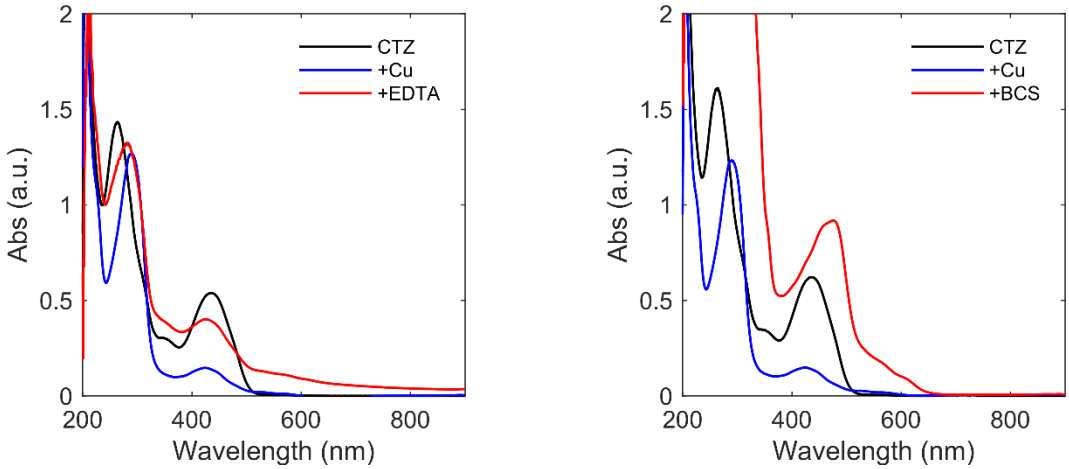



**Figure 6.7:** Electronic absorption spectra of 50 μM CTZ with and without addition of 50 μM CuSO4 in ethanol. Spectral changes upon addition of Cu(II) suggest oxidation of CTZ. Addition of 500 μM EDTA does not restore the CTZ spectrum pointing to an irreversible reaction, and addition of 500 μM BCS confirms the presence of Cu(I) in solution resulting from the redox reaction.

A metal screen with biologically relevant metals showed that Fe(III) was the only other metal ion to have a similar effect as Cu(II) on the absorption spectra (Figure 8).

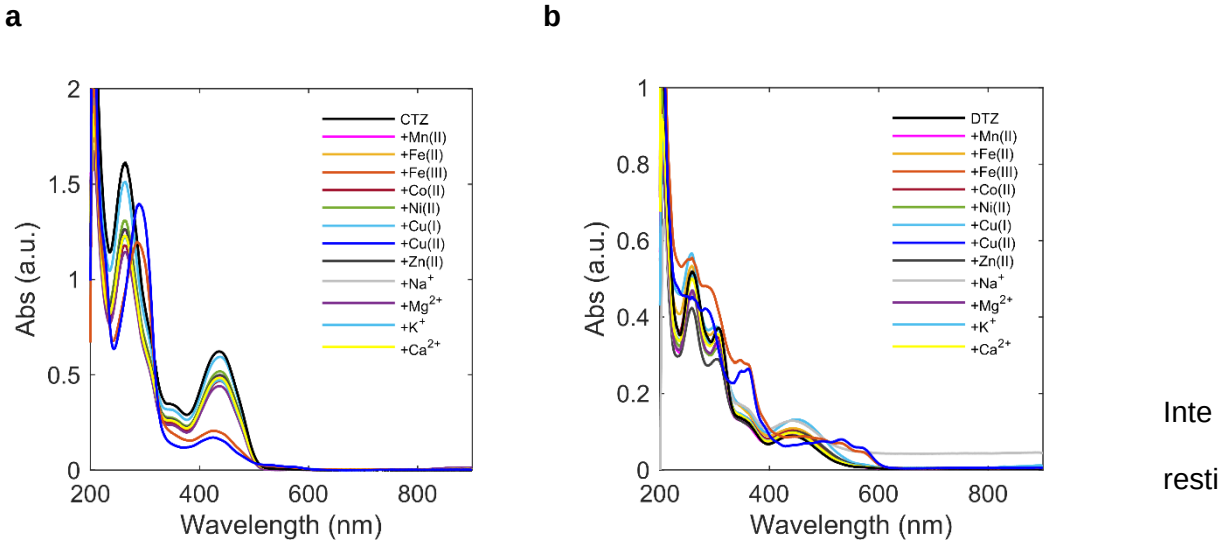



**Figure 6.8:** Electronic absorption spectra of 50 μM (A) CTZ and (B) DTZ alone (black) and upon addition of 50 μM biologically relevant metal ions. Significant spectral changes occur in both CTZ and DTZ spectra upon addition of Cu(II) and Fe(III) ions indicating interactions between the luciferins and these metal ions.

Interestingly, in enzyme studies

analogous to those presented in Figure 2 but performed with Fe(III), no inhibition of light output was observed (Figure 9).

