Chemoenzymatic Synthesis of UDP-GlcNAc and UDP-GalNAc Derivatives for Chemoenzymatic Labeling

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CHEMOENZYMATIC SYNTHESIS OF UDP-GLCNAC AND UDP-GALNAC DERIVATIVES FOR CHEMOENZYMATIC LABELING

by

YUAN ZHENG

Under the Direction of Peng George Wang, PhD

ABSTRACT

Glycans are macromolecules that contain several classes. Glycans can play an important role in biological activities. Studying the cell surface glycans can provide a very powerful way to understand the fundamental process. Also it could help to regulate expected cell response. Thus it is very necessary to have a method to detect cell-surface glycans efficiently.

An efficient method for glycan detection is necessary. Metabolic glycan labeling and chemoenzymatic glycan labeling are most commonly used. Chemoenzymatic glycan labeling is a rapid and sensitive method which also has high specificity. This method can be applied in both vitro and vivo. However the availability of unnatural sugar nucleotides functioned by bioorthogonal groups is the main limitation for chemoenzymatic labeling.

In this thesis, UDP-GlcNAc and UDP-GalNAc derivatives were prepared for further chemoenzymatic labeling by using chemoenzymatic synthesis method.
INDEX WORDS: Chemoenzymatic Glycan Labeling, Metabolic Labeling, Sugar Nucleotide, Bioorthogonal Chemistry, Click Chemistry
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FOR CHEMOENZYMATIC LABELING

by

YUAN ZHENG

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of
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CHEMOENZYMATIC LABELLING OF UDP-GLCNAC AND UDP-GALNAC DERIVATIVES FOR CHEMOENZYMATIC LABELING

by

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Committee: Jun Yin
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Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
May 2017
DEDICATION

First I would like to thank to my family, especially to my mother for her love, suggestions, and financial support. She gives me great comfort and encouragement when I was depressed. She always cheers me up. She is my light in my life.

To my brother Ke; my aunties, Yuxian and Fuying; my uncles Xiaobing and Jiankun and my cousin Lucy. They always give me full of support and love.

To my father, who protect me and accompany with me from the heaven all the time.

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<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>Ac</td>
<td>Acetyl</td>
</tr>
<tr>
<td>br</td>
<td>Broad (NMR)</td>
</tr>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>Bz</td>
<td>Benzoyl</td>
</tr>
<tr>
<td>°C</td>
<td>degree Cesius</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>GT</td>
<td>glycosyltransferase</td>
</tr>
<tr>
<td>h</td>
<td>hour (s)</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5′-triphosphate</td>
</tr>
<tr>
<td>L</td>
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</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>m</td>
<td>micro</td>
</tr>
<tr>
<td>M</td>
<td>moles per liter</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
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<td>methly</td>
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<td>ethyl</td>
</tr>
<tr>
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<td>thin layer chromatography</td>
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</tr>
<tr>
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<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo dalton</td>
</tr>
<tr>
<td>PPI</td>
<td>pyrophosphate</td>
</tr>
<tr>
<td>PPA</td>
<td>Yeast inorganic pyrophosphatase</td>
</tr>
<tr>
<td>MHz</td>
<td>megahertz</td>
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<tr>
<td>GalNAc-1-P</td>
<td>N-acetylgalactosamine-1-Phosphate</td>
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1 INTRODUCTION

Glycans are macromolecules that made up of ten monosaccharides: glucose (Glc), sialic acid (Sia), galactose (Gal), mannose (Man), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), fucose (Fuc) and xylose (Xyl). Glycans mainly have three categories: O-linked glycans, N-linked glycans and glycolipids.

It is well-known that glycans play important roles in many fields, such as cell-cell interaction, cell-pathogen interactions, immunity, stability of proteins and cancer. For example, glycans can be used to detect blood types, because blood types are based on the glycan structures.

Studying the cell surface glycans can provide a very powerful way to understand the fundamental process. Also it could help to regulate expected cell response. Thus it is very necessary to have a method to detect cell-surface glycans efficiently.

In previous studies, glycans detection was using lectins and antibodies. Lectins and antibodies can be used in the glycan detection mainly because of the specificities they have. At the same time, lectins and antibodies binding glycans has high affinity, which is also benefit the detection among variety of cell surface glycans.

