



SCHOOL OF SCIENCE

Synthesis and Properties of  
Cobalamin Derivatives  
Incorporating Halogenated Alkenyl  
Ligands

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## Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed:

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## 1 Thesis Abstract

Synthesis of organocobalamins has been shown to be a complex process due to the conditions required to coordinate the organic molecules to the cobalt metal centre. It has been found that due to the light and air sensitive nature of typical organocobalamins, a strict air-free and dark environment is required to successfully synthesise the desired compounds. There is an abundance of literature reported on alkyl and alkynylcobalamins compared with alkenylcobalamins. The attempted synthesis of halogenated vinylcobalamins was conducted using two different methods to coordinate a halogenated alkenyl ligand to the cobalt metal centre of vitamin B<sub>12</sub>. The reduction method used sodium borohydride and a reverse addition of the reduced Co(I) species to the ligand resulted in several different complexes being produced. The final attempt of this method resulted in the formation of a new compound, shown by the new aromatic peaks observed in the <sup>1</sup>H NMR spectrum. Unfortunately, this new compound was unable to be fully characterised due to decomposition, and the LC-MS results proved inconclusive. The reduction-free method used copper(I) acetate to remove the cyanide ligand from the beta axial site and a base, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), to deprotonate the trichloroethene ligand. In the final attempt of this method, it was found that a room temperature reaction for 4 hours resulted in the observation of several new compounds. The <sup>1</sup>H NMR spectrum from this synthesis indicated a new major complex was present in the product mixture, with five new aromatic proton peaks (7.24 ppm, 6.85 ppm, 6.82 ppm, 6.25 ppm, and 6.05 ppm). However, once again LC-MS analysis of the product mixture was inconclusive.

## 2 Introduction

### 2.1 Background

Vitamin B<sub>12</sub> was first isolated from bacteria by Mary Shorb as part of Karl Folkers research team in 1947 and successfully administered to treat pernicious anaemia<sup>1</sup>. Since then, this structurally complex vitamin has been the subject of intense research along with its naturally occurring derivatives. The vibrant red colour of the vitamin B<sub>12</sub> complex arises from the conjugation of the corrin ring surrounding the cobalt metal centre, as seen in Figure 1<sup>2</sup>. The lower (alpha) axial ligand coordination to the metal centre acts as a sophisticated switch, controlling the reactivity of the metal centre. For many B<sub>12</sub>-dependent

enzyme reactions the lower axial ligand nucleotide is substituted by the imidazole of the side chain of a histidine in the active site of the protein<sup>3</sup>. Produced by bacteria, and obtained through the diet to be used by humans and animals in metabolic processes present in every cell, B<sub>12</sub> or cobalamin, is an exceptional molecule<sup>4</sup>.

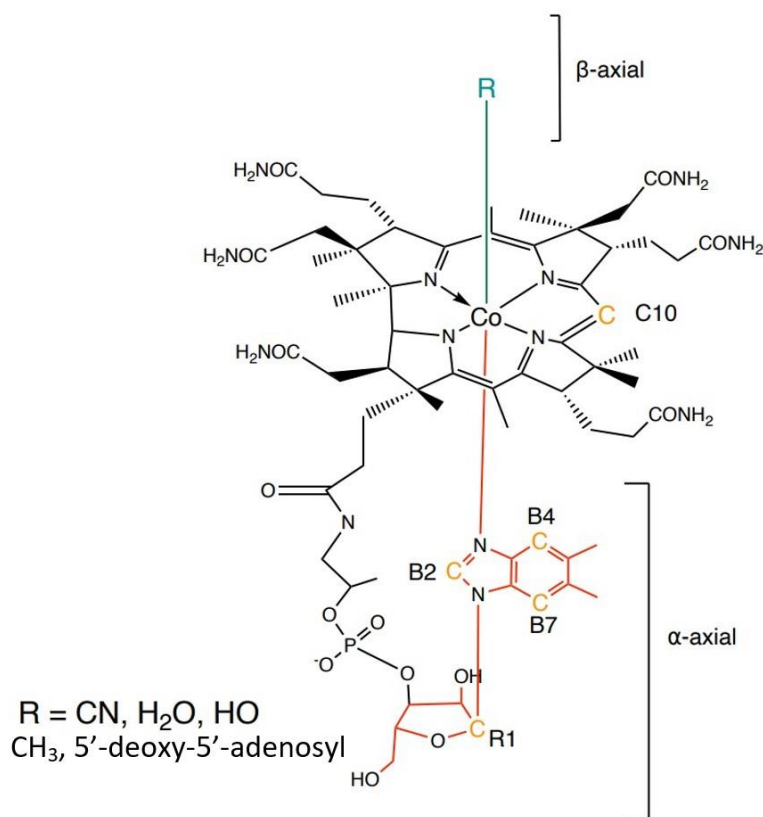


Figure 1: Structural Formula of Cobalamins. Vitamin B<sub>12</sub>: R = CN, Aquacobalamin: R = H<sub>2</sub>O, Hydroxycobalamin: R = OH, Methylcobalamin: R = CH<sub>3</sub>, Adenosylcobalamin: R = 5'-deoxy-5'-adenosyl).

Interest in organocobalamin derivatives with a Co-C bond at the beta axial site stems from the biologically active methylcobalamin and adenosylcobalamin cofactors. As both are required to activate B<sub>12</sub>-dependent enzymes, they have been studied extensively and were found to be challenging to isolate from biological samples due to their instability under aerobic conditions and their photosensitivity<sup>5</sup>. From these alkylcobalamins, interest in the stability of the related alkenylcobalamin and alkynylcobalamin analogues arose. It was found that alkylcobalamins were the least stable with respect to Co-C bond homolysis, while alkynylcobalamins were typically stable under aerobic conditions<sup>5</sup>. This is likely due to the difference in the stabilities of the radicals formed once the Co-C bond is cleaved.

Cobalamins have also recently been investigated for their potential to deliver therapeutic drugs or antimicrobial agents<sup>6</sup>. The essential micronutrient cobalamin is transported into every cell using B<sub>12</sub>-specific uptake proteins, allowing efficient delivery of the cargo<sup>7</sup>. For this reason, formation of a difficult alkenylcobalamin was explored for future applications of synthesis of a semi-stable therapeutic agent.

## 2.2 This Research Project

The aim of this study is to develop a method to synthesise halogenated vinylcobalamin complexes of cobalamins. In this research project, two specific methods were investigated for their suitability to synthesise a halogenated vinylcobalamin. The classic reduction method uses a reducing agent such as NaBH<sub>4</sub> to reduce the metal centre of cob(III)alamin from Co(III) to Co(I)<sup>8</sup>. This is done to make the cobalamin more reactive with the desired ligand. Cob(I)alamin has been referred to as a “super nucleophile” and rapidly attacks alkyl halides with expulsion of a halide via an S<sub>N</sub>2 mechanism<sup>3</sup>. The second method used was a reduction-free method using a sterically hindered base to deprotonate the halogenated alkenyl ligand, which should then be able to substitute the solvent ligand at the beta-axial site of cob(III)alamin. Future possible applications of new alkenylcobalamins include intracellular delivery of therapeutic or antimicrobial agents.

## 3 Literature Review

### 3.1 The Structure and Biological Importance of Vitamin B<sub>12</sub> Derivatives (Cobalamins)

Cobalamin (Cbl) is an enzyme cofactor used by animals and humans which is produced by bacteria<sup>1</sup>. “Vitamin B<sub>12</sub>” refers to the most common vitamer cyanocobalamin which is not the enzymatically active form and is therefore considered a provitamin<sup>9,10</sup>. There are two organometallic B<sub>12</sub> cofactors which are biologically active, methylcobalamin and adenosylcobalamin (or coenzyme B<sub>12</sub>).

Cbl is a structurally complex molecule which features two major structural components; there is a corrin ring containing four pyrrole-like subunits which coordinate to the metal cobalt centre at the four equatorial sites of the octahedral complex, as well as a 5,6-

dimethylbenzimidazole (DMB) moiety which coordinates to the cobalt at the lower (alpha) axial site, Figure 1<sup>3</sup>. When this 5,6-dimethylbenzimidazole moiety is coordinated to the cobalt centre, the cobalamin is in the 'base-on' form<sup>3</sup>. In this form, the coordination of the DMB group affects the flex of the corrin ring, exhibiting a *trans* effect and influence on ligands coordinated at the beta axial site, Figure 1. Cobalamins are additionally stabilised by high binding affinity ligands at the beta-axial position<sup>2</sup>. Without the presence of these axial ligands, the Co(III) metal centre is prone to reduction to Co(II) or even Co(I)<sup>2</sup>. In humans, cobalamins are reduced to cob(II)alamin in a reaction catalysed by the enzyme CblC, methylmalonic aciduria type C and homocystinuria, a Cbl-deligase<sup>9,10</sup>. This repair enzyme functions through modification of cob(III)alamin to cob(II)alamin via the reductive de-ligation of the cyanide ligand<sup>9,10</sup>.

The two B<sub>12</sub> derivatives which are biologically active are methylcobalamin and adenosylcobalamin. They are utilised by three classes of enzymes in mammals and bacteria: AdoCbl-dependent isomerases, MeCbl-transferases, B<sub>12</sub>-dependent reductive dehalogenases<sup>3,11</sup>. B<sub>12</sub> derivatives are typically in a 'base-off' form when bound to the enzyme, with a histidine sidechain substituting the DMB at the alpha-axial site of the cobalamin<sup>3</sup>. For AdoCbl-dependent isomerases homolytic cleavage of the Co-C bond between the 5'-deoxyadenosyl ligand and the cobalt metal centre of the cobalamin occurs to generate an adenosyl radical<sup>3</sup>. The 5'-deoxyadenosyl radical then abstracts a hydrogen atom of the substrate initiating isomerisation<sup>3</sup>. This general mechanism is utilised by the three subclasses of AdoCbl-dependent isomerases. The first class rearranges the carbon skeleton, as seen in glutamate mutase, methylmalonyl-CoA mutase, isobutyryl mutase, and others<sup>11</sup>. The second class of enzyme, utilising the 'base-on' form, eliminates the presence of a heteroatom, as seen in diol dehydratase, glycerol dehydratase, and others<sup>11</sup>. The third class of enzymes facilitate the intramolecular amino group migration, as seen in L-β-lysine 5,6-aminomutase and D-ornithine 4,5-aminomutase. The mammalian enzyme methylmalonyl-CoA mutase plays a key role in the tricarboxylic acid cycle, an essential metabolic pathway involved in carbohydrate, protein, and fat metabolism<sup>11</sup>.



## 3.2 Synthesis and Stability of Beta-Axial Ligand Derivatives of Cobalamins

### 3.2.1 Synthesis of cobalamins with inorganic ligands at the beta-axial site

Inorganic ligands typically bind to the beta-axial site of cobalamin via an oxygen, nitrogen, or sulfur donor atom<sup>12</sup>. The synthesis of these cobalamin complexes can be done under mild reaction conditions. For example, the inorganic ligand is dissolved in an aqueous buffer solution such as MES buffer solution (0.1 M, pH ~6). This is then added dropwise to the starting material hydroxycobalamin hydrochloride (HOCbl·HCl) in a MES buffer solution (0.1 M, pH ~6)<sup>12</sup>. The reaction is allowed to proceed for ~30 minutes in an ice bath (0°C), and the product precipitated out into chilled acetone (-20°C) and washed with acetone and diethyl ether(-20°C)<sup>12</sup>. This method has been used by other research groups using different salts for the buffer solution or a different pH to suit the pK<sub>a</sub> of the donor ligand as well as the hydroxycobalamin<sup>13</sup>. This is a simple ligand substitution reaction, where the aqua ligand of the reactant is substituted by the inorganic ligand. The cobalt centre remains in the Co(III) oxidation state. Inorganic cobalamins are simple to synthesise compared with organocobalamins.

### 3.2.2 Synthesis of cobalamins with a beta-axial alkyl, alkenyl or alkynyl ligand

The most commonly used method to synthesise organocobalamins, with a Co-C bond at the beta-axial site, involves the reduction of the cob(III)alamin Co(III) metal centre using a strong reducing agent such as sodium borohydride, zinc, sodium formate, or chromium(II) acetate<sup>14</sup>. These produce the similar effect of reducing the cobalt(III) centre to cobalt(I). This results in the beta axial ligand being released and the generation of the highly nucleophilic cob(I)alamin intermediate. The cob(I)alamin intermediate is able to react with an alkyl, alkenyl, or alkynyl halide to produce the desired organocobalamin by eliminating a halide from the ligand. There are also electrochemical methods which allow for the selective or controlled reduction of the cobalt centre from Co(III) to Co(II) or Co(I) using electrodes and an agar salt bridge<sup>8</sup>. Finally, there is a modern method which is called “reduction-free” as it displaces the cyanide ligand of cyanocobalamin using a copper(I) catalyst with the deprotonated alkyl ligand present in solution<sup>15</sup>.

When synthesising alkylcobalamins, reduction methods have been most commonly used as the cob(I)alamin intermediate is highly reactive with electrophiles, such as alkyl halides, to produce the desired alkylcobalamin derivative<sup>14</sup>. The reduction of the cobalt metal centre has been performed using Adam's catalyst, which catalyses the reduction of Co(III) to Co(II)<sup>8</sup>. In this method, oxygen must be absent to prevent the oxidation back to the Co(III) state, which is true for all organocobalamin syntheses via the Cbl(I) or Cbl(II) intermediates. This method has not been reported to oxidise the metal centre to the fully reduced Co(I) or B<sub>12</sub>S state of cobalamin<sup>8</sup>. Hill et al<sup>16</sup> discuss the reduction of cob(III)alamins by reducing agents. In acidic conditions the reduction by sodium proceeds to the Cbl(II) state, however under alkaline conditions Cbl(I) is formed<sup>16</sup>. Chromous salts were shown to produce Cbl(II) by Boos et al<sup>17</sup>, and the effects of pH were further investigated by Beaven and Johnson, with chromous salts behaving similarly to sodium borohydride under certain conditions<sup>18</sup>. Thiols have also been used to facilitate the reduction of B<sub>12</sub> to Cbl(II) in alkaline solutions<sup>16</sup>. Cbl(II) is unreactive with an alkylating agent, such as alkyl halides, unless it is reduced by a thiol<sup>8</sup>. In order to reach this fully reduced state, an excess of the reducing agent is required and can result in irreversible reduction of B<sub>12</sub><sup>8</sup>. The addition of copper catalyses this reaction but it also results in the reduction of the desired alkylcobalamin product which should be stable towards the reducing agent<sup>8</sup>. For this reason a cobalt salt is recommended in order to prevent irreversible reduction of the cobalamin and reducing of the desired alkylcobalamin<sup>8,14</sup>. The reduction method has been used to synthesise alkyl, alkenyl, and alkynylcobalamins.

The "reduction-free" method for the synthesis of cobalamins with beta-axial alkynyl ligands does not require the reduction of the cobalt metal centre in order to synthesise the new organocobalamin. It utilises a Cu(I) salt to stretch the Co-C bond between the cobalt of cyanocobalamin and the carbon from the cyanide<sup>15</sup>. A base is reacted with the desired alkynyl ligand to deprotonate the ligand, which will then coordinate to the cobalt metal centre in the reaction mixture<sup>15</sup>. Although this is how the mechanism is proposed in the literature, it has not been conclusively proven. The cyanide ligand of cyanocobalamin is removed by adding Cu(I) acetate, due to the large binding constant of Cu(I) with cyanide, to form the Cu(CN)<sub>2</sub><sup>-</sup> precipitate<sup>19</sup>. The benefits of this reaction are the simplicity of this reaction leading to fewer side products, observed by Brenig et al<sup>20</sup>. This procedure is very

successful for the synthesis of alkynylcobalamin complexes<sup>21</sup>. Although these complexes are very stable, with the beta-axial ligands being difficult to substitute under biological conditions, these ligands can be removed in acidic media<sup>21</sup>. This procedure was successfully used to synthesise phenylethynylcobalamin. This synthesis method also allowed for the production of various alkynylcobalamin compounds, such as 4-nitrophenylalkylidenecobalamin, 4-methoxyphenylalkylidenecobalamin, and 2-TMS-ethylpropiolate alkynylcobalamin<sup>15</sup>.

### 3.2.3 Stability of the organocobalamins in solution

The stability of cobalamin compounds in solution has been the subject of intense research, including their photostability, heat sensitivity, and pH sensitivity<sup>5,15,22,23</sup>. The corrin is typically significantly more stable than the ligand coordinated at the beta-axial site. The stability is influenced by the flex of the corrin ring, which can result in steric hinderance at the beta-axial site and a lengthening of the Co-C bond<sup>23</sup>. When these studies are conducted, the results are often compared with other cobalamins.

The most well-studied cobalamin is methylcobalamin, one of the two biologically active organocobalamins. Alkenylcobalamins are considered highly unstable due to the saturated carbon centre coordinated to the cobalt centre. Methylcobalamin has a Co-C bond length of 1.979 Å and a bond dissociation energy of 37 kcal mol<sup>-1</sup><sup>24,25</sup>. The reported half-life of methylcobalamin is 3.1 minutes at pH 9.27 under aerobic conditions, and ~20 hours under anaerobic conditions with both showing first order decomposition kinetics, using a xenon lamp and a mercury lamp, respectively<sup>5,22</sup>. Kinetic studies have been conducted by observing the changes in absorbance at a specific wavelength, 311 nm, after irradiation with light (temperature and pH are controlled in each comparison)<sup>5,22</sup>. An aerobic photolysis study showed that there is little difference between the quantum yield of methylcobalamin at pH 7 vs pH 1<sup>22</sup>. Due to the apparent increased stability of methylcobalamin under anaerobic conditions, it is likely that the Cbl(II) radical species which is formed after bond homolysis reacts with oxygen to form aquacobalamin rather than radical recombination to reform the Co-C bond. Ethylcobalamin has a longer Co-C bond length of 2.023 Å. It has been shown in an aerobic photolysis study that ethylcobalamin has a higher quantum yield than methylcobalamin. This is likely due to the longer Co-C bond length<sup>22</sup>. As the higher the

quantum yield of the compound, the more molecules that have been destroyed, and therefore the more labile the ligand as it is able to cleave from the cobalamin.

There is much less information available about the stability of alkenylcobalamins.

Vinylcobalamin was first synthesised in the 1960's, with its X-ray crystal structure being reported by Van der Donk et al. in 2005, together with (*Z*)-chlorovinylcobalamin<sup>23</sup>. These alkenylcobalamins have been reported as intermediates in the B<sub>12</sub>-catalysed reductive dichlorination of perchloroethylene and trichloroethylene by bacteria<sup>26</sup>. The Co-C bond length of vinylcobalamin is shorter than that of chlorovinylcobalamin (1.911 Å and 1.951 Å, respectively)<sup>23</sup>. This is likely due to the chloride causing steric repulsion with the corrin ring and possibly from the chlorovinyl ligand having a weaker  $\sigma$ -donating capacity<sup>23</sup>. A photolysis study under aerobic conditions was conducted for these compounds, and it was found that both vinylcobalamin and chlorovinylcobalamin photolyzed ~20x slower than methylcobalamin under the same conditions<sup>23</sup>. Under anaerobic conditions, it has been shown that vinylcobalamin decomposes considerably faster than that of methylcobalamin, 2.1 hours vs 20 hours<sup>5</sup>. This is likely due to the vinyl radical from vinylcobalamin being more stable than the methyl from methylcobalamin, allowing for a more efficient geminate cage recombination of the radical<sup>23</sup>. If the radical is stable, it promotes Co-C bond cleavage. This is influenced by oxygen as it is able to oxidise the Cbl(II) species and thus prevent Co-C bond re-formation, to form superoxide and Cbl(III), which results in aquacobalamin. These findings in the bond length affecting the stability of the organocobalamin are consistent with the (*Z*)-chlorovinylcobalamin being easier to reduce compared with vinylcobalamin ( $E^\circ = -1.11$  V and  $-1.48$  V respectively)<sup>23</sup>. Additionally, the structurally related cobaloximes showed that the redox potential increased by approximately +0.15 V for each additional chloride present on the sp<sup>2</sup> carbon of the vinylcobaloxime<sup>23</sup>. Another alkenylcobalamin which has been synthesised and analysed by X-ray crystallography is  $\beta$ -phenylvinylcobalamin<sup>27</sup>. Its isomer  $\alpha$ -phenylvinylcobalamin was also formed in this reaction however, it could not be crystallised<sup>27</sup>. The Co-C bond length of  $\beta$ -phenylvinylcobalamin was 2.004 Å, determined by X-ray crystallography. After 3 hours of exposure to visible light both products were shown to degrade to hydroxycobalamin<sup>27</sup>.

