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The Chelation of Metal Ions by Vicibactin, a Siderophore Produced by Rhizobium leguminosarum ATCC 14479

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The Chelation of Metal Ions by Vicibactin, a Siderophore Produced by *Rhizobium leguminosarum* ATCC 14479

By

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Abstract

Vicibactin is a small, high-affinity iron chelator produced by *Rhizobium leguminosarum* ATCC 14479. Previous work has shown that vicibactin is produced and secreted from the cell to sequester ferric iron from the environment during iron-deplete conditions. This ferric iron is then transported into the cell to be converted into ferrous iron. This study uses UV-Vis spectroscopy as well as ion trap-time of flight mass spectroscopy to determine that vicibactin does form a complex with copper(II) ions, however, at a much lower affinity than for iron(III). Stability tests have shown that the copper(II)-vicibactin complex is stable over time. The results of this study show that vicibactin could be used in order to remove copper(II) ions from the soil or other media if they are present in toxic amounts. It also suggests that vicibactin's purpose for the rhizobia could be expanded to include both copper sequestering and to reduce extracellular copper concentrations to prevent toxicity.

Introduction

The Need for Metal Ions-

Many metal ions are vital for the survival of all living things. Metal ions serve as cofactors for many enzymes in the metabolic processes and growth of organisms. Most organisms require metal ions from the first row of transition metals. These metal ions have a high capacity for oxidation-reduction reactions making them useful in catalytic processes. Metal ions in this category include nickel, copper, and iron. Among these, iron is one of the most crucial metals in bacterial growth. (Palmer et al. 2016)

Iron-

Iron is a critical trace metal in numerous cellular processes. It serves as a cofactor in reduction-oxidation enzymes such as cytochromes, in enzymes responsible for neutralizing oxygen radicals such as catalase and superoxide dismutase, and as a cofactor in the oxygen-carrying molecule hemoglobin. (Egli 2003) It has also been shown to be a terminal electron acceptor in anaerobic and facultative bacteria, being reduced from its natural state (Fe^{3+}) to its soluble state (Fe^{2+}) (Chi et al. 2007).

Iron is a fairly abundant metal found in nature especially in the Earth's crust, however, because of its oxidation state it can be difficult to obtain sufficient amounts. This is due to it being found primarily in its ferric state, which is insoluble and unusable. In order for organisms to use iron in metabolic processes it must be converted to its ferrous state, which makes it soluble. The concentration of ferrous iron found in nature is approximately 10^{-18} M, while most bacteria require internal concentrations of approximately 10^{-6} M (Raymond et al. 2003). This problem is solved by many bacteria by producing small organic compounds which are secreted from the cell, bind to ferric ions, and return them to the cell to be converted into metabolically usable iron. These high affinity iron chelators are referred to as siderophores and are produced by many bacteria, fungi, and even higher order organisms.

Siderophores-

Siderophores are traditionally known as iron(III) transport compounds. These are small molecules which are normally subdivided into three classes, hydroxamates, catechols, and miscellaneous. This classification system is based upon the chemical structure of the iron binding chemical functional groups of the siderophore. The chemical type of siderophore can be determined using various tests which are specific

for each type of siderophore. For example, the Atkins' Assay is used to identify the presence of hydroxamate-type siderophores while Arnow's Assay is used in the case of catecholate-type siderophores. Siderophores can be found in a wide range of organisms, spanning from pathogenic bacteria, soil bacteria, and fungi, as well as plants and animals. (Shwyn et al. 1987) One group of bacteria which produces siderophores is the genus *Rhizobia*, a commonly found soil bacteria.

Siderophore Chemistry-

Siderophores are, in general, low molecular weight organic chelators with a very high affinity and specificity for iron in its ferric (Fe^{3+}) or insoluble state. This high selectivity for ferric iron is due to optimal selection of metal binding groups and the stereochemical arrangement of these groups. They are classified based on their binding subunits into hydroxamate, catecholate, or alpha-hydoxycarboxylic acid type siderophores. The denticity of the siderophore is also very important for its ability to chelate iron. Most siderophores are produced with the optimum denticity, hexadentate, to bind optimally to the six coordination sites of ferric iron. This high denticity also serves as a mechanism of specificity as the hexadentate siderophore prefers ligands with higher numbers of coordination sites. This leads to a low affinity for low denticity ligands and increases the siderophores specificity. This is because of the nature of the binding interaction between the siderophore and the iron(III) ion. Often the ion is bound in the center of the molecule where it is relatively stable. However, if fewer binding interactions occur the iron(III) ion could be left on the external surface of the siderophore leading to a much more insecure complex. (Boukhalfa et al. 2002) This specificity and optimal binding conditions lead to the very high stability of a siderophore-

iron complex. The stability constants of some siderophores have been measured as some of the highest constants in iron complexes. The uptake of this complex by the cell is complicated by the fact that these complexes have a hydrophilic nature which means they cannot be taken up by diffusion. In gram (-) bacteria the siderophore is taken up through a process which begins with a specific protein receptor on the cell surface. This receptor allows the siderophore to be taken up into the periplasmic space using the proton motive force of the periplasmic space and a series of proteins referred to as the Ton system. (Chakraborty 2013) This outer membrane receptor's structure has been shown to be a "barrel and plug" domain with a beta-barrel structure and a N-terminal globular domain which serves as a plug. (Ferguson et al. 1998; Locher et al. 1998; Buchanan et al. 1999) After the siderophore has entered the cell, the dissociation of the iron molecule from the siderophore is typically a slow process due to the high affinity for iron of the siderophore. The first step in this dissociation involves the dissociation of a bidentate moiety from the chelated iron. This step involves replacing a binding unit from the first coordination shell of ferric iron with water molecules. This vacant binding unit lowers the affinity of the siderophore to iron which may be involved in further dissociation of the complex. This is often catalyzed by a reductase enzyme, which reduces the ferric iron to its ferrous state and frees one of the binding units on the siderophore from the ion. (Boukhalfa et al. 2000; Boukhalfa et al. 2000, 2001) After this first step each siderophore undergoes a unique ligand dissociation process. Some of these processes are single pathway processes while others have multiple pathways. The uptake of various metal ions has also been tested using the siderophore desferrioxamine B. This study measured the stability constants of these complexes. The

study showed that the siderophore fully wrapped around the metal ion to form the complex. Therefore, the size of the metal was the largest factor in the stability of the complexes formed. (Hernlem et al. 1995)

Rhizobia-

Rhizobium is a genus of bacteria often found to establish a symbiotic relationship with numerous leguminous plants. Many rhizobia produce small nodule structures on the roots of the legumes they colonize. This nodule only forms when high quantities of rhizobia are present, which requires a large amount of iron to satiate the needs of the high volume of bacteria. In the scarcity of iron, it has been shown that often either nodule initiation or later development of the nodule are negatively impacted. (Carson et al. 1992) Inside these nodules the nitrogen fixation process occurs in which atmospheric nitrogen is converted into ammonia, which the plants can absorb and use as a nitrogen source. The nitrogen fixation process is carried out by an enzyme called nitrogenase, which is strongly inhibited by oxygen and requires very small amounts of molybdenum as a cofactor. Because of this oxygen intolerance the nodules produced by rhizobia contain leghemoglobin, in which iron is an essential metal. The leghemoglobin molecules bind any available oxygen in the nodule creating an anaerobic environment. The impacts on both nodulation and nitrogen fixation efficiency amplify the scarcity of natural, usable iron for the *Rhizobium* bacteria. A certain *Rhizobium* species, *Rhizobium leguminosarum* ATCC 14479, produces a siderophore known as vicibactin to gather iron for the cell and to address this problem.

