The Conjugation of Protein and Peptide Therapeutics to Vitamin B\textsubscript{12} for the Oral Treatment of Diabetes

A Capstone Project Submitted in Partial Fulfillment of the Requirements of the Renée Crown University Honors Program at Syracuse University

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Abstract

Currently patients with diabetes receive most of their treatments, including insulin, subcutaneously. Developing a method to orally deliver proteins, peptides, and potentially other therapeutics has the ability to increase patient compliance by making treatments easier to administer. Utilizing the Vitamin B$_{12}$ uptake pathway is one proposed method of oral delivery of protein therapeutics.

We investigated a way to synthesize a carboxylic acid derivative of Vitamin B$_{12}$ (B$_{12}$) that could increase the ease of its conjugation to proteins and peptides. The 5'-position of the ribose tail of B$_{12}$ was oxidized using excess 2-iodoxybenzoic acid (IBX) and 2-hydroxypyridine (HYP). The synthesis of this B$_{12}$ derivative allows for peptides, proteins, and other molecules to be conjugated to the 5'-position of the ribose tail of B$_{12}$ using an amide bond instead of a carbamate bond. Conjugation of a simple amine-containing organic molecule, benzylamine, to this B$_{12}$ derivative with a 40% yield supported the synthesis of the derivative and its potential to increase the yield of B$_{12}$ conjugate formation.

We also investigated a method to recombinately express C-peptide using a SUMO system and *Escherichia coli*. The ability to deliver C-peptide orally using the vitamin B$_{12}$ uptake pathway would be an easy treatment to potentially reduce diabetic neuropathy, a complication present in many patients with diabetes.
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1 Diabetes

1.1 Diabetes

Diabetes is a group of diseases categorized by high levels of blood glucose. This can be a result of defective insulin production, use, or a combination of both. The two main types of diabetes are type 1 and type 2.¹

1.1.1 Type 1 Diabetes

The onset of type 1 diabetes is due to the destruction of pancreatic beta cells by the body’s immune system. Because these cells are the body’s only source of insulin production, people with type 1 diabetes must receive insulin via an outside source, usually an injection or a pump. Type 1 diabetes usually develops during childhood or young adulthood and accounts for about 5% of all diagnosed cases of diabetes.¹

1.1.2 Type 2 Diabetes

Type 2 diabetes usually begins when the body’s cells cannot use insulin properly. The demand for insulin in the body then rises, which eventually causes the pancreas to lose its ability to produce it. Aging, obesity, and family history are all associated with the onset of type 2 diabetes.¹

1.2 Diabetes in the United States

The Center for Disease Control and Prevention (CDC) reports diabetes as the seventh most common cause of death. It affects 25.8 million people or 8.3% of the population in the United States. Diabetes is also the primary cause of kidney failure and is a major contributor to stroke and heart disease. The CDC estimates that in 2010...
in the United States about 1.9 million people over the age of 20 were newly diagnosed with diabetes.¹

1.3 Current Treatments

Diet, insulin, and oral medication are the main forms of treatment used to regulate blood glucose levels in people with diabetes. People with type 1 diabetes must have insulin to sustain life. However, many people with type 2 diabetes are able to manage their disease by just eating healthy, exercising, and losing weight in combination with oral medication. 12% of adults diagnosed with diabetes take insulin only, 14% take insulin and oral medication, 58% take only oral medication, 16% do not take either one. Self-management and education are also key aspects of the treatment of diabetes.¹

2 Vitamin B₁₂

2.1 Structure

B₁₂, a water-soluble organometallic compound, is the largest and most complex of all of the vitamins.² B₁₂ consists of a corrin ring with the metal cobalt(III) at its center.³ Cobalt has 6 coordination sites, 4 being occupied by nitrogens in the corrin ring. The fifth site is occupied by a ligand on the β side. In the active forms of B₁₂ in the human body, the β ligand is a methyl group or a 5-deoxyadenosyl group and the compounds are called methylcobalamin and 5-deoxyadenosylcobalamin, respectively. In the form of B₁₂ usually present in dietary supplements, the β ligand is a cyanide group and the compound is called cyanocobalamin. In the body, cyanocobalamin is converted to its active forms.⁴ The 6th coordination site is occupied by 5,6-dimethylbenzimidazole, which is linked to a 5 carbon sugar. This 5 carbon
sugar is linked to a phosphate group that is attached to an amide group in the corrin ring. This structure can be seen in Figure 1 below.

Figure 1 Structure of Vitamin B₁₂

2.2 B₁₂ Uptake Pathway

B₁₂ is an essential vitamin that cannot be synthesized by the human body. The average adult diet consists of an intake of 1-5 µg of B₁₂ a day. Once ingested, proteases and the acidic environment of the gastrointestinal tract begin to break B₁₂’s peptide bonds in order to extract it from food.

B₁₂’s uptake pathway (Figure 2) begins with the binding of B₁₂ to salivary haptocorrin (HC). The acidic conditions (pH<3) cause HC to have a high affinity for B₁₂. HC is then able to protect B₁₂ from acid hydrolysis, which would normally occur in an environment with such a low pH. Then the HC and B₁₂ complex moves from the stomach into the first section of the small intestine or the duodenum. The pH in the
duodenum is higher than that of the stomach (>5.5), decreasing HC’s affinity for B_{12}. Enzymes from the pancreas also begin to digest HC, which frees the B_{12} to then bind to intrinsic factor (IF). IF, a second transport protein, is produced in the gastric mucosa and the pancreas. The IF and B_{12} complex binds to its receptor, cubilin, to be transported across the intestinal enterocyte. B_{12} then appears in the blood bound to transcobalamin II (TC II). The cells take up this complex of TC II and B_{12}. B_{12} is finally freed from this complex after a lysozyme degrades TC II.\textsuperscript{5}

Figure 2: Representation of B_{12} uptake pathway
3 The Use of Vitamin B\textsubscript{12} in Oral Delivery

3.1 Background

Many therapeutic peptides and proteins are currently given intravenously. Although oral administration of these therapeutics would be an ideal alternative route of delivery in order to increase patience compliance and comfort, two main barriers stand in the way. First of all, the acidic environment of the gastrointestinal tract would degrade any protein or peptide, leaving it in an inactive form. Second of all, the body does not have a method or pathway to absorb these peptides and proteins from the gastrointestinal tract into the bloodstream. \(^5\) If these two problems could be solved, then the oral delivery of peptides and proteins would be a feasible method to deliver medications, improving the lives of many. For example, the ability to deliver insulin orally would increase the ease at which it can be administered to those with diabetes. This could significantly increase patient compliance, lowering the complications and deaths each year caused by diabetes.