Antivitamins are organic compounds which counteract the physiological activity of vitamins or a group of structurally related compounds which are metabolically active and contribute to overall health<sup>9,28</sup>. Vitamins and antivitamin should be considered pairs, due to the antivitamin's inhibitory role with respect to the vitamin in a metabolic system<sup>9,28</sup>. The inhibitory function occurs due to the structural similarity between the two, allowing the antivitamin to act as a Type I competitive multifunctional inhibitor, as well as a Type II indirect inhibitor through the reduction of effective vitamin activity<sup>9,28</sup>. Antivitamins of B<sub>12</sub> are currently of considerable interest, particularly antivitamin with stable beta-axial ligands in biological systems, as they are still able to be taken up into cells unhindered and are unable to be modified into a metabolically active vitamer by removal of the  $\beta$ -axial ligand by the CblC enzyme<sup>9</sup>. Arylcobalamins have been studied as potential B<sub>12</sub> antivitamin analogues due to their thermal and chemically inert properties, making them an effective type I antivitamin in a cellular environment<sup>9</sup>. Additionally, alkynylcobalamins have been investigated as possible B<sub>12</sub> antivitamin<sup>9</sup>. These were found to be exceptionally stable<sup>9</sup>. Both aryl and alkynylcobalamins are resistant to reduction in a reaction catalysed by the CblC enzyme due to strong bonding between the Co centre and unsaturated C centre<sup>9</sup>. Additionally, they appear to be structurally similar to natural cobalamins, as the general structure is unchanged and they are still able to bind to the desired proteins<sup>9</sup>.

Arylcobalamins are unstable in the presence of light and therefore considered a conditional antivitamin<sup>9,10</sup>. This was found to be a common feature of most arylcobalamins, and proposed as a sort of feature where the use of laser photolysis with visible light can enable a controlled unlocking of the beta axial antivitamin ligand<sup>9,10</sup>. Ethylphenyl-cobalamin was administered to mice to induce functional B<sub>12</sub> deficiency<sup>9,10</sup>. Although the exact cellular location of the inhibition was not directly established, it was proposed that using a combination of B<sub>12</sub> antivitamin could aid in the identification of the main inhibitory effect and cellular distributions<sup>9</sup>.

Alkynylcobalamins were also investigated as antivitamin. Ethynylcobalamin has been shown to be very stable in aerobic photolysis experiments, with a half-life of 25 days<sup>5</sup>. A strong bond between the Co and C centres is a feature of alkynylcobalamins and this is what prompts the suggestion of alkynylcobalamins being possible antivitamin, as well as some

variations of alkynylcobalamins being unable to be processed by the CblC enzyme<sup>28</sup>.

Phenylethynylcobalamin was synthesised and found to be so stable as an antivitamin that it has been referred to as 'locked' cobalamin<sup>9</sup>. Proteolytic detachment of the ligand occurs in the pH 2 – pH 5 range. This compound has a high resistance to thermolytic cleavage, and is resistant to photolytic cleavage in an aerated solution exposed to daylight for 75 hours<sup>9,29</sup>. It binds to the B<sub>12</sub> transport system proteins similarly to cyanocobalamin, and is likely therefore to be taken up into cells, and is resistant to  $\beta$ -axial ligand removal and reduction to Cbl(II) by the enzyme CblC<sup>9,29</sup>. Stability tests were conducted to compare between alkynylcobalamins, where 4-nitrophenylalkylidenecobalamin was more stable than the rest both thermally and proteolytically, and methoxyphenylalkylidenecobalamin was the least thermally stable of the compounds synthesised<sup>15</sup>.

The application of antivitamins to *in vivo* situations could be a reusable modification able to target all cells by acting as a possible antibiotic or cellular growth inhibitor<sup>28</sup>. An example of this is the addition of ethylphenylcobalamin to a bactericidal sulfonamide cocktail to target hospital-resistant gram-negative bacteria<sup>28</sup>. Additionally, the use of antivitamins as an anticancer alternative has been explored due to its ability to inhibit cell growth<sup>28</sup>. This extends beyond the initial thoughts of simply a method to model B<sub>12</sub> deficiency and shows the vast potential of antivitamins depending on the modifications made to the complete corrinoid.

### 3.3 Characterisation Techniques of Cobalamins

Characterisation of cobalamin compounds can be done using nuclear magnetic resonance spectroscopy (NMR), high performance liquid chromatography (HPLC), UV-Vis spectroscopy, and mass spectrometry (MS). These are all fairly straightforward methods which are able to indicate definitively the presence of new compounds.

UV-Vis spectroscopy has been used to characterise a wide range of cobalamin species. The wavelength maxima are highly dependent on the  $\beta$ -axial ligand<sup>30</sup>. UV-Vis spectroscopy is typically used in kinetic studies of  $\beta$ -axial ligand substitution and the photodecomposition of cobalamin derivatives as the absorption maxima shift. Although this is a successful method to identify a single compound, if a product is difficult to separate from the starting material

or additional side products occur, the resulting spectra could give inconclusive results and requires the product mixture to be characterised using other methods.

While UV-Vis spectroscopy has limited success with respect to product mixtures,  $^1\text{H}$  NMR spectroscopy is extremely powerful to identify cobalamin impurities<sup>31</sup>. It was found that the aromatic region of  $^1\text{H}$  NMR spectra of  $\text{B}_{12}$  derivatives is highly dependent on the  $\beta$ -axial ligand, with distinct chemical shifts in  $\text{D}_2\text{O}$  for the five protons which resonate in the aromatic region, as seen in Figure 1 identified in orange and labelled C10, B2, B4, B7, and R1 protons<sup>31</sup>. This is also beneficial for identifying cobalamin derivatives which are unable to survive HPLC separation due to having labile  $\beta$ -axial ligands. No two cobalamins have the same chemical shifts for all five proton peaks in the aromatic region, and a significant difference of the C10 chemical shift<sup>31</sup>.

Reverse phase HPLC is useful to separate  $\text{B}_{12}$  compounds and LC-MS is useful in determining the exact mass of each product in complex mixtures. Organocobalamins have a higher retention time than inorganic cobalamins<sup>32</sup>. This is due to the  $\beta$ -axial ligand interacting strongly with the aliphatic side chains of the silica column, requiring a higher acetonitrile percentage to elute<sup>15</sup>. Unfortunately, some compounds do not remain intact during this process and are therefore unable to be analysed by LC-MS. This arises from a lack of stability of the  $\beta$ -axial bonds, resulting in the substitution of the ligand with a solvent molecule<sup>31</sup>. Despite being unable to determine the exact mass of compounds with labile  $\beta$ -axial ligands, there remains an indication of new compounds based on new retention times. LC-MS is ideal for stable cobalamin compounds.

When the characterisation methods listed above are used in conjunction with each other, they are typically able to unequivocally characterise organocobalamin complexes. X-ray crystallography is also a powerful method to characterise cobalamins<sup>15,23,25,27</sup>.

### 3.4 This Research Project

The chemistry and properties of organocobalamins incorporating alkyl and alkynyl ligands at the beta-axial site is well-studied. There is much less known about organocobalamins with

alkenyl ligands. These compounds are intermediates in the B<sub>12</sub>-catalysed reductive dehalogenation of bacteria<sup>26,33</sup>. The studies with vinylcobalamin have been reported since the 1960's<sup>5</sup>. The X-ray structures of vinylcobalamin, chlorovinylcobalamin, and phenylvinylcobalamin have been reported, and the mechanisms by which B<sub>12</sub>-dependent bacteria reductively dehalogenate chlorinated alkenes has also been investigated<sup>26,27,33</sup>.

Alkenylcobalamins can be thought of as an intermediate between alkylcobalamins and alkynylcobalamins, such as their stability in solution<sup>5,23</sup>. Alkylcobalamins are unstable and typically photolysed in minutes in the presence of light, whereas alkynylcobalamins are stable, able to withstand days of light exposure and in some cases unable to be photolysed without manipulation of pH conditions<sup>5,15,22</sup>.

The aim of this research was to develop synthetic procedures for and study the properties of organocobalamins incorporating alkene ligands. The organocobalamins were characterized using NMR spectroscopy, and LC-MS. These complexes have the potential to be used for the delivery of therapeutic, with the parent complex being more photochemically stable than alkylcobalamins but also capable of undergoing CblC catalyzed Co-C bond cleavage in mammalian cells to release therapeutics attached via the alkene to the cobalamin moiety.

## 4 Methods

### 4.1 Chemicals

All solvents used were purchased from Fischer Scientific or Merck. Anhydrous dimethylacetamide (99%), and anhydrous acetone (99%) were purchased from Merck and stored under nitrogen. Ethyl bromide was purchased from Merck. Hydroxocobalamin (HOCbl.HCl) was purchased from AK Scientific and stored at 4 °C. Ultra-pure water was obtained from a Purite purewater 200 system. NMR solvents were purchased from Eurisotop; deuterium oxide (99.8% D), methanol-d<sub>4</sub> (99.8% D), and dimethylsulfoxide-d<sub>6</sub> (99.9% D) and stored at 4 °C. All spectra were referenced to trimethylsilylpropanoic acid (TSP) purchased from Merck and stored at 4 °C.



## 4.2 Instrumentation

An MBraun Labmaster 130 nitrogen glove box was used for the synthesis described in sections 4.5 and 4.6, with O<sub>2</sub> and H<sub>2</sub>O less than 5 ppm. NMR spectroscopy was carried out using a Bruker Ascend 400 NMR spectrometer and spectra analysed using TopSpin version 4.1.3.

An Agilent 1260 Infinity Quaternary LC System (Santa Clara, CA 95051 USA) was used for LC-MS. The components of the instrument are as follows: 1260 quaternary pump (model number: G1311B), 1260 infinity ALS sampler (model number: G1329B), 1200 series autosampler thermostat FC/ALS/Therm (model number: G1330B), 1260 infinity TCC column component (model number: G1316A), 1260 infinity diode array detector (DAD) (model number: G4212B), connected to a 6420 triple quadrupole LC-MS system with multimode ionisation source (model number: G1948B) operating in positive electrospray ionisation mode. The autosampler was maintained at 4°C. Mass to charge data was analysed between 500 to 1500 m/z. Gas was maintained at a temperature of 300°C, with a flow of 6 L/min, nebuliser pressure at 15 psi and capillary voltage at 4000 V. Solvent flow was 0.5 mL/min. A Luna C18 (4.6 × 100 mm, 3.5 µm) column was used for the analysis. The LC-MS system used MassHunter Workstation software (Agilent Technologies Inc. 2012, CA 95051, United States). Post-run analyses were done using Qualitative Analysis B.07.00.

HPLC analysis was conducted using a Shimadzu HPLC system consisting of a Prominence degassing unit (model number: DGU-20A5), Prominence pump (model number: LC-20AT), Prominence autosampler (model number: SIL-20AC HT), connected to a UV-visible detector (model number: SPD-20A). The column was maintained at 35°C. Peaks were detected at 361 nm. A Luna C18(2) 100 Å column (model number: 00G-4252-N0) was used.

## 4.3 General Methods

Preparations of anaerobic solutions of ultra-pure water were conducted by transferring water (25 mL) into a small round bottom flask and securing the top with a rubber septum cap as well as a clamp band around the septum cap. Argon gas was then gently bubbled through the solution via a needle, with another needle being used to ensure there is not an

increase in pressure. This was done for 30 minutes, before turning off the gas cylinder and removing needles piercing the septum cap.

Degassing of additional solutions taken into the glovebox were conducted using the Schlenk line. The chemical was transferred into a Schlenk test tube, where the top was secured with a septum cap and clamp band. An anaerobic solution was prepared using the freeze-thaw technique (3 times). This was done for small volumes of chemicals as it is the most efficient method and ensures minimal evaporation of the solvent. This method was used to prepare anaerobic trichloroethylene (TCE) and anaerobic ethyl bromide.

Liquid Chromatography-Mass Spectrometry (LC-MS) was used to analyse the compounds from the attempted synthesis of a halogenated vinylcobalamin using reduction method (method 3). The mobile phase of this method consisted of 1% formic acid in milli-Q water (Solvent A) and acetonitrile (Solvent B). Elution parameters were set as follows; 0-2 min 95:5 A:B, 2-22 min 95:5 A:B to 70:30 A:B, 22-25 min 70:30 A:B to 10:90 A:B, 25-30 min isocratic elution of 10:90 A:B, 30-32 min 10:90 A:B to 95:5 A:B. The LC-MS method used to analyse the trichloroethenylcobalamin attempted synthesis using a reduction-free method (method 4) consisted of the mobile phase of this method consisted of 1% formic acid in milli-Q water (Solvent A) and acetonitrile (Solvent B). Elution parameters were set as follows; 0-2 min 90:10 A:B, 2-22 min 90:10 A:B to 60:40 A:B, 22-25 min 60:40 A:B to 10:90 A:B, 25-30 min isocratic elution of 10:90 A:B, 30-32 min 10:90 A:B to 90:10 A:B.

## 4.4 Synthesis of Ethylcobalamin

### 4.4.1 Method 1

The initial part of the synthesis was carried out in the glovebox. An anaerobic solution of HOCbl was prepared using water (3.00 mL) and HOCbl·HCl (30.0 mg, 0.0190 mmol). An anaerobic solution of NaBH<sub>4</sub> (1.19 M) was prepared by the addition of anaerobic water (0.137 mL) to NaBH<sub>4</sub> (6.16 mg). An aliquot of the NaBH<sub>4</sub> solution (0.100 mL, 0.114 mmol, 5.00 equiv) was added to the HOCbl solution with stirring. This was allowed to react at room temperature until the solution was charcoal in colour (~10 - 20 minutes), indicating the presence of cob(I)alamin. The excess NaBH<sub>4</sub> was quenched via the addition of anhydrous

acetone (0.100 mL). The reaction vial was covered with aluminium foil and the lights were turned off. Ethyl bromide (0.00710 mL, 0.0952 mmol, 6.00 equiv) was added dropwise to the HOCbl solution with stirring. This reaction was left to stir for 30 minutes at room temperature and the flask subsequently was removed from the glovebox. Part of this solution was used to test whether the EtCbl product can be isolated by precipitation. An aliquot was added dropwise into acetone (ice water bath, 0 °C) to precipitate the product. The mixture was then filtered through a fine fritted sintered filter attached to a Buchner funnel. It was unsuccessful as the precipitate was too fine and passed through the filter with the acetone. The remaining product was therefore placed in the freezer and roto-evaporated down 3 days later. Once dry, the  $^1\text{H}$  NMR spectrum was obtained. The mass of the product mixture was not determined.

The procedure was repeated using the same stoichiometric ratio of reagents. The masses and volumes of reagents were: HOCbl·HCl (30.0 mg, 0.0190 mmol),  $\text{NaBH}_4$  (4.3 mg),  $\text{NaBH}_4$  solution (0.100 mL, 0.114 mmol, 5.00 equiv), EtBr (0.00710 mL, 0.0952 mmol, 6.00 equiv).

#### 4.4.2 Method 2

The reduction of HOCbl·HCl was conducted as described in Method 1 in the glovebox, with the same stoichiometric ratio of reagents and the entire reaction scaled up by a factor of 3. The masses and volumes of the reagents were: HOCbl·HCl (90.0 mg, 0.0571 mmol),  $\text{NaBH}_4$  (12.8 mg),  $\text{NaBH}_4$  solution (0.300 mL, 0.338 mmol, 5.00 equiv). Three separate flasks of EtBr were prepared. Each flask of EtBr (0.00710 mL, 0.0952 mmol, 5.00 equiv) had different volumes of anaerobic water, giving (0.952  $\text{mol L}^{-1}$ , 0.190  $\text{mol L}^{-1}$ , and 0.0635  $\text{mol L}^{-1}$  EtBr). The flasks were covered in aluminium foil and the lights were turned off. An aliquot of the Cbl(I) solution (3.00 mL) was added dropwise to each EtBr solution in the glovebox while stirring. The product mixtures were left to react with stirring for 30 minutes and removed from the glovebox. One of the flasks of EtCbl (0.190  $\text{mol L}^{-1}$ ) was added dropwise into anhydrous chilled acetone (30.0 mL, ice-water bath, 0 °C), to precipitate the product. The mixture was then filtered through a fine sintered filter attached to a Buchner funnel. It was again unsuccessful as the precipitate was too fine and passed through the filter with the acetone. The remaining flasks were then transferred to the freezer and roto-evaporated to

dryness the following day. Once dry,  $^1\text{H}$  NMR spectra were obtained from two of the EtCbl mixtures ( $0.952\text{ molL}^{-1}$ , and  $0.0635\text{ molL}^{-1}$ ).

#### 4.5 Attempted Synthesis of a Halogenated Vinylcobalamin from Cob(I)alamin and Trichloroethylene

##### 4.5.1 Method 1

The reduction of  $\text{HOCbl}\cdot\text{HCl}$  to cob(I)alamin by  $\text{NaBH}_4$  was conducted in the glovebox with anaerobic solutions as described section 4.4.1, with the same stoichiometric ratio of reagents. The masses and volumes of the reagents were:  $\text{HOCbl}\cdot\text{HCl}$  (30.0 mg, 0.0190 mmol),  $\text{NaBH}_4$  (4.6 mg),  $\text{NaBH}_4$  solution (0.100 mL, 0.114 mmol, 5.00 equiv). Once this was complete, the reaction vial was covered with aluminium foil and the lights were turned off. The Cbl(I) solution was added dropwise to a septum capped vial in the glovebox containing trichloroethylene (TCE, 0.00860 mL, 0.0952 mmol) using a syringe. The resultant solution was left to stir for 15 minutes and removed from the glovebox. The sample was then transferred to the freezer and roto-evaporated to dryness the following day. Once dry, an  $^1\text{H}$  NMR spectrum was obtained.

##### 4.5.2 Method 2

The reduction of  $\text{HOCbl}\cdot\text{HCl}$  was conducted as described above in Method 1 in the glovebox, with the same stoichiometric ratio of reagents and the entire reaction scaled up by a factor of 2. The masses and volumes of the reagents were:  $\text{HOCbl}\cdot\text{HCl}$  (60.0 mg, 0.0190 mmol),  $\text{NaBH}_4$  (9.40 mg),  $\text{NaBH}_4$  solution (0.200 mL, 0.238 mmol, 5.00 equiv). Once cob(I)alamin had been formed, the reaction vial was covered with aluminium foil and the lights were turned off. An aliquot of the Cbl (I) solution (3 mL, 0.0190 mmol) was slowly added dropwise to a vial containing of TCE (0.00860 mL, 0.0952 mmol) and acetone (0.500 mL) or an acetone/water mixture (0.250 mL/0.250 mL) respectively. The dropwise addition of Cbl(I) was carried out over a  $\sim 30$ -minute time period. The resultant product mixtures were left to stir for 15 minutes and removed from the glovebox. The samples were transferred to the freezer and roto-evaporated to dryness the following day. Once dry, a sample was taken and the  $^1\text{H}$  NMR spectrum of each product was obtained.

#### 4.5.3 Method 3

The reduction of  $\text{HOCbl}\cdot\text{HCl}$  was conducted as described above in Method 1 in the glovebox, with the same stoichiometric ratio of reagents. The masses and volumes of the reagents were:  $\text{HOCbl}\cdot\text{HCl}$  (30.0 mg, 0.0190 mmol),  $\text{NaBH}_4$  (14.3 mg),  $\text{NaBH}_4$  solution (0.100 mL, 0.114 mmol, 5.00 equiv). Once cob(I)alamin had been formed, the reaction vial was covered with aluminium foil and the lights were turned off. An aliquot of the  $\text{Cbl(I)}$  solution (3 mL, 0.0190 mmol) was slowly added dropwise to a vial containing of TCE (0.00860 mL, 0.0952 mmol) and acetone (0.500 mL) or an acetone/water mixture (0.250 mL/0.250 mL) respectively. The dropwise addition of  $\text{Cbl(I)}$  was carried out over a  $\sim 30$ -minute time period. The resultant product mixtures were left to stir for 2 hours and removed from the glovebox. The sample was dropped onto cotton wool in a separating funnel containing diethyl ether (1 x 25 mL) and washed with ethyl acetate (2 x 25 mL). This was then released using methanol and roto-evaporated down to dryness. Once dry, a sample was taken and  $^1\text{H}$  NMR spectrum was obtained.