But this method was suffered from several reported disadvantages, such as the antibodies which are using for binding glycans, especially IgM isotype are difficult to raise, besides the binding affinity is always very low. At the same time, most lectins do not have strong specificity. Those two main drawbacks make the study of the glycans much more difficult.

Later on, chemical methods became popular for the detection of glycans. It has been used for a long time. However chemical methods cannot be sued widely because the toxicity of chemicals. For instance, in order to detect sialic acid, researchers often use alcian blue and
periodic acid, which are all damaging the carbohydrate chains.\textsuperscript{14} Thus this method cannot be used for furthermore applications.

Metabolic labeling was then came up, this is a powerful tool for the detection of glycans.\textsuperscript{15} In order to achieve the goal of glycoprotein enrichment, using metabolic labeling combined with biorthogonal method has many advantages.\textsuperscript{16} Compared with antibodies or lectins poor specificities and weak substrate binding, the chemical reporter will directly bind with glycan, glycosylation-specific enrichment will be achieved by using specific probes.\textsuperscript{11} However metabolic labeling suffered low efficiency,\textsuperscript{17} toxicity for cellular system, and limitations in human sample’s glycoprotein enrichment.\textsuperscript{18-19}

There are some popular detection methods were then reported. Such as detection of fucosylated,\textsuperscript{20-21} sialylated\textsuperscript{22} and mucin-type O-glycans\textsuperscript{23} in living cells by using metabolic labeling.\textsuperscript{12} However metabolic labeling is not available for detection of disaccharides or trisaccharides, because those higher order glycans contains monosaccharides, some precursors could enter varieties of glycan biosynthetic pathways which has many glycosyltransferases are involved, so the specificity of the linkage is difficult to be controlled.\textsuperscript{12,19} Besides there are not enough research could prove that metabolic labeling could be used in human, so rather than metabolic labeling, a good detection method still need to be developed.

In order to solve those limitations of chemical methods detection and metabolic labeling methods, a new method called chemoenzymatic labeling was came up.\textsuperscript{24} Chemoenzymatic glycan labeling is a technique that using glycosyltransferases and unnatural sugar nucleotide linked together, at the same time, take advantage of bioorthogonal chemical tags in order to achieve specific glycan labeling in biological samples.\textsuperscript{25} According to the strategy, chemoenzymatic labeling method is able to detect glycans both on the cell surface and in cell
lysates. As I mentioned previously, chemical methods and metabolic labeling cannot label higher orders of glycans, while chemoenzymatic labeling can solve this limitation perfectly because of the high specificity of glycosyltransferases.

The first chemoenzymatic glycan labeling method was reported by Hsieh-Wilson’s group in 2004. Their idea was using a mutant galactosyltransferase (Y289L GalT) to transfer a ketone onto an O-GlcNAc modified protein. After transferred, biotin was attached on the ketone, and then using streptavidin (HRP) for the detection of the O-GlcNAc glycosylated protein. This method is more efficient and specific to detect the O-GlcNAc modified protein compared to traditional methods.

Bertozzi and co-workers came up with a glycan imaging technology only using small molecule by two-step reaction. First, an unnatural glycan with chemical group metabolically into target sugar chain. Second, a covalent reaction occurred between chemical group and imaging probe, in order to be detected.

The study of the glycosylation has many challenges; the biggest one is because of the difficulties of synthesis of unnatural glycan and the complexity of glycans structures. The existing methods have many limitations which including the low affinity, while chemoenzymatic labeling is a new method could solve those limitations.