The utilization of the B\textsubscript{12} uptake pathway described in section 2.2 has been a method of oral delivery of peptides and protein receiving increasing attention over the past decade. If proteins and peptides can be conjugated to B\textsubscript{12}, B\textsubscript{12}’s mechanism for delivery within the body naturally has ways to protect the proteins and peptides from the acidic environment in the gastrointestinal tract and allow them to be absorbed into the bloodstream. \(^5\)

3.2 Conjugation Sites

When coupling B\textsubscript{12} and a peptide, protein, or any other molecule, it is important that both entities maintain their biological function in the body. The
functional sites of the peptide, protein, or molecule must still be intact in order to work properly once it reaches the bloodstream. In addition, the sites on B\textsubscript{12} recognized by the proteins in its uptake pathway must still be recognizable. There are three major sites on B\textsubscript{12} that are believed to not interfere with its recognition in its uptake pathway.\textsuperscript{5}

1) The e peripheral propionamide on the corrin ring
2) The ribose 5'-hydroxy group
3) The phosphate unit on the α tail

These sites are highlighted in figure 3 below.

Figure 3: Potential sites of conjugation on B\textsubscript{12}
4 C-peptide

4.1 Background

C-peptide is a protein that contains 31 amino acids. It is part of the proinsulin (Figure 4) produced from pre-proinsulin translated off the ribosome and is synthesized by the beta cells of the pancreas. After C-peptide is cleaved from proinsulin, Insulin is released into the body in its active form, along with an equimolar amount of C-peptide. For a long time, C-peptide was thought to be biologically inert and therefore people with diabetes only received injections of supplemental insulin, not C-peptide. However, recent studies have suggested that C-peptide may serve a regulatory role in the body by contributing to vascular homeostasis in various tissues. Upon the administration of C-peptide, a number of cellular responses occur in insulin dependent patients with diabetes, such as a “rise in blood flow to the kidneys, muscle, skin, and nerves in the diabetic state.” For patients with diabetes, the lack of C-peptide in combination with high blood glucose levels reduces microvascular circulation, which can eventually lead to complications like neuropathy. ^5,6

![Diagram of Human Proinsulin](image)

Figure 4: Representation of Human Proinsulin
5 Site-Selective Oxidation of Vitamin B\textsubscript{12}

5.1 Background

As mentioned prior, there are several potential sites for conjugation to B\textsubscript{12}, however conjugation has most successfully occurred at (1) the 5’-hydroxyl group of the ribose and (2) the \(e\) propionamide of the peripheral corrin ring (Figure 5). Fortunately, neither site interferes with the proteins of the B\textsubscript{12} uptake pathway. Although the carboxylic acid derivative of the \(e\) propionamide group has been made, it is low yielding and difficult to purify. This is partially attributed to the simultaneous synthesis of the \(b\)-, \(d\)-, and \(e\)- acid derivatives. The 5’-hydroxyl group of the ribose of B\textsubscript{12} is a versatile site that could potentially be used for the synthesis of a variety of B\textsubscript{12} conjugates. However, limited chemistry with the hydroxyl group can be performed and the conjugates synthesized using this site are done through a carbamate or ester bond shown in Figure 6. This presents stability issues and the potential for degradation. The selective oxidation of the 5’-hydroxyl group of B\textsubscript{12} to a carboxylic acid was done using 2-iodoxybenzoic acid (IBX). This has the potential to increase the chemistry available at B\textsubscript{12}’s 5’-position and the ability to form conjugates through a more stable amide bond (Figure 6).\textsuperscript{8,9}
Figure 5: Sites available for conjugation to B_{12}. This figure also shows potential B_{12} derivatives to Carboxylic Acids. The numbering is used for NMR.
5.1.1 2-Iodobenzoic Acid (IBX)

Hypervalent iodine reagents, like IBX, are used in many oxidation reactions. Most iodonanes are advantageous for oxidations due to their availability, ability to regenerate, mild reaction conditions, and stability in the presence of oxygen and moisture. In literature it was found that the combination of IBX and N-Hydroxysuccinimide (NHS) produces activated esters and carboxylic acids from alcohols. NHS is used as an activating agent that helps to bring the oxidation state from the alcohol to the aldehyde all the way to the desired to the carboxylic acid. Although the use of IBX for an oxidation reaction is seen in literature, this is the first
time it has been utilized for a molecule like $B_{12}$, expanding its array of possible reactions.\textsuperscript{10}

5.2 Synthesis of 5’-Carboxylic Acid of $B_{12}$ Ribose Tail

Beginning with the method developed by Giannis \textit{et al.}, a reaction to synthesize the $B_{12}$ carboxylic acid derivative was created. This reaction uses an O-nucleophile, NHS, in conjunction with IBX to oxidize primary alcohols. The initial use of NHS with IBX for the oxidation of the 5’-hydroxyl group on the ribose of $B_{12}$, resulted in a mixture of products from which the carboxylic acid could not be isolated. This mixture of products was likely due to the simultaneous formation of the activated ester along with the carboxylic acid. The activated ester has been reported to be the dominant product of this reaction.\textsuperscript{10} 2-hydroxypyridine (HYP) was then used in place of the NHS as the O-nucleophile, which provide better results. The proposed reaction mechanism can be seen in Figure 7.
Figure 7: Proposed mechanism of the synthesis of B_{12}CA using IBX and HYP

B_{12} (10.0 mg, 0.007 mmol), IBX (5.37 mg, 0.019 mmol), and HYP (3.51 mg, 0.037 mmol) were dissolved in 1 mL of dimethyl sulfoxide (DMSO). Using these reactants, a series of various experiments were conducted in order to optimize this
reaction. Conditions such as the co-oxidant, temperature, and the time were varied. These results can be seen in Figure 8 below.

<table>
<thead>
<tr>
<th>Co-oxidant</th>
<th>Temperature (°C)</th>
<th>Time (hours)</th>
<th>% Yield</th>
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<tbody>
<tr>
<td>None</td>
<td>50</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>HYP</td>
<td>50</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
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<td>50</td>
<td>3</td>
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</tr>
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</tr>
<tr>
<td>HYP</td>
<td>60</td>
<td>2</td>
<td>30</td>
</tr>
</tbody>
</table>

Figure 8: Reaction Optimization

After many reactions, the optimal conditions were determined to be 5 equivalents of HYP for every 2.6 equivalents of IBX in DMSO at 60°C. As shown in Figure 8, it resulted in a 30% yield of the 5’-carboxylic acid derivative of B\textsubscript{12} (B\textsubscript{12}CA) over a two hour time period. It was also noted that using a temperature above 60°C or a time deviating from two hours for this reaction increased the amount of byproducts and/or degradation of B\textsubscript{12}, making the B\textsubscript{12}CA difficult to purify and reducing its yield. In addition, using an IBX:HYP ratio that differed from 2.6:5 equivalents resulted in an increase of byproducts, and therefore a decrease in percent yield of the B\textsubscript{12}CA. When comparing the synthesis of the B\textsubscript{12}CA to that of the e-
carboxylic acid derivative (Figure 5), the purification process was much easier and the yield was almost three times as high. 8,11

In order to prepare the reaction product for purification, it was precipitated out of the DMSO into diethyl ether. The ether was decanted and the solid product was allowed to air dry. The solid product was then re-dissolved in water.