Vicibactin-

Vicibactin is a small, tri-hydroxymate type siderophore with a molecular weight of 774.3647, which is produced by *R. leguminosarum* ATCC 14479 in iron-deficient conditions. (Wright 2010) It binds to the iron by folding onto the molecule with the three hydroxamic acid groups binding to the iron molecule. The tri-hydroxymate nature of the siderophore makes it very conducive to the binding of ferric iron in a 1:1 stoichiometric ratio. After being transported back into the cell reductase enzymes can convert the ferric iron to its ferrous state to make it usable in metabolic processes.

The Vicibactin and Iron Complex-

After vicibactin has sequestered iron from the extracellular environment it is considered ferric-vicibactin. This complex shows an absorption maximum at 450 nm and a molar absorption coefficient of 1510 M^{-1} cm^{-1}. This is atypically low for a tri-hydroxamate type siderophore, with most having a molar absorption coefficient between 2500-3000 M^{-1} cm^{-1}. It has been proposed that this is because of a decrease in iron coordination by the ligands. It has been proposed that this value indicates that fewer than six ligands are attached to iron. (Dilworth et al. 1998) Later work proposed an exact structure for free vicibactin shown in Fig. 1. This molecule was shown to have an exact mass of 774.3647. When the molecule became ferriated its molecular weight was shown to be 828.2 which shows the addition of one iron molecule and the loss of two hydrogens. The chemical formula of the vicibactin molecule was shown to be $C_{33}H_{54}N_6O_{15}$. (Wright 2010)

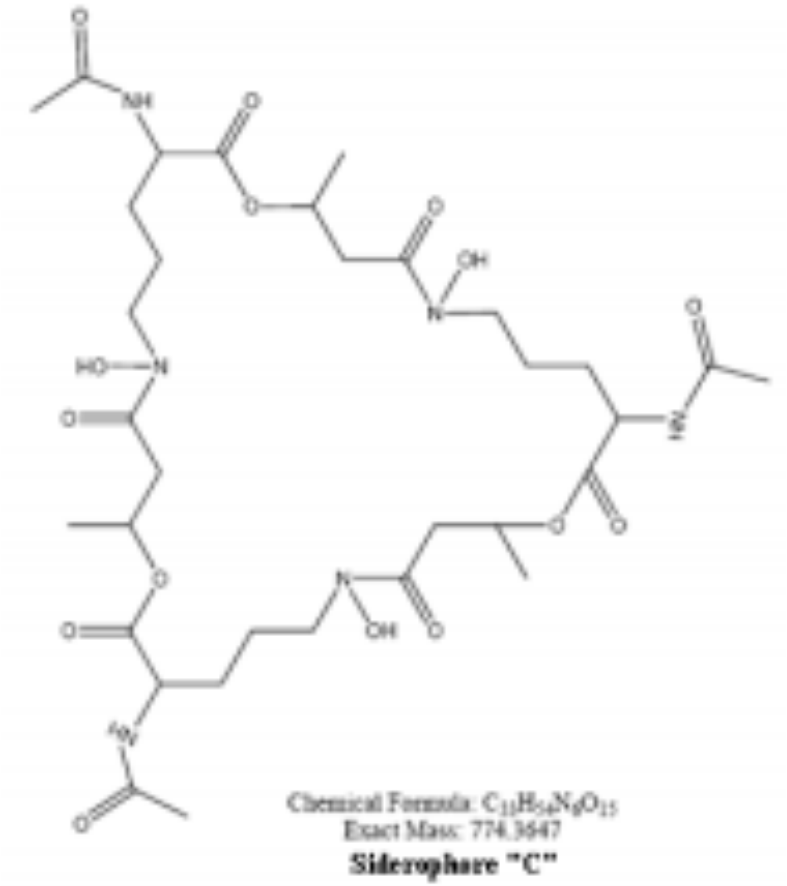

Figure 1: The deferriated structure of vicibactin as characterized by Bill Wright in 2010.

In previous studies, vicibactin has shown to have an uptake rate of 0.19 nmol/min^{-1} with *R. leguminosarum* strain WSM710 and 0.21 nmol/min^{-1} with strain MNF7101. (Dilworth et al. 1998) After uptake, the removal of iron from the siderophore has been hypothesized to occur by one of two different processes. The first proposed hypothesis involves intracellular electron donors reducing the iron back to its ferrous state. This causes the release of the iron facilitated by an increase in ligand exchange kinetics as well as a decrease in complex stability. (Harrington et al. 2009) This hypothesis proposes that the iron-free vicibactin is then recycled by the cell. The second model involves the breakdown of the ferric-vicibactin complex by enzymatic processes.

However, this model has currently only been observed in catecholate type siderophores. (Abergel et al, 2009) It has been proposed that the three hydroxamic acid groups serve as electron donors to allow the iron to bind. This produces a binding pocket in the center of the symmetrical molecule. (Wright 2010)

Past Studies-

It has been proven that some siderophores can form complexes with other metal ions besides iron. For example, desferrioxamine B, a siderophore produced by *Streptomyces pilosus*, has been shown to form complexes with Ga^{3+}, Al^{3+} and In^{3+} with affinities less than but similar to the complex formed with iron. (Hernlem et al. 1996) Pyoverdine and pyochelin, both products of *Pseudomonas aeruginosa,* were screened with sixteen different metal ions and showed complexation with all of them. (Braud et al. 2009) It has also been shown that the siderophore produced by *R. leguminosarum bv phaseoli* can protect alfalfa (*M. sativa*) seeds from copper toxicity. Previous work with vicibactin has shown that the siderophore can be used to reduce aluminum toxicity in the bacteria. However, the mechanism through which this occurs is still unknown. (Rogers et al. 2001)

Experimental Significance-

Previous studies have shown vicibactin's affinity for iron as a specific chelator for the bacterial cell. However, vicibactin's use in sequestering other metal ions for the cell has not been studied closely. Metal ions besides iron are useful for many cellular processes including nitrogen fixation. Studying vicibactin's ability to chelate other metal ions could possibly elucidate a wider purpose for vicibactin in the cell's metabolic processes. Also, heavy metals are persistent environmental contaminants due to their

inability to be degraded. These contaminants enter both flora and fauna, as well as the human population through contamination of food and water sources. (Lenn-tech 2004) These contaminating metals often exist as sulphides, in which the anion to which the metal ion binds is sulfur, and oxides, in which the anion for the compound is an oxygen ion (O^{2-}). The major causes of this contamination stem from natural and human mechanisms. Mining is the largest source of heavy metal contamination and can persist for hundreds of years after a mine ends its operational life. (Duruibe et al. 2007) These contaminants can be washed into animal grazing areas through a process called acid mine drainage. The animals which graze on plants contaminated by these metals can accumulate the toxic metals in both their tissues and their milk. This can lead to human contamination causing various biochemical disorders. (Duruibe et al. 2007) Vicibactin's ability to chelate these metal ions, either through in vivo methods when *R. leguminosarum* colonizes toxic soils, or in vitro if purified vicibactin is added to contaminated soil, could prove to be a successful way to remove these toxins from soil or possibly clean these mining areas to prevent drainage.

Present Work-

The siderophore vicibactin was produced and purified from an iron-deficient culture of *R. leguminosarum* ATCC 14479. The activity of the siderophore was proven through two assay tests, the first of which was the Atkins' Assay, a colorimetric assay for hydroxamate-type siderophores, which uses iron(III) perchlorate and .1M perchloric acid, this solution creates a wine-red color when added to vicibactin. (Atkin et al. 1970) The second assay used, the CAS assay, is a universal chemical assay used for the detection of siderophores. It uses the dye Chrome Azurol S to chelate iron and form a

blue color. When vicibactin is added to the CAS assay the dye is stripped of iron and becomes a yellow color. (Shwyn et al. 1987) The optimum ferric binding pH of vicibactin was shown using spectrophotometric tests. This pH, along with standard physiological pH, was used to test the ability of vicibactin to bind to other metal ions. Results indicate that vicibactin could have a very low affinity for copper but no other metal ion has shown binding capabilities with vicibactin.