In the past decades, bioorthogonal reactions has a big impact in chemical biology, also has many applications on the detection of cell surface glycans. Bioorthogonal reaction can be widely used because the size of the bioorthogonal probes is not big, those probes will not change glycan structure, and will not do harm to the living cells or disturb their native environment. Because there are so many claims to the reaction conditions, therefor in the past decades, only few biorthogonal reactions were well developed, including Staudinger ligation.
catalyzed azide-alkyne cycloaddition (also known as CuAAC),\textsuperscript{32} strain-promoted alkyne-azide cycloadditions (also known as Cu-free click chemistry or SPAAC).\textsuperscript{33} Those reactions have been widely used for labeling glycans in living cells or cell lysates.\textsuperscript{28}

![Figure 1.1 Staudinger Ligation](image)

Staudinger ligation was the first bioorthogonal reaction reported by Staudinger and co-worker in 1919.\textsuperscript{31} This is a photo-type bioorthogonal ligation reaction that transform azides into primary amines by phosphines.\textsuperscript{34} This reaction was used to target biomolecules not only in living cells but also living animals.\textsuperscript{26} Later on, some other improvements were made for this reaction, such as use modified reagent. Accompany with those advantages, Staudinger ligation suffered from the slow kinetics and the phosphine reagents are easy oxidized during the reaction progress, thus this reaction is not a perfect candidate for the glycan detection.\textsuperscript{12}

![Figure 1.2 Copper-Catalyzed Click Chemistry](image)

To date until now, the most popular and common bioorthogonal method is copper-catalyzed click chemistry.\textsuperscript{27, 32} This is a copper-catalyzed cycloaddion reaction between alkyne and azide to form a 5 membered ring.\textsuperscript{35} In early 2000s, Sharpless group and Meldal group
discovered nearly at the same time that this reaction could be accelerate by copper dramatically, and this reaction can be occurred in aqueous system.\textsuperscript{12, 31} Although this copper-catalyzed click reaction has been widely used in chemical biology, however, copper is a toxic reagent which will do harm to living cells or the biological system, so this method has limitations in many applications.\textsuperscript{12, 36} And the toxicity of Cu(I) makes this method not good enough to be further used in many area, such as diagnosis in human diseases.\textsuperscript{12} In order to solve this problem, some researchers tried to decrease the amount of Cu (I) usage to decrease the damage to the living cells.\textsuperscript{12, 33}

\begin{center}
\begin{align*}
\begin{array}{c}
\text{R}_1, \text{R}_2 = \text{biomolecules, bioactive molecules, fluorophores, affinity tags, etc.}
\end{array}
\end{align*}
\end{center}

\textit{Figure 1.3 Strain-Promoted Azide-Alkyne Cycloaddition}

After limited success, a safer method is still needed without devastating living cells. Bertozzi and co-worker further developed a copper free click chemistry which also named Strain-promoted azide-alkyne cycloaddiion (SPAAC).\textsuperscript{33} Instead of linear alkyne, they used strained cyclooctynes. Without using toxic Cu(I), this form of alkyne can react with azide quickly and highly reactively in biological system.\textsuperscript{37} This method do no harm to living cells and living animals, thus it has been widely used and remain the most popular and common click reaction. Because of its advantages, it also served as standard method of chemoenzymatic glycan labeling.\textsuperscript{12}
1.1 Purpose of the Study

Glycans on cell surface always play an important role in many area, such as cell death, cell migration, and immune response etc. Finding efficient methods for the detection of glycans on cell surface is a major problem that researchers are facing. Although chemoenzymatic labeling of specific glycans is a good method to address this problem, but lack of those related sugar nucleotides or sugar nucleotide derivatives is the impediment to research’s progress. So it is extremely important for us to synthesis those sugar nucleotide derivatives.

There are some common monosaccharides in mammalian cells: glucose (Glc), sialic acid (Sia), galactose (Gal), mannose (Man), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), fucose (Fuc) and xylose (Xyl).

![Figure 1.4 Symbolic Representations of Common Monosaccharides in Mammalian Cells](image)

GlcNAc and GalNAc are commonly existing in both bacteria and mammalian cells. They are C-4 anomers which has very important significance in structure and functionality. GlcNAc is structural units in backbone of bacteria’s cell walls. GlcNAc is an important component of glycosaminoglycan (GAG) and hyaluronic acid (HA). GlcNAc take part in many biological pathways, the most identical pathway is regulating signal transduction pathway by glycosylation of O-GlcNAc protein. GalNAc is a component of two glycosaminoglycans: chondroitin sulfuric acid and dermanan sulfate. It is also the first glycosyl of mucin type glycosylation.
Isolation and purification of defined sugar nucleotides from nature is very difficult, thus it is necessary to synthesize sugar nucleotides and their derivatives. In the past decades, researchers already gained a great progress related to the both chemical and enzymatic synthesis of sugar nucleotides. Compared with enzymatic synthesis, chemical synthesis suffered from several protection and de-protection steps.