The purification process was done using high-performance liquid chromatography (HPLC) and an Agilent SAX analytical column (5 µm, 9.4 X 250 mm), which utilized ion exchange chromatography. Solvent A was ultra purified water and solvent B was a 50:50 mixture by volume of Acetonitrile (MeCN) and phosphate buffer pH 2. Note that an increase in pH for solvent B resulted in a decrease in the amount of purified B12CA.

With a flow rate of 1 mL/minute, 100% of solvent A was allowed to flow through the HPLC and column for 5 minutes. This was followed by a gradient from 100% solvent A, 0% solvent B to 65% solvent A, 35% solvent B over 5 minutes. The HPLC profile can be seen in Figure 9 below. 8,11
Figure 9: HPLC spectra of $B_{12}$CA purification, monitored at 360 nm. The retention time of the unreacted $B_{12}$ and aldehyde was about 5 minutes and the $B_{12}$CA was about 12 minutes.

The peak at a retention time of 5 minutes was identified as a mixture of unreacted $B_{12}$ and the ribose 5'-aldehyde $B_{12}$ derivative. The peak with a retention time of about 12 minutes was identified as $B_{12}$CA, the desired product. The peak at 12 minutes, which was red in color, was collected and neutralized. It was mixed with amberlite XAD4 resin and rocked overnight in order to remove any salt. The amberlite resin turned red, suggesting the binding of the $B_{12}$CA. The resin was collected and washed with water to remove any excess salt. The resin was placed in Methanol (MeOH), which would release the $B_{12}$CA. The MeOH solution containing the $B_{12}$CA was collected and lyophilized to get rid of the MeOH and leave a red powder product.\textsuperscript{8,11}
5.3 Identification of B\textsubscript{12}CA

Various methods were chosen to support the identification of the synthesized product as B\textsubscript{12}CA.

### 5.3.1 Matrix Assisted Laser Desorption Ionization (MALDI)

Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-ToF) mass spectrometry was done using a Bruker Autoflex II Smartbeam machine with laser intensity from 50-73%. A small sample of the purified product (B\textsubscript{12}CA) was taken and mixed with a matrix containing α-cyano-4-hydroxycinnamic acid (CHCA) in a 1:1 volume ratio of water and acetonitrile with 0.1% trifluoroacetic acid (TFA). Angiotensin I was also added for use as an internal control, which has a mass to charge ratio (m/z) of 1296. The mass to charge ratio expected for a product containing B\textsubscript{12}CA is m/z=1343.5 [M-CN]\textsuperscript{+}. This peak was present in the MALDI profile as shown in Figure 10, supporting the synthesis of B\textsubscript{12}CA.
Figure 10: MALDI-ToF of the reaction product. At m/z=1296.7 the expected angiotensin peaks can be seen and at m/z=1343.5 the expected B$_{12}$CA peaks can be seen.

MADLI-ToF was also performed for the HPLC fractions with a retention time of 5 minutes, which was speculated to be the unreacted B$_{12}$ and 5'-aldehyde B$_{12}$ derivative. The large group of peaks in Figure 11 is a mixture of the expected MALDI-ToF pattern for B$_{12}$ with m/z=1329.5 and the 5'-aldehyde B$_{12}$ derivative with m/z=1327.5. This supports the identification of the HPLC fractions with a retention time of 5 minutes.$^{8,11}$
Figure 11: MALDI-ToF of the HPLC peak at 5 seconds. The expected angiotensin peaks can be seen at m/z=1296.7, B_{12} at m/z=1329.5, and the 5'-aldehyde B_{12} derivative at m/z= 1327.5.

\section*{5.3.2 Electron Absorption Spectroscopy (EAS)}

Electron Absorption Spectroscopy (EAS) was used to determine the yield of the B_{12}CA product. The spectrum is shown in Figure 12 below. The yield was calculated using Beer-Lambert law in which A= εlc, where A is the absorption, ε is
the extinction coefficient, \( l \) is the path length, and \( c \) is the concentration. For B\(_{12}\)CA the extinction coefficient used was \( \varepsilon = 27500 \text{ M}^{-1}\text{cm}^{-1} \).

Figure 12: Electronic Absorption Spectrum of B\(_{12}\)CA taken in 35% B from HPLC run

5.3.3 1D and 2D Nuclear Magnetic Resonance Analysis of B\(_{12}\)CA

Using \(^1\text{H}\) Nuclear Magnetic Resonance (NMR) (Figure 13) and \(^1\text{H}-^{13}\text{C}\) heteronuclear (HSQC) (Figure 14), confirmation of the formation of B\(_{12}\)CA was further supported. Looking at the \(^1\text{H}\) NMR for the B\(_{12}\)CA and comparing it to that of a standard B\(_{12}\) Cyanocobalamin \(^1\text{H}\) NMR (Figure 15), it can be seen that 2 protons were lost in the formation of B\(_{12}\)CA. These protons are located at \( \delta_{\text{H}} = 3.75 \) and 3.92 in the B\(_{12}\) Cyanocobalamin \(^1\text{H}\) NMR. In order to determine which two protons were lost, a comparison of HSQC of standard B\(_{12}\) Cyanocobalamin and B\(_{12}\)CA was done (Figure 16). The signal that disappears at \( \delta_{\text{H}} = 63.3 \) in the HSQC of B\(_{12}\)CA indicated that the protons lost in going from B\(_{12}\) to B\(_{12}\)CA were attached to the R5 carbon.\(^8\)\(^,\)\(^11\)
Figure 13: 500 MHz $^1$H NMR of B$_{12}$CA

Figure 14: 500 MHz $^1$H-$^{13}$C HSQC NMR of B$_{12}$CA
Figure 15: 500 MHz $^1$H NMR of B$_{12}$

Figure 16: Zoomed in portion of 500 MHz $^1$H-$^{13}$C HSQC NMR of B$_{12}$ (left) and B$_{12}$CA (right). Note the loss of the protons at $\delta_H=3.75$ and 3.92 when the carbon at R5 ($\delta_H=63.3$) is oxidized.
5.4 Conjugation of Benzylamine to B₁₂CA

Benzylamine was chosen to conjugate to the B₁₂CA. Benzylamine is a simple amine-containing organic molecule and was chosen because of its easily identifiable diagnostic peaks present in an NMR profile. This would help to characterize the R5 carbon even further. In addition, it would help to confirm the synthesis of the B₁₂CA by showing that a simple amine could be conjugated to the carboxylic acid at the 5’ position on the ribose of B₁₂.