Materials and Methods

Bacterial Growth-

A stock culture of *Rhizobium leguminosarum* ATCC 14479 was used to streak for isolated colonies on a modified Mannitol Yeast Agar media containing the Congo Red dye. This dye is used when growing rhizobia due to the difference in uptake rates of the dye between the rhizobia and other bacteria. This allows any contaminants in the culture to be easily identified by the red coloration of the colony caused by the uptake of the dye. This media contained (w/v) 1% mannitol, 0.05% K_2HPO_4, 0.02% $MgSO_4*7H_2O$, 0.01% NaCl, 0.1% yeast extract, and 3% Bacto-agar. The pH of the medium was brought to 6.8 using 6M NaOH. Congo Red dye, 1% 1.00mL, was added and the media was autoclaved. (Hammonds 2008) The rhizobia were incubated at 30°C for 5 days. A colony from this plate was then inoculated in two test tubes containing 5mL of a modified Manhart and Wong media. This media contained (w/v): 0.0764% K_2HPO_4, 0.1% KH_2PO_4, 0.15% Glutamate, 0.018% $MgSO_4$, 0.013% $CaSO_4*2H_2O$, and 0.6% Dextrose. (Wright 2010) This media was pH adjusted to 6.8 using 6M NaOH and autoclaved. After the media had cooled 1.00mL of filter sterilized, concentrated vitamin solution was added. The contents of this vitamin solution can be found in table 1.

Concentrated Vitamin and Trace Metal Solution (1000x)	
Component	mg per 100 mL ddH_2O
H_3BO_3	145
$CuSO_4*5H_2O$	4.37
$MnCl_2*4H_2O$	4.3
$ZnSO_4*7H_2O$	108
$Na_2MoO_4*2H_2O$	250
$CoCl_2*6H_2O$	10
$Na_2EDTA*2H_2O$	550
Riboflavin	10
ρ-aminobenzoic Acid	10
Nicotinic Acid	10
Biotin	12
Thiamine HCl	40
Pyridoxine HCl	10
Calcium Panthenate	50
Inositol	50
Vitamin B12	10

Table 1- Concentrated Vitamin Solution used in MMW Media (Wright 2010)

This vitamin solution does not contain iron, stimulating the production of vicibactin by the rhizobia. Both nalidixic acid and tetracycline were added at concentrations of 30mg/ml and 10mg/ml respectively to prevent contamination of the media. These two tubes were incubated for 4 days on a shaker at 30°C and used as starter cultures for two 1L flasks containing 250mL of identical MMW media. After 5 days of incubation on a shaker at 30°C the OD of flask A was 1.236A and flask B was 1.031A at 600nm. This turbidity indicated an adequate amount of both bacterial growth and vicibactin production. The cultures were transferred into acid washed 250mL bottles and centrifuged at 10,000 rpm and -4°C for 35 minutes. The supernatant was collected in two acid-washed 1L Wheaton bottles.

Detection of Vicibactin Production-

The first assay used to detect the presence of vicibactin was the Chrome Azurol S (CAS) Assay. This is a universal chemical assay used for the general detection of

siderophores. (Shwyn et al. 1987) The agar contains Chrome Azurol S dye that is blue when bound to ferric iron in the media. Wells are cut into the plates and filled with 0.5µL of the vicibactin-containing supernatant. Over 24 hours, the vicibactin stripped this iron from the agar causing the dye to change to a yellow color.

The second assay used is the Atkins' Assay. This is a colorimetric assay specifically used for hydroxamate-type siderophores. It is a solution containing 5mM $Fe(ClO_4)_3$ in 0.1M $HClO_4$. (Atkin et al. 1970) 0.5mL of the supernatant was added to 2.5mL of the Atkins' reagent. After 5 minutes at room temperature the appearance of a wine-red color indicated the presence of vicibactin. The intensity of the color formed is a useful tool when to determine the concentration of vicibactin present in solution. The absorbance of the solution is measured at 450nm and compared against a blank containing uninoculated MMW media mixed with the Atkins' reagent. This absorbance can then be used with the Beer-Lambert Law to determine the concentration of vicibactin present in solution.

Purification of Vicibactin-

After the presence of vicibactin in the supernatant is confirmed the supernatant must be purified to ensure nothing but vicibactin remains. This is done through a series of chromatography columns. The first column through which the supernatant was run was a 5 x 30 cm column packed with 160g of Amberlite XAD-2 to a height of 8cm. This column separated cyclic compounds from linear ones. The column was cleaned with three bed volumes of methanol and then equilibrated with 4 bed volumes of ddH_2O. A bed volume is approximately 160mLs. After the column was equilibrated the supernatant was acidified to a pH of 2.0 using 6M HCl and then run through the column

1L at a time. After each liter of supernatant, the column was washed and eluted with two bed volumes of ddH_2O and approximately 500mL of methanol. The elution was carried out in 10 fractions of 50mL each and some of these fractions contained the vicibactin. All of these fractions were tested using the Atkins' Assay to determine which fractions contained vicibactin. These tests indicated that fractions 2-7 contained vicibactin. These were then lyophilized and resuspended in 5mLs of methanol. This supernatant was then run through a 1.5x50 cm column containing 25g of Sephadex LH-20, which separates compounds based upon their size and hydrophobicity, packed to a height of 45cm. The column was equilibrated with 3 bed volumes (approximately 80mLs per bed volume) of methanol. The supernatant was loaded into the column and collected in 250 drop aliquots using a fraction collector. Fifty five of these fractions were collected and tested for the presence of vicibactin. Approximately 10mL of the supernatant showed the presence of vicibactin and was collected in a 15mL tube. This supernatant was evaporated using a roto-vapor and resuspended in a 3mL of methanol. Finally, this supernatant was purified using reverse phase high pressure liquid chromatography using deaerated ddH_2O and filtered 100% methanol as the mobile phases. The column was equilibrated with three bed volumes of ddH_2O and then the supernatant was injected in 0.75mL fractions. The vicibactin was eluted from the column at a methanol concentration of 48%. This yielded approximately 1.5mL of pure vicibactin per run at a concentration of approximately 1.16mM. All purification methods were performed according to the protocol developed by Bill Wright. (2010)

<u>Vicibactin Concentration Determination-</u>

After the purification process was complete another Atkins' Assay was run to determine the concentration of purified vicibactin. Purified vicibactin, 0.5mL, was added to 2.5mL of the Atkins' reagent and allowed to incubate at room temperature for 5 minutes. After incubation, the absorbance of the solution was tested at 450 nm using a blank of methanol and the Atkins' reagent. This absorbance was used in the Beer-Lambert law to determine the concentration of vicibactin.

pH Optimization of Chelating Conditions-

In order to perform chelation tests between vicibactin and various metal ions the optimal binding pH for vicibactin had to be determined. This was done using Atkins' reagents modified to various pHs using 6M NaOH. The pH of standard Atkins' reagent was found to be 1.70. The pH values of 3.0, 5.0, and 7.0 were also tested. The binding conditions were tested by performing an Atkins' Assay with each of the solutions. The absorbance of each solution was tested at 450nm and the solution with the highest absorbance was assumed to be the optimum binding pH for vicibactin within the range tested.