The most common enzyme used in chemoenzymatic synthesis is glycosyltransferases. It is an efficient enzyme that could specific sugar moiety from a door to an acceptor. The advantage of using glycosyltransferases is the high specificity and high efficiency.

**Synthesis of sugar nucleotide**

![Synthesis Pathway of Sugar Nucleotide](image)

Sugar nucleotide also named nucleotide sugar, there are 9 most common sugar in mammalian cells: UDP-Glu, UDP-Gal, UDP-GlcNAc, UDP-GalNAc, UDP-Xyl, UDP-GlcA, GDP-Man, GDP-Fuc and CMP-Sialic acid (Figure 1). Sugar nucleotides and their derivatives in very important due to its role in research of glycoconjugate biosynthesis pathway. Thus, synthesis of natural and unnatural sugar nucleotides is very significant in the field of biochemistry and pharmaceutical chemistry.
2 EXPERIMENT

Chemical synthesis of UDP-GlcNAc and UDP-GalNAc derivatives is a reaction contains multiple steps and purification steps which suffered from low yield and tedious process. On the contrary, glycoslytransferases can achieve an efficient biosynthesis pathway. Especially, one pot multienzyme system provide a very efficient approach for the production of UDP-GlcNAc and UDP-GalNAc derivatives.

2.1 Expression and purification of NahK, GlumU, AGX1

As reported, N-acetylhexosamin kinase (NahK) is an efficient transferase which was found in Bifidobacterium longum. GalNAc/GlcNAc derivatives act as substrate could react with ATP phosphate donor and then synthesis GlcNAc-1-P/ GalNAc-1-P by using this enzyme efficiently. AGX1 is an uridylyltransferase from human which was reported has a good activity to synthesis UDP-GalNAc/ UDP-GalNAz from GalNAc-1-P/ GlcNAc-1-P.

For protein production, adding NahK enzyme strain and 50 µg/mL Kanamycin into 6ml LB medium, incubated at 37 °C for overnight. Transfer culture into 2L LB medium with Kanamycin, and culture for about 10 h. Adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to the expression medium, then culture at 16 °C overnight. After expression, the cells were collected by centrifuge at 4,000 rpm for 30 mins. Discard supernatant, use 35mL lysis buffer to re-suspended pellet. Cell was fully lysed by sonication in ice-water bath. Then centrifuge suspension by centrifuge at 13,500 rpm at 4°C for 30 mins. Discard pellet, collect the supernatant.

The supernatant was immediately loaded in to a column which contains Ni²⁺ affinity beads twice. Washing the column by wash buffer, and then use a higher imidazole gradient elution buffer to elute the protein.
2.2 Enzymatic synthesis of UDP-GlcNAc derivatives

With the GlcNAc derivatives ready for use, I performed one-pot multienzymatic reaction to synthesis UDP-GlcNAc derivatives by using NahK, AGX1 or GlmU and PPA.

![Synthesis Pathway of UDP-GlcNAc Derivatives](image)

Figure 2.1 Synthesis Pathway of UDP-GlcNAc Derivatives

This is an effective route which put substrate and all enzymes mix together for UDP-GlcNAc derivatives. Table below shows the reaction system.

Table 2.1 Small Reaction System for UDP-GlcNAc synthesis

<table>
<thead>
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<th>50μl Reaction system</th>
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<tr>
<td>GlcNAc derivatives</td>
<td>20mM</td>
</tr>
<tr>
<td>ATP</td>
<td>20mM</td>
</tr>
<tr>
<td>UTP</td>
<td>20mM</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>20mM</td>
</tr>
<tr>
<td>Mg2+</td>
<td>10mM</td>
</tr>
<tr>
<td>H2O</td>
<td></td>
</tr>
<tr>
<td>Adjust Ph (1M NaOH)</td>
<td></td>
</tr>
<tr>
<td>NahK&amp;AGX1(or GlmU)&amp;PPA</td>
<td>3ul</td>
</tr>
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</table>

After overnight reaction, UDP-GlcNAc derivative was formed. Then P-2 column was used for purification.