5.4.1 Synthesis of Conjugate

The B₁₂CA and benzylamine were dissolved in dimethylformamide (DMF) containing 1% water, along with 10 molar equivalents of ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 20 molar equivalents 1-hydroxybenzotriazole (HOBt). This was left at room temperature for 1 hour to allow the reaction to occur. During this time, the B₁₂CA-benzylamine (B₁₂CABA) conjugate was synthesized with a 40% yield. The reaction is shown in figure 17 below.

Figure 17: The reaction of B₁₂CA with benzylamine (1) to form B₁₂CABA using EDC and HOBt in DMF for 1 hour at room temperature.
5.4.2 Purification of Conjugate

Using the same HPLC method established to purify the B_{12}CA from the original reaction, the B_{12}CABA was purified. This involved ion exchange with an SAX column, a flow rate of 1 mL/minute, and 100% of solvent A flowing through the HPLC and column for 5 minutes. This was followed by a gradient from 100% solvent A, 0% solvent B to 65% solvent A, 35% solvent B over 5 minutes. Solvent A was ultra purified water and solvent B was a 50:50 mixture by volume of Acetonitrile (MeCN) and phosphate buffer pH 2. The profile obtained is shown in Figure 18.

![HPLC profile of B_{12}CABA](image)

Figure 18: HPLC profile of B_{12}CABA

5.4.3 Identification of B_{12}CABA

The B_{12}CABA was plated for MALDI, again using CHCA in a 1:1 volume ratio of water and acetonitrile with 0.1% TFA. Angiotensin I was used for an internal control. The MALDI-ToF profile of B_{12}CABA showed a mass to charge ratio of m/z=1432.5, which is what was expected if the conjugations was successful (Figure 19). A close up of the benzylamine MALDI peaks can be seen in Figure 20.
Figure 19: MALDI-TOF of the B_{12}CABA. The expected angiotensin peaks can be seen at m/z=1296.7 and B_{12}CABA at m/z=1432.6
Next, $^1$H NMR (Figure 21) of the B$_{12}$CABA was done to further support the synthesis of the B$_{12}$CABA. Looking at the $^1$H NMR for the B$_{12}$CABA and comparing it to that of B$_{12}$CA, the appearance of the set of peaks just below $\delta_H = 7.5$ is diagnostic of the addition of the benzylamine.
Figure 21: 500 MHz $^1$H NMR of B$_{12}$CABA

$^1$H-$^{13}$C HSQC NMR (Figure 22) and $^1$H-$^{13}$C HMBC NMR (Figure 23) were also performed on the B$_{12}$CABA to study the connectivity to the R5 carbon. From the HMBC, it can be seen that the R5 carbon ($\delta_C=173.8$ ppm) of B$_{12}$CA is connected to the two protons of C$_A$ of the benzyl amine ($\delta_H=4.42$ and 4.50 ppm). The protons of C$_A$ also show direct connectivity to C$_B$ ($\delta_C=140.8$ ppm) and C$_C$ ($\delta_C=131.4$ ppm) of the benzylamine. The numbering assignments of the atoms as well as the signal assignments of the B$_{12}$CABA for HSQC and HMBC can be seen in Figure 24. $^8,^{11}$
Figure 22: 500 MHz $^1$H-$^{13}$C HSQC NMR of B$_{12}$CABA

Figure 23: 500 MHz $^1$H-$^{13}$C HMBC NMR of B$_{12}$CABA
Figure 24: A: Numbering assignments of $B_{12}$CABA, B: Signal assignments of the HSQC of $B_{12}$CABA, C: Signal assignments of the HMBC of $B_{12}$CABA

5.5 Conclusion

In conclusion, we have successfully made a derivative of $B_{12}$ that could increase the ease at which $B_{12}$ conjugates are made. We have not only shown that IBX can be used for the oxidation of $B_{12}$, but this oxidation is site specific of the ribose 5’-hydroxyl group. The $B_{12}$CA is synthesized with a yield that triples the synthesis of the carboxylic acid derivative of the propionamide or any other reported carboxylic acid derivative of $B_{12}$. It is also much easier to purify and quicker to make. The formation of a derivative of $B_{12}$ that has a carboxylic acid instead of a hydroxyl group available for conjugation increases the chemistry that can be performed at the 5’ site. This will allow higher yielding and more stable conjugates to be formed in the future. This conjugate will be of significant use in the field of $B_{12}$ conjugation chemistry.$^{8, 11}$
6 Expression of C-peptide

6.1 Introduction

As mentioned prior, administration of C-peptide has the potential to reduce diabetic neuropathy, a major complication that arises in many people with diabetes. Currently a subcutaneous administration of C-peptide is being studied for use in patients with diabetes\(^7\), however the ability to deliver C-peptide orally has the potential to be more convenient for patients with diabetes and therefore increase patient compliance. Utilization of the B\(_{12}\) uptake pathway by conjugating C-peptide to the 5’-carboxylic acid derivate of B\(_{12}\) we have created (B\(_{12}\)CA), could allow the oral delivery of C-peptide.

6.2 Recombinant Expression of C-peptide

6.2.1 Sumo System

In order to create a conjugate of B\(_{12}\) and C-peptide, the first step was to establish a method of expressing recombinant C-peptide. The system chosen to do so with was a Small Ubiquitin-like Modifier (SUMO) system, made by the Lucigen Corporation. The SUMO protein contains 100 amino acids and aids in the expression of difficult target proteins by enhancing the protein’s solubility and expression. The SUMO system contains a 6xHis-Sumo tag, which is used for purification. The SUMO system also does not require purification of the PCR product or restriction enzymes prior to ligation.
6.2.2 Amplification of C-peptide

The target protein, C-peptide was amplified using Polymerase Chain Reaction (PCR). Three different PCR reactions were done with a 1:10, 1:5, and 1:1 volume to volume ratio of DNA to water. The DNA used was a pUC57 plasmid containing the target gene C-peptide, which was ordered. The primers were also designed (Figure 25) based on the instructions in the Lucigen SUMO manual in order to synthesize the C-peptide PCR product with the correct flanking sequences needed for the ligation into the SUMO containing vector.