Metal Ion Chelation Tests-

The method chosen to test whether vicibactin could chelate metal ions besides iron was to use the Atkins' Assay, which was modified. The standard metal salt, iron(III) perchlorate, was replaced with a metal salt for the chosen metal to be tested. The metal salts used for testing were copper(II) sulfate, copper(II) perchlorate, lead(II) acetate, lead(IV) dioxide, ammonium molybdate, mercuric chloride, gallium nitrate, and manganese(II) chloride. These were all added individually in 5mM, 50mM, 0.5M and 1M concentrations in order to determine the lowest concentration required for binding, if any

occurred. Five hundred microliters of the reagent were added to 100 microliters of vicibactin in a cuvette, and then the volume was increased to 1mL using deionized water. This served as the test for each solution while 500 microliters of reagent brought to 1.00mL of volume using deionized water was used as a control. The blank for each test was 1.00mL of 0.1M perchloric acid, the solute of the Atkins' Assay. The modified assay without vicibactin and vicibactin alone were both used as controls for each test while the modified assay and vicibactin combined served as the test. Any shift in the test assay as compared to the vicibactin only absorption frequency was considered a positive test for interaction between the compounds. All of these solutions were standardized to a pH of 3.0 in accordance with the results of the pH optimization test.

Mass Spectroscopy-

Mass spectroscopy analysis was performed with an ion trap-time of flight mass spectrometer. Two 20 μL samples were injected using flow injection. The first of these samples was purified, unbound vicibactin. The second was purified, unbound vicibactin added to 1M copper perchlorate Atkins' reagent in the appropriate ratio of one part vicibactin to five parts reagent. These samples were first analyzed using both positive and negative electrospray interactions (ESI). It was determined that the peaks were more visible using positive ESI so this was used for further analysis.

Stability Tests-

Stability tests were performed using 500 microliters of a modified Atkins' Assay with 0.5M metal ion present in 0.1M perchloric acid. The two metal ions tested were copper(II) perchlorate and iron(III) perchlorate. The blank used in UV-Vis spectroscopy was 600 microliters of 0.1M perchloric acid. Purified vicibactin (100 microliters) was

added to each modified Atkins' Assay. A sample of both iron and copper assays were kept at room temperature while a separate sample was kept at 4° Celsius. All 4 assays were tested every 30 minutes for 6 hours and then again at 24 hours. The copper samples were tested at 358nm (the observed absorbance peak of the copper-vicibactin complex) while the iron samples were tested at 450nm.

Results and Discussion

pH Optimization Tests-

pH of Atkins' Assay	Absorbance
1.70 (Stock)	1.113
3.0	1.132
5.0	1.099
7.0	1.122

Table 2: Results of the pH optimization tests performed on the binding interaction between ferric iron ions and vicibactin.

The results of the pH optimization tests for the binding of vicibactin and iron are shown in table 3. The pH of 3.0 was chosen as the optimal binding conditions under which to perform all subsequent tests. However, the difference in the values recorded was very small. It is unclear whether a pH of 3.0 was truly the optimum binding condition or if this was merely random variability. The results of an optimum pH of 3.0 seem contradictory to the known interaction between vicibactin and iron. It would seem that the higher H^+ ion concentration would block the three hydroxyl sites on vicibactin to which the ferric iron binds. It would seem much more plausible that a pH closer to neutral and physiological conditions would provide a higher concentration of bound

molecules. The reason no pH over 7.0 was tested was due to the acidic nature of the Atkins' Assay. The recommended solvent in the assay is 0.1M perchloric acid, and this assay run with neutral or basic solvents is untested. Studies on the binding kinetics of vicibactin and iron at neutral and basic pH's should be explored.

Unbound Vicibactin Absorbance Peak-

Unbound vicibactin was tested to determine the control peak which all other tests would be compared to. This test yielded an absorbance frequency peak from 349-356nm. Any shifts in this peak could indicate interaction between vicibactin and a metal ion. It was hypothesized that this shift would occur at a higher frequency, placing the peak closer to the visible range of the spectrum. This was expected because of the shift which occurs in the standard Atkins' Assay in which the peak shifts from the 349-356 peak of vicibactin to a peak of 450nm.

Metal Ion Chelation Tests-

Concentration of $Cu(ClO_4)_2$	Peak Absorbance Wavelength of Assay and Vicibactin at pH of 3.0
5mM	350-356nm
50mM	356-361nm
.5M	356-360nm
1M	356-358nm

Table 3: Peak absorbance wavelengths of vicibactin and modified Atkins' Assay using different concentrations of copper perchlorate in the assay.

Table 3 shows the results of testing different concentrations of copper perchlorate in a modified Atkins' Assay with vicibactin. At 5mM the peak is nearly

identical to the unbound vicibactin peak. However, at concentrations of 50mM to 1M the peak shifts approximately 5nm towards the visible spectrum at between 356-361nm and 356-358nm. These results indicate that there is a possible interaction between vicibactin and copper. A test of only the copper perchlorate assay showed no peak in the vicibactin range but a large peak around 750nm which causes the blue color of the copper perchlorate. This test confirmed the peak visible above 50mM was caused by vicibactin. However, the small shift in the peak did not produce a colored solution similar to the standard Atkins' Assay. These results cannot confirm binding between vicibactin and the copper(II) ions. They merely suggest that there is an interaction between vicibactin and the copper(II) ions. These results were further validated through mass spectroscopy of both unbound, pure vicibactin and a vicibactin and copper perchlorate Atkins' Assay solution to determine if the interaction was binding or otherwise. Following indication of an interaction between vicibactin and copper (II) ions a dose-response test was performed to help further confirm there was an interaction. Figure 2 shows the results of this dose response test.

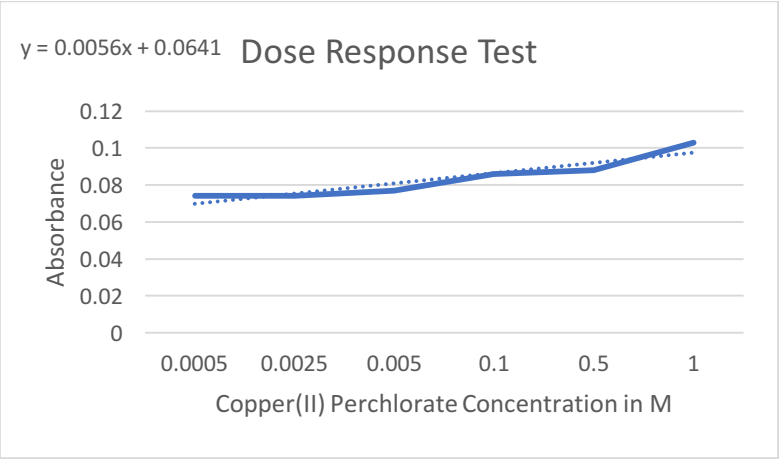

Figure 2: Dose-response test of the vicibactin and copper complex using 6 different concentrations of copper perchlorate. The linear regression formula is shown in the top-left corner of the figure.

The dose-response test was then analyzed using a linear regression to determine the statistical significance of the results. The r-value of this regression was .9539 showing statistical significance. However, this r-value is very close to being insignificant. Using a wider range of concentrations would be useful in further confirming significance of the results.

Metal Species	Control Absorbance Peak Wavelength of Metal Ions in Atkins' Assay	Absorbance Peak Wavelength of Metal Ions and Vicibactin
Pb^{2+}	288nm	288nm
Hg^{2+}	288nm	288nm
Mn^{2+}	293nm	298nm
Ga^{3+}	309nm	302nm
Mo^{4+}	324nm	324nm

Table 4: Results of tests using various metal ions in a modified Atkins' Assay throughout the concentration range of 5mM, 50mM, .5M, and 1M. All solutions had a pH of 3.0. One wavelength is provided for all four concentrations because this value did not change with different concentrations of metal ion.