2.3 Enzymatic synthesis of UDP-GalNAc derivatives

With the GalNAc derivatives ready for use, we performed one-pot multienzymatic reaction to synthesis UDP-GalNAc derivatives. I tried both NahK and GlmU to find out the most efficient one, and further did large scale reactions.
This is an effective route which put substrate and all enzymes mix together for UDP-GalNAc derivatives. This method takes less time than the chemical synthesis of UDP-GalNAc derivatives, which can be widely use for the synthesis of unnatural sugar nucleotides derivatives.

*Figure 2.2 Synthesis Pathway of UDP-GalNAc Derivatives*
3 RESULTS

The synthesized UDP-GalNAc/UDP-GlcNAc and their derivatives are all confirmed by NMR. Overall, I synthesized 10 UDP-GlcNAc derivatives and 7 GalNAc derivatives in total which are ready for future chemoenzymatic labeling.

3.1 Enzyme activity study of NahK and AGX1

The enzyme activity of NahK and AGX1 was determined by four small scale reactions. All four reaction systems were performed in a 50 ul reaction system mixture, each of the mixture containing 10mM substrate, 15mM ATP, 15mM UTP, 1.5mM Mg$^{2+}$, 2.5mM Tris-HCL buffer (pH 8.0). Adjust pH to around 8.0. Adding water to the first reaction system up to 50ul. Adding 1ug NahK and water into the second reaction system up to 50ul. Adding 1ug AGX1 and water into the third reaction system up to 50ul. Adding 1ug NahK, 1ug AGX1 and water to the fourth reaction system.

Thus from TLC analysis, we can observe that the starting materials was mostly consumed in 12h, and a spot of the product UDP-GalNAc and byproduct ADP, UDP was showed clearly. (GlcNAc/GalNAc has no fluorescence under the short wavelength UV light, meanwhile sugar nucleotides has fluorescence spot under the UV light).

3.2 Chemoenzymatic synthesis of UDP-GlcNAc derivatives

In order to synthesis UDP-GlcNAc derivatives, highly efficient one pot multienzyme method was used.

This reaction is a 15ml reaction system, contains 10mM GlcNAc or its derivatives, 15mM UTP, 15 Mm ATP, 10mM Mg$^{2+}$, 5mM Tris-HCL, using NaOH adjust pH to 8.0, then add 1mL NahK and 1mL AGX1. The reaction progress was determined by Thin-layer Chromatography (EtOAc: MeOH: H$_2$O: HOAC=5:2:1.4:0.4).
After overnight reaction at 37°C, and the reaction fully finished (determined by TLC), using NaOH to adjust pH to 9.0, adding 120U of Alkaline phosphatase to hydrolyze by-products UDP and UDP, and the remaining ATP and UTP at 37°C for overnight. Once all those byproducts were hydrolyzed, equal amount of Ethonal was added to precipitate all the remaining enzymes. The mixture was centrifuged at 6000 rpm for 20 mins, the supernatant were collected and concentrated to 5ml. The concentrated samples were loaded into P2 column for purification. The fractions that contains UV-visible spots are pure product we need. The fractions contains product were collected and concentrated by oil pump. The concentrated product was formed to white powder by lyophilization.
Table 3.2: Compound Design and reaction yield of UDP-GlcNAc Derivatives

<table>
<thead>
<tr>
<th>Entry</th>
<th>Starting material</th>
<th>Enzymes</th>
<th>Product</th>
<th>Yield</th>
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<td><img src="image1" alt="Chemical Structure" /></td>
<td>NahK, GlmU and PPA</td>
<td><img src="image2" alt="Chemical Structure" /></td>
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<tr>
<td>a7</td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>NahK, GlmU and PPA</td>
<td><img src="image4" alt="Chemical Structure" /></td>
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<td><img src="image5" alt="Chemical Structure" /></td>
<td>NahK, GlmU and PPA</td>
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<tr>
<td>a9</td>
<td><img src="image7" alt="Chemical Structure" /></td>
<td>NahK, GlmU and PPA</td>
<td><img src="image8" alt="Chemical Structure" /></td>
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<tr>
<td>a10</td>
<td><img src="image9" alt="Chemical Structure" /></td>
<td>NahK, AGX1 and PPA</td>
<td><img src="image10" alt="Chemical Structure" /></td>
<td>48</td>
</tr>
</tbody>
</table>

In summary, there are 10 UDP-GlcNAc derivatives were synthesized successfully, all the products were then confirmed by NMR spectrum.