### 4.6 Synthesis of Phenylethynylcobalamin Using a Reduction-Free Method

#### 4.6.1 Method 1

This synthesis was carried out under aerobic conditions.  $\text{CNCbl}$  (50.0 mg, 0.0369 mmol) was weighed out and transferred in a round-bottom reaction flask (25.0 mL) with a stir bar. Copper(I) acetate (4.50 mg, 0.0369 mmol) was weighed out and transferred into the same reaction flask. The flask was submerged in an oil bath (40 °C) and dimethylformamide (DMF, 5.00 mL) added slowly while stirring and the solution stirred until the solution appeared uniform without large clumps,  $\sim 45$  minutes. DMF (0.500 mL), phenylacetylene (0.0405 mL, 0.369 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 0.0299 mL, 0.180 mmol) were added to a small vial and the contents mixed by shaking. This solution was slowly added dropwise to the other reaction flask. The reaction was allowed to proceed overnight in the oil bath (40 °C). After  $\sim 20$  hr, the product mixture was precipitated into a closed separating funnel plugged with cotton wool containing diethyl ether (50 mL). Upon opening the tap of the separating funnel, the solvent mixture passed through whereas the fine precipitate of the product was retained on the cotton wool. The cotton wool was then washed with diethyl ether (1 x 50 mL) and ethyl acetate (2 x 50 mL). The precipitate was then removed

using methanol and the solution taken to dryness by rotary evaporation. The sample was then analysed by  $^1\text{H}$  NMR spectroscopy.

#### 4.6.2 Method 2

This synthesis was carried out as described in the method above. The masses and volumes of reagents used were: CNCbl (50.0 mg, 0.0369 mmol), DMF (5.00 mL), copper(I) acetate (4.5 mg, 0.0369 mmol), DMF (0.500 mL), phenylacetylene (0.0405 mL, 0.369 mmol), DBU (0.0299 mL, 0.180 mmol). However, before adding the DMF/phenylacetylene/DBU solution to the reaction flask, the reaction mixture of CNCbl and copper(I) acetate was left to stir overnight in an oil bath. After stirring overnight, the remaining reagents were added, and the reaction was allowed to proceed for ~3 hours. The precipitation method was conducted as described in the method above. The sample was analysed by  $^1\text{H}$  NMR spectroscopy.

#### 4.6.3 Method 3

This synthesis was conducted under anaerobic conditions using a Schlenk line. CNCbl (50 mg, 0.0369 mmol) was weighed out and transferred into the round-bottom reaction flask (25.0 mL) with a stir bar. Copper(I) acetate (4.5 mg, 0.0369 mmol) was weighed out and transferred into the reaction flask. The reaction flask was placed on the Schlenk line and evacuated 3 times to ensure the mixture is air-free. This was then placed on an oil bath (40 °C) with dimethyl sulfoxide (DMSO, 2.00 mL) added slowly while stirring and stirred to dissolve for 10 minutes. In a small vial, DMSO (0.5 mL) was added, as well as phenylacetylene (0.0405 mL, 0.369 mmol) and 1,8-diazabicyclo(5.4.0)undec-7-ene (0.0299 mL, 0.180 mmol) using air-free techniques. Once given a slight shake, the contents of the vial were slowly added to the reaction flask. The product mixture was allowed to react for ~4 hours. The reaction mixture was exposed to air and the precipitation of the product was conducted as described in section 4.6.1. The precipitate was then removed using methanol and the solution was evaporated down to dryness using rotary evaporation and placed on the high vac overnight. The sample was analysed using  $^1\text{H}$  NMR spectroscopy.

## 4.7 Attempted Synthesis of Trichloroethylcobalamin Using a Reduction-Free Method

### 4.7.1 Method 1

This synthesis was carried out under anaerobic conditions using a Schlenk line. CNCbl (50.0 mg, 0.0369 mmol) and copper(I) acetate (4.50 mg, 0.0369 mmol) were transferred into a round-bottom reaction flask (25 mL) with a stir bar. This was then evacuated three times on the Schlenk line (vacuum followed by N<sub>2</sub> each time). The flask was submerged in an oil bath (40 °C) and anhydrous dimethyl sulfoxide (2.00 mL) added slowly, with stirring. Anaerobic DMSO (0.500 mL), trichloroethylene (0.0332 mL, 0.369 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (0.0299 mL, 0.180 mmol) were added to a vial and the solution shaken to aid mixing. The solution was then added dropwise to the CNCbl/Cu(I)acetate/DBU solution, under nitrogen. The reaction was allowed to proceed for ~5 hours. The reaction mixture was exposed to air and the product precipitated onto cotton wool in a separating funnel containing diethyl ether (50 mL, see section 4.6.1). The precipitate on the cotton wool was washed with diethyl ether (1 x 50 mL) and ethyl acetate (2 x 50 mL) and the product mixture removed using methanol and taken to dryness using rotary evaporation. The sample was analysed by <sup>1</sup>H NMR spectroscopy.

### 4.7.2 Method 2

This synthesis was carried out as described in the method above. The masses and volumes of reagents used were: CNCbl (50.0 mg, 0.0369 mmol), copper(I) acetate (4.51 mg, 0.0369 mmol), trichloroethylene (0.0332 mL, 0.369 mmol), DBU (0.0299 mL, 0.180 mmol). In this synthesis, the solvent for the reaction was changed from anhydrous DMSO to anhydrous dimethylacetamide (1.50 mL, 0.500 mL). Additionally, the reaction was allowed to react for ~20 hours in an oil bath at (40 °C). The reaction mixture was exposed to air and precipitated out into a centrifuge tube containing diethyl ether (15 mL) at 5000 RPM for 20 minutes. This was then washed and centrifuged with diethyl ether (1 x 15 mL) and extracted with ultra-pure water (15 mL) in the centrifuge. The product was evaporated down to dryness using rotary evaporation. The sample was analysed using <sup>1</sup>H NMR spectroscopy.

#### 4.7.3 Method 3

This synthesis was carried out as described in the method above. The masses and volumes of reagents used were: CNCbl (50.0 mg, 0.0369 mmol), copper(I) acetate (4.54 mg, 0.0369 mmol), trichloroethylene (0.0332 mL, 0.369 mmol), DBU (0.0299 mL, 0.180 mmol). In this synthesis the reaction was conducted in an ice bath (0 °C), with a nitrogen balloon attached once the addition of all reagents had occurred. This reaction mixture was allowed to proceed for ~20 hours with stirring, with aliquots taken at 20 minutes and 4 hours. The product mixture was precipitated out using the centrifuge method described in section 4.7.2. The product mixture was exposed to air and precipitated out into a centrifuge tube containing diethyl ether (15 mL) at 5000 RPM for 20 minutes. This was then washed and centrifuged with diethyl ether (1 x 15 mL) and extracted with ultra-pure water (15 mL) in the centrifuge. The product was evaporated down to dryness using rotary evaporation. The samples were analysed by  $^1\text{H}$  NMR spectroscopy.

#### 4.7.4 Method 4

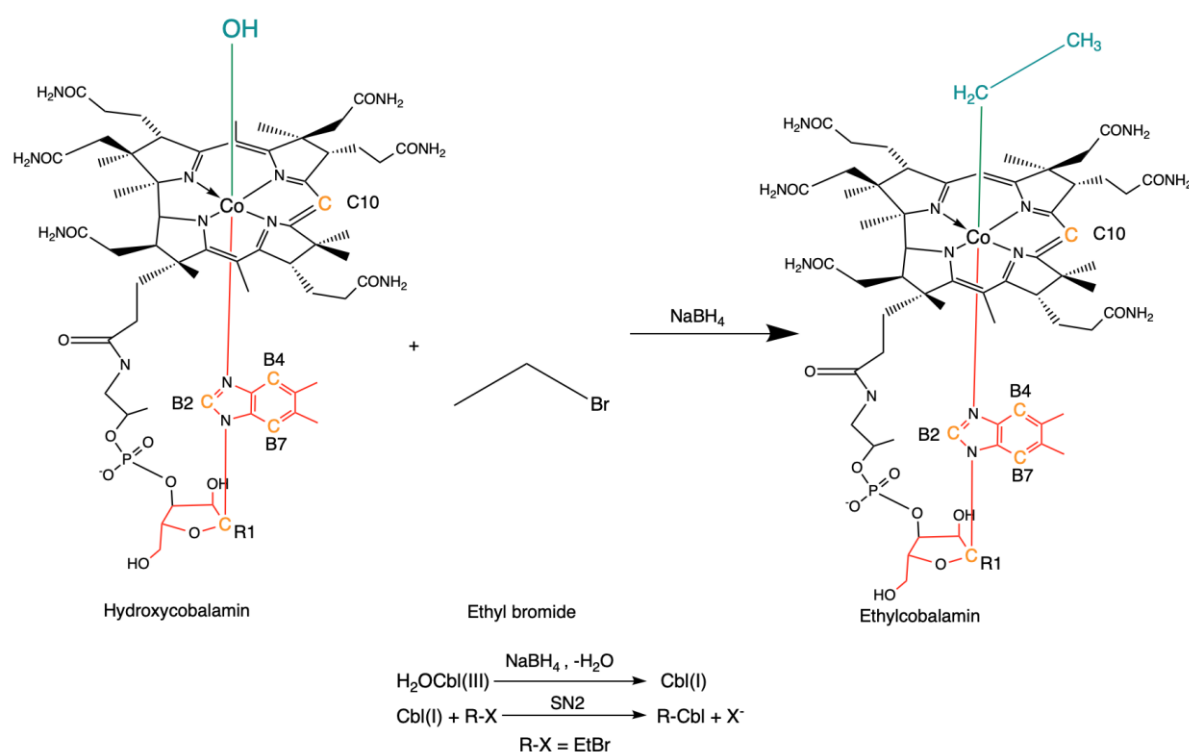
This synthesis was carried out as described in the method above. The masses and volumes of reagents used were: CNCbl (50.0 mg, 0.0369 mmol), copper(I) acetate (4.57 mg, 0.0369 mmol), trichloroethylene (0.0332 mL, 0.369 mmol), DBU (0.0299 mL, 0.180 mmol). In this synthesis the reaction was conducted at room temperature, with a nitrogen balloon attached after the addition of all reagents. This reaction was allowed to proceed with stirring for ~20 hours, with aliquots taken at 20 minutes and 4 hours. The reaction mixture was precipitated out using the centrifuge method described in section 4.7.2. The reaction mixture was exposed to air and precipitated out into a centrifuge tube containing diethyl ether (15 mL) at 5000 RPM for 20 minutes. This was then washed and centrifuged with diethyl ether (1 x 15 mL) and extracted with ultra-pure water (15 mL) in the centrifuge. The product was evaporated down to dryness using rotary evaporation. The samples were analysed by  $^1\text{H}$  NMR spectroscopy.



## 5 Results

### 5.1 Synthesis of Ethylcobalamin

This synthesis was carried out to gain experience in the synthesis of organocobalamin derivatives, prior to attempting the synthesis of alkenylcobalamins incorporating halogenated alkene ligands. The synthesis was conducted according to a literature procedure<sup>33</sup>. In this reaction aqua/hydroxycobalamin ( $\text{H}_2\text{OCbl}/\text{HOCbl}$ ,  $\text{Co}^{3+}$ ) is reduced to cob(I)alamin ( $\text{Co}^+$ ) in water using  $\text{NaBH}_4$ . Unreacted  $\text{NaBH}_4$  was quenched by acetone and ethyl bromide was added to produce ethylcobalamin ( $\text{EtCbl}$ ) via nucleophilic substitution. The synthesis was conducted in the glove box to ensure an air-free environment. This was done as the cob(I)alamin formed is air sensitive and reverts to the starting material when exposed to air. Additionally, aluminium foil and blinds on the windows were used to prevent the formed product from deteriorating as alkylcobalamins have been shown to be light sensitive<sup>5,22,23</sup>.



Scheme 1: Synthesis of Ethylcobalamin.

The product was characterised by  $^1\text{H}$  NMR spectroscopy. In the aromatic region cobalamin compounds have five characteristic shifts which are dependent on the beta axial ligand bound to the cobalt centre<sup>31</sup>. The major characteristic peaks in the aromatic region were determined to be 7.17 ppm, 7.03 ppm, 6.29 ppm, 6.25 ppm, and 6.08 ppm, as seen in Figure

2. These chemical shifts are in excellent agreement for the reported values of the B7 (7.17 ppm, singlet), B4 (6.29 ppm, singlet), and B2 (7.03 ppm, singlet) from the nucleotide base, the C10 (6.08 ppm, singlet) from the corrin ring, and the R1 (6.25 ppm, doublet) of ethylcobalamin published in the literature<sup>34</sup>. Also present are minor peaks which can be attributed to hydroxycobalamin whose five characteristic peaks are the B7 (7.17 ppm, singlet), B4 (6.50 ppm, singlet), and B2 (6.74 ppm, singlet) from the nucleotide base, the C10 (6.07 ppm, singlet) from the corrin ring, and the R1 (6.25 ppm, doublet) from the ribose ring in the literature<sup>31</sup>. The estimated conversion from H<sub>2</sub>OCbl/HOCbl to EtCbl was 65%, determined from the areas of the C10 peaks.

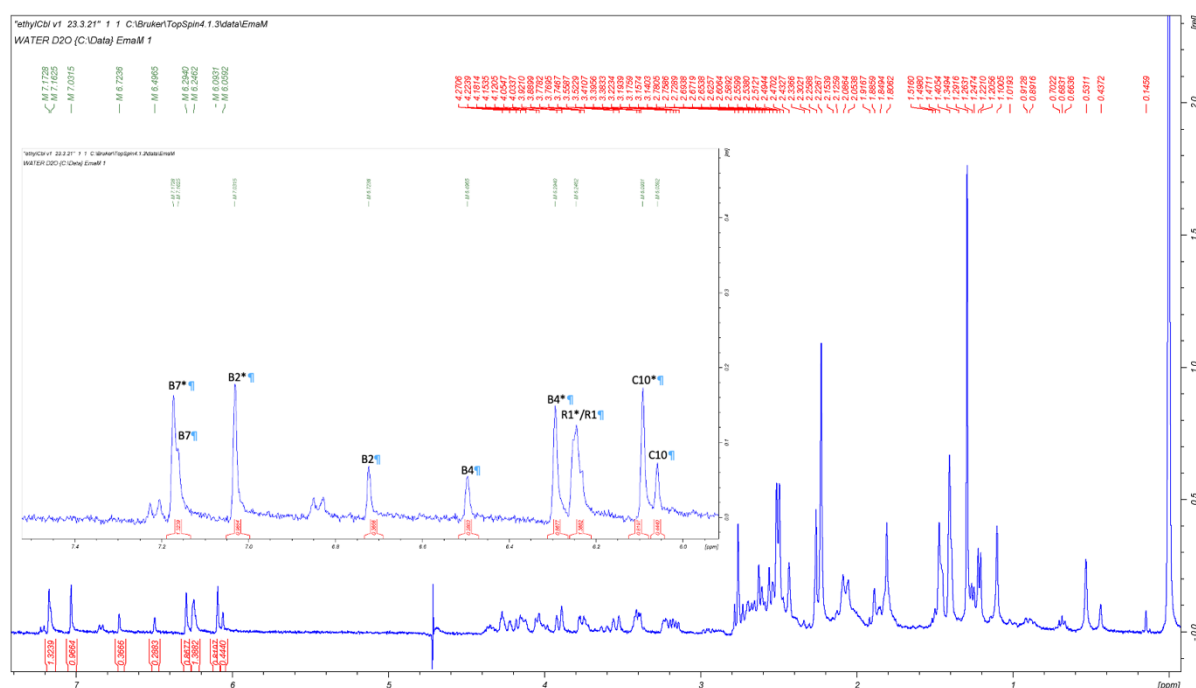


Figure 2: <sup>1</sup>H NMR spectrum of the crude ethylcobalamin product mixture (D<sub>2</sub>O, TSP as reference). The inset shows the aromatic region the peaks have been assigned to EtCbl 7.17 (B7\*, singlet, 1H), 7.03 (B2\*, singlet, 1H), 6.29 (B4\*, singlet, 1H), 6.24 (R1\*, overlapping doublet, 1H), 6.09 (C10\*, singlet, 1H) and HOCbl 7.16 (B7, singlet, 1H), 6.72 (B2, singlet, 1H), 6.49 (B4, singlet, 1H), 6.24 (R1, overlapping doublet, 1H), 6.06 (C10, singlet, 1H).

The synthesis was repeated to achieve a higher conversion of the reactant to product. The <sup>1</sup>H NMR spectrum is shown in Figure 3. As captioned, the peaks labelled with a \* icon indicate the product peaks present in solution. A higher conversion of H<sub>2</sub>OCbl to EtCbl was achieved (89% conversion), as indicated by the peak areas. The increase in conversion can be attributed to a variety of factors due to the nature of this synthesis. Firstly, the light sensitivity of the product formed. If the desired product is exposed to light or left in solution

for a long period of time it degrades to the starting material, as reported in the literature<sup>22</sup>. As this was a known issue, extra precautions and care were taken to limit the exposure of light in the second attempt to achieve a higher conversion to product. This was done by immediate placement of the product mixture into the dark freezer upon discovering the product did not precipitate into the acetone as desired, and by additionally keeping the aluminium foil on the round bottomed flask containing the product mixture during rotary evaporation. Secondly, when the sample remains in solution the product may also degrade to the starting material. Therefore, since precipitation of the product was unsuccessful, to prevent decomposition, the product solution was frozen until rotary evaporation was possible. In the first trial, after the attempted precipitation failed, the product mixture was left in solution for a longer time period as additional options were explored prior to placing it in the freezer. These small changes could contribute to the 24% difference in conversion between the two trials. Additionally, due to the increase in conversion to give a percentage yield expected for this synthetic procedure, this literature method was modified to assess whether a reverse addition of the reagents would still result in a successful synthesis of the product.

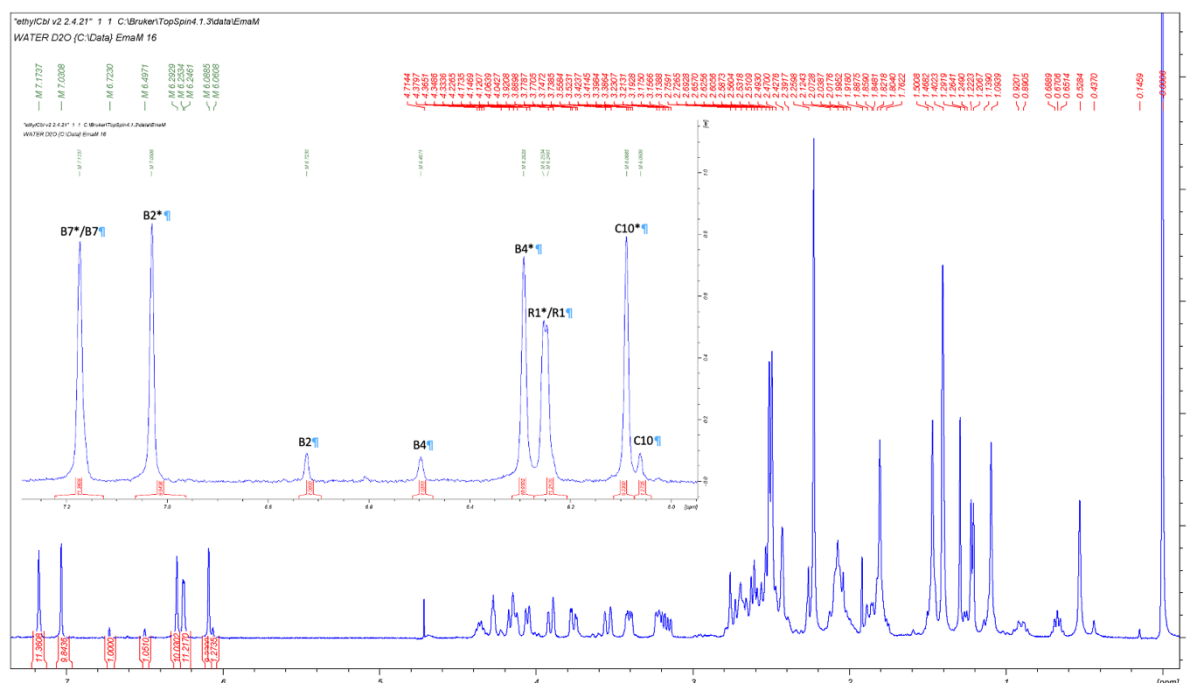


Figure 3: <sup>1</sup>H NMR spectrum of the crude ethylcobalamin product mixture (D<sub>2</sub>O, TSP as reference). The inset shows the aromatic region. The peaks have been assigned to EtCbl 7.17 (B7\*, singlet, 1H), 7.03 (B2\*, singlet, 1H), 6.29 (B4\*, singlet, 1H), 6.24 (R1\*, overlapping

doublet, 1H), 6.09 (C10\*, singlet, 1H) and HOCbl 6.72 (B2, singlet, 1H), 6.49 (B4, singlet, 1H), 6.24 (R1, overlapping doublet, 1H), 6.06 (C10, singlet, 1H).