Tests of 5 other metal ions yielded no discernible interactions. Lead(II), mercury(II), and molybdenum(IV) all had no change in their absorbance frequency peaks after the addition of vicibactin. Both manganese(II) and gallium(III) both showed slight peak shifts, with manganese moving towards the visible spectrum and gallium moving away from it. However, it is not believed that either of these shifts involve

vicibactin. The gallium shift can be ruled out because of the direction in which the peak shifted. Any interaction with vicibactin would move the peak towards the visible spectrum, as seen in both the copper and iron assays. Instead, this peak shifts away from the visible spectrum. The manganese shift can be discounted because of its location. Any interaction with vicibactin would presumably cause the peak to shift to a region higher than that of unbound vicibactin. This was observed in both the copper and iron assays as well. Instead, this peak is still much below the unbound vicibactin spectrum and is assumed to be unrelated. The nature of these two peak shifts is unknown.

Mass Spectroscopy-

Mass spectroscopy was performed to analyze the masses of both unbound vicibactin, which was the control, and vicibactin in a 1M copper (II) perchlorate solution. The control test showed a peak with a mass to charge ratio (m/z) of 751.2541. This peak is shown in figure 3. Because positive ESI was used during this analysis the mass of the sample was 750.2541.

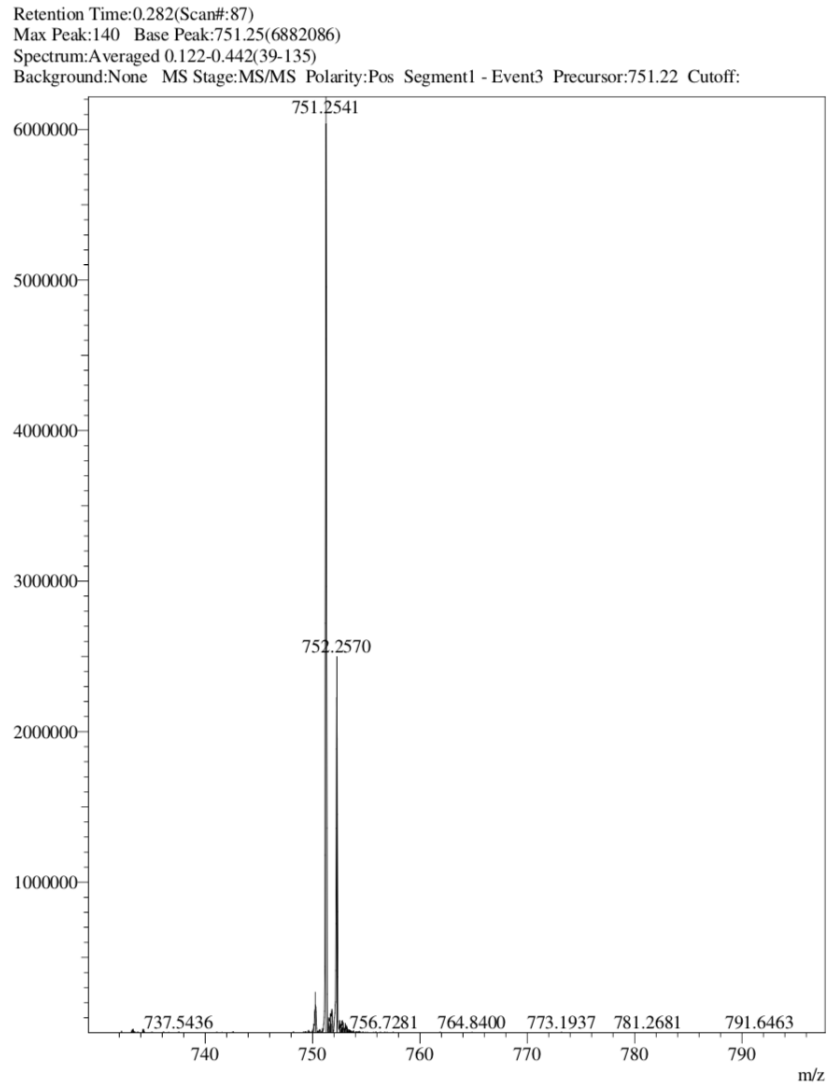

Figure 3: Mass spectra of the main peak observed in an unbound vicibactin sample.

This mass is very similar to a previously characterized degradation product (figure 4) of vicibactin with a mass of 750.3647. The doublet shown on the spectra, with the second peak at 752.2570 is indicative of the vicibactin molecule with the carbon-13 isotope. This isotope has a relative abundance in nature of approximately 1.1%. This relative abundance, multiplied by the number of carbons in the molecule (33) should give a peak with an intensity of approximately 1/3rd of the carbon-12 peak. This is what is observed in the spectrum.

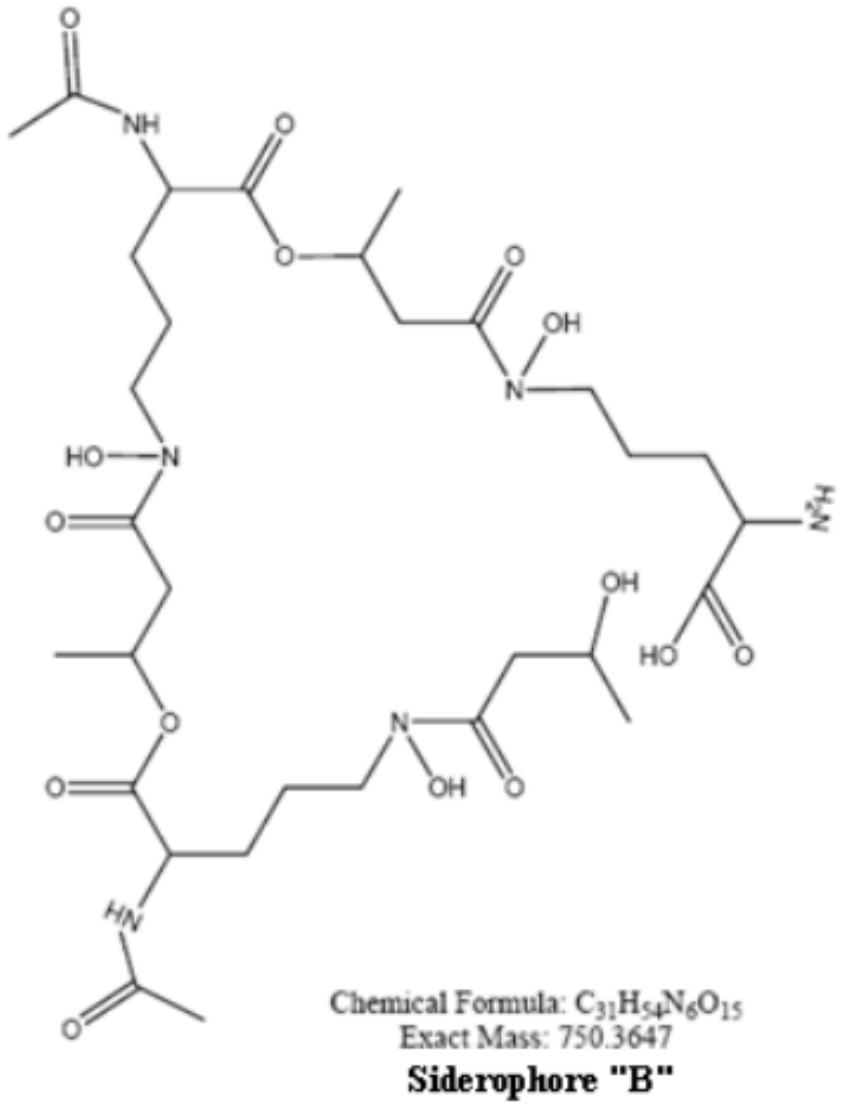

Figure 4: The largest major degradation product of vicibactin, characterized by Bill Wright in 2010.