3.2.1 $^1$H-NMR and $^{13}$C-NMR analysis of compound 1

$^1$H NMR (400 MHz, D2O) δ 7.92 (d, J = 8.1 Hz, 1H), 6.01 – 5.90 (m, 2H), 5.51 (dd, J = 7.2, 3.2 Hz, 1H), 4.36 – 4.30 (m, 2H), 4.25- 4.20 (m, 2H), 4.17 – 4.14 (m, 2H), 4.06 – 4.01 (m, 2H), 3.91 – 3.86 (m, 1H), 3.83 – 3.76 (m, 3H), 3.54 (t, J = 9.6 Hz, 1H); $^{13}$C NMR (100 MHz, D2O) δ 170.90, 166.18, 151.72, 141.59, 102.56, 94.32, 94.26, 88.52, 83.08, 82.99, 73.71, 72.97, 70.75, 69.54, 69.36, 64.92, 60.21, 53.66, 53.58, 51.50; $^{31}$P NMR (162 MHz, D2O) δ -11.28 (d, J = 17.2 Hz), -13.00 (d, J = 17.0 Hz).
Figure 3.1 The $^1$H-NMR of Compound 1

Figure 3.2 The $^{13}$C-NMR of Compound 1
3.2.2 $^1$H-NMR and $^{13}$C-NMR analysis of compound 2

$^1$H NMR (400 MHz, D2O) δ 5.21 (d, J = 3.3 Hz, 1H), 3.93 – 3.88 (m, 1H), 3.88 – 3.86 (m, 1H), 3.79 – 3.72 (m, 2H), 3.60 (t, J = 6.3 Hz, 3H), 3.50 – 3.44 (m, 2H), 2.61 – 2.56 (m, 2H);

$^{13}$C NMR (100 MHz, D2O) δ 174.10, 166.23, 151.79, 141.66, 102.64, 94.55, 94.49, 88.56, 83.20, 83.11, 73.79, 73.04, 70.92, 69.63, 69.52, 65.02, 64.96, 60.32, 53.66, 53.58, 47.08, 34.83; $^{31}$P NMR (162 MHz, D2O) δ -11.29 (d, J = 20.7 Hz), -13.05 (d, J = 20.7 Hz).

Figure 3.3 The $^1$H-NMR of Compound 2
3.2.3  $^1$H-NMR and $^{13}$C-NMR analysis of compound 3

$^1$H NMR (400 MHz, D2O) δ 7.98 (d, J = 8.1 Hz, 1H), 6.04 – 5.94 (m, 2H), 5.53 (dd, J = 6.7, 2.8 Hz, 1H), 4.39 – 4.35 (m, 2H), 4.30 – 4.21 (m, 3H), 4.04 (d, J = 10.5 Hz, 1H), 3.95 (d, J = 10.0 Hz, 1H), 3.90 – 3.74 (m, 3H), 3.57 (t, J = 9.6 Hz, 1H), 2.61 -2.58 (m, 2H), 2.52 (d, J = 7.7 Hz, 2H), 2.39 (s, 1H); $^{13}$C NMR (100 MHz, D2O) δ 175.15, 166.22, 151.78, 141.65, 102.66, 94.63, 94.57, 88.54, 83.65, 83.19, 83.10, 73.80, 73.01, 70.86, 70.04, 69.61, 69.52, 64.99, 64.94, 60.30, 53.54, 34.27, 14.33; $^{31}$P NMR (162 MHz, D2O) δ -11.33 (d, J = 20.5 Hz), -13.13 (d, J = 20.5 Hz).
Figure 3.5 The $^1$H-NMR of Compound 3