Alkenylcobalamins incorporating halogenated alkene ligands are unlikely to be stable in the presence of excess cob(I)alamin<sup>23</sup>. In the classic reduction method, used in section 4.4.1, the alkyl halide was added to a solution of the highly nucleophilic Cbl(I), to form the desired alkylcobalamin product. The newly formed alkylcobalamin is therefore exposed to excess Cbl(I) in solution. This would not be ideal if the product reacts with Cbl(I). A modified method for synthesising organocobalamins was therefore tested, in which Cbl(I) was slowly added (dropwise over ~10 minutes) to a solution of EtBr in water, so that exposure of the organocobalamin to the Cbl(I) was minimised. If successful, it was anticipated that this method could be used for the synthesis of alkenylcobalamins with halogenated alkene ligands.

In this modified procedure, method 2, aquacobalamin ( $\text{Co}^{3+}$ ) was reduced to cob(I)alamin ( $\text{Co}^+$ ) in water using excess  $\text{NaBH}_4$ , and the excess  $\text{NaBH}_4$  was quenched using acetone. The cob(I)alamin solution was added dropwise to a solution of EtBr in water. A solution of ethyl bromide in water was used since the volume of EtBr required was so small (7.1  $\mu\text{L}$ ). In this experiment three trials were carried out simultaneously with three different concentrations of EtBr in water.

Figure 4 shows the  $^1\text{H}$  NMR spectrum for the synthetic procedure where the ethyl bromide concentration was  $0.952 \text{ mol L}^{-1}$ . Figure 5 shows the  $^1\text{H}$  NMR spectrum of the product mixture with  $0.0635 \text{ mol L}^{-1}$  ethyl bromide in water. The major aromatic peaks in Figure 4 and Figure 5 are 7.17 ppm, 7.01 ppm, 6.27 ppm, 6.25 ppm, and 6.07 ppm. These peaks can be assigned to the five characteristic peaks for ethylcobalamin, the B7 (7.17 ppm, singlet), B4 (6.29 ppm, singlet), and B2 (7.03 ppm, singlet) from the nucleotide base, the C10 (6.08 ppm, singlet) from the corrin ring, and the R1 (6.25 ppm, doublet), as reported in the literature within  $0.02 \text{ ppm}^{34}$ . There are also two minor peaks in Figure 4 and Figure 5 present at 6.72 ppm, and 6.49 ppm. These minor peaks match that of the starting material hydroxycobalamin, B4 (6.50 ppm, singlet), and B2 (6.74 ppm, singlet) from the nucleotide base, as reported in the literature<sup>31</sup>. The other expected peaks of the B7 (7.17 ppm, singlet), R1 (6.25 ppm, doublet), and C10 (6.07 ppm, singlet) may be overlapping with the

ethylcobalamin peaks in Figures 4 and 5. No  $^1\text{H}$  NMR spectrum was obtained using  $0.190\text{ mol L}^{-1}$  ethyl bromide as the sample was lost as a result of an attempt to precipitate the product. It has previously been found that cobalamins can be precipitated by dripping the solution into chilled acetone<sup>34</sup>. However, in this case the precipitate was too fine for the sintered filter used and passed through with the acetone. The precipitation was trialled a second time as this would isolate the product nicely rather than just using rotary evaporation to remove a small volume of water. Unfortunately, this was found to be unsuccessful, and the sample was discarded. This experiment indicated that the reverse addition method was successful and could be used to proceed with synthesis of other organocobalamins.

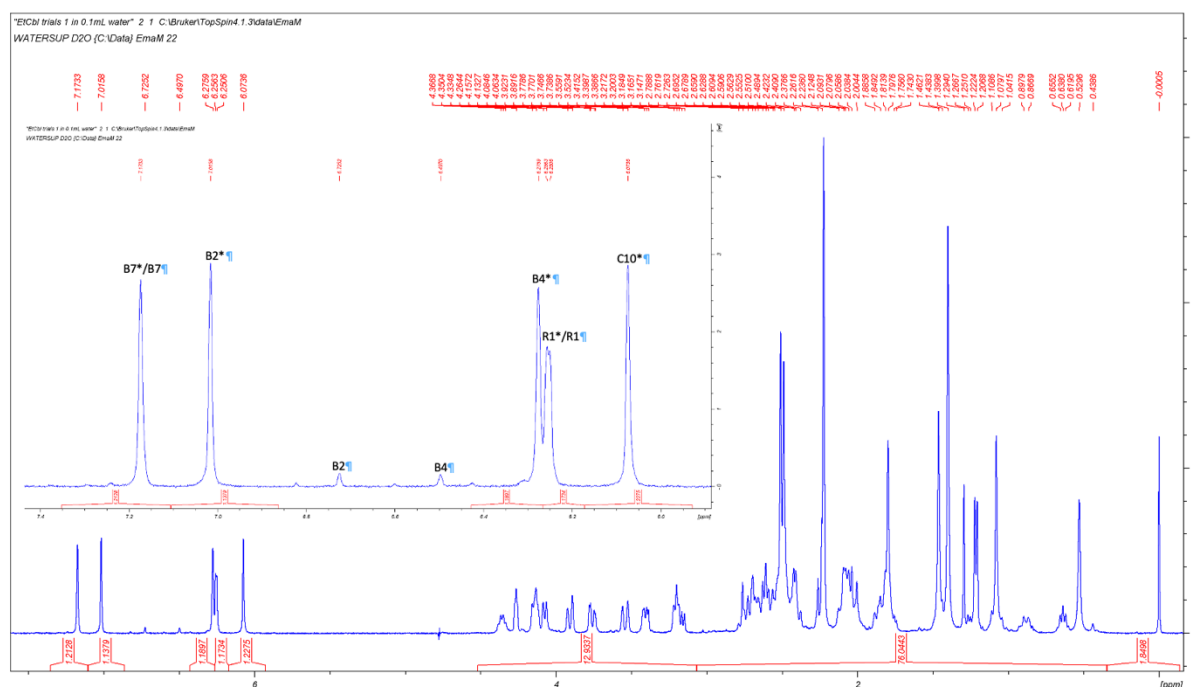


Figure 4:  $^1\text{H}$  NMR spectrum of the crude ethylcobalamin product obtained upon the addition of Cbl(I) to ethyl bromide ( $0.952\text{ mol L}^{-1}$ ) in  $\text{D}_2\text{O}$  (TSP as reference). The inset shows the aromatic region with assigned peaks at 7.17 (B7\*/B7, singlet, 2H), 7.01 (B2\*, singlet, 1H), 6.72 (B2, singlet, 1H), 6.49 (B4, singlet, 1H), 6.27 (B4\*, singlet, 1H), 6.25 (R1\*/R1, overlapping doublets, 2H), 6.07 (C10\*, singlet, 1H).

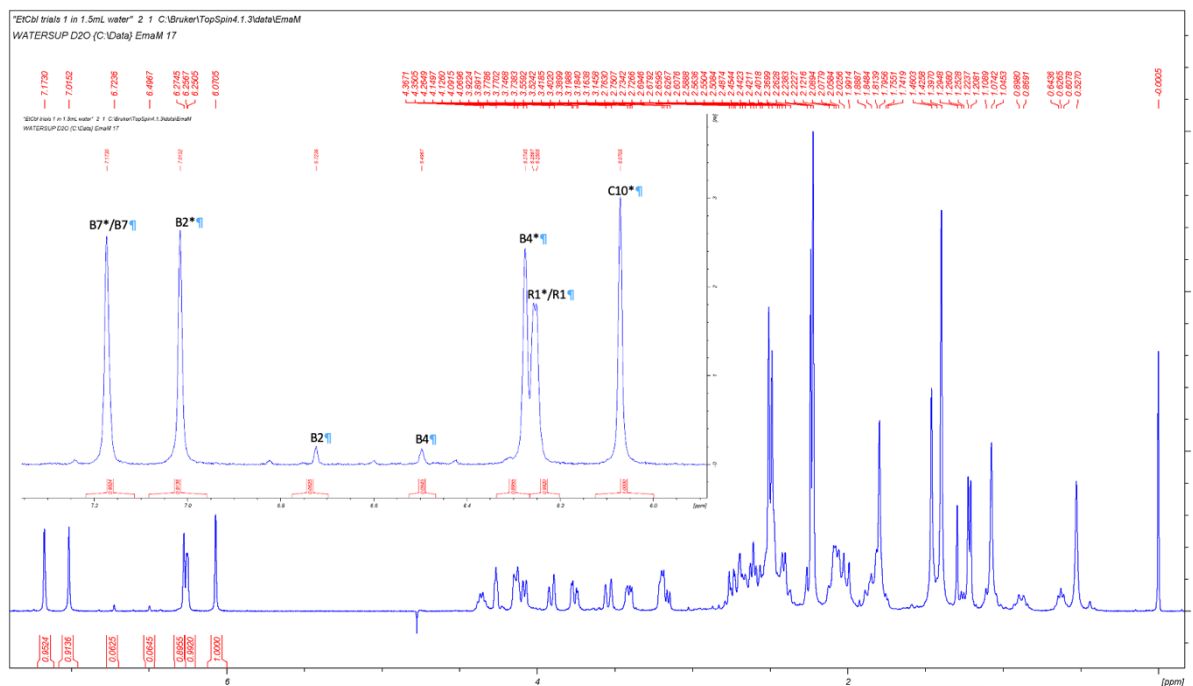
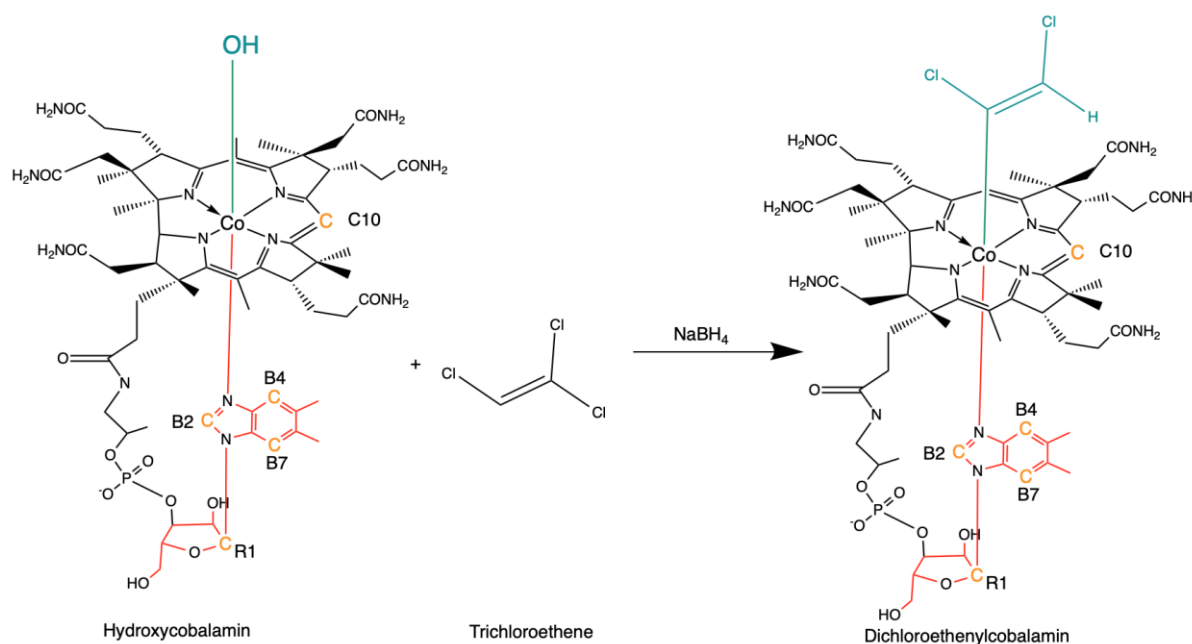


Figure 5:  $^1\text{H}$  NMR spectrum of the crude ethylcobalamin product obtained upon the addition of Cbl(I) to ethyl bromide ( $0.0635 \text{ mol L}^{-1}$ ) in  $\text{D}_2\text{O}$  (TSP as reference). The inset shows the aromatic region with assigned peaks at 7.17 (B7\*/B7, singlet, 2H), 7.01 (B2\*, singlet, 1H), 6.72 (B2, singlet, 1H), 6.49 (B4, singlet, 1H), 6.27 (B4\*, singlet, 1H), 6.25 (R1\*/R1, overlapping doublets, 2H), 6.07 (C10\*, singlet, 1H).

## 5.2 Attempted Synthesis of a Halogenated Vinylcobalamin from Cob(I)alamin and Trichloroethylene

In this synthesis the reduction of cob(III)alamin to cob(I)alamin was conducted as described above. Aqua/hydroxycobalamin ( $\text{Co}^{3+}$ ) was reduced to cob(I)alamin ( $\text{Co}^+$ ) in water using  $\text{NaBH}_4$ , and excess  $\text{NaBH}_4$  was quenched by acetone. The Cbl(I) solution was then added dropwise to a mixture of trichloroethylene and water, as shown in Scheme 2. This method was developed from section 4.1.2. Previous research conducted by others to synthesise this species showed that adding a halogenated alkene to Cbl(I) results in  $\text{B}_{12}$  catalysed dechlorination<sup>33</sup>. This is due to the cob(I)alamin species being able to react with haloalkenylcobalamin. In addition to dichlorovinylcobalamin, chlorovinylcobalamin was formed, with only chlorovinylcobalamin able to be isolated and analysed<sup>33</sup>. Additional investigations into the formation of only dichlorovinylcobalamin had been trialled, but it was proposed that the additional chloride substituents on the vinyl ligand create a reduction potential close to that of  $\text{Co}^{\text{I}}/\text{Co}^{\text{II}}$ <sup>33</sup>.



Scheme 2: Synthesis of Dichloroethenylcobalamin, Reduction Method.

Figure 6 shows the  $^1\text{H}$  NMR spectrum of the product mixture (method 1). The major peaks present in the aromatic region are at 7.17 ppm, 6.73 ppm, 6.50 ppm, 6.25 ppm, and 6.07 ppm. This matches that of hydroxycobalamin whose five characteristic peaks have been reported as 7.17 ppm (B7, singlet), 6.50 ppm (B4, singlet), and 6.74 ppm (B2, singlet) from the nucleotide base, the 6.07 ppm (C10, singlet) from the corrin ring, and the 6.25 ppm (R1, doublet) from the ribose ring in the literature<sup>31</sup>.

Therefore, this method was not successful. This could possibly be attributed to trichloroethene being insoluble in the small volume of water used, which was the primary solvent in this synthesis. As solubility is often essential for a successful synthesis, the solubility of trichloroethene in a variety of solvents was separately investigated. Vials were prepared of methanol (1 mL), ethanol (1 mL), acetone (1 mL), and water (1 mL). Trichloroethene (TCE, 0.2 mL) was then added to each vial, and shaken. The best solubility was observed in acetone, with a homogenous, clear solution obtained. There was clouding observed with some samples due to TCE only being miscible with methanol and ethanol, and there was a slight solvent layering observed for water and TCE<sup>35</sup>. Dissolving TCE in acetone was advantageous acetone was used to quench the excess  $\text{NaBH}_4$ , so dissolving TCE in acetone should not impact the synthesis. From these trials it was determined that either

acetone or an acetone/water mixture would allow for trichloroethene to be miscible in the reaction solution.

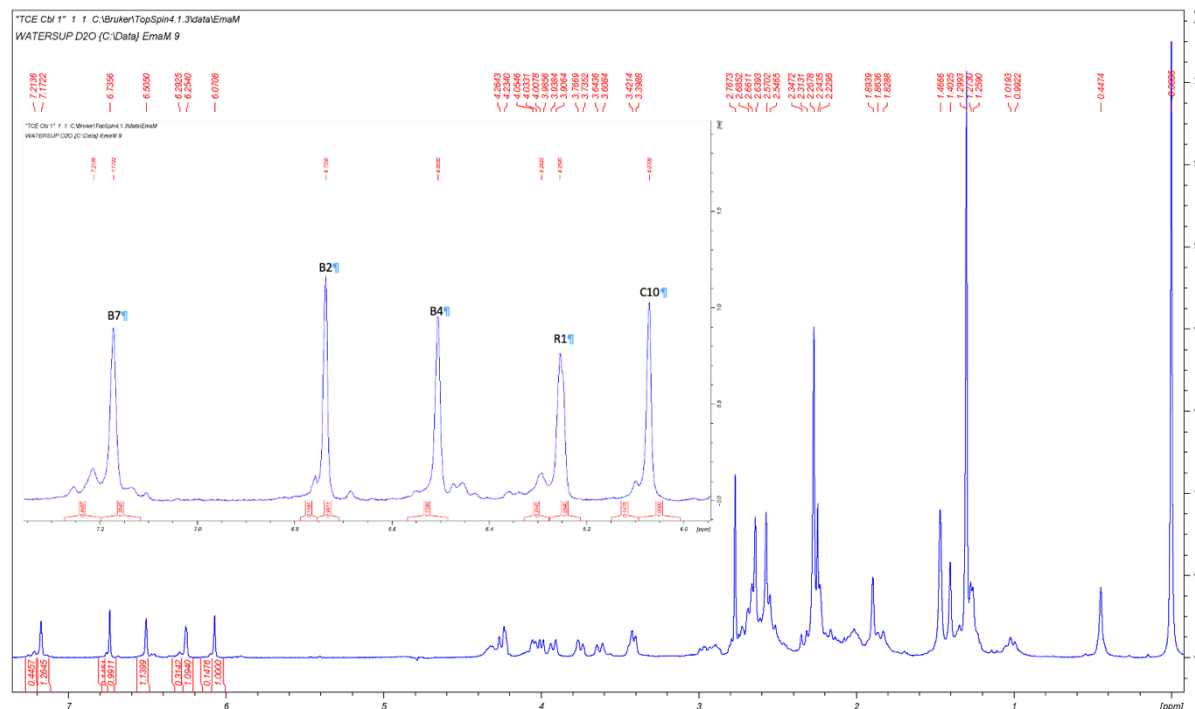


Figure 6:  $^1\text{H}$  NMR spectrum of the crude product mixture obtained by reacting Cbl(I) with TCE/water mixture ( $\text{D}_2\text{O}$ , TSP as reference). The inset shows the aromatic region with the peaks at 7.17 (B7, singlet, 1H), 6.73 (B2, singlet, 1H), 6.50 (B4, singlet, 1H), 6.25 (R1, doublet, 1H), 6.07 (C10, singlet, 1H).

In the second attempt to synthesise an alkenylcobalamin incorporating a halogenated alkene, half of the cob(I)alamin solution was added to a solution of TCE ( $0.190 \text{ mol L}^{-1}$ ,  $0.0952 \text{ mmol}$ ) in acetone ( $0.500 \text{ mL}$ ), and the other half was added to a solution of TCE ( $0.190 \text{ mol L}^{-1}$ ,  $0.0952 \text{ mmol}$ ), acetone ( $0.250 \text{ mL}$ ), and water ( $0.250 \text{ mL}$ ). The  $^1\text{H}$  NMR spectra of the crude product mixtures are given in Figures 7 and 8. The major peaks at 7.17 ppm, 6.73 ppm, 6.50 ppm, 6.25 ppm, and 6.07 ppm can be assigned to hydroxycobalamin (7.17 ppm, 6.74 ppm, 6.50 ppm, 6.25 ppm, 6.07 ppm)<sup>31</sup>. However, the minor peaks at 7.21 ppm, 7.25 ppm, 6.47 ppm, 6.45 ppm, 6.75 ppm, 6.68 ppm, 6.29 ppm, 6.35 ppm, and 5.90 ppm are observed in both Figure 7 and Figure 8, within 0.02 ppm. From chemical shifts and the peak integrations (0.2 and 0.09), it appears that there are two different minor cobalamin species present in the crude product mixtures, as cobalamins typically have 5 peaks in the aromatic region of the  $^1\text{H}$  NMR spectrum<sup>31</sup>.



Therefore, the synthesis was moderately successful as it was evident there were new products in the mixture, which was an improvement upon the previous synthesis. One of these could possibly be attributed to the desired product, dichlorovinylcobalamin, as there were no obvious peaks from the vinylic protons in the 5-6 ppm range. There were two small singlet peaks visible in the 5-6 ppm range which could be from a vinylic proton. However, the integration of both peaks is below 0.01, and since each vinylic proton should integrate for 1H, these peaks cannot be from the products discussed above. These peaks could be from a non-cobalamin species as this was a crude product mixture. Both sample mixtures resulted in similar new compound values with a small difference in the percentages of the two new compounds. This could possibly be attributed to a slight difference in the speed which the cob(I)alamin was added to the TCE solutions. Cob(I)alamin is able to perform reductive dechlorination with halogenated vinylcobalamins<sup>23</sup>. The low yield of new compounds could possibly be attributed to the short reaction time. Therefore, possible improvements on this synthesis could be an increase in the time that the reactants are allowed to react to assess if there could be an improvement in the new compound yield. As this synthesis does not use a cobalt(II) salt which would help catalyse this reaction, it may be likely that the reaction takes longer<sup>8</sup>. Another possible reason for the low yield of new compound could be that the alkylcobalamin which is produced in the experiment is not stable under the reaction conditions and is reduced to cob(II)alamin or cob(I)alamin, which once exposed to air, reverts to aqua/hydroxycobalamin. From the results of this synthesis, it is evident that another trial should be attempted with the minor products investigated to determine their composition and a longer reaction time to see the effect it has on product yields.