A second previously characterized degradation product with a mass of 492.2431 was also found in the control sample. The peak found during mass spectroscopy analysis showed a *m/z* of 493.1748 with a previously characterized mass of 492.2431, this peak is shown in figure 5 as well as the structure of the degradation product in figure 6. (Wright, 2013) A second major peak, seen at *m/z* of 475.1697 is indicative of the

vicibactin degradation product after the loss of a water molecule. The structure of this degradation product has not been characterized but this dehydration can be assumed because of the loss of a mass of 18.

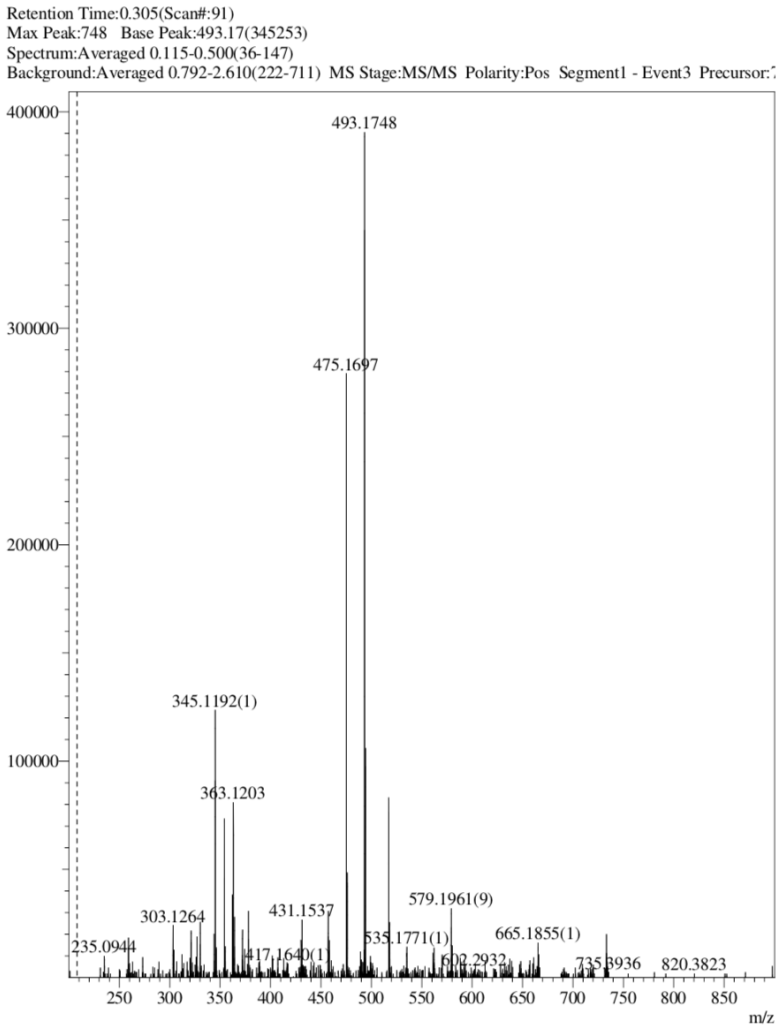

Figure 5: Mass spectra of the second degradation product of vicibactin with the previously identified fragment at m/z of 493.1748, as well as a second, dehydrated degradation product seen at 475.1679.

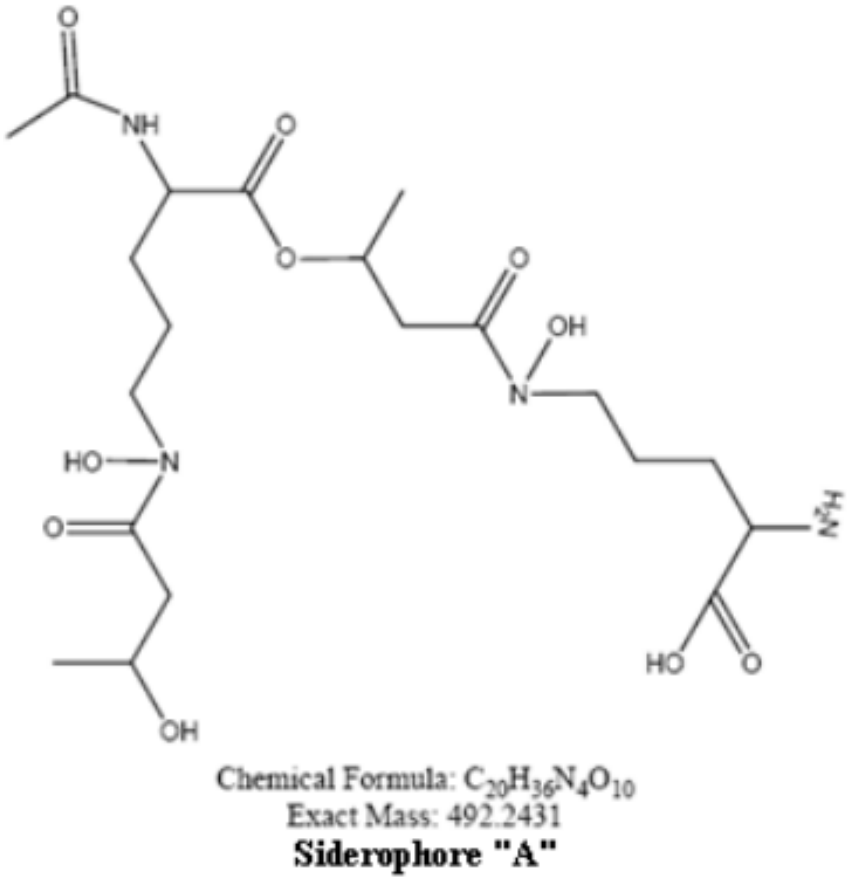

Figure 6: The second degradation product characterized by Bill Wright in 2010.

However, a peak correlating with the full vicibactin molecule was not found. This peak was expected to be in the range of 775 *m/z*, however, no peaks were shown in this area. The degradation of the vicibactin is most likely attributable to processes in purification, as well as the time left in its crude form. Current literature states that it is unknown whether these products occur only as a result of post-secretion degradation or if degradation products are also secreted from the cell. Tests have shown that the larger degradation product (mass of 750.2541) retains its activity with chelating iron. (Wright,

2010) Analysis of the second sample showed a disappearance of the larger degradation products shown in the test containing no copper(II) ions (figure 7).