Figure 3.6 The $^{13}$C-NMR of Compound 3
3.2.4 \textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR analysis of compound 4

\textsuperscript{1}H NMR (400 MHz, D2O) \( \delta \) 7.93 (d, \( J = 8.1 \) Hz, 1H), 5.97 – 5.90 (m, 2H), 5.48 (dd, \( J = 6.9, 3.0 \) Hz, 1H), 4.34 - 4.30 (m, 2H), 4.25 – 4.13 (m, 2H), 3.97 (d, \( J = 10.5 \) Hz, 1H), 3.90 (d, \( J = 9.8 \) Hz, 1H), 3.85- 3.74 (m, 3H), 3.52 (t, \( J = 9.6 \) Hz, 1H), 2.42 (t, \( J = 7.4 \) Hz, 1H), 2.33 (s, 1H), 2.22 (t, \( J = 6.0 \) Hz, 1H), 1.81 – 1.74 (m, 2H); \textsuperscript{13}C NMR (100 MHz, D2O) \( \delta \) 176.69, 166.17, 151.71, 141.61, 102.58, 94.52, 94.46, 88.49, 84.83, 83.10, 83.01, 73.74, 72.94, 70.77, 69.75, 69.53, 69.47, 64.87, 60.21, 53.51, 53.43, 34.54, 24.14, 17.14; \textsuperscript{31}P NMR (162 MHz, D2O) \( \delta \) -11.41 (d, \( J = 20.3 \) Hz), -13.13 (d, \( J = 20.3 \) Hz).

Figure 3.7 The \textsuperscript{1}H-NMR of Compound 4
3.2.5 \textit{H-NMR and }\textit{C-NMR analysis of compound 5}

\textit{H NMR (400 MHz, D2O)} $\delta$ 7.96 (d, J = 8.1 Hz, 1H), 5.97 (m, 2H), 5.56 (dd, J = 6.9, 1.9 Hz, 1H), 4.68 (s, 2H), 4.38 - 4.34 (m, 2H), 4.28 - 4.18 (m, 3H), 3.92 - 3.84 (m, 2H), 3.81 - 3.74 (m, 3H), 3.56 - 3.52 (m, 1H), 2.89 (s, 1H); \textit{C NMR (100 MHz, D2O)} $\delta$ 166.24, 157.52, 151.80, 141.60, 102.62, 94.88, 94.82, 88.37, 83.22, 83.13, 78.41, 75.68, 73.81, 72.94, 71.29, 69.61, 69.37, 64.90, 64.84, 60.25, 55.51, 55.42, 52.94; \textit{P NMR (162 MHz, D2O)} $\delta$ -11.34 (d, J = 20.7 Hz), -13.09 (d, J = 20.7 Hz).
Figure 3.9 The $^1$H-NMR of Compound 5

Figure 3.10 The $^{13}$C-NMR of Compound 5
3.2.6 $^1$H-NMR and $^{13}$C-NMR analysis of compound 6

$^1$H NMR (400 MHz, D2O) δ 7.94 (d, J = 8.1 Hz, 1H), 5.95 – 5.93 (m, 2H), 5.49 (dd, J = 7.0, 3.2 Hz, 1H), 4.35 – 4.32 (m, 2H), 4.26 – 4.24 (m, 1H), 4.21 – 4.16 (m, 4H), 4.02- 3.98 (m, 1H), 3.92– 3.88 (m, 1H), 3.86– 3.76 (m, 5H), 3.53 (t, J = 9.6 Hz, 1H), 2.85 (t, J = 2.3 Hz, 1H), 2.64 (t, J = 6.1 Hz, 2H); $^{13}$C NMR (100 MHz, D2O) δ 174.19, 166.22, 151.76, 141.58, 102.59, 94.52, 94.46, 88.43, 83.15, 83.10, 83.05, 79.22, 75.84, 73.75, 72.94, 70.86, 69.54, 69.46, 69.40, 65.67, 64.88, 64.86, 60.23, 57.73, 53.61, 53.52, 35.55; $^{31}$P NMR (162 MHz, D2O) δ -11.41 (d, J = 20.8 Hz), -13.15 (d, J = 20.8 Hz).