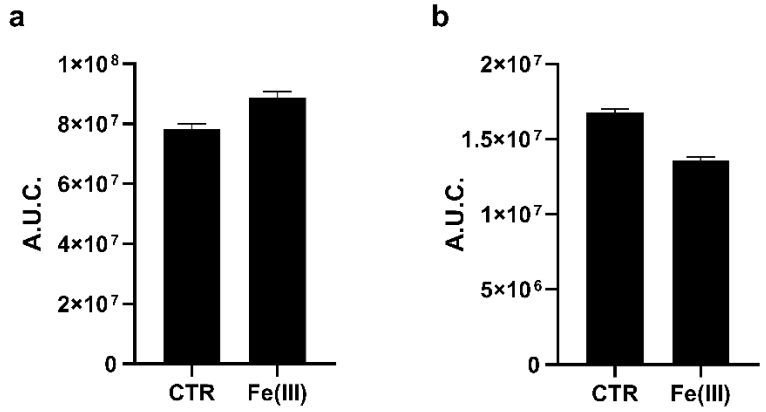



**Figure 6.9:** Calculated area under the curve over twenty minutes of kinetic luminescence measurements of 10 µM (A) CTZ or (B) DTZ in the absence or presence of Fe(III) ions (100 µM) with recombinant Nluc (0.4 µg/mL).

The same electronic absorption spectroscopy studies were performed with CTZ resulting in similar spectral changes, suggesting the Cu(II) interaction is not a DTZ dependent effect and is likely occurring at the shared imidazopyrazinone core.

The irreversible nature of the interaction as well as the similarity in effect with Fe(III) led us to investigate potential redox events involving Cu(II). To determine if Cu(II) is reduced to Cu(I) in the presence of DTZ, we used a colorimetric Cu(I) chelator, bathocuproine disulfonic acid (BCS), to confirm the presence of Cu(I) in solution (Figure 10).

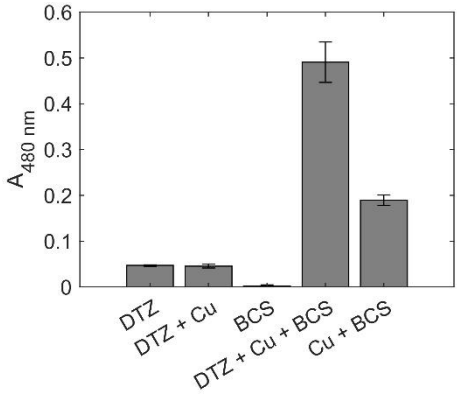



**Figure 6.10:** Bathocuproine disulfonic acid (BCS) is a copper(I)-specific colorimetric chelator that absorbs at 480 nm when complexed. Addition of 500 µM BCS to a solution of 50 µM DTZ and 50 µM CuSO¬4

results in increased absorbance at 480 nm indicating reduction of Cu(II) by DTZ. Error bars represent STD (n = 3).

Indeed BCS shows a significant increase in absorption at 480 nm in the presence of DTZ and $CuSO_4$ suggesting that DTZ is able to reduce Cu(II) to Cu(I). These spectroscopic data point to a redox-driven chemical reaction over Cu(II) coordination as the cause of the observed enzyme inhibition.

We thus tested the hypothesis that Cu(II) addition may drive DTZ oxidation towards a luciferase-inert compound. Reverse-phase LC-MS analysis of DTZ in the presence of Cu(II) showed the emergence of a peak exhibiting a ten-minute increase in retention time relative to DTZ alone (Figure 11).

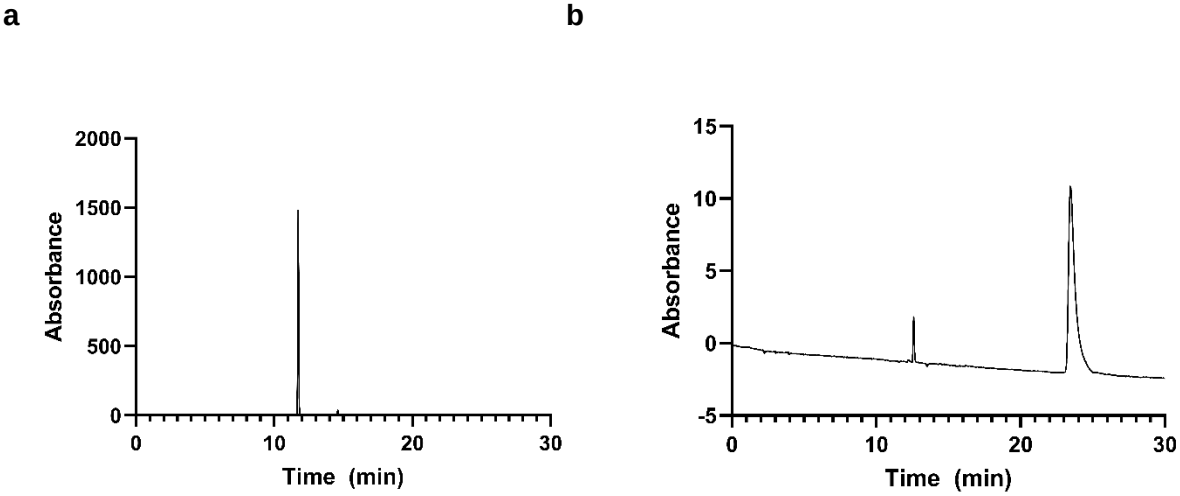



**Figure 6.11:** Chromatograms of (a) DTZ and (b) dhDTZ reveal a 10 minute longer retention time.

The increased retention time suggests that the oxidized product is more non-polar than DTZ. The mass of the species in this peak (m/z = 376.1, Figure 12), corresponds to a loss of 2 protons corroborating an oxidation reaction.