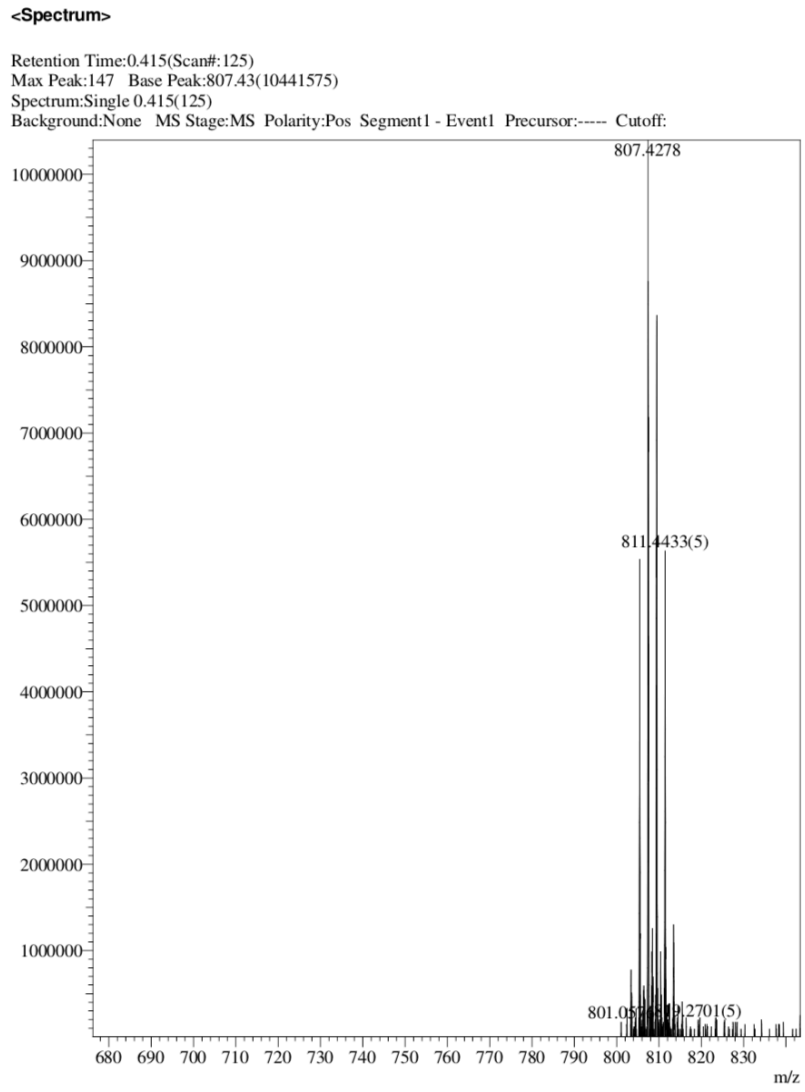

Figure 7: Mass spectra of the sample containing Cu^{2+} showing no peaks in the range of the large degradation product.

In this spectrum (figure 8), there were six peaks similar to the expected mass of a vicibactin-copper complex.

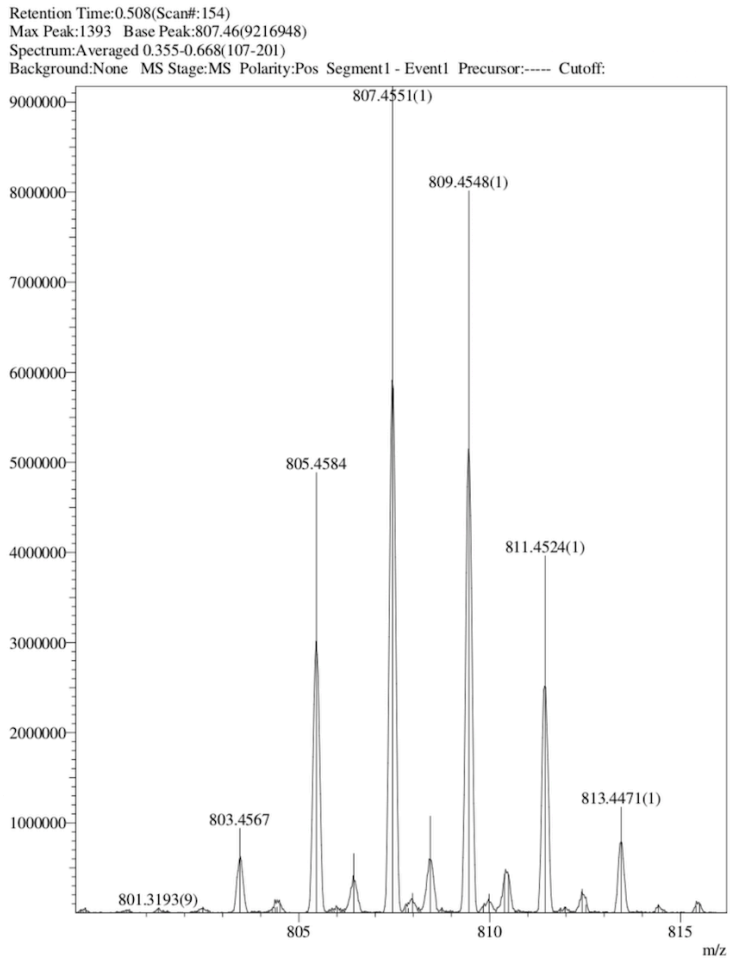

Figure 8: Mass spectra of the six peaks associated with an assumed vicibactin-copper complex.

However, the peak which was identical to the projected weight, approximately 813, was one of the smallest peaks and did not show isotope peaks. The dominant peaks were at *m/z* 807.4551 and 809.4548. This spectrum gives evidence to the formation of a vicibactin-copper complex in the disappearance of the major degradation product but it is unclear why the complex's peak is not one isolated peak at *m/z* 813 but instead is a range of six peaks. Previous studies have shown anomalous fragmentation patterns which can occur with siderophores and other organic molecules in mass spectroscopy when complexed with copper. Studies performed with ferrioxamine, ferrrichrome, and

iron(III) rhodotoluate have shown that specific siderophores have specific sites at which cleavage is most likely to occur during mass spectroscopy. (Gledhill 2001) It was shown that all three tested siderophores cleaved most readily at C-N bonds, which is also where the pyoverdins and enterobactin cleaved. (Kilz 1999 and Berner 1991) Because of the mass lost it is not believed that vicibactin cleaved at one of these bonds as suggested by the previous study. Instead, it is believed that oxidation and reduction processes occurred in the highly charged conditions under which mass spectroscopy must occur. This is supported by a study done on metal ion chelation by flavonoids, which are organic molecules produced by plants. This study showed increasing numbers of hydroxyl groups greatly intensified the number of radicals lost. This led to the inconsistent fragmentation patterns. (Davis 2004) Vicibactin has three hydroxyl groups which could lead to increased radicalization that could possibly account for the mass loss seen in the spectra. This could be a potential explanation for the anomalous peaks seen in the spectra. It has also been shown that in other studies with flavonoids that the predicted number of electrons involved, based on the number of hydroxyl groups, could be lower than the actual number involved. However, no explanation for this phenomenon is provided. (Fernandez et al. 2002) A final possibility to explain the anomalous results would be a cascade of H_2 losses at various ionization states during mass spectroscopy. The mass difference between each peak is very close to two for every peak in the range. The vicibactin degradation product observed has a high number of hydrogens (55) which could be lost in pairs during the analysis.

Stability Tests-

Tests performed to determine the stability of both the vicibactin-iron and vicibactin-copper complexes showed that both complexes were stable over time at both room temperature and 4° Celsius.