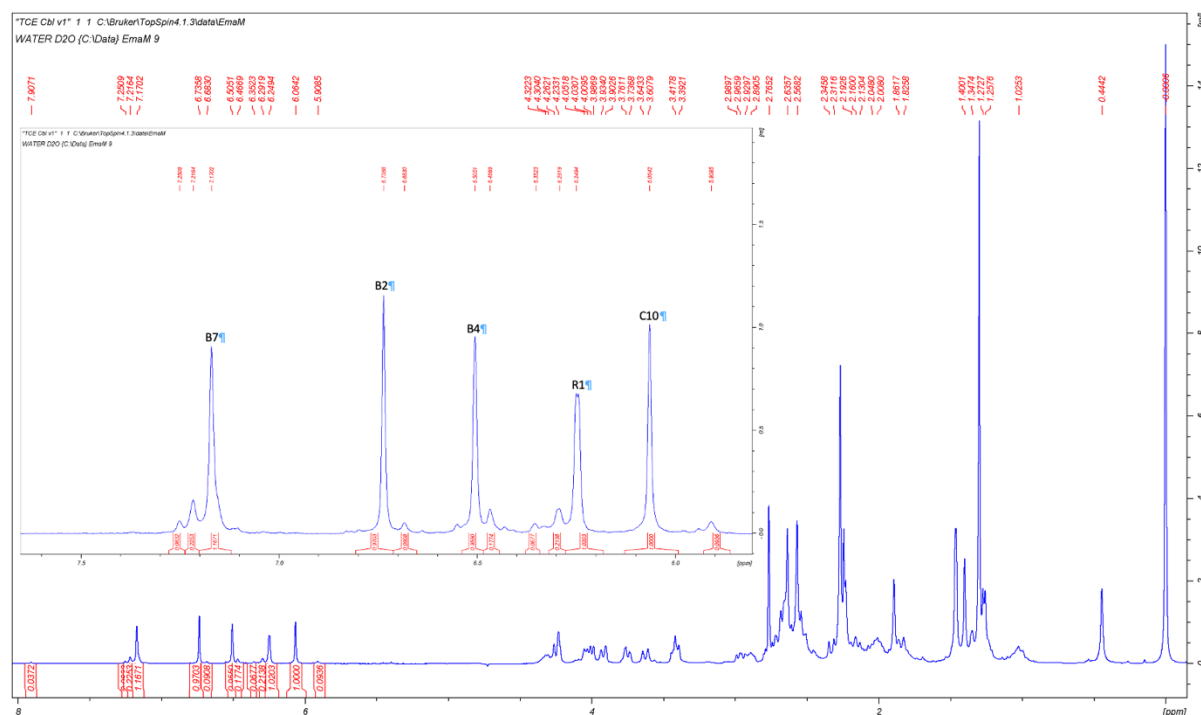


Figure 7: <sup>1</sup>H NMR spectrum of crude product mixture obtained from method 2, with TCE dissolved in acetone (D<sub>2</sub>O, TSP as reference). The inset shows the aromatic region with the major peaks at 7.17 (B7, singlet, 1H), 6.73 (B2, singlet, 1H), 6.50 (B4, singlet, 1H), 6.25 (R1, doublet, 1H), 6.07 (C10, singlet, 1H) assigned to HOCbl. Minor aromatic peaks observed at 7.25 ppm, 7.21 ppm, 6.47 ppm, 6.45 ppm, 6.75 ppm, 6.68 ppm, 6.29 ppm, 6.35 ppm, and 5.90 ppm.

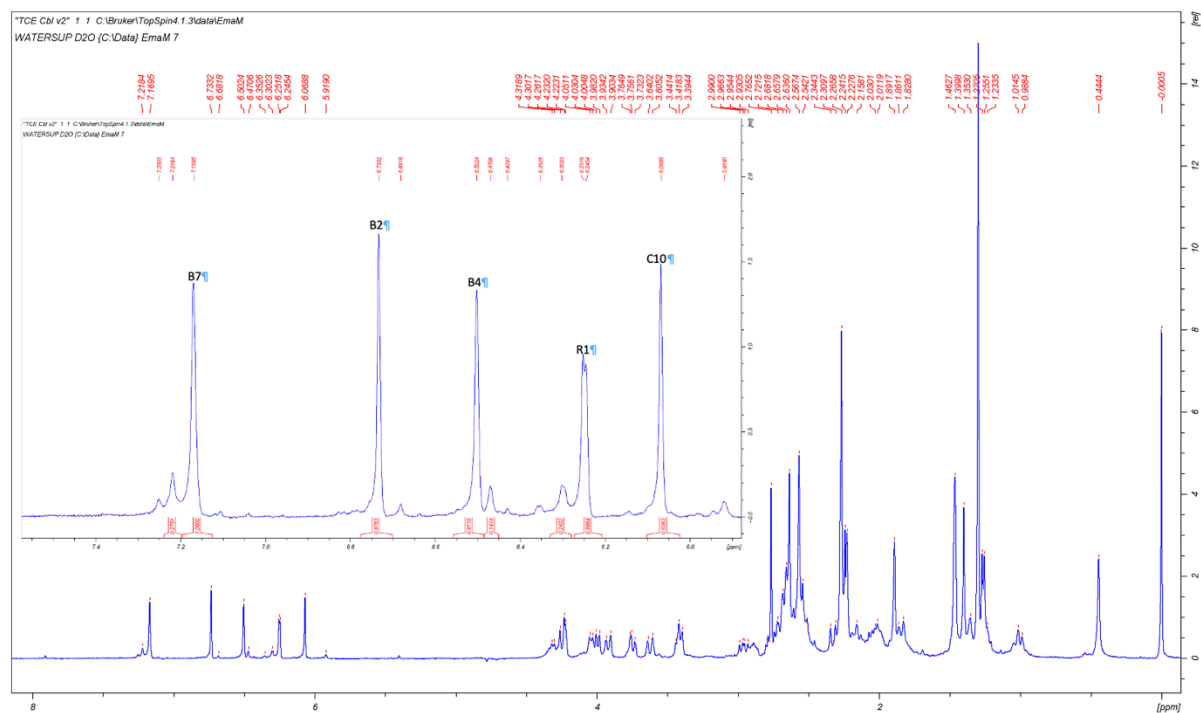


Figure 8: <sup>1</sup>H NMR spectrum of crude product obtained from method 2, with TCE dissolved in acetone/water mixture (D<sub>2</sub>O, TSP as reference). The inset shows the aromatic region with the major peaks at 7.17 (B7, singlet, 1H), 6.73 (B2, singlet, 1H), 6.50 (B4, singlet, 1H), 6.25 (R1, doublet, 1H), 6.07 (C10, singlet, 1H) assigned to HOCbl. Minor aromatic peaks observed at 7.25 ppm, 7.21 ppm, 6.47 ppm, 6.45 ppm, 6.75 ppm, 6.68 ppm, 6.29 ppm, 6.35 ppm, and 5.90 ppm.

at 7.25 ppm, 7.21 ppm, 6.47 ppm, 6.45 ppm, 6.75 ppm, 6.68 ppm, 6.29 ppm, 6.35 ppm, and 5.90 ppm.

In the third attempt, method 3, aqua/hydroxycobalamin ( $\text{Co}^{3+}$ ) was reduced to cob(I)alamin ( $\text{Co}^+$ ) in water using  $\text{NaBH}_4$ , with excess  $\text{NaBH}_4$  quenched by acetone, and this solution was added dropwise to a solution of TCE (0.00860 mL, 0.0952 mmol) in acetone (0.500 mL), over a period of ~30 minutes. The reaction mixture was stirred for a further 2 hours before the solvent was removed.

This  $^1\text{H}$  NMR spectrum was taken in methanol- $\text{d}_4$ , to compare directly with the chemical shifts of the vinyl protons of chlorovinylcobalamin and vinylcobalamin reported in literature<sup>23</sup>. From the  $^1\text{H}$  NMR spectrum obtained, Figure 9, it is evident that the major product is hydroxycobalamin (peaks at 7.13 ppm, 6.85 ppm, 6.60 ppm, 6.16 ppm, 6.11 ppm). This was determined spiking the product mixture with authentic hydroxycobalamin. Minor peaks were observed at 7.23 ppm, 7.16 ppm, 6.55 ppm, 6.21 ppm, and 5.93 ppm. Cobalamin complexes have 5 peaks in the aromatic region and each peak arises from one proton of the compound. The aromatic peaks of chlorovinylcobalamin and vinylcobalamin have not been reported in literature. However, the vinylic protons have been reported as 5.73 ppm and 5.03 ppm for chlorovinylcobalamin, and 5.59 ppm for vinylcobalamin<sup>23</sup>. As the peaks in the aromatic region and the vinyl region are each representing one proton, the peak areas were compared to attempt to assign the minor product to an identified species. The peak areas of the vinyl protons for chlorovinylcobalamin (5.73 ppm (0.078) and 5.03 ppm (0.01)) do not appear to match that of the minor aromatic peak areas 7.23 ppm (0.19), 7.16 ppm (0.30), 6.55 ppm (0.16), 6.21 ppm (0.17), and 5.93 ppm (0.14). Therefore, although chlorovinylcobalamin is present in the crude product mixture, it is a minor product species.

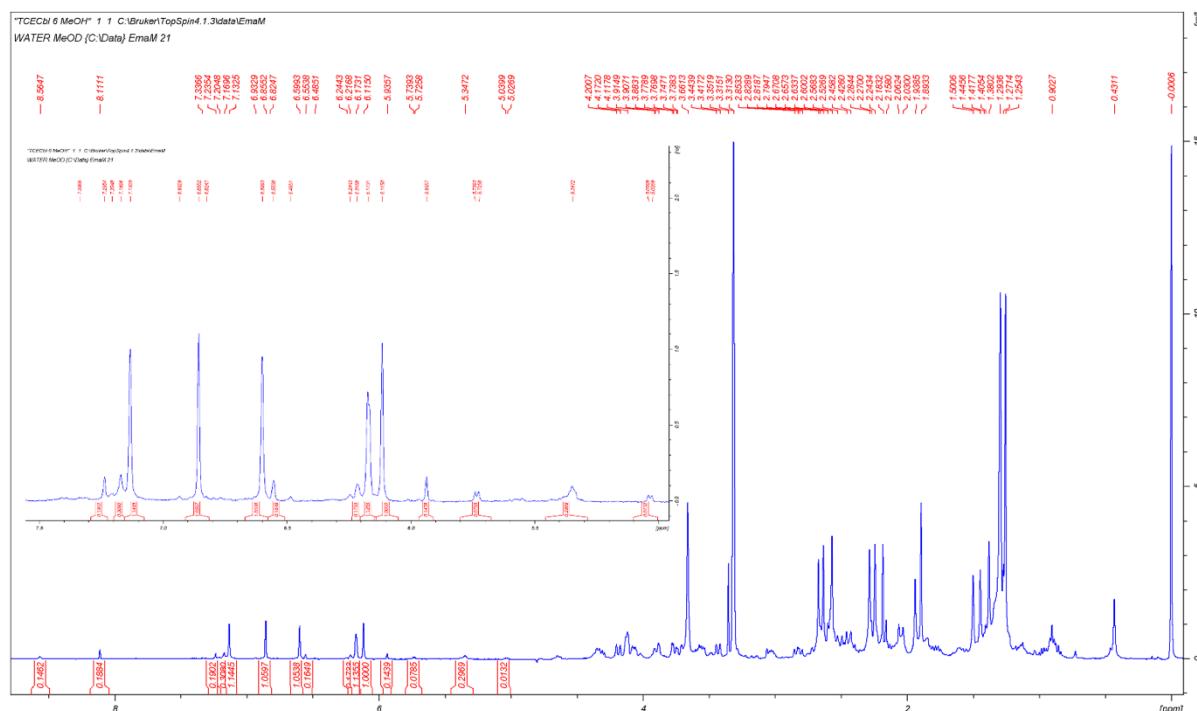


Figure 9:  $^1\text{H}$  NMR spectrum of crude product mixture obtained from method 3 (methanol-d<sub>4</sub>, TSP as reference). The inset shows the aromatic region with the major peaks at 7.13 (singlet, 1H), 6.85 (singlet, 1H), 6.59 (singlet, 1H), 6.17 (doublet, 1H), 6.11 (singlet, 1H) assigned to hydroxycobalamin. Minor peaks observed at 7.23 ppm, 7.16 ppm, 6.55 ppm, 6.21 ppm, and 5.93 ppm.

A second  $^1\text{H}$  NMR spectrum of the product mixture was recorded in  $\text{D}_2\text{O}$  which contained acidic MES buffer to ensure there would only be aquacobalamin present in solution ( $\text{pK}_\text{a}$   $\text{H}_2\text{OCbl}^+ = 7.8$ )<sup>36</sup>. This can be seen in Figure 10. Unfortunately, there was no longer any evidence of vinyl peaks in solution. It was found that when dissolved in buffer, a very basic solution was obtained. To lower the pH down to the desired value (pH 6), a solution of HCl (1 M) was added dropwise. This may have resulted in decomposition occurring, resulting in the  $^1\text{H}$  NMR spectra shown below, with what appears to be three major species in solution, which was not consistent with the  $^1\text{H}$  NMR spectrum of the product mixture shown in Figure 9.

This required further analysis and a sample was therefore prepared for LC-MS analysis. The UV Chromatograms taken at 254 nm and 361 nm were not shown as they did not clearly show all products present in the mixture. Therefore, the resulting total ion count chromatogram (TIC) is shown in Figure 11. Peaks were observed at 8.78, 9.06, 9.33, 17.58, 21.15, 21.55 minutes. The peak at 8.78 minutes was assigned to H<sub>2</sub>OCbl. A m/z value of 664.9 was observed for this peak, which corresponds to [Cbl(II) + H]<sup>2+</sup>, C<sub>62</sub>H<sub>89</sub>O<sub>14</sub>N<sub>13</sub>CoP. The ESI source reduces H<sub>2</sub>OCbl(III) to Cbl(II)<sup>+37</sup>. With unstable ligands a similar result may be observed, as during the separation process along the column, the ligand is removed, resulting in observed [Cbl + H]<sup>2+</sup>. This can be seen in the peak at 9.33 minutes which had an m/z value of 665.0, corresponding to [Cbl(II) + H]<sup>2+</sup>, C<sub>62</sub>H<sub>89</sub>O<sub>14</sub>N<sub>13</sub>CoP. The peaks at 9.06 and 17.58 had observed m/z values of 673.2 and 673.0 which could potentially match that of the aquacobalamin lactone (calculated m/z for [(H<sub>2</sub>OCbl(lactone)) + H<sub>2</sub>O + H]<sup>2+</sup>, C<sub>62</sub>H<sub>88</sub>O<sub>16</sub>N<sub>12</sub>CoP = 673.3; calculated m/z for [(H<sub>2</sub>OCbl(lactone)) + H<sub>2</sub>O]<sup>+</sup>, C<sub>62</sub>H<sub>87</sub>O<sub>16</sub>N<sub>12</sub>CoP = 1345.6). There is evidence of chlorovinylcobalamin possibly being in solution, as shown by the peaks observed at 21.15 and 21.55 minutes. The observed m/z values were 695.9 and

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695.8, which could potentially match that of the monochlorovinylcobalamin (calculated  $m/z$  for  $[\text{MCECbl} + 2\text{H}^+]^{2+}$ ,  $\text{C}_{64}\text{H}_{92}\text{CoN}_{13}\text{O}_{14}\text{PCl} = 695.9$ ; calculated  $m/z$  for  $[\text{MCECbl} + \text{H}^+]^+$ ,  $\text{C}_{64}\text{H}_{91}\text{CoN}_{13}\text{O}_{14}\text{PCl} = 1391.6$ ). Although it is not shown in the chromatogram in Figure 11, there was an abundance of species eluting at higher acetonitrile concentrations, in the washing part of the method. This LC-MS would ideally be repeated; however, this was not possible due to time constraints.

Therefore, the synthesis was slightly successful as there is a set of peaks which do not match that of the starting material and there is a shift in major species shown to a possible halogenated vinylcobalamin product. Further investigation is required to determine the structure of the products present in this reaction mixture.

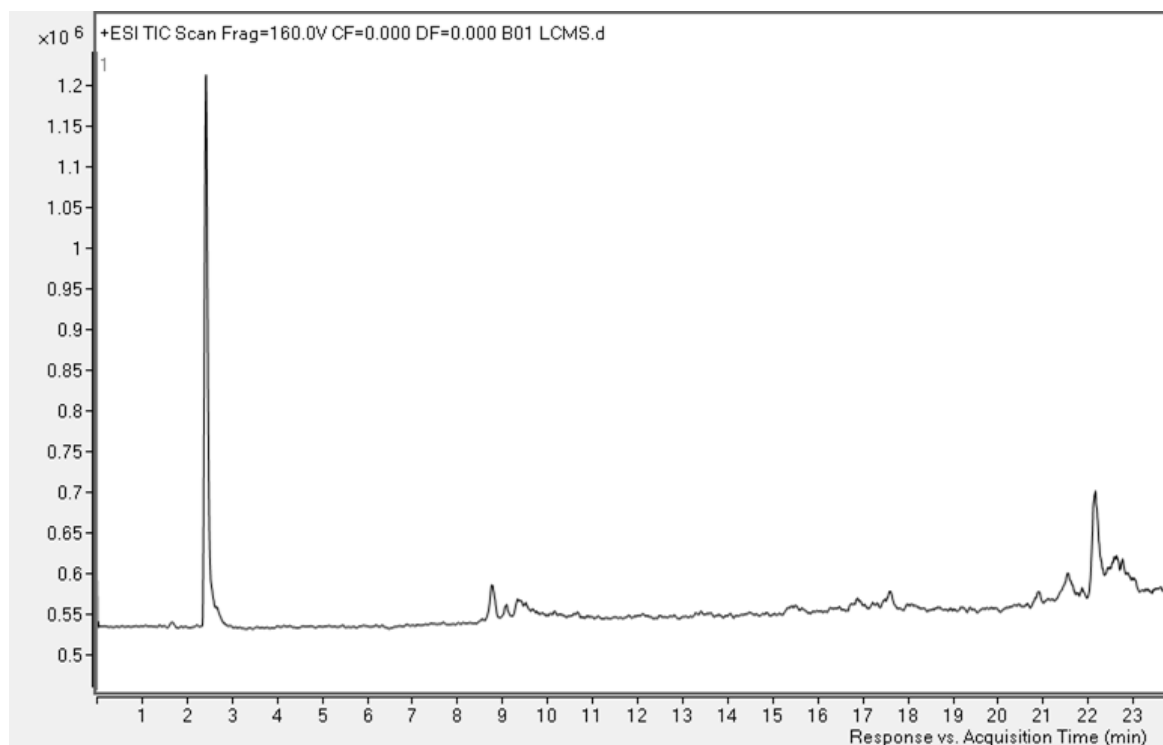


Figure 11: LC-MS Total Ion Chromatography of crude product from attempted synthesis of a halogenated vinylcobalamin, Method 3.