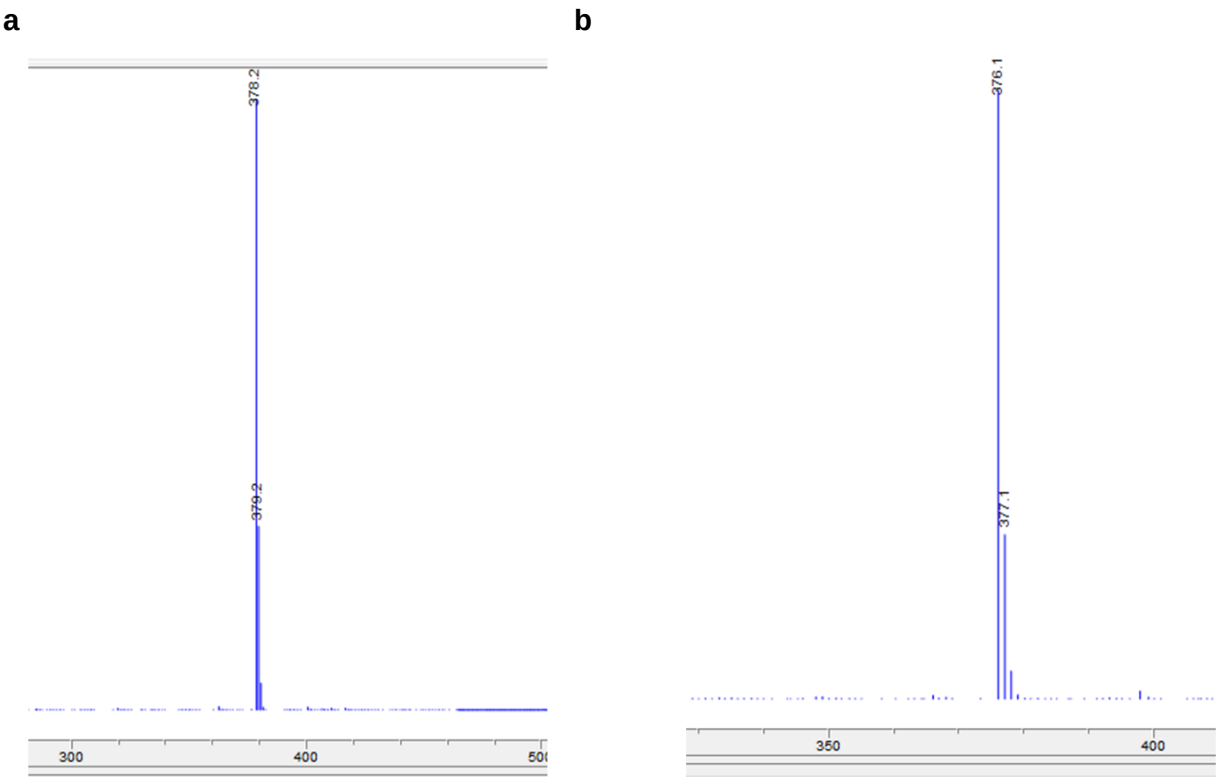



**Figure 6.12:** Mass spectra of (a) DTZ and (b) dhDTZ show an m/z of 378.2 and 376.1 for DTZ and dhDTZ respectively.

A similar phenomenon was observed with CTZ where the oxidized product was retained on the column for three minutes (Figure 13) compared to native CTZ and had a mass (m/z 422.1, Figure 14) that corresponds to the loss of two protons.