Forward Primer: 5’-CGCGAACAGATTGGAGGTGCGGAAGATCTGCAAGTG-3’
Reverse Primer: 5’-GTGGCGCCCGCTCTATTATTGCAGAGAGCCTTCCAG-3’

Figure 25: Primers for C-peptide Insertion into the SUMO system

The PCR mixture consisted of 10 µL of the different DNA to water ratios described above, 20 µL of sterile water, 5 µL of a 10 µM concentration of the forward primer, 5 µL of a 10 µM concentration of the reverse primer, 5 µL of thermopol buffer, 4 µL of dNTPs, and 1 µL of deep vent enzyme. The PCR conditions were 34 cycles of 94°C for 1 minute, 60°C for 1 minute and 74 °C for 20 seconds.

The 3 different PCR products were then run down a DNA agarose gel (0.5 grams of agarose, 50 mL of 1X TBE buffer, 2.5 µL of Ethidium Bromide) for 70 minutes at 70 Volts (Figure 26). C-peptide plus the flanking sequences added for ligation contains about 100 base pairs and can be seen in all 3 lanes of the varied PCR reactions near the 0.100 kilobase marker, supporting its synthesis.
Figure 26: Agarose Gel of PCR product. C-peptide plus the flanking sequences added contain about 100 base pairs and can be seen at around the 0.100 kilobase marker in all 3 lanes.

6.2.3 Ligation

The Lucigen Corporation Sumo kit contained a linearized pETite N-His SUMO Kan vector, which includes the SUMO sequence. On each end of the linearized sequence the flanking sequences are complementary to that of the C-
peptide PCR product made, which helps to ligate the C-peptide and the vector. The ligation was done by adding 2 µL of the pETite vector linearized DNA and 3 µL of the PCR product to thawed chemically competent HI-Control 10G cells. Lucigen Corporation optimizes these cells for a high efficiency transformation. The cells were allowed to sit on ice for 30 minutes, then they were heat shocked by being placed in water at 42°C for 45 seconds. 960 µL of recovery media was added to the cells and then the cells were left on ice for 2 minutes. Finally the cells were placed at 37°C for 1 hour. The cells were then plated on LB Kanamycin (Kan) Agar plates and incubated at 37°C overnight. Single colonies that grew on the plates were selected and grown in 5 mL of LB and 5 µL of Kan overnight at 37°C, 250 RPM. The plasmid was then isolated and sent for sequencing. The sequence confirmed that a successful ligation had occurred between the C-peptide and the pETite N-His SUMO Kan vector.

6.2.4 Induction

Following the successful ligation, SUMO C-peptide was expressed from the pETite N-His SUMO Kan vector. Single colonies were selected and grown in 5 mL of LB and 5 µL of Kan overnight at 37°C, 250 RPM. In the morning, 0.5 mL of this culture was taken and placed in 50 mL LB broth with 50 µL of Kan. It was grown at 37°C, 250 RPM until the OD$_{600}$ was 0.4-0.6. The cultures were then induced with either 5 µL of 1 mM isopropylthiogalactoside (IPTG) or 5 µL of 0.1 mM IPTG, giving the final concentration of IPTG in either culture as 0.1 mM and 0.01 mM, respectively. Samples of each culture were taken prior to the addition of IPTG, at 1 hour after, 4 hours, and overnight. The samples were poured into falcon tubes, centrifuged, and decanted. Each sample was redissolved in 20 mM tris-HCl, 150 mM
NaCl pH 7.2. The samples were then lysed by being sonicated on ice with 10 pulses, 2 minutes of incubation on ice, two times. They were then centrifuged and the supernatant, containing the proteins, was collected.

### 6.2.5 Purification

The supernatant following the lysis was purified using Fast Protein Liquid Chromatography (FPLC). A nickel resin HisTrap column was used, along with 20 mM Hepes, 0.5 M NaCl, pH 7.2 for solvent A and 20 mM Hepes, 0.5 M NaCl, pH 7.2 with 250 mM Imidizole for solvent B. 100% A was run through the column, followed by 100% B. The histag in the sumo system first bound to the column during the initial flow through of solvent A. It was then eluted as solvent B flowed through the column and imidizole displaced it. The FPLC profile is shown in Figure 27.

Figure 27: FPLC profile of SUMO C-peptide system.
6.2.6 Cleavage of SUMO

In order to cleave the SUMO protein from C-peptide, the eluted from the FPLC purification was collected and boiled for 5 minutes with 1.5 µL of Dithiothreitol (DTT). 0.5 µL of the SUMO protease was added. The mixture was left at 30°C for 1 hour. The SUMO protease recognizes SUMO’s tertiary structure and cleaves at its terminal carboxyl group. In theory, it should leave the target protein (C-peptide) without any extra residues. However, an SDS-Page gel was done (Figure 28) and showed that the C-peptide was not being cleaved from the SUMO system. If C-peptide was cleaved from the SUMO protein, then after the digestion there should be two bands, one for each the SUMO and C-peptide. In the gel in Figure 28, there is only one band that did not change position after the digestion. SUMO is about 18 kDa and C-peptide is about 3 kDa.
Figure 28: SDS-Page gel. Lane 1: Low range protein ladder markers, Lane 2: Pre-induction control, Lane 3: Overnight induction crude, Lane 4: FPLC flow through, Lane 5: FPLC eluted, Lane 6: After Dialysis, Lane 7: Digestion

Different conditions were changed in order to try and get the cleavage to occur. This included varying the temperature, the use of DTT and urea, and the protease concentration and type. However, after many attempts at cleavage of the SUMO protein and C-peptide, it was decided that a different method of cleavage was necessary.
6.2.7 The Addition of Factor Xa

Because the SUMO protease recognizes SUMO’s tertiary structure in order to cleave the protein, it was decided a protease was needed that recognized a definitive sequence and not a particular folding pattern. Factor Xa protease was chosen, which cleaves after Arginine in the cleavage site Isoleucine-Glutamic Acid-Glycine-Arginine (IEGR). New primers were designed to incorporate the factor Xa site into C-peptide (Figure 29).