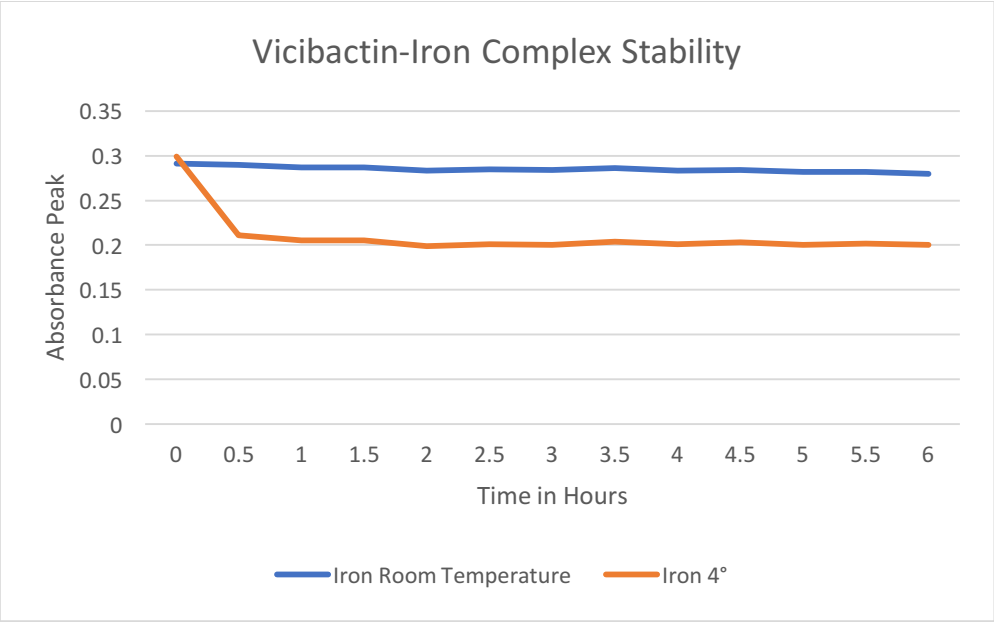

Figure 9: Stability of the vicibactin-iron complex at both room temperature and 4 degrees Celsius over 6 hours.

The absorbance peak at both 24 and 48 hours was also tested for both the room temperature and 4° Celsius iron complexes with the 24-hour values being .249 and .234 respectively. This data shows that the complex is stable out to 24 hours. The copper complex showed similar stability to the iron complex over both the 6-hour time interval and the 24-hour time interval. The absorbance peak for the 24-hour interval was .024 and .025 for the room temperature and the 4° Celsius tests respectively. The cause for the unevenness of the graph for the room temperature test is unknown. There is a possibility experimental error occurred when the absorbance values were tested but the

experimental methods were assumed to be sound. This unevenness does not detract from the conclusions regarding the stability of the complex.

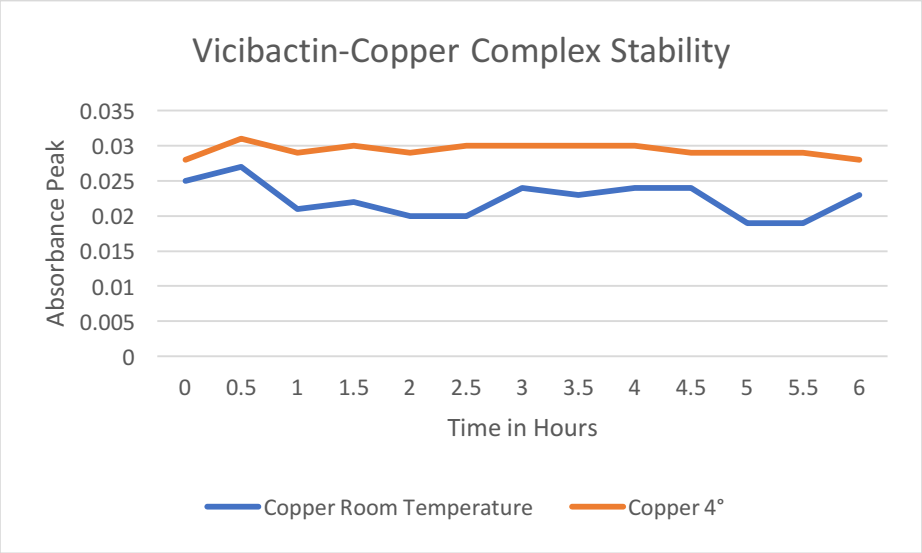

Figure 10: Stability of the iron-copper complex at both room temperature and 4° Celsius over 6 hours.

These tests show that the complex vicibactin forms with copper has comparable stability to that of iron. The stability observed across the temperature range was expected for the iron complex as vicibactin is shown to have incredibly high affinity for iron. Also, for the molecule to remain effective for the bacteria under environmental conditions it must be able to chelate iron at a wide range of temperature. The comparable stability of the copper complex over time at various temperature ranges could indicate that vicibactin is also produced to chelate copper for the cell.

Implications of Results-

The results of this work have shown that vicibactin does form a complex with copper which has a lower affinity but a comparable stability to the complex with iron. This could be useful for the *R. leguminosarum* for various reasons. The terminal

electron receptor in cellular respiratory chain is the enzyme cytochrome c oxidase. This enzyme uses both iron and copper ions as electron carriers with the copper center composed of either one or two copper ions. (Tsukihara et al. 1995) Because of the necessity of this enzyme in efficient energy production copper is necessary in low amounts in the cell. However, excess levels of copper ions in the cell have shown to be toxic to the cell. It has been shown that these ions perturb cellular redox reactions and produce hydroxyl radicals. (Waldron et al. 2009) Because of the toxicity of these necessary ions in excess, the management of the concentration of these ions is crucial for the cell. Vicibactin could be produced by the bacteria not only to gather copper for cellular processes but also to chelate free copper ions to reduce the toxicity. It has been indicated that the siderophore produced by *R. leguminosarum bv. Phaseoli* can protect alfalfa seeds from copper toxicity and this could be the mechanism through which this occurs. (Rogers et al. 2001) If this is the case, it could be presumed that vicibactin could also be used to clean toxic levels of copper ions from contaminated soil or other media. The stability of the complex indicates that isolated vicibactin could be introduced into the soil *in vitro* to reduce the toxicity of the ions. However, a more efficient and long-term solution could be the introduction of legumes with an established symbiotic relationship with *R. leguminosarum* to contaminated soil. This would bypass the time-intensive process of isolating and purifying vicibactin as the bacteria would constantly introduce new vicibactin to the soil. The impact of the increased copper-vicibactin complexes on both the bacteria and the plant would have to be further explored. It is unknown whether these complexes would enter the bacterial cell and cause internal toxicity. Any of these proposed methods for soil remediation should be explored.

Conclusion

This work has shown that vicibactin, a small, iron-chelating molecule produced by *R. leguminosarum*, shows the ability to chelate copper as well as iron. This has been shown using UV-Vis spectroscopy in a modified Atkins' Assay as well as mass spectroscopy analysis using an ion trap-time of flight mass spectrometer with both positive and negative electrospray interactions. A shift of the peak absorbance of the modified Atkins' Assay when vicibactin was added indicated that a possible interaction between vicibactin and copper was occurring. A dose-response test was performed to confirm the significance of this interaction. Finally, mass spectroscopy was performed to determine if the interaction occurring was vicibactin chelating the copper ion. The control test showed multiple degradation products of vicibactin, some of which are known to retain their ability to chelate iron. However, the intact vicibactin molecule was not observed. When the vicibactin was added to a modified Atkins' Assay containing 1M copper perchlorate these degradation products disappeared. The peaks observed were slightly lower in mass than the expected mass of the copper-vicibactin complex. It is believed that the hostile conditions vicibactin was exposed to in the mass spectrometer led to the loss of hydrogen ions during analysis, which accounts for the slight shift in mass observed. This work shows that vicibactin may be produced by *R. leguminosarum* not only to sequester iron, but also to possibly sequester copper for cellular processes or reduce the toxicity of intolerable concentrations of extracellular copper ions. This study also shows that vicibactin could possibly be a potential tool in environmental copper ion clean-up. The nature of the molecular rearrangement which occurs during mass spectroscopy should be explored, possibly through nuclear magnetic resonance

analysis. Also, the affinity of vicibactin to both copper and iron should be explored so that these two values can be compared.

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