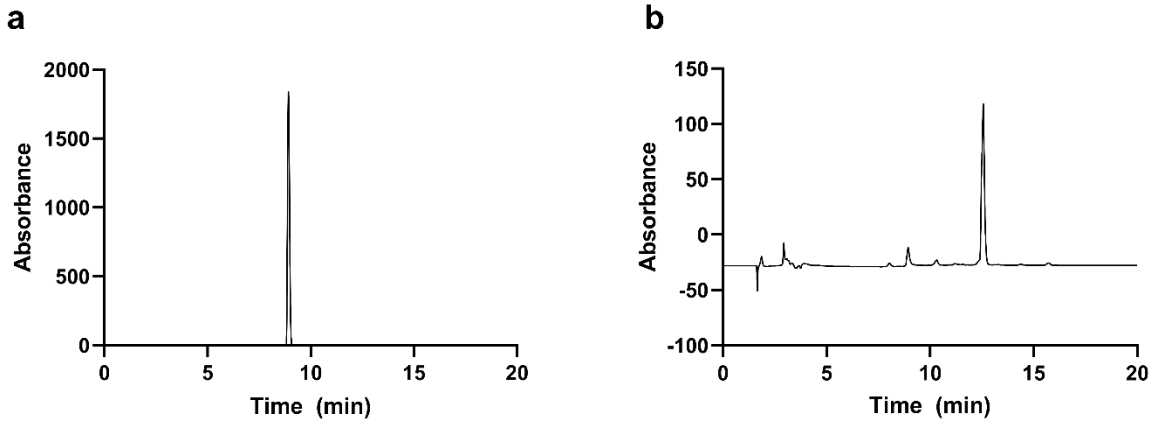



**Figure 6.13:** Chromatograms of (a) CTZ and (b) dhCTZ reveal a three minute longer retention for dhCTZ.

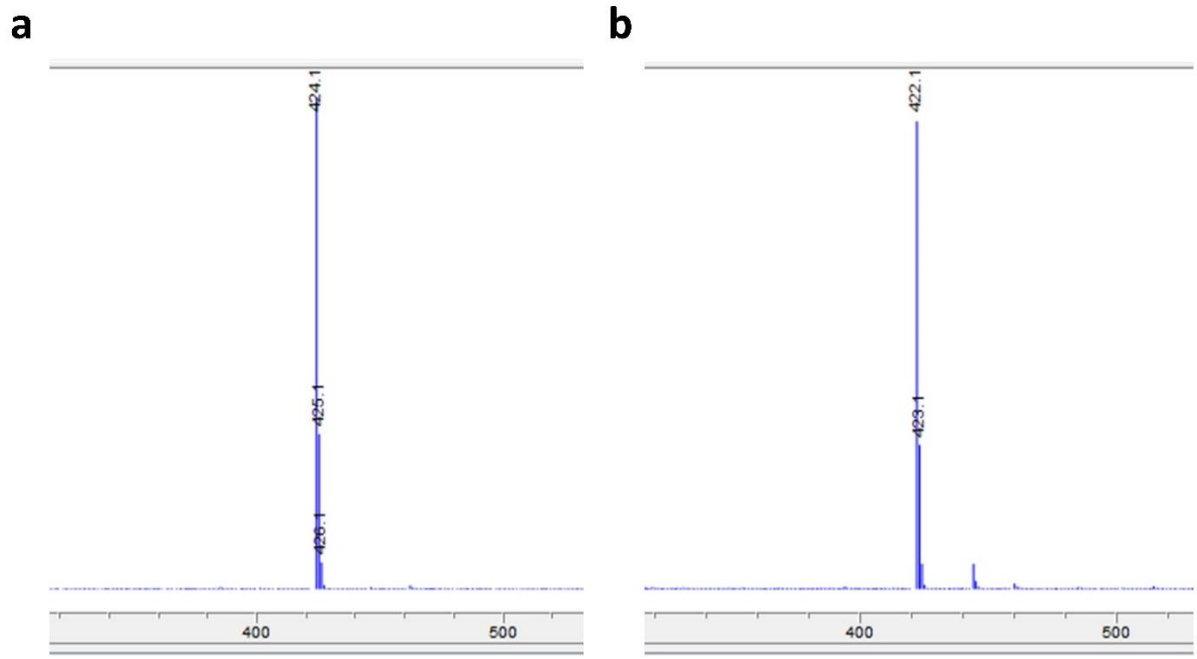



**Figure 6.14:** Mass spectra of (a) CTZ and (b) dhCTZ show an m/z of 424.1 and 422.1 for CTZ and dhCTZ respectively.

We applied NMR spectroscopy to observe any changes in the 1H spectra (Figure 15). DTZ was reacted with excess Cu(II) until full conversion to oxidized DTZ was observed by LC-MS. As Cu(II) is paramagnetic and may cause universal peak broadening, a liquid extraction with DCM/$H_2O$ was performed to remove the metal ion from the sample prior to NMR analysis.

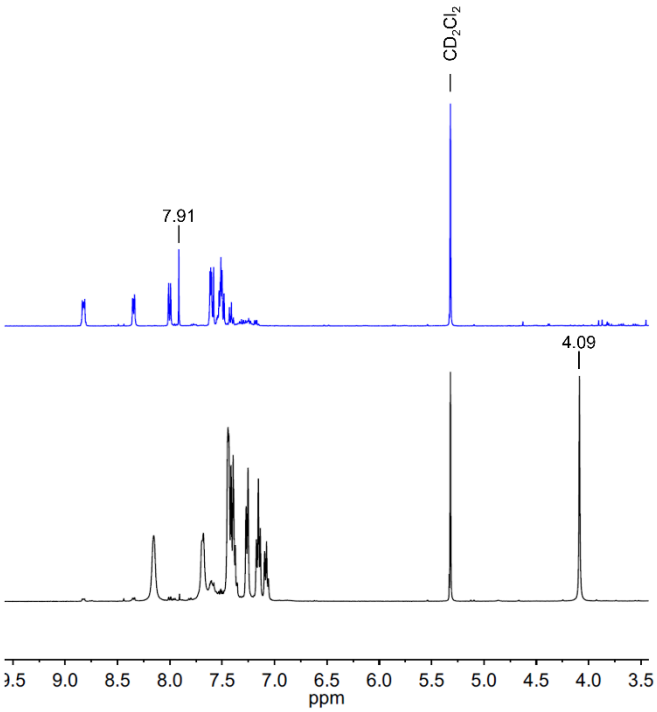



**Figure 6.15:** 1H NMR spectra of oxidized DTZ (top) and DTZ (bottom) measured in CD2Cl2 as solvent shows a loss of methylene proton signals at 4.09 ppm and appearance of a vinyl proton signal at 7.91 ppm after oxidation by Cu(II).

The most notable changes in the NMR spectra were the absence of methylene proton signals from the benzyl group of DTZ at 4.09 ppm and the appearance of a peak at 7.91 ppm. This coupled with the LC-MS data suggests the formation of a vinyl proton which could only occur at the benzylic carbon of DTZ. Therefore, we hypothesized that Cu(II) oxidized DTZ to dehydrated DTZ (dhDTZ, Figure 16). Similar LC-MS and NMR experiments (see supplementary information) using native CTZ suggest the same oxidation reaction occurring to form dehydrated coelenterazine (dhCTZ). The proton NMR obtained after reaction of CTZ with copper is similar to previously reported spectra of dhCTZ further supporting the hypothesis that copper is able to oxidize coelenterazine to dehydrated coelenterazine.[19]