Forward Primer:
5’CGCGAACAGATTGGAGGTATTGAAGGCCGCAAGCGGAAG3’

Reverse Primer:
5’ GTGGCGGCCGCTCTATTATTGCAGAGAGCCTTCCAG3’

Figure 29: Primers for C-peptide with Factor Xa

The PCR product was amplified as in section 6.2.2 and ligated as in section 6.2.3 into HI-control 10G cells. After many attempts, it was clear that the ligation was not occurring. Instead, a DNA sequence was ordered that contained SUMO, a Factor Xa cleavage site, and C-peptide (Figure 30). Two different restriction sites were also added to each end in order to digestion and ligate this DNA sequence into a vector, pET-15b. Based on the restriction sites available in pET-15b, Nde1 and BamH1 were chosen as the restriction enzymes/sites. The DNA sequence arrived in pUC57.
A: *Nde*I His8 SUMO Factor Xa C-peptide*Bam*HI Stop Codon

B:  
CATATG CATCACCATCACCATACCATCACGATATG CATCACGATCATCACCACC
ATCACGGGTCCCTGCAAGGACTCAAGAAGCTAAAGCTAAGCCA
GAGGTCAAGCCAGAAGTCAAAGCCTGAGACCTCAGACATCAATTTAAAGGT
GTCGATGGATCTTCAGAGATCTTCTTCAAGATCATAAAAGACCCACTCC
TTAAAGAAGGGCTGATGGAAGCGTCCGCTAAAGACAGGGTTAAGGAAA
TGGACTCTCTTAAGTGTTGAACTGGGACGATTTGAAATTCGAAGCTGATTC
AGAGCTCTAAGATTTGCAATGGAAGGATTAGATATTATTAAAAAGGCGTAC
ACCAGCAACAGATTTGAGGTATTGAAGGCGCGGAAGCAGGAAGATCTG
CAGGGTTGCGCAGGTGGGAACCTGGGACGGGCGGCCCGGGCCGGGCGAGCC
TGCGAGCCGCTGGCGCTGGAAGGCGGAGCGCTGCAGCCGATCCUAA

Figure 30: Ordered DNA sequence containing SUMO, Factor Xa, and C-peptide. A shows a representation of the parts of the sequence and B shows the actual DNA sequence.

Once the DNA was received, both the DNA sequence and pET-15b were digested to prepare for ligation. 4 µL of pET-15b, 1 µL *Nde*I protease, 2 µL buffer 4, and 11 µL of sterile water were mixed in one eppendorf tube. 4 µL of the ordered DNA sequence in pUC57, 1 µL *Nde*I protease, 2 µL buffer 4, and 11 µL of sterile water were mixed in another eppendorf tube. Both were placed at 37°C for 30 minutes. Then to the pET-15b eppendorf tube, 1 µL Calf Intestinal Phosphatase (CIP) and 1 µL of *Bam*HI were added To the DNA sequence eppendorf tube, 1 µL of sterile water and 1 µL of *Bam*HI were added. Both reactions were placed back at 37°C for 60 minutes.

Using an agarose gel and the E.Z.N.A kit, the digested DNA (C-peptide) and vector were purified and extracted. Molar ligations were performed in the following ratios: 1:1 2 µL C-peptide/1 µL vector, 3:1 6 µL C-peptide/1 µL vector, 5:1 10 µL C-
peptide/1 µL vector, 10:1 20 µL C-peptide/1 µL vector. To each of these, 2 µL of buffer, 1 µL of T4 ligase, and enough sterile water to make each reaction mixture 21 µL were added to complete the ligation mixtures.

3 µL of each ligation mixture was then added to BL21(DE3) cells thawed on ice to begin the transformation. The cells with the added ligation mixture were incubated on ice for 30 minutes and then heat shocked for 45 seconds at 42°C. 960 µL of recovery media was added and the cells were allowed to sit on ice for 2 minutes. The cells were then plated on LB Ampicillin (AMP) Agar plates and grown overnight at 37°C.

All of the plates grew as expected and twelve singles colonies were selected, grown overnight in 5 mL LB, 5 µL AMP at 37°C, 250 rpm. The plasmid was isolated from two of the cultures that had grown and sent for sequencing. The sequence confirmed that a successful ligation had occurred between the C-peptide sequence and the pET-15b vector.

The colonies with the correct sequences were selected, induced, and lysed as in section 6.2.4. An SDS-Page gel was ran (Figure 31) of the various time points (pre-induction, 1 hour after, 4 hours, overnight) and IPTG concentrations (0.1 mM and 0.01 mM). The gel did not show a band near 20-21 kDa, which is where the Sumo C-peptide should appear. After multiple attempts, the cells did not appear to be lysing properly, so a more effective method of lysing the cells is currently being developed by increasing the length of sonication.
Figure 31: SDS-Page gel of SUMO, Factor Xa, and C-peptide system after induction and lysis. Lane 1: Precision Plus Protein Standards, Lane 2: Pre-induction control .01 IPTG, Lane 3: 1 hour .01 IPTG, Lane 4: 4 hours .01 IPTG, Lane 5: O/N .01 IPTG, Lane 6: PIC 0.1 IPTG, Lane 7: 1 hour 0.1 IPTG, Lane 8: 4 hours, 0.1 IPTG, Lane 9: O/N 0.1 IPTG

6.3 Conclusion

Although the cleavage of C-peptide from the SUMO system has not yet been achieved, this work shows that the recombinant expression of C-peptide is possible utilizing Escherichia Coli cells. The ability to recombinantly express C-peptide is a low cost alternative to other methods currently available. Once a method of cleavage
is developed, C-peptide can be expressed and we can begin utilizing it for oral delivery experiments using $B_{12}$.

7 Future Work

7.1 C-peptide Expression and Confirmation

Once the cells are lysed properly, a purification method to isolate the SUMO Xa C-peptide will be developed. Using the Factor Xa protease, we will cleave C-peptide from the SUMO system. We will then purify the C-peptide using Factor Xa binding beads.

After the recombinant expression and purification of C-peptide is achieved, we are going to run diagnostic tests that support its synthesis. This will include a Western blot and an Enzyme-linked immune sorbent assay (ELISA), which uses antibody recognition to confirm C-peptide’s presence. Also, Circular Dichroism (CD) will be conducted to determine the folding state and MALDI-ToF will be performed to support the presence of C-peptide by using its mass to charge ratio.

7.2 Oral Delivery of C-peptide

Using the 5'-carboxylic acid $B_{12}$ derivative we have made, we are going to conjugate C-peptide to $B_{12}$. In order to make the oral delivery of C-peptide feasible, C-peptide cannot lose its function when conjugated to $B_{12}$. C-peptide needs to be conjugated using its carboxyl terminus. Because a carboxylic acid and carboxylic acid cannot be coupled, a linker will be used. We will use ethylenediamine to link the 5’-carboxylic acid to the carboxyl terminus of C-peptide. Although this will add two carbons, it should allow C-peptide to maintain its function in the body.
8 References


Capstone Summary

Diabetes is a group of disease categorized by high levels of blood glucose (sugar). Insulin is a hormone produced by the body that regulates blood glucose levels. Therefore, diabetes can be a result of defective insulin production, use, or a combination of both. The two main types of diabetes are type 1 and type 2.