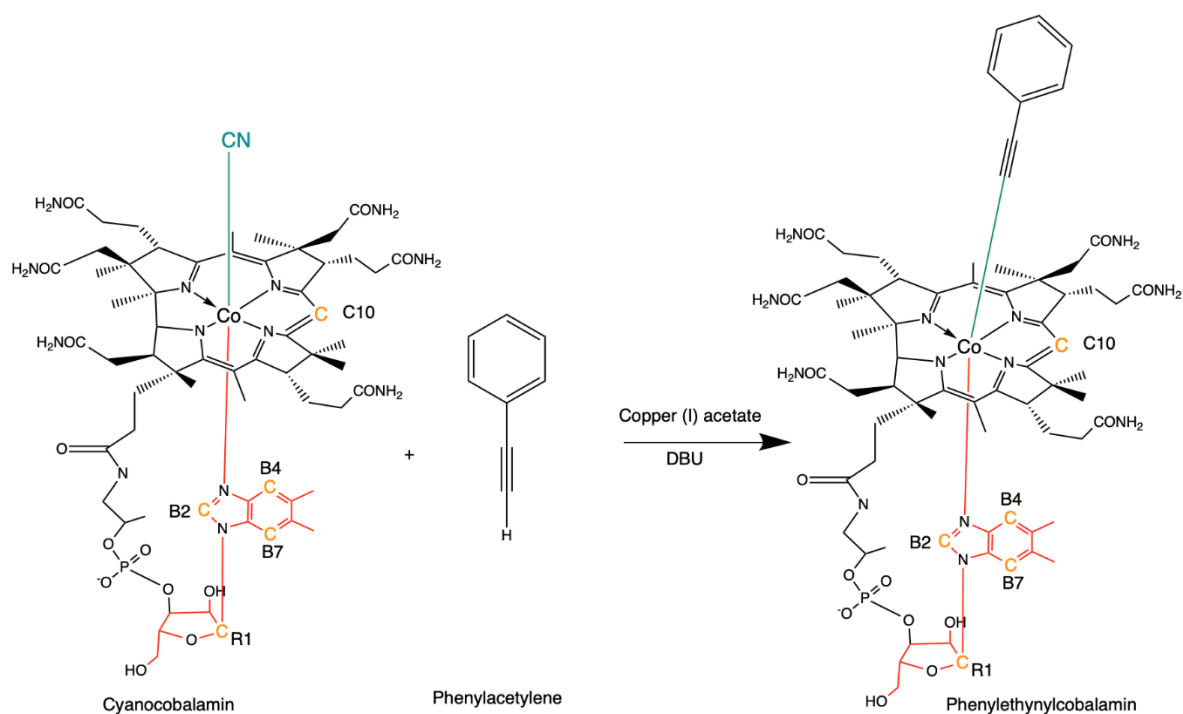
Table 1: Calculated  $m/z$  of possible products found in LCMS results; TCECbl = Trichloroethenylcobalamin, DCECbl = Dichloroethenylcobalamin, MCECbl = Monochloroethenylcobalamin.

Complex	Possible Molecular Ion	Molecular Formula	$\text{M}^+ + 1$	$\text{M}^{2+} + 2$
DCECbl	$[\text{DCECbl} + \text{H}^+]^+$	$\text{C}_{64}\text{H}_{89}\text{CoN}_{13}\text{O}_{14}\text{PCl}_2 + \text{H}$	1424.518	712.259

	$[\text{DCECbl} + 2\text{H}^+]^{2+}$	$\text{C}_{64}\text{H}_{89}\text{CoN}_{13}\text{O}_{14}\text{PCl}_2 + \text{H}_2$	1425.525	712.7625
	$[\text{DCECbl} + \text{Na}^+]^+$	$\text{C}_{64}\text{H}_{89}\text{CoN}_{13}\text{O}_{14}\text{PCl}_2 + \text{Na}$	1446.5	723.25
	$[\text{DCECbl} + 2\text{Na}^+]^{2+}$	$\text{C}_{64}\text{H}_{89}\text{CoN}_{13}\text{O}_{14}\text{PCl}_2 + \text{Na}_2$	1469.489	734.7445
	$[\text{DCECbl} + \text{Na}^+ + \text{H}^+]^{2+}$	$\text{C}_{64}\text{H}_{89}\text{CoN}_{13}\text{O}_{14}\text{PCl}_2 + \text{Na} + \text{H}$	1447.507	723.7535
MCECbl	$[\text{MCECbl} + \text{H}^+]^+$	$\text{C}_{64}\text{H}_{90}\text{CoN}_{13}\text{O}_{14}\text{PCl} + \text{H}$	1390.557	695.2785
	$[\text{MCECbl} + 2\text{H}^+]^{2+}$	$\text{C}_{64}\text{H}_{90}\text{CoN}_{13}\text{O}_{14}\text{PCl} + \text{H}_2$	1391.564	695.782
	$[\text{MCECbl} + \text{Na}^+]^+$	$\text{C}_{64}\text{H}_{90}\text{CoN}_{13}\text{O}_{14}\text{PCl} + \text{Na}$	1412.539	706.2695
	$[\text{MCECbl} + 2\text{Na}^+]^{2+}$	$\text{C}_{64}\text{H}_{90}\text{CoN}_{13}\text{O}_{14}\text{PCl} + \text{Na}_2$	1435.528	717.764
	$[\text{MCECbl} + \text{Na}^+ + \text{H}^+]^{2+}$	$\text{C}_{64}\text{H}_{90}\text{CoN}_{13}\text{O}_{14}\text{PCl} + \text{Na} + \text{H}$	1413.546	706.773
VinylCbl	$[\text{VinylCbl} + \text{H}^+]^+$	$\text{C}_{64}\text{H}_{91}\text{CoN}_{13}\text{O}_{14}\text{P} + \text{H}$	1356.596	678.298
	$[\text{VinylCbl} + 2\text{H}^+]^{2+}$	$\text{C}_{64}\text{H}_{91}\text{CoN}_{13}\text{O}_{14}\text{P} + \text{H}_2$	1357.603	678.8015
	$[\text{VinylCbl} + \text{Na}^+]^+$	$\text{C}_{64}\text{H}_{91}\text{CoN}_{13}\text{O}_{14}\text{P} + \text{Na}$	1378.578	689.289
	$[\text{VinylCbl} + 2\text{Na}^+]^{2+}$	$\text{C}_{64}\text{H}_{91}\text{CoN}_{13}\text{O}_{14}\text{P} + \text{Na}_2$	1401.567	700.7835
	$[\text{VinylCbl} + \text{Na}^+ + \text{H}^+]^{2+}$	$\text{C}_{64}\text{H}_{91}\text{CoN}_{13}\text{O}_{14}\text{P} + \text{Na} + \text{H}$	1379.585	689.7925

### 5.3 Reduction-free Synthesis of Phenylethynylcobalamin

All attempts at synthesising halogenated alkenylcobalamins using TCE were not especially successful. A different approach was therefore explored, which avoided using the highly nucleophilic cob(I)alamin intermediate. A controlled experiment was carried out to check that this synthetic procedure was successful in our hands. The successful synthesis of phenylethynylcobalamin using a reduction-free method had previously been reported<sup>15</sup>. They achieved a yield of 96% using a cyanide scavenger, copper(I) acetate, and a base 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in anhydrous dimethylacetamide (DMA), as shown in Scheme 3.



Scheme 3: Synthesis of Phenylethynylcobalamin.

Unfortunately, the solvent dimethylacetamide could not be obtained until towards the end of this project, due to lengthy delays in ordering of the chemical by AUT as a result of lockdown, and shipping delays associated with COVID19. For this reason, the attempted synthesis of phenylethynylcobalamin was initially trialled using DMF in place of DMA as this was the initially trialled solvent used in the literature reporting the reduction-free method<sup>15</sup>. In this synthesis copper(I) acetate is used in catalytic amounts to stretch and ultimately cleave the Co – C bond of cyanocobalamin. The base DBU is used to remove the terminal proton from the ligand phenylacetylene so that it may coordinate to the cobalt metal centre of cobalamin. The work-up of this reaction involved the precipitation of the crude product mixture onto cotton wool in a separating funnel containing in diethyl ether. The product on the cotton wool was washed twice with ethyl acetate. This was done as the newly formed cobalamin complex should be insoluble in these solvents and precipitates nicely onto cotton wool, so organic reagents and solvents can be easily removed. Using methanol to remove the product from the cotton wool is also beneficial as the remaining copper salt formed is insoluble in water and alcohol and is trapped as a fine precipitate in the cotton wool. This method was found in the literature<sup>6</sup>.



The  $^1\text{H}$  NMR spectrum of the crude product mixture is shown in Figure 12. The chemical shifts of the product were compared to the starting material cyanocobalamin, with its characteristic aromatic peaks being the B7 (7.28 ppm, singlet), B4 (6.51 ppm, singlet), and B2 (7.10 ppm, singlet) from the nucleotide base, the C10 (6.09 ppm, singlet) from the corrin ring, and the R1 (6.36 ppm, doublet) from the ribose ring<sup>31</sup>. The chemical shifts of the major product present in the mixture matched that of the starting material, with minor product peaks at 7.57 ppm, 7.49 ppm, 7.28 ppm, 9.98 ppm, 6.56 ppm, 6.26 ppm, and 6.01 ppm, as seen in Figure 12. The  $^1\text{H}$  NMR spectrum was recorded in  $\text{D}_2\text{O}$  so as to provide a direct comparison with other cobalamin compounds synthesised in this report as well as the reported CNCbl aromatic peaks<sup>31</sup>. Therefore, this synthesis was not successful. This lack of success in this synthesis was attributed to the starting material cyanocobalamin having limited solubility in DMF. For this reason, another attempt was carried out to maximise the amount of CNCbl dissolved in DMF.

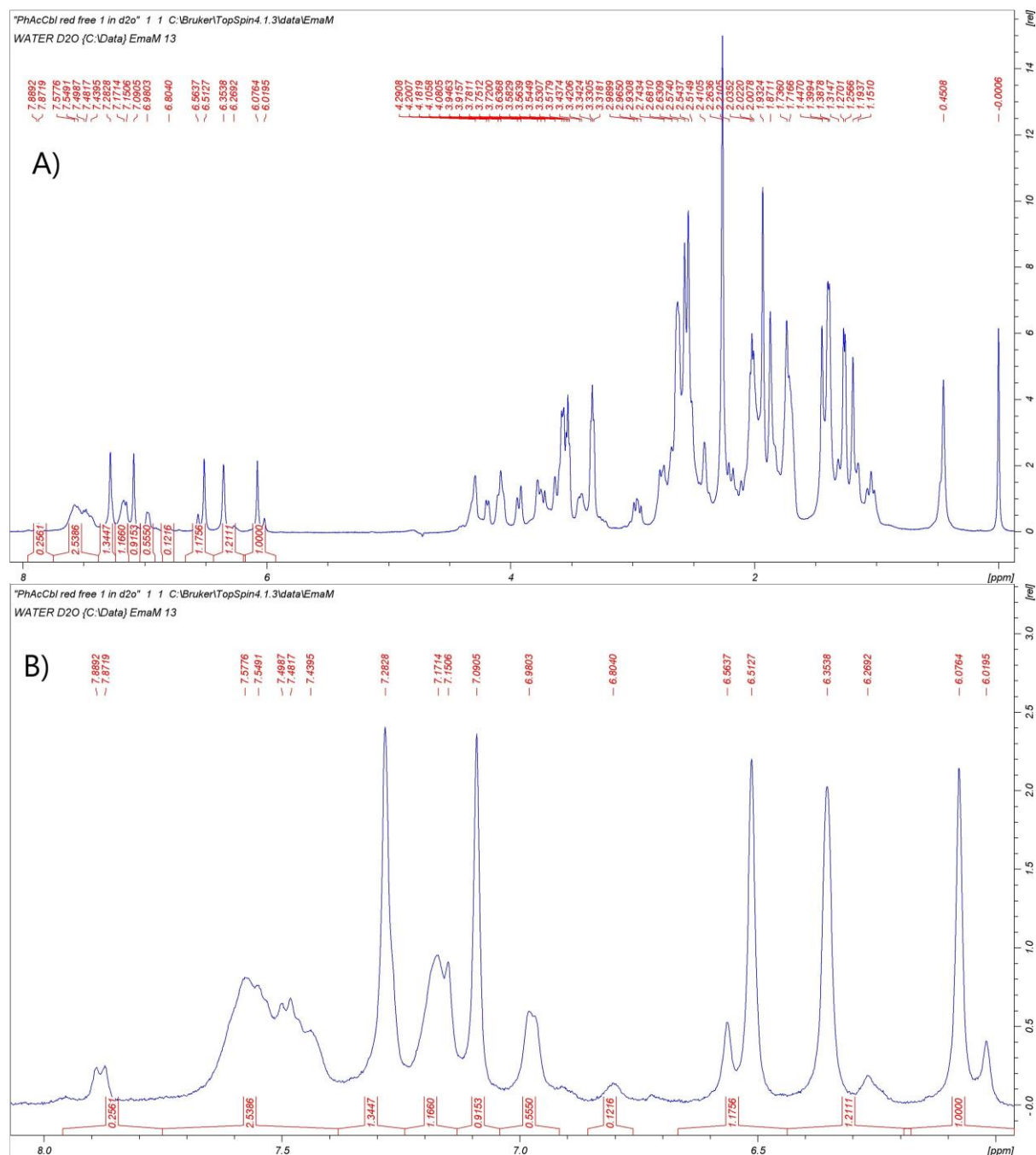


Figure 12: A)  $^1\text{H}$  NMR spectrum of crude product mixture of the first attempted synthesis of Phenylethynylcobalamin, Method 1 ( $\text{D}_2\text{O}$ , TSP as reference). B)  $^1\text{H}$  NMR spectrum shows the aromatic region of A with the major peaks at 7.28 (singlet, 1H), 7.09 (singlet, 1H), 6.51 (singlet, 1H), 6.35 (doublet, 1H), and 6.07 (singlet, 1H) which can be assigned to  $\text{CNCbl}^{31}$ . Minor peaks observed at 7.57 ppm, 7.49 ppm, 7.28 ppm, 9.98 ppm, 6.56 ppm, 6.26 ppm, and 6.01 ppm.

In the second attempt, Method 2, cyanocobalamin and copper(I) acetate were stirred in DMF overnight in an oil bath ( $40^\circ\text{C}$ ) until the mixture was homogenous. After this the vial of DMF, DBU, and phenylacetylene was added, allowing for phenylethynylcobalamin to be formed.



spectrum of the crude product mixture was also recorded in DMSO to directly compare with literature chemical shifts for phenylethynylcobalamin<sup>29</sup>. The characteristic peaks of the desired phenylethynylcobalamin product are 7.69 (singlet, 1H), 7.57 (singlet, 2H), 7.48 (singlet, 2H), 7.30 (singlet, 1H), 7.26 (singlet, 1H), 7.11 (singlet, 1H), 7.08 (singlet, 1H), 7.05-6.99 (multiplet, 3H), 6.98-6.94 (multiplet, 1H), 6.93 (singlet, 1H), 6.88 (singlet, 1H), 6.74 (singlet, 1H), 6.74-6.70 (multiplet, 1H), 6.63 (singlet, 1H), 6.50 (singlet, 1H), 6.48 (singlet, 1H), 6.33-6.27 (multiplet, 1H), 6.25 (singlet, 1H), 6.06 (broad singlet, 1H), and 5.83 ppm (singlet, 1H)<sup>29</sup>. The chemical shift peaks of phenylacetylene in DMSO-d<sub>6</sub> have been reported as 7.50, 7.48, 7.40, 7.38, 7.36, and 4.17 ppm<sup>39</sup>. The product present in the <sup>1</sup>H NMR in Figure 14 had peaks present of 7.83 (singlet), 7.67 (singlet), 7.60 (singlet), 7.55 (multiplet), 7.48 (singlet), 7.31 (singlet), 7.28 (singlet), 7.10 (singlet), 7.08 (singlet), 7.07 (singlet), 7.05-7.01 (multiplet), 6.99-6.96 (multiplet), 6.91 (singlet), 6.86 (singlet), 6.82(singlet), 6.79(singlet), 6.76 (singlet), 6.75 (singlet), 6.73-6.71 (multiplet), 6.62 (singlet), 6.51 (singlet), 6.48 (singlet), 6.38 (singlet), 6.32 (multiplet), 6.25 (singlet), 6.21 (singlet), 6.04 (singlet), 5.90 (singlet), and 5.83 (singlet). This is in agreement with the literature values for the product phenylethynylcobalamin, within experimental error, there are several additional peaks which is likely due to impurities present in the sample.

This synthesis was moderately successful, as shown by the <sup>1</sup>H NMR spectra in Figure 14. However, the solvent was difficult to completely remove. The <sup>1</sup>H NMR spectrum was recorded using DMSO-d<sub>6</sub> to directly compare with the literature. This method could likely be improved on by replicating what was done in literature which uses DMA as the solvent as it results in a high yield of product<sup>15</sup>. Another possible reason for an incomplete reaction could be that the reaction requires longer to react. As there was evidence of a of the desired product present in the <sup>1</sup>H NMR spectrum, it was decided that this method was promising enough to use for the synthesis of trichloroethenylcobalamin using a “reduction-free” method.

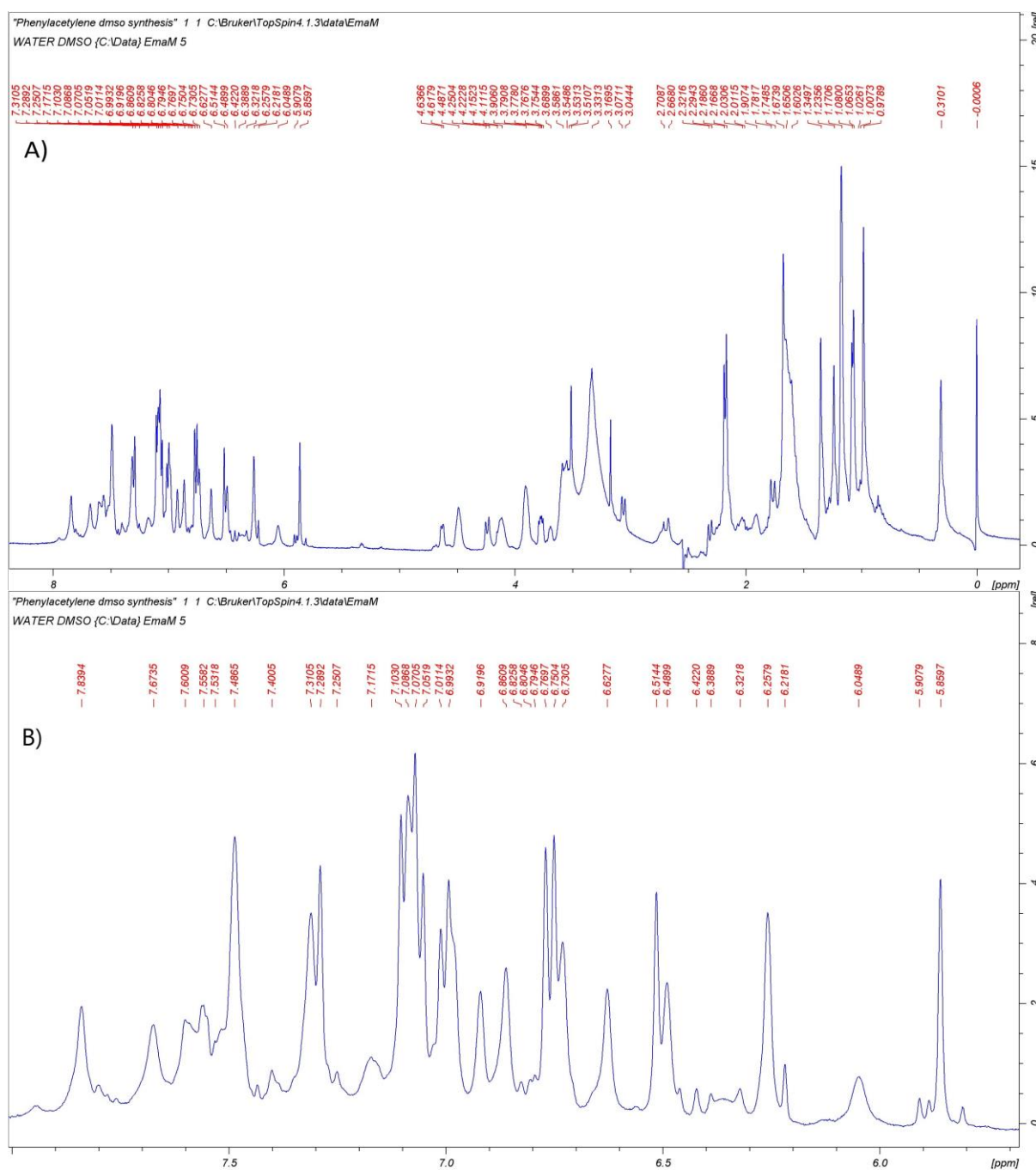
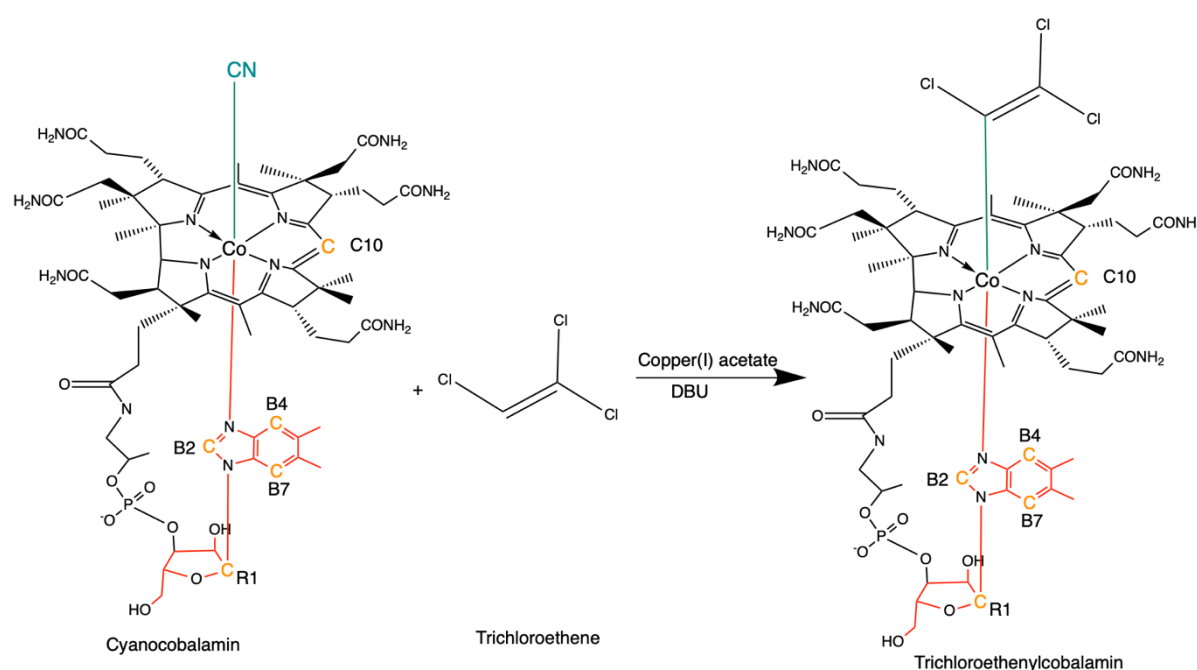


Figure 14: A)  $^1\text{H}$  NMR spectrum of crude product mixture of the third attempted synthesis of phenylethynylcobalamin, Method 3 (DMSO- $d_6$ , TMS as reference). B) Shows the aromatic region of A with the major peaks at 7.83 (singlet), 7.67 (singlet), 7.60 (singlet), 7.55 (multiplet), 7.48 (singlet), 7.31 (singlet), 7.28 (singlet), 7.10 (singlet), 7.08 (singlet), 7.07 (singlet), 7.05-7.01 (multiplet), 6.99-6.96 (multiplet), 6.91 (singlet), 6.86 (singlet), 6.82 (singlet), 6.79 (singlet), 6.76 (singlet), 6.75 (singlet), 6.73-6.71 (multiplet), 6.62 (singlet), 6.51 (singlet), 6.48 (singlet), 6.38 (singlet), 6.32 (multiplet), 6.25 (singlet), 6.21 (singlet), 6.04 (singlet), 5.90 (singlet), and 5.83 (singlet).