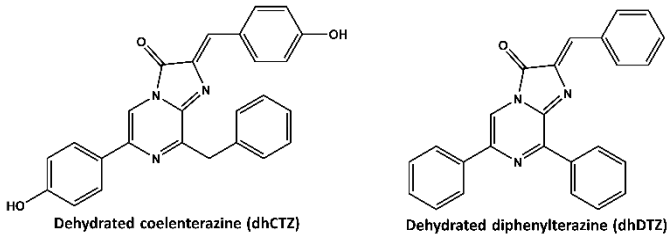



**Figure 6.16:** Structure of dehydrated diphenylterazine (dhDTZ) and dehydrated coelenterazine (dhCTZ).

Indeed, copper mediated oxidation of similar molecules containing dihydropyrazine moieties have been previously reported in the literature.[20,21] Brook et al. noted the structural uniqueness of 1,4-dihydropyrazines related to the cyclic 8π electron system they possess imparting antiaromatic character.[20] This electron richness allows them to readily undergo the loss of an electron generating a radical cation. In 2016, Li et al. reported a new Cu(II) sensor that is based on the oxidation of a dihyropyrazine containing compound.[21] Taken together, this strongly supports the hypothesis that Cu(II) is able to oxidize coelenterazine and related marine luciferases generating the dehydrated forms depicted in Figure 6.

Interestingly, dehydrated coelenterazine (dhCTZ) has previously been observed in nature and reported in the literature independent of any Cu(II)-mediated mechanism. The first report of a dehydrated coelenterazine was in 1977 from Inouye et. al where dhCTZ was isolated from the liver of the squid, Watasenia scintillans, suggesting perhaps the dehydrated form plays a role in storage.[22] Here researchers noticed that the isolated compound was two mass units less then CTZ and found that reduction with $NaBH_4$ restored CTZ. Similarly, when we treated dhDTZ with $NaBH_4$, we were able to restore light output with Nanoluciferase (Figure 17).

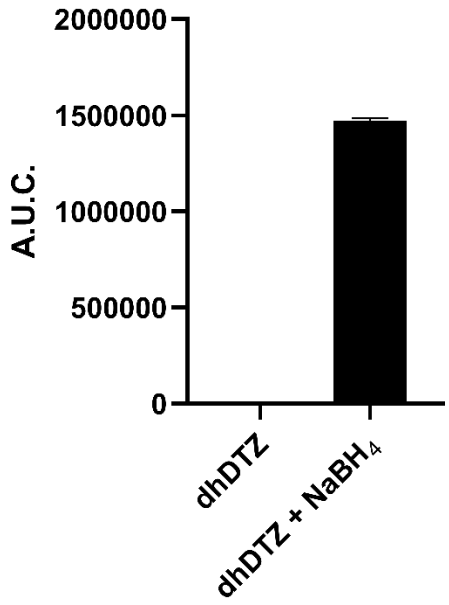



**Figure 6.17:** Calculated area under the curve over fifteen minutes of kinetic luminescence measurements of 10 µM dhDTZ with or without preincubation with NaBH4 (100 µM) in the presence of recombinant Nluc (0.4 µg/mL).

Furthermore, in 1993, dehydrated coelenterazine was found in the light organs of another luminous squid, Symplectoteuthis oualaniensis.[23] In this report, the authors further found that dehydrocoelenterazine had no luminescent activity but acetone and dithiothreitol adducts formed by addition to the vinyl group had luminescent activity. Thus, the authors proposed a mechanism in which protein-dhCTZ adducts served to stabilize the chromophore in the protein binding pocket. Indeed, fifteen years later in 2008 Isobe et al. confirmed that cysteine-390 is the binding site of the photoprotein symplectin where Cys390 forms an adduct at the vinyl group of dhCTZ to yield the luminescent chromophore.[24]

More recently in 2008, a second marine organism, Pholas dactylus, which is a marine bivalve mollusk was proposed to utilize dhCTZ for its luminescent activity.[25] Indeed, a year later the same group successfully showed that similar to symplectin, pholasin's prosthetic group was also formed through an adduct formation of dhCTZ at a free cysteine in the protein.[26] Interestingly the luciferase is a 150-kDa copper containing enzyme characterized as a peroxidase.[27] Taken

together with our findings, we propose that the copper center may have functions beyond peroxidase activity. For example, it is possible that CTZ is converted to dhCTZ by the copper-containing luciferase before being loaded into pholasin to form the active adduct.

In conclusion, we have determined for the first time that the copper-dependent inhibition of non-homologous marine luciferases originates from Cu(II)-mediated oxidation of the shared imidazopyrazinone structure rather than from interactions with the enzyme. This is, to our knowledge, the first example of a metal mediated transformation of marine luciferin substrates. This information not only provides valuable insight towards the development of future biotechnologies with said marine luciferin/luciferase systems but also opens the door to further questions and possibilities for the evolution and molecular mechanisms surrounding native marine bioluminescent systems.

## 6.3 Materials and Methods

**Materials**. *Gaussia, Renilla,* and Nano- luciferases were all purchased commercially (Promega, Raybiotech, & Nanolight technologies). Diphenylterazine (DTZ) was synthesized as previously described.[16] Coelenterazine (CTZ) was purchased from GoldBio. $^{1}H$ and $^{13}C$ NMR spectra were collected at room temperature in $CD_2Cl_2$ on a 400 Bruker Avance III HD Nanobay NMR spectrometer. All chemical shifts are reported as δ ppm relative to the residual solvent peak at 5.32 ($CD_2Cl_2$) for 1H. Electrospray ionization mass spectral analyses were performed using an LC-MSD system (Agilent Technologies 1260 Infinity II coupled with an Agilent Technologies InfinityLab LC/MSD).