Currently patients with diabetes receive most of their treatments/therapeutics, including insulin, subcutaneously via an injection or pump. Although oral administration of these therapeutics would be an ideal alternative route of delivery in order to increase patience compliance and comfort, two main barriers stand in the way. First of all, the acidic environment of the stomach and digestive system would degrade any protein or peptide that was ingested, leaving it unable to function properly in the body. Second of all, the body does not have a method or pathway to absorb these peptides and proteins into the bloodstream so they would just stay in the digestive system and would not function in the body. If these two problems could be solved, then the oral delivery of peptides and proteins would be a feasible method to deliver medications, improving the lives of many. The ability to deliver insulin orally would increase the ease at which it can be administered to those with diabetes. This could significantly increase patient compliance, lowering the complications and deaths each year caused by diabetes.

Utilizing the Vitamin B\textsubscript{12} uptake pathway is one proposed method of oral delivery of protein therapeutics. The utilization of the B\textsubscript{12} uptake pathway has been a method of oral delivery of peptides and protein receiving increasing attention over the
past decade. If proteins and peptides can be linked or conjugated to B₁₂, B₁₂’s pathway and mechanism for delivery within the body naturally has ways to protect the proteins and peptides from the acidic environment in the gastrointestinal tract and allow them to be absorbed into the bloodstream.

When coupling B₁₂ and a peptide, protein, or any other molecule, it is important that both entities maintain their biological function in the body. The functional sites of the peptide, protein, or molecule must still be intact in order to work properly once it reaches the bloodstream. In addition, the site on B₁₂ recognized by the proteins in its uptake pathways must still be recognizable. Therefore, vitamin B₁₂ only has select positions that a protein can be conjugated to.

Conjugation has most successfully occurred at (1) the 5’-hydroxyl group of the ribose and (2) the e propionamide of the peripheral corrin ring. The 5’-hydroxyl group of the ribose of B₁₂ is a versatile site that could potentially be used for the synthesis of a variety of B₁₂ conjugates. However, limited chemistry with a hydroxyl group can be performed and the conjugates synthesized using this site are done through a carbamate or ester bond, which present stability issues. Also, it creates potential for degradation or breaking of the bonds used to conjugate the two entities. One way to solve this problem would be to change the hydroxyl group on B₁₂ into a group, such as a carboxylic acid, that allows for a greater variety of chemistry as well as a more stable bond.

We investigated a way to synthesize a derivative of Vitamin B₁₂ (B₁₂) that could increase the ease of its conjugation to proteins and peptides. The 5’-position of the ribose tail of B₁₂ was oxidized to a carboxylic acid using excess 2-iodoxybenzoic
acid (IBX) and 2-hydroxypyridine (HYP). The synthesis of this B\textsubscript{12} derivative allows for peptides, proteins, and other molecules to be conjugated to the 5’-position of the ribose tail of B\textsubscript{12} using a more stable amide bond instead of a carbamate bond.

In conclusion, we have successfully made a form or derivative of B\textsubscript{12} that could increase the ease at which B\textsubscript{12} conjugates are made. We have shown that IBX can be used to specifically oxidize the 5’ site of B\textsubscript{12}. The B\textsubscript{12} derivative synthesized was done with a yield that triples the synthesis of any other reported carboxylic acid derivative of B\textsubscript{12}. It is also much easier to purify and quicker to make. The formation of a derivative of B\textsubscript{12} that has a carboxylic acid instead of a hydroxyl group available for conjugation increases the chemistry that can be performed at the 5’ site. This will allow higher yielding and more stable conjugates to be formed in the future. This conjugate will be of significant use in the field of B\textsubscript{12} conjugation chemistry.

C-peptide is a protein that is part of the proinsulin. Proinsulin is the form of insulin before it is activated for use as a hormone in the body. After C-peptide is cleaved from proinsulin, Insulin is released into the body in its active form, along with the same amount of C-peptide. For a long time, C-peptide was thought to be biologically inactive and did not have a significant role in the body. People with diabetes therefore only received injections of supplemental insulin, not C-peptide. However, recent studies have suggested that C-peptide may serve a regulatory role in the body by contributing to vascular homeostasis in various tissues. For patients with diabetes, the lack of C-peptide in combination with high blood glucose levels reduces circulation, which can eventually lead to complications like neuropathy. Neuropathy is the disease or dysfunction of nerves. The administration of C-peptide has the
potential to reduce diabetic neuropathy. Diabetic neuropathy most commonly leads to limb amputations. Currently a subcutaneous administration of C-peptide is being studied for use in patients with diabetes, however the ability to deliver C-peptide orally has the potential to be more convenient for patients with diabetes and therefore increase patient compliance. Utilization of the B$_{12}$ uptake pathway by conjugating C-peptide to the 5′-carboxylic acid derivate of B$_{12}$ we have created (B$_{12}$ CA), could allow the oral delivery of C-peptide.

We have successfully created a method to recombinantly express C-peptide using Escherichia coli or a type of bacteria. We have expressed C-peptide using a SUMO system. The SUMO system is added in order to purify C-peptide and extract it for use. The problem we are currently facing is cleaving or cutting C-peptide from the SUMO system. Currently we are investigating alternative methods to solve this problem. After this is completed, the next step will be conjugating C-peptide to B$_{12}$ for oral delivery studies.
Site-Selective Oxidation of Vitamin B₁₂ Using 2-Iodoxybenzoic Acid

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Abstract: Reaction of vitamin B₁₂ (B₁₂) with excess 2-iodoxybenzoic acid and 2-hydroxyypyridine leads to selective oxidation of the 5'-hydroxyl group of the ribose tail of B₁₂ in a 30% isolated yield. The acid derivative was purified in one step by HPLC chromatography and characterized by MALDI-TOF mass spectrometry, ¹H NMR and 2D (HSQC and HMBC) NMR. The new carboxylic acid derivative is perfectly suited to make stable amide-based B₁₂ bioconjugates.

Key words: vitamins, oxidation, 2-iodoxybenzoic acid, carboxylic acids, cobalamin

Interest in using vitamin B₁₂ (B₁₂) for the oral delivery of peptides and proteins¹ has encouraged us to further explore the available conjugate sites of B₁₂. Modifications of B₁₂ that allow for more facile, higher yielding or stable bioconjugate formation with continued recognition by B₁₂ dietary uptake and transport proteins have been explored by numerous groups.²⁻⁴ Several functional groups are available on B₁₂ for conjugation, including the 5'-hydroxyl group of the ribose tail, the cobalt(III) ion, the phosphate moiety, and suitably modified propionamides off the corrin ring.² All such modifications suffer from issues including reduced or complete loss of interaction with one or more of the B₁₂ binding proteins, stability issues that result from the production of ester or carbamate bonds, low yielding conjugation reaction due to the nature of the functional group, or require problematic purification of the modified B₁₂ for subsequent conjugation.²⁻⁴ These limitations have been well investigated⁶ and a review of such sites has been reported,⁷ however, in brief for purposes of perspective for the work described herein: Conjugation resulting in the recognition of all three transport proteins has been most successful with B₁₂ at two major sites: (i) the peripheral corrin ring e-propionamide, and (ii) the 5'-hydroxyl group of the ribose unit of the a`-'tail'. The side chain e-carboxylic acid derivative (Figure 1) allows for a variety of modifications,⁸ but the synthesis of the derivative is low yielding and requires laborious purification, the result, in part, of the formation of b- and d-carboxylic acid isomers in addition to the desired e-isomer.⁹ The 5'-hydroxyl group has been cited as being a more versatile site according to structure-activity relationships, however, the conjugates have typically been synthesized as mentioned above, through carbamate or ester bonds.