## 5.4 Attempted Synthesis of Trichloroethenylcobalamin Using a Reduction-Free Method

This method was developed from Method 3 of the phenylethynylcobalamin synthesis, section 4.6.3. This synthesis was conducted under anaerobic conditions using the Schlenk line. Cyanocobalamin and copper(I) acetate were dissolved in anhydrous DMSO with stirring in an oil bath (40°C). Once the mixture was homogenous a solution of DBU, TCE, and DMSO was added dropwise with stirring and the mixture was left to react for ~5 hours. The reaction is shown in Scheme 4. The product mixture was then washed as described in 4.7.1, and the precipitate was removed using methanol. Rotary evaporation was used to bring the product down to dryness.



Scheme 4: Synthesis of Trichloroethenylcobalamin, Reduction-Free Method.

The  $^1\text{H}$  NMR spectrum is given in Figure 15. There was a mixture of two major cobalamin products present in the product mixture, as each cobalamin species has 5 aromatic peaks, with an overlap of the R1 doublet at 6.35 ppm. One of the major products present has peaks which match that of cyanocobalamin (7.28 ppm (B7, singlet), 6.51 ppm (B4, singlet), and 7.10 ppm (B2, singlet) from the nucleotide base, the 6.09 ppm (C10, singlet) from the corrin ring, and the 6.36 ppm (R1, doublet) from the ribose ring)<sup>31</sup>. However, what is interesting to note is that there are other peaks present in the solution which could possibly be attributed

to the desired trichloroethenylcobalamin. These peaks are 7.24 ppm, 6.85ppm, 6.82 ppm, 6.25 ppm, and 6.05 ppm.

This synthesis was concluded to be partially successful due to the presence of a new compound in the product mixture not attributed to the cyanocobalamin starting material or aqua/hydroxycobalamin. This synthesis could be possibly be optimised through the use of the solvent recommended in literature for this synthesis<sup>15</sup>. Due to shipping and lockdown delays the recommended solvent had not yet arrived when this synthesis was attempted. For this reason, subsequent synthesis attempts were conducted using the recommended anhydrous dimethylacetamide as the solvent for the reaction, which had just arrived.

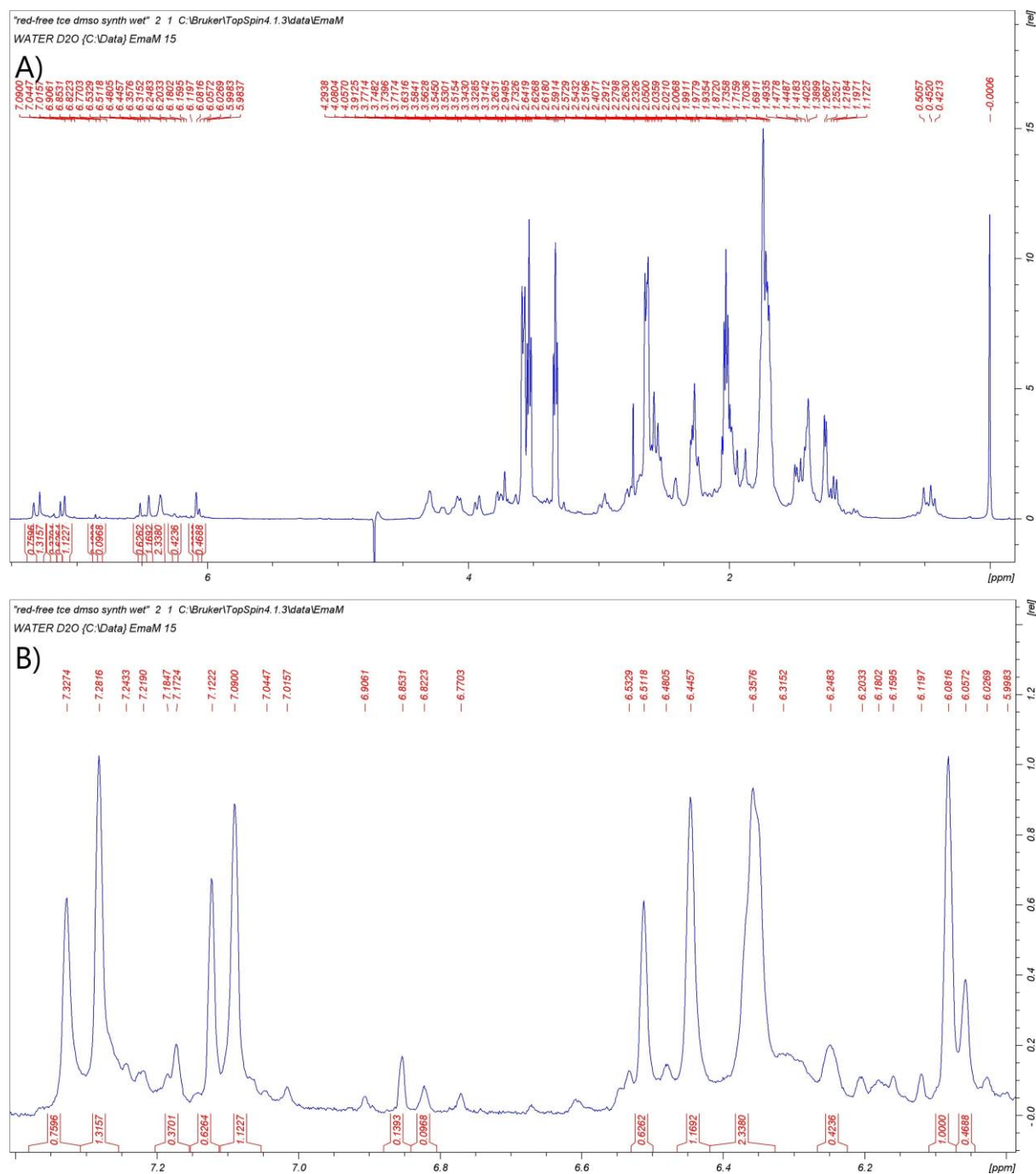


Figure 15: A) <sup>1</sup>H NMR spectrum of the crude product mixture for the attempted synthesis of trichloroethenylcobalamin, reduction-free Method 1 (D<sub>2</sub>O, TSP as reference). B) <sup>1</sup>H NMR spectrum shows the aromatic region of A with the major peaks at 7.32 (singlet, 1H), 7.28(singlet, 1H), 7.12 (singlet, 1H), 7.09(singlet, 1H), 6.51(singlet, 1H), 6.44(singlet, 1H), 6.35 (doublet, 1H), 6.08 (singlet, 1H), and 6.05 (singlet, 1H).

In the second attempt (Method 2), the solvent DMA was used as recommended in the literature<sup>15</sup>. This synthesis was conducted using air-free techniques on the Schlenk line. Cyanocobalamin and copper(I) acetate were added to anhydrous DMA while stirring. The



remaining reagents DBU, TCE, DMA were added to the reaction flask dropwise. This reaction was allowed to proceed overnight (~20 hours). The precipitation in this attempt was modified from the original separating funnel technique used previously, to the centrifuge method conducted in the literature<sup>15</sup>. This method was faster and enabled the product mixture to be brought to dryness in a shorter period of time.

The results from the second attempt are shown in Figure 16. From this <sup>1</sup>H NMR spectrum it is challenging to determine the number of cobalamin species present in solution. There appears to be approximately four species in the product mixture, with major peaks at 7.40, 7.36, 7.32, 7.30, 7.28, 7.24, 7.19, 7.17, 7.12, 7.01, 6.52, 6.49, 6.48, 6.46, 6.40, 6.36, 6.35, 6.33, 6.12, and 6.10 ppm. The number of species present in the product mixture can be estimated by the number of major peaks present in the aromatic region as intact cobalamin derivatives typically have 5 characteristic aromatic peaks. The starting material cyanocobalamin is probably in the product mixture as peaks were observed at 7.28, 6.51, 7.10, 6.09, and 6.35 ppm<sup>31</sup>.

From these results it is difficult to determine whether this synthesis was successful. A possible contributor to the large number of peaks observed could be the reaction mixture being heated by an oil bath (40°C). Unlike phenylethynylcobalamin, trichloroethenylcobalamin may be less stable at higher temperatures. For this reason, a new procedure was trialled at a lower reaction temperature.

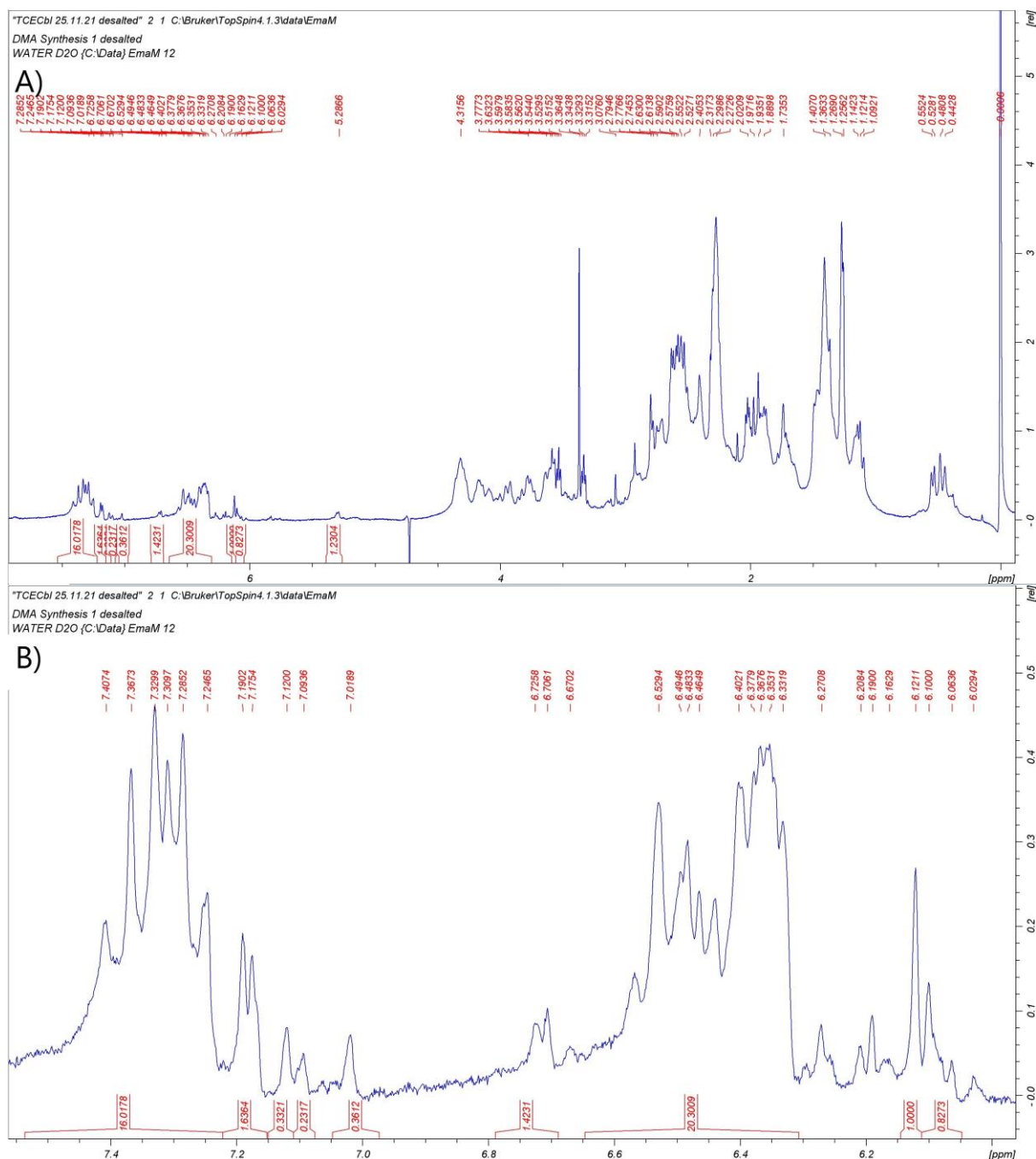


Figure 16: A) <sup>1</sup>H NMR spectrum of the crude product mixture obtained from the attempted synthesis of trichloroethenylcobalamin reduction-free, Method 2 (D<sub>2</sub>O, TSP as reference). B) <sup>1</sup>H NMR spectrum shows the aromatic region of A with the major peaks at 7.40, 7.36, 7.32, 7.30, 7.28, 7.24, 7.19, 7.17, 7.12, 7.01, 6.52, 6.49, 6.48, 6.46, 6.40, 6.36, 6.35, 6.33, 6.12, and 6.10 ppm.

In the third attempt, Method 3, the synthesis was conducted using an ice bath and left to react overnight. The ice bath slowly warmed up to room temperature overnight. This was the only variable changed, with aliquots taken at 20 minutes, 4 hours, and 20 hours. The product sample removed at 4 hours contained only 2 cobalamin products, as determined by

$^1\text{H}$  NMR spectroscopy. The product sample taken at 20 minutes was predominantly starting material, and the 20-hour aliquot was unchanged from the 4-hour aliquot.

The  $^1\text{H}$  NMR spectrum of the crude product mixture after 4 hours is shown in Figure 17. The major peaks present are 7.28 (singlet, 1H), 7.09 (singlet, 1H), 6.51 (singlet, 1H), 6.35 (doublet, 1H), and 6.08 (singlet, 1H). These major peaks match that of cyanocobalamin<sup>31</sup>. However, what is of interest are the minor peaks present in the solution at 7.16 (singlet, 1H), 7.15 (singlet, 1H), 6.73 (singlet, 1H), 6.45 (singlet, 1H), 6.24 (singlet, 1H), and 6.06 (singlet, 1H). These peaks are different than that of HOCbl (7.16, 6.51, 6.44, 6.26, and 6.22 ppm)<sup>31</sup>. These could potentially be attributed to the desired product trichloroethenylcobalamin. From these results it is demonstrated that the temperature of the reaction mixture has a great effect on the observed products synthesised. However, due to the low yield of this reaction mixture, it is evident that a higher temperature may be better. A new synthesis was therefore attempted at a slightly higher temperature to attempt to produce a higher yield of the second minor product observed in the synthesis.

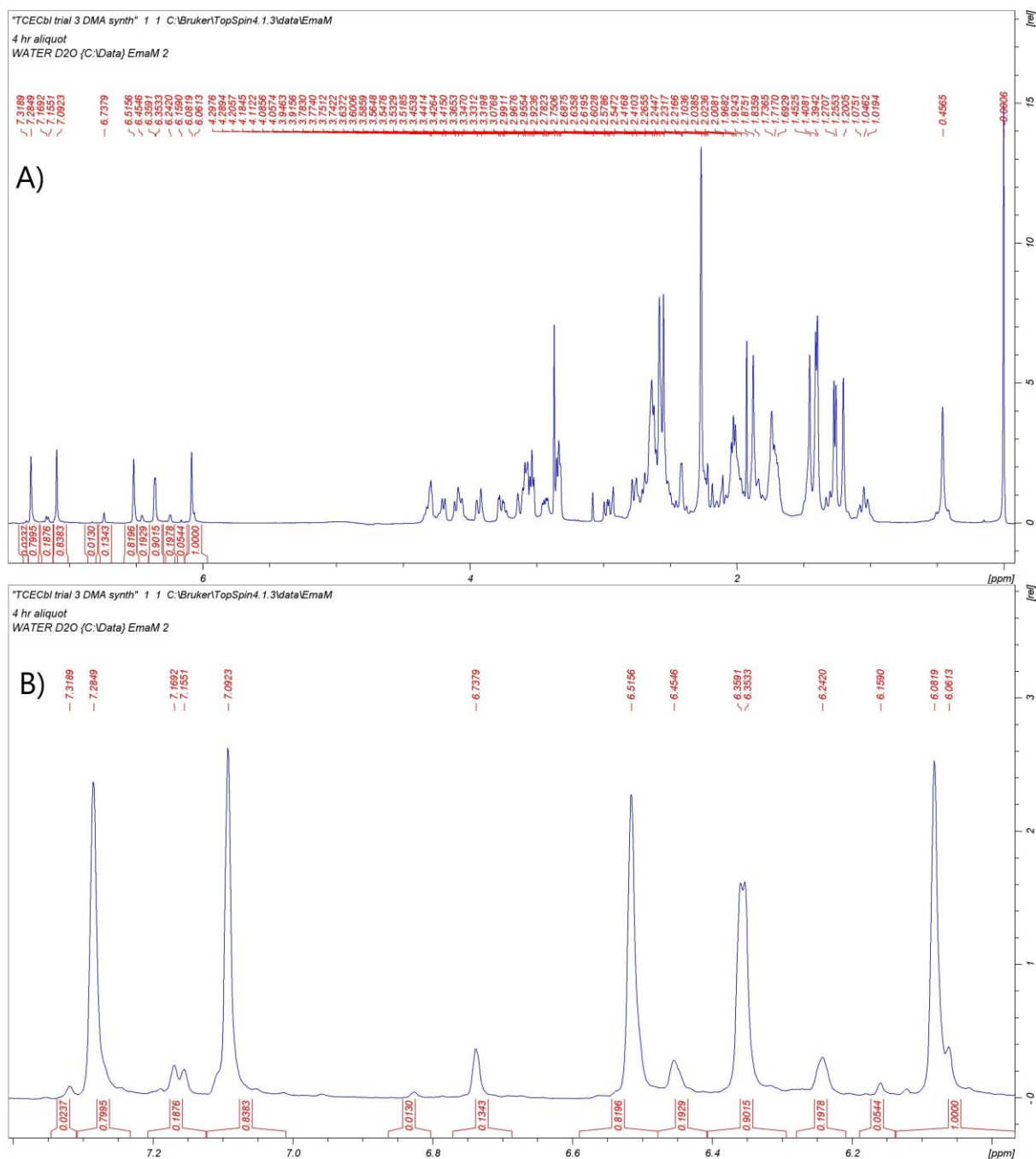


Figure 17: A) <sup>1</sup>H NMR spectrum of crude product mixture obtained from the third attempted synthesis of trichloroethenylcobalamin reduction-free, Method 3, (D<sub>2</sub>O, TSP as reference). B) <sup>1</sup>H NMR spectrum shows the aromatic region of A with the major peaks at 7.28 (singlet, 1H), 7.09 (singlet, 1H), 6.51 (singlet, 1H), 6.35 (doublet, 1H), and 6.08 (singlet, 1H) and minor peaks at 7.16 (singlet, 1H), 7.15 (singlet, 1H), 6.73 (singlet, 1H), 6.45 (singlet, 1H), 6.24 (singlet, 1H), and 6.06 (singlet, 1H).