**Luminescence Measurments**. All luminescence measurements were performed similarly to previously reported methods.[16] In short, metal ion solutions were prepared in water, luciferin stock solutions were prepared in pure ethanol and diluted in phosphate buffer, and luciferase

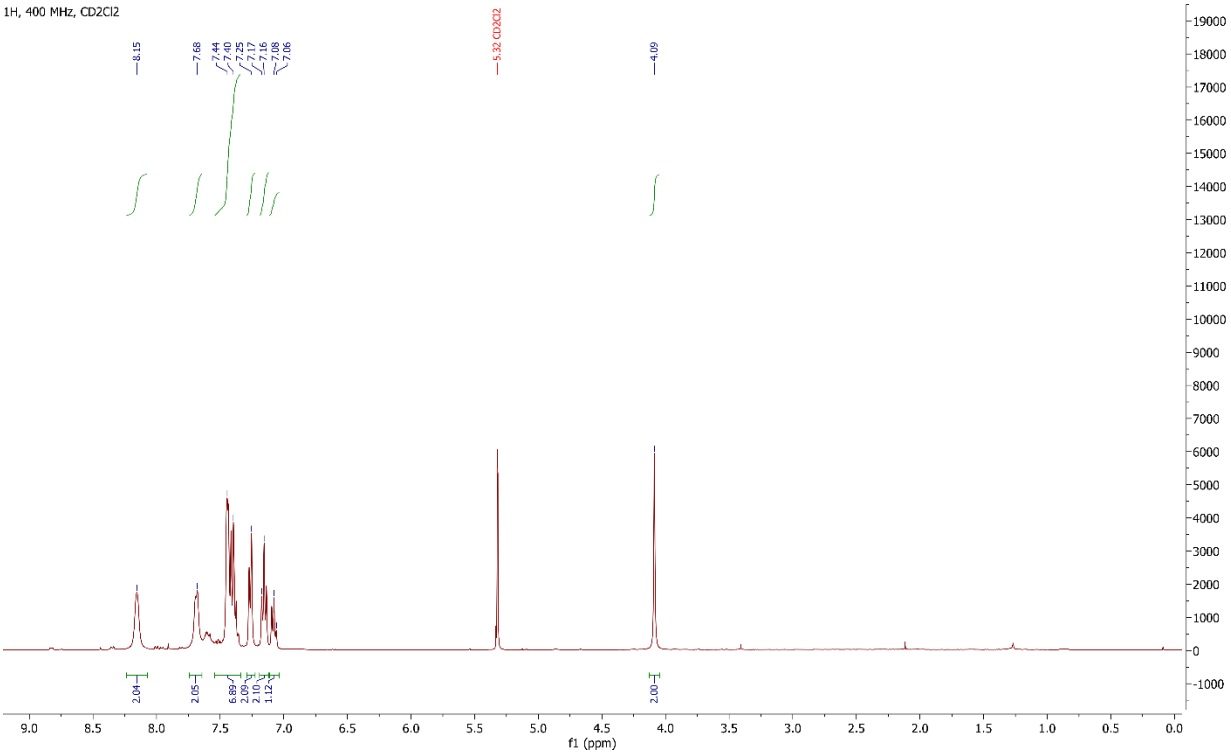
solutions were prepared freshly in phosphate buffer. Readings were measured on a SpectraMax i3x plate reader.

**Reaction of DTZ with Cu(II).** DTZ (245 mg, 0.650 mmol) was dissolved in methanol and to this was added $CuSO_4$ (161 mg, 0.650 mmol). The solution was stirred at room temperature until complete conversion was observed by LCMS after five hours. After this the reaction was poured into water and extracted into dichloromethane. The combined dichloromethane extracts were then washed with saturated sodium chloride, collected, and dried over magnesium sulfate. After filtration, the crude product was concentrated under reduced pressure. The crude mixture was then purified by silica gel column chromatography using DCM and then was recrystallized in acetonitrile to afford dhDTZ as dark purple powder (100 mg, 40% yield). $^1$H NMR (400 MHz, $CD_2Cl_2$) δ 8.81- 8.83 (m, 2H), 8.33-8.35 (m, 2H), 7.99-8.01 (d, 2H), 7.91 (s, 1H), 7.60-7.62 (t, 3H), 7.58 (s, 1H), 7.48-7.55 (m, 5H), 7.39-7.43 (m, 1H). $^{13}$C NMR (101 MHz, $CD_2Cl_2$) δ 151.76, 138.44, 135.57, 135.10, 134.29, 133.89, 133.48, 131.62, 131.25, 130.30, 129.60, 129.20, 129.04, 128.83, 128.51, 128.30, 128.22, 125.30, 110.73. Low-resolution mass spectrometry (LRMS) (m/z): $[M+H]^+$ calculated for $C_{25}H_{17}N_3O$ 376.1; found, 376.1.