The goal of this work was to create a simple route for the production of a 5'-carboxylic acid derivative of B₁₂ with facile purification to ultimately produce stable amide-linked bioconjugates at this optimal position for conjugation, especially in regard to intrinsic factor binding (critical for oral uptake of B₁₂ bioconjugates).¹⁰ Herein, we report the use of the hypervalent iodine reagent, 2-iodoxybenzoic acid (IBX) for the selective oxidation of the 5'-hydroxyl group of B₁₂ to the corresponding carboxylic acid. This is the first time IBX has been utilized for the oxidation of such a molecule as B₁₂, and expands the range of use of this environmentally friendly oxidizing agent.¹¹ The B₁₂ derivative was synthesized through a modification of the method developed by Giannis et al., which incorporates the addition of an O-nucleophile to aid IBX in the transformation of a primary alcohol into a carboxylic acid (Scheme 1).¹² The use of N-hydroxysuccinimide (NHS) was initially attempted but resulted in the formation of a product mixture from which it was not possible to isolate the carboxylic acid, likely due to the formation of the activated NHS ester, which has been reported as a dominating product of this reaction.¹² Therefore, 2-hydroxypyridine (HYP) was used as the O-nucleophile. To optimize the reaction conditions, a series of experiments were performed in which the co-oxidant, temperature, and time were varied (Table 1). The best result was obtained in the

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presence of 2.6 equivalents of IBX and 5 equivalents of HYP in dimethyl sulfoxide (DMSO) at 60 °C.

These conditions resulted in the oxidation of B12 to the 5'-carboxylic acid derivative B12CA in two hours with a 30% yield (see the Supporting Information). It was determined that a 30% yield was optimal for the IBX reaction in this case. An increase in temperature, IBX/HYP ratios, or time resulted in an increase in the amount of byproducts and/or decomposition of the B12 (especially at temperatures in excess of 60 °C), reducing the yield and complicating purification. The 30% yield is approximately three times that previously reported for the preparation of the e-carboxylic acid derivative, with far easier separation (see below).

The crude reaction mixture was precipitated from DMSO by ether addition and the precipitate was redissolved in water prior to purification by ion exchange chromatography using a SAX column. After a 5 minute hold at 100% water, a gradient from 100% water to 35% MeCN–phosphate buffer (pH 2; 50:50 v/v) over 5 minutes was necessary for the separation and elution of B12CA (Figure 2). Slight increases in pH above pH 2 resulted in a reduction in the amount of pure isolated product. HPLC purification allowed the recovery and reuse of B12 starting material, albeit with the oxidized 5'-aldehyde also present (Figure 2). B12CA was identified as the peak at 13.6 minutes retention time. The purity of B12CA was at least 95%, as assayed by RP-HPLC. MALDI-TOF mass spectrometry analysis of B12CA revealed the anticipated molecular ion peak (m/z = 1343.5 [M –– CN]+; Figure 3).

Electronic absorption spectroscopy (EAS) was used to establish the oxidation state of the metal center as Co(III), with no indication of derivation of the axial ligands bound to the metal center (εCA361 = 27500 M⁻¹cm⁻¹; see Figure 4)

The structure of B12CA was determined on the basis of 1H NMR (see Figure S2) and 1H–13C heteronuclear (HSQC and HMBC) correlations (see Figures S3 and S4). The 1H NMR spectra of B12CA revealed the loss of two protons (δH = 3.75 and 3.92 ppm) when compared to the spectrum of B12 (CNCbl; see Figures S2 and S5). A comparison of the HSQC of B12 and B12CA indicates the protons that are absent in B12CA were originally attached to the R5 carbon (δC = 63.5 ppm; see Figure S6). HMBC was attempted to further establish the presence of a carbonyl at the R5 position (see Figure S4), however, it was not possible to assign the R5 carbon from the HMBC spectra because it

### Table 1

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<th>Entry</th>
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2).
showed no connectivities to any of the ribose protons, and so was assigned by HSQC.\textsuperscript{13}

A simple amine-containing organic molecule, benzylamine, was conjugated to the derivative to help with the identification of the R5 carbon by NMR analysis. In the presence of 10 equivalents of ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 20 equivalents 1-hydroxybenzotriazole (HOBt) in N,N-dimethylformamide (DMF) with 1% water, the desired conjugate was produced at 40% yield over the first hour (Scheme 2), thus establishing proof-of-concept for the new \textit{B}\textsubscript{12}CA.

The conjugate was purified by using the same purification method established for the carboxylic acid (Figure 5) and characterized by MALDI mass spectrometry (Figure S7) as well as 1D (Figure S8) and 2D NMR (Figures S9 and S10) spectroscopy.

With the addition of the benzylamine, connectivity to the R5 carbon was established by HMBC (Figure 6). Specifically, the protons of C\textsubscript{A} (\(\delta\textsubscript{H} = 4.42\) and \(4.50\) ppm) showed direct connectivity to the R5 carbon (\(\delta\textsubscript{C} = 173.8\) ppm) as well as to C\textsubscript{B} (\(\delta\textsubscript{C} = 140.8\) ppm) and C\textsubscript{C} (\(\delta\textsubscript{C} = 131.4\) ppm) of the benzylamine. The complete assignment of \textit{B}\textsubscript{12}CA and \textit{B}\textsubscript{12}CABA can be found in Table S1.

In conclusion, we have developed a convenient site-specific oxidation of \textit{B}\textsubscript{12} using IBX and HYP. This new carboxylic acid derivative is highly suited to the production...
of bioavailable amide bound bioconjugates. The one-step purification is simple and the synthesis results in a 30% yield within two hours, which is far in excess of any previously reported B<sub>12</sub> carboxylic acid derivative. The resulting derivative has the potential to form high-yielding conjugates and we believe will be of considerable use in the field of B<sub>12</sub> conjugation.

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Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synlett

References and Notes

Reference(s)


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