In the fourth attempt, Method 4, the only variable changed from the previous synthesis was the temperature of the reaction. The reaction was conducted at room temperature and allowed to react overnight. Aliquots were taken at 20 minutes, 4 hours, and 20 hours. <sup>1</sup>H

NMR spectra of these samples showed the aliquot taken at 4 hours was the most promising. The sample removed after 20 minutes showed predominantly starting material (cyanocobalamin), and the 20 hours sample decomposed due to laboratory closure for the Christmas holidays. Despite the sample being dry, the product was hygroscopic and picked up water present in the atmosphere, allowing for decomposition to occur.

The  $^1\text{H}$  NMR spectrum of the product sample taken after 4 hours is shown in Figure 18. MES buffer (pH 6) was used as the NMR solvent to ensure there is not a mixture of aquacobalamin and hydroxycobalamin present in solution ( $\text{pK}_a \text{H}_2\text{OCbl}^+ = 7.8$ )<sup>36</sup>. From the  $^1\text{H}$  NMR spectrum displayed it is evident that there are three major cobalamin species in solution (~15 major peaks in the aromatic region). Five of these peaks can be attributed to the aromatic peaks of cyanocobalamin; B7 (7.28 ppm, singlet), B4 (6.51 ppm, singlet), and B2 (7.10 ppm, singlet) from the nucleotide base, the C10 (6.09 ppm, singlet) from the corrin ring, and the R1 (6.36 ppm, doublet) from the ribose ring within 0.02 ppm<sup>31</sup>. Another five can be attributed to aquacobalamin; B7 (7.16 ppm, singlet), B4 (6.44 ppm, singlet), and B2 (6.51 ppm, singlet) from the nucleotide base, the C10 (6.26 ppm, singlet) from the corrin ring, and the R1 (6.22 ppm, doublet) from the ribose ring within 0.02 ppm<sup>31</sup>. The remaining peaks are 7.24 ppm, 6.85 ppm, 6.82 ppm, 6.25 ppm, and 6.05 ppm. Importantly, these chemical shifts match the unknown cobalamin compound in the previous trial of the reduction-free trichloroethenylcobalamin Method 1, Figure 15. Unfortunately, this could not directly be compared with the reduction trial due to the modification of the pH resulting in a deteriorated product.

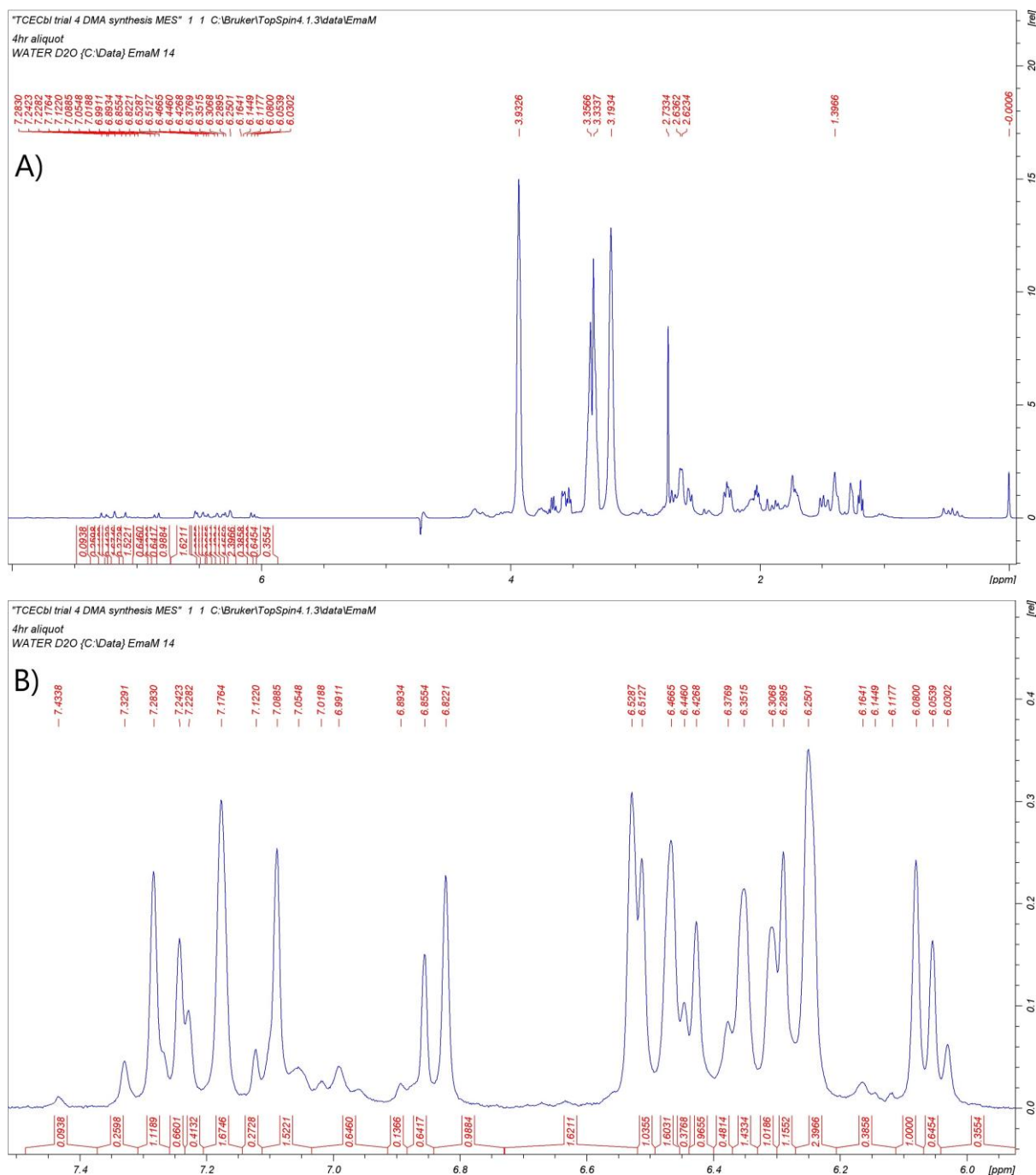


Figure 18: A) <sup>1</sup>H NMR spectrum of crude product mixture obtained from the fourth attempted synthesis of trichloroethenylcobalamin reduction-free Method 4 (D<sub>2</sub>O, TSP as reference). B) <sup>1</sup>H NMR spectrum shows the aromatic region of A with the major peaks at 7.28 (singlet), 7.24 (singlet), 7.17 (singlet), 7.08 (singlet), 6.85 (singlet), 6.82 (singlet), 6.52 (singlet), 6.51 (singlet), 6.46 (singlet), 6.42 (singlet), 6.35 (doublet), 6.30 (doublet), 6.28 (singlet), 6.25 (doublet), 6.08 (singlet), 6.05 (singlet).

For this reason, this synthesis was determined to be very promising. A sample was therefore prepared for LC-MS analysis. The resulting total ion count chromatogram (TIC) can be seen in Figure 19. Peaks were observed at 3.17, 3.92, 8.85, 9.22 and 11.05 minutes. The peak at 3.17 minutes was assigned to H<sub>2</sub>OCbl. A m/z value of 664.9 was observed for this peak,

which corresponds to  $[\text{Cbl(II)} + \text{H}]^{2+}$ ,  $\text{C}_{62}\text{H}_{89}\text{O}_{14}\text{N}_{13}\text{CoP}$ . The ESI source reduces  $\text{H}_2\text{OCbl(III)}$  to  $\text{Cbl(II)}^+$ <sup>37</sup>. The peaks at 3.92 and 8.85 minutes could unfortunately not be assigned to a species. The peak at 9.22 minutes had observed  $m/z$  values of 678.4 and 1355.5 and can be assigned to the reactant cyanocobalamin (calculated  $m/z$  for  $[\text{CNCbl} + 2\text{H}]^{2+}$ ,  $\text{C}_{63}\text{H}_{90}\text{CoN}_{14}\text{O}_{14}\text{P} = 678.3$ ; calculated  $m/z$  for  $[\text{CNCbl} + \text{H}]^+$ ,  $\text{C}_{63}\text{H}_{89}\text{CoN}_{14}\text{O}_{14}\text{P} = 1355.6$ ). The peak at 11.05 minutes had observed  $m/z$  values of 678.4 and 1356.5 and can be assigned to vinylcobalamin (calculated  $m/z$  for  $[(\text{CH}_2=\text{CH})\text{Cbl} + 2\text{H}]^{2+}$ ,  $\text{C}_{64}\text{H}_{93}\text{CoN}_{13}\text{O}_{14}\text{P} = 678.8$ ; calculated  $m/z$  for  $[(\text{CH}_2=\text{CH})\text{Cbl} + \text{H}]^+$ ,  $\text{C}_{64}\text{H}_{92}\text{CoN}_{13}\text{O}_{14}\text{P} = 1356.6$ ). Even though these two latter species have essentially the same  $m/z$  values, other LC-MS experiments showed that  $\text{CNCbl}$  elutes with  $\sim 18\%$   $\text{CH}_3\text{CN}$ , whereas others have shown that cobalamins with alkene groups at the beta axial site elute at higher percentages of  $\text{CH}_3\text{CN}$ <sup>40</sup>. As shown in the chromatogram in Figure 19, there appears to be an abundance of species eluting at higher acetonitrile concentrations, in the washing part of the method. This LC-MS would ideally be repeated; however, this was not possible due to time constraints.

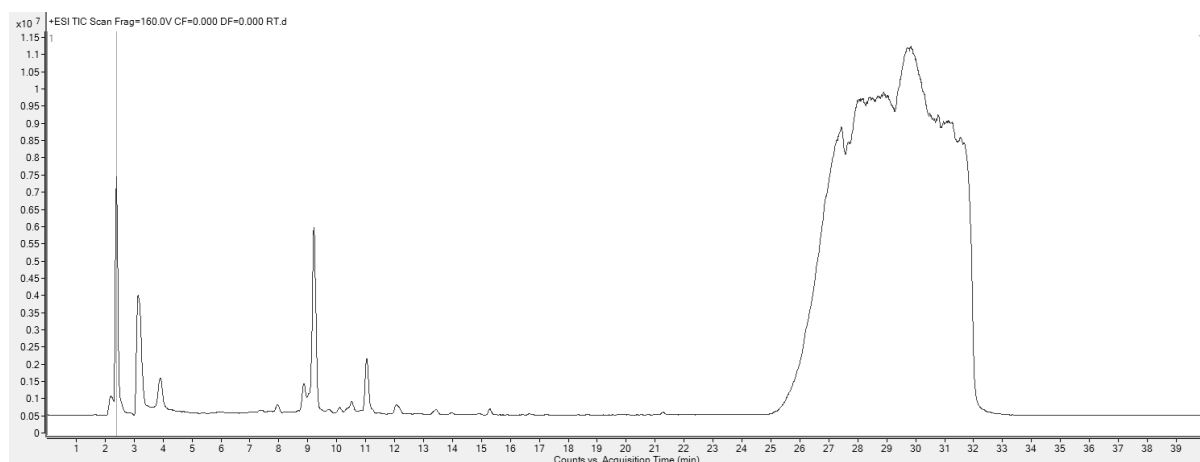


Figure 19: LC-MS Total Ion Chromatography of crude product from attempted synthesis of trichloroethenylcobalamin, Method 4.

## 6 Discussion

Prior to attempting the synthesis of a halogenated alkenylcobalamin via the commonly used reduction method, the alkylcobalamin ethylcobalamin was first synthesized using this procedure, following a literature method. In this method aquacobalamin is first reduced to cob(I)alamin using  $\text{NaBH}_4$  followed by the addition of EtBr to the cob(I)alamin solution form EtCbl. With the first attempted synthesis as described in section 4.4.1, although evidence for the presence of EtCbl in the product mixture was found using  $^1\text{H}$  NMR spectroscopy, a lower yield of EtCbl was observed, which was believed to be a result of not enough care taken to exclude air and light during the synthesis. This instability of alkylcobalamins has repeatedly been demonstrated in literature, as well as this research<sup>1,6,14,16,17,22</sup>. Upon repeating this method, a much better conversion, 89%, of aquacobalamin to EtCbl was observed.

A variation of this method has been used for the synthesis of chlorovinylcobalamin and vinylcobalamin, using chloroacetylene as a reagent to produce the desired species<sup>23</sup>. It was proposed by this research team that trichloroethenylcobalamin could not be synthesized using this method, due to ability of cob(I)alamin to react with the newly formed trichloroethenylcobalamin during the synthesis, resulting in successive reductive dechlorination to give dichloroethenylcobalamin, monochloroethenylcobalamin, and vinylcobalamin<sup>23</sup>. For this reason, we decided to modify this method so cob(I)alamin would instead be added dropwise into a solution of the halogenated alkyl or alkenyl species, rather than the usual procedure in which the halogenated compound is added to a solution of cob(I)alamin. This would prevent the organocobalamin product from being exposed to excess cob(I)alamin, since alkyl and alkenyl halides react rapidly with cob(I)alamin. The synthesis of EtCbl using this reverse addition method was first tested, and to our delight the procedure was successful.  $^1\text{H}$  NMR spectroscopy showed that 95% EtCbl was present in the product mixture.

Unfortunately, synthesising a dihalogenated vinylcobalamin by the slow addition of cob(I)alamin to trichloroethene was unsuccessful, with essentially no reaction observed. This was found to be largely due to issues with the solubility of the ligand in the reaction solvent. The subsequent attempts proved much more successful; that is, in the product



mixture cobalamins were observed which were not the starting material. The dropwise addition of the cob(I)alamin was difficult to control. For this reason, this synthesis was difficult to recreate. A possible improvement in this synthesis could be to use a dropping funnel or investigate whether the addition of a cobaltous salt would improve the synthesis. By adding a cobalt(II) salt, it could allow for a lesser equivalents of reducing agent and prevent the irreversible reduction of the cobalamin species. Further experiments using this method need to be conducted to optimise this synthetic procedure and to determine whether or not the halogenated vinylcobalamin species is formed. Unfortunately, the percentage conversion of reactant to products remained too low to conclusively determine whether the desired compound was a product.

Since the synthesis of a dihalogenated vinylcobalamin by reacting TCE with Cbl(I) was unsuccessful, a “reduction free” method was instead explored. In this method, a sterically hindered base is used to deprotonate the alkene, which then substitutes the axial ligand of the cobalamin. Copper(I) acetate was used to assist in the removal of the cyanido ligand from cyanocobalamin, in a reaction driven by the insolubility of the resultant  $\text{Cu}(\text{CN})_2^-$  precipitate. Prior to attempting this method with TCE, a control experiment to synthesise phenylethynylcobalamin using this reduction-free method was attempted, as described in the literature<sup>15</sup>. Unfortunately, the best solvent for this synthesis, anhydrous dimethylacetamide (DMA), was not available until towards the end of this research, held up as a result of COVID19 - associated ordering and shipping delays. In the first attempt, DMA was replaced by DMF, since this solvent had also been successful, although lower yields of phenylethynylcobalamin were obtained compared with DMA<sup>15</sup>. However, the syntheses using DMF were not successful with essentially only cyanocobalamin being present in the product mixture, most likely as a result of the limited solubility of  $\text{CNCbl}$  in DMF, even with overnight stirring of  $\text{CNCbl}$  in DMF in an oil bath (40°C). It was therefore decided to instead use DMSO, since  $\text{CNCbl}$  is much more soluble in this solvent. This synthesis was successful, with the  $^1\text{H}$  NMR spectrum of the product mixture being dominated by peaks matching those reported for phenylethynylcobalamin reported in the literature<sup>15</sup>.

We then moved on to attempting to synthesise trichloroethenylcobalamin using this reduction-free method. Both DMSO and DMA were tested as solvents.  $^1\text{H}$  NMR spectroscopy of the product mixture is very informative, as cobalamin species have five characteristic peaks in the aromatic region, with no two species having identical chemical shifts for all five of these protons. Interestingly, multiple products were present in the reaction mixture. For this reason, the reaction temperature was lowered to attempt to limit the number of products formed. The moderately successful attempts of Methods 1 and 4 were unable to be reproduced. A new compound was present in the product mixture with  $^1\text{H}$  NMR peaks in the aromatic region at 7.24, 6.85, 6.82, 6.25, and 6.05 ppm. This was promising as previous trials had given different results and either a variety of peaks present in the aromatic region, indicating the presence of many cobalamin derivatives, or simply the cobalamin starting material used in the synthesis. This reaction could possibly be optimised by being conducted in the glovebox, which would provide a stricter air-free environment. Additionally, the product mixture would benefit from immediate analysis, as it was shown that samples decompose over time. Unfortunately carrying out experimental work after 5 pm was not allowed during this time period due to COVID19-associated restrictions concerning building access, so the solvent could not always be removed from the product solution until the following day. Further trials of this synthesis are required to reproduce results and optimise the reaction procedure.

The cobalamin compounds produced in the attempted synthesis of trichloroethenylcobalamin were unable to be conclusively identified. The results of the  $^1\text{H}$  NMR spectra obtained showed a mixture of cobalamin products. Throughout this project, it was realised that there was a new peak present in the aromatic region of multiple crude product mixtures. This was at 6.05 ppm (singlet, 1H) and was found in later synthesis trials, and is likely to arise from a C10 proton. At pH 6 aquacobalamin, not hydroxycobalamin, is present in solution ( $\text{pK}_a \text{H}_2\text{OCbl}^+ = 7.8$ )<sup>36</sup>. By adjusting the pH so the dominant species is aquacobalamin, a peak at 6.05 ppm in  $\text{D}_2\text{O}$  was useful with respect to showing that a new compound was present in solution. Although there were 5 aromatic peaks which were frequently present in different synthesis attempts (7.24, 6.85, 6.82, 6.25, and 6.05 ppm), the C10 peak had the least overlap with other species and was therefore used to confirm a promising new compound in the product mixture.

In an attempt to identify the cobalamin species, LC-MS was used to separate and analyse the compounds present in the promising product mixtures. Unfortunately, there was no conclusive evidence for the presence of the desired trichloroethenylcobalamin in the LC-MS chromatograms. Cobalamins can occasionally be prone to reduction by the ESI source. For example, during LC-MS aquacobalamin is reduced to  $\text{Cbl(II)}^+$  by the ESI source<sup>37</sup>. The difficulties in isolating and conclusively determining the presence of the desired product could likely stem from the instability of the product arising from steric hindrance from the chloride substituents of the vinyl ligand which may interact with sidechains of the corrin macrocyclic ring<sup>23,33</sup>. This effect has been demonstrated by the differences in the Co-C bond length between (Z)-chlorovinylcobalamin and vinylcobalamin, where the chlorovinylcobalamin bond length was longer, similar to that of methylcobalamin<sup>23</sup>. This has also been demonstrated by the inability to isolate dichlorovinylcobalamin<sup>23</sup>. For this reason, small influences could result in the ligand being displaced.

These attempted syntheses have shown to be considerably more complicated than initially expected. Factors such as light and air sensitivity from an unstable beta-axial ligand influenced the conversion from starting material to product<sup>5,14,22,23</sup>. Additionally, the desired product may be unstable due to steric interactions between the ligand substituents and the corrin ring sidechains<sup>23</sup>. The pitfall of this research was the inability to conclusively show the formation of the desired product. Further experiments are required. The ability of the novel alkenylcobalamin derivative to be synthesised through the modification of existing organocobalamin synthetic methods could pave the way for the production of a new alkenylcobalamin - based therapeutic agent. The methods which were used in the attempted synthesis of this novel alkenylcobalamin were a variation on the tried-and-true  $\text{NaBH}_4$  reduction method in which the organic halide is added to a solution of cob(I)alamin, shown to be highly successful in the synthesis of most organocobalamins to date. As well as this, a newer reduction-free method was also used which has been used for the successful synthesis of alkynylcobalamin derivatives<sup>15,33</sup>. Based on the reported  $\text{pK}_a$  value of the ligand used, trichloroethene (the values of this  $\text{pK}_a$  value vary from 18-29) it was thought that each method could potentially result in a successful synthesis, if the product was not too unstable<sup>33,41,42</sup>.

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