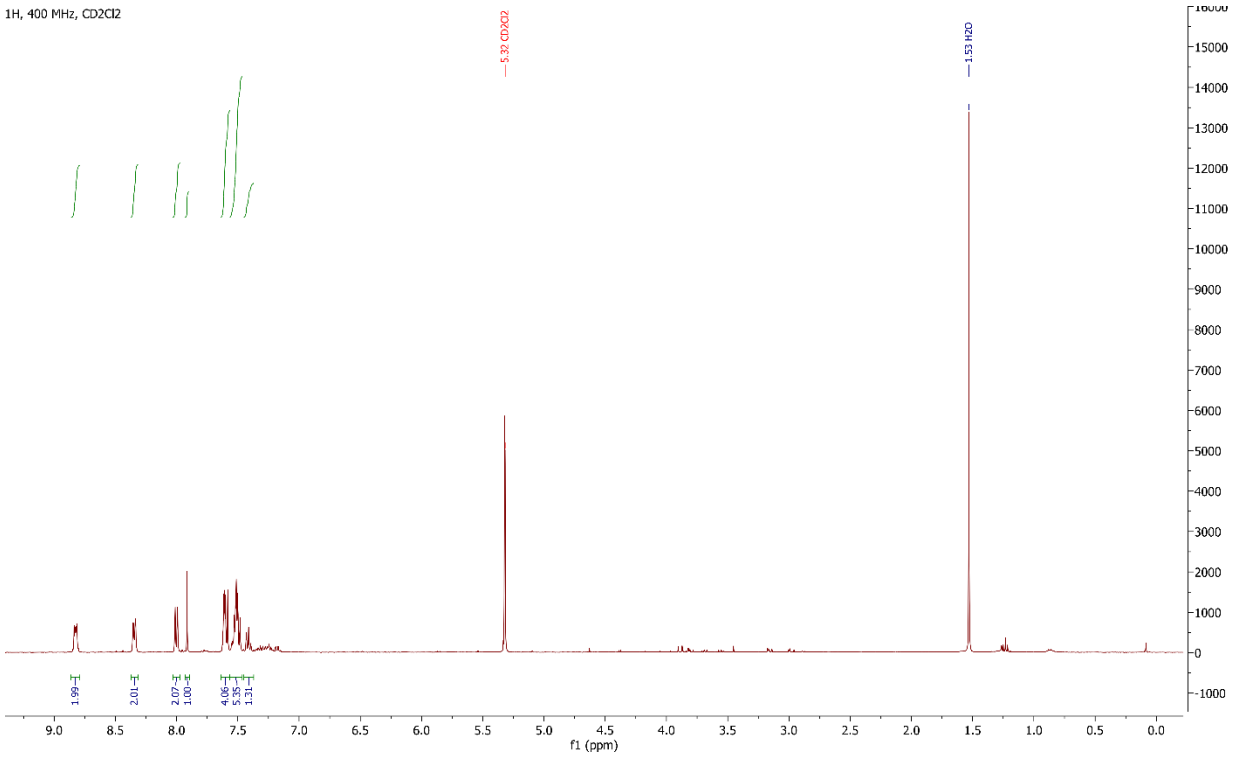
**Reaction of CTZ with Cu(II).** CTZ (10 mg, 0.024 mmol) was dissolved in methanol and to this was added $CuSO_4$ (4 mg, 0.024 mmol). The solution was stirred at room temperature until complete conversion was observed by LCMS after five hours. After this the reaction was poured into water and extracted into dichloromethane. The combined dichloromethane extracts were then washed with saturated sodium chloride, collected, and dried over magnesium sulfate. After filtration, the product was concentrated under reduced pressure to obtain dhCTZ as a dark red solid (9 mg, 90% yield). $^1$H NMR (400 MHz, DMSO-d6) δ 9.63 (br.), 8.30-8.32 (d, 2H), 7.91 (s, 1H), 7.76-7.78 (d, 2H), 7.48-7.51 (m, 3H), 7.32-7.36 (t, 2H), 7.22-7.25 (t, 1H), 6.93-6.95 (d, 2H), 6.80-6.82 (d, 2H), 4.31 (s, 2H). Low-resolution mass spectrometry (LRMS) (m/z): $[M+H]^+$ calculated for $C_{26}H_{19}N_3O_3$ 422.1; found, 422.1.

**Electronic absorption spectroscopy**. UV-Vis studies were performed on a Shimadzu UV-1900i at room temperature using quartz cuvettes (Starna) with a pathlength of 1 cm. All spectra were referenced to water and buffer subtracted. Stock solutions of 1 mM luciferin in ethanol, 1 mM metal salts in water, 10 mM EDTA in water, and 10 mM BCS in water were prepared. The final concentrations were 50 μM luciferin, 50 μM metal salts, 500 μM EDTA, and 500 μM BCS using ethanol as the buffer to prevent precipitation of the luciferin. Excess BCS was used to detect the presence of copper(I) in solution, and excess EDTA chelated free copper ions in solution.

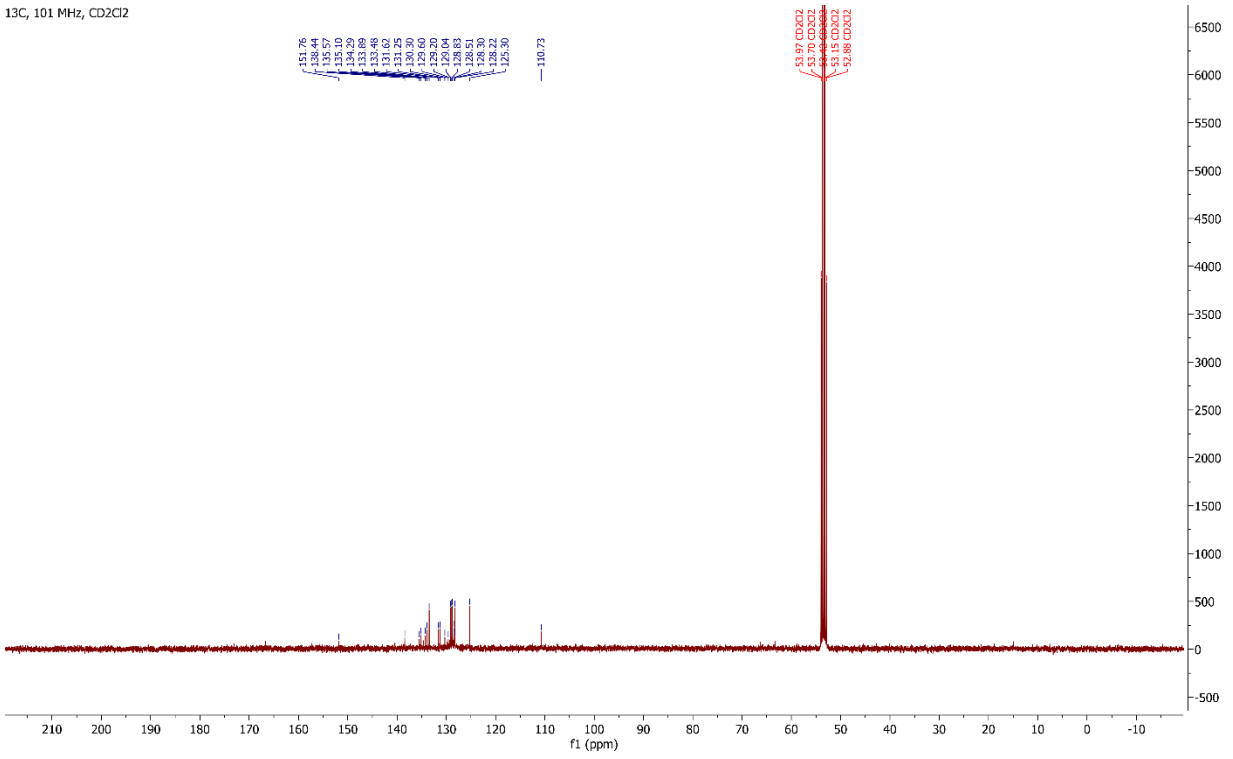
**$^1$H NMR of DTZ**



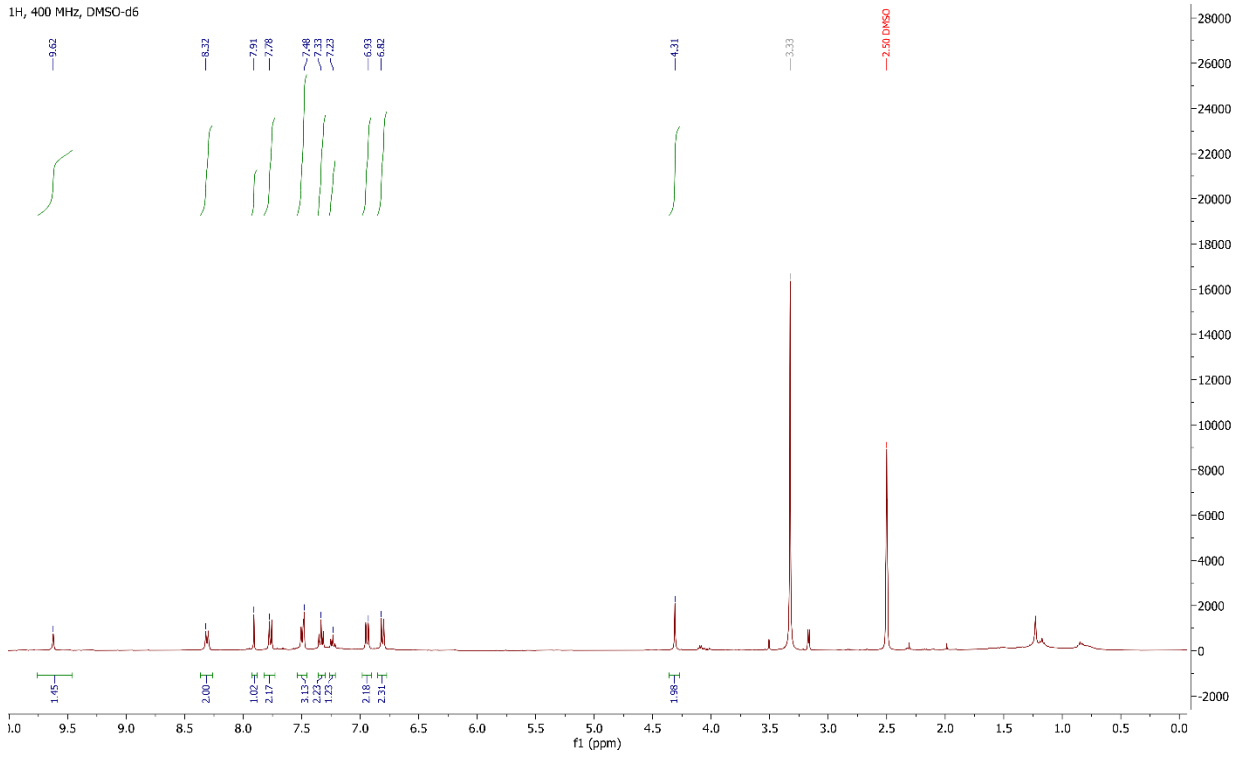

**$^1$H NMR of dhDTZ**




## $^{13}$C NMR of dhDTZ




## $^{1}$H NMR of dhCTZ


1H, 400 MHz, DMSO-d6
9.62
8.32
7.91
7.78
7.48
7.33
7.23
6.93
6.82
4.31
3.33
2.50 DMSO
1.45
2.00
1.02
2.17
3.13
2.23
1.23
2.18
2.31
1.98
f1 (ppm)

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