Figure 3.11 The $^1$H-NMR of Compound 6
Figure 3.12 The $^{13}$C-NMR of Compound 6

3.2.7 $^1$H-NMR and $^{13}$C-NMR analysis of compound 7

$^1$H NMR (400 MHz, D2O) δ 7.95 (d, J = 8.1 Hz, 1H), 5.97 – 5.91 (m, 2H), 5.92 – 5.81 (m, 1H), 5.49 (dd, J = 6.6, 2.7 Hz, 1H), 5.06 (dd, J = 26.7, 13.8 Hz, 2H), 4.37 – 4.35 (m, 2H), 4.27 – 4.19 (m, 3H), 3.95 (dd, J = 23.7, 10.0 Hz, 1H), 3.88 – 3.77 (m, 3H), 3.54 (t, J = 9.6 Hz, 1H), 2.45 – 2.42 (m, 2H), 2.37 – 2.33 (m, 2H); $^{13}$C NMR (100 MHz, D2O) δ 176.78, 166.20, 151.75, 141.64, 137.14, 115.45, 102.62, 94.64, 94.58, 88.49, 83.19, 83.10, 73.76, 72.97, 70.78, 69.58, 69.53, 64.94, 60.27, 53.54, 53.45, 34.84, 29.25; $^{31}$P NMR (162 MHz, D2O) δ -11.43 (d, J = 20.6 Hz), -13.22 (d, J = 20.5 Hz).
Figure 3.13 The $^1$H-NMR of Compound 7

Figure 3.14 The $^{13}$C-NMR of Compound 7
3.2.8 $^1$H-NMR and $^{13}$C-NMR analysis of compound 8

$^1$H NMR (400 MHz, D2O) δ 7.96 (d, J = 8.4 Hz, 1H), 5.98- 5.96 (m, 2H), 5.60 – 5.37 (m, 1H), 4.39 – 4.35 (m, 2H), 4.29- 4.18 (m, 3H), 4.00 (d, J = 10.4 Hz, 1H), 3.94 (d, J = 9.1 Hz, 1H), 3.85 (m, 3H), 3.56 (t, J = 9.6 Hz, 1H), 2.87 (dd, J = 12.6, 6.4 Hz, 2H), 2.62 (t, J = 6.7 Hz, 2H), 2.24 (s, 3H); $^{13}$C NMR (100 MHz, D2O) δ 214.03, 175.68, 166.18, 151.75, 141.64, 102.60, 94.58, 94.52, 88.55, 83.17, 83.07, 73.78, 73.01, 70.92, 69.60, 69.48, 65.00, 64.95, 60.31, 53.64, 53.55, 38.20, 29.36, 29.17; $^{31}$P NMR (162 MHz, D2O) δ -11.33 (d, J = 18.4 Hz), -13.04 (d, J = 19.0 Hz).

Figure 3.15 The $^1$H-NMR of Compound 8
Figure 3.16 The $^{13}$C-NMR of Compound 8

3.2.9 $^1$H-NMR and $^{13}$C-NMR analysis of compound 9

$^1$H NMR (400 MHz, D2O) δ 7.96 (d, J = 8.0 Hz, 1H), 5.98- 5.96 (m, 2H), 5.53 (d, J = 4.0 Hz, 1H), 4.38- 4.36 (m, 2H), 4.28 – 4.20 (m, 3H), 4.05 (jd, J = 10.3 Hz, 1H), 3.93 (d, J = 9.8 Hz, 1H), 3.83 (m, 3H), 3.56 (t, J = 9.5 Hz, 1H), 2.81- 2.75 (m, 4H); $^{13}$C NMR (100 MHz, D2O) δ 173.14, 166.22, 151.77, 141.62, 102.62, 94.57, 94.46, 88.53, 83.06, 73.75, 73.02, 70.90, 69.58, 69.53, 69.46, 64.95, 60.29, 59.31, 53.57, 30.44, 12.69; $^{31}$P NMR (162 MHz, D2O) δ -11.37 (d, J = 17.7 Hz), -13.06 (d, J = 17.5 Hz).
Figure 3.17 The $^1$H-NMR of Compound 9

Figure 3.18 The $^{13}$C-NMR of Compound 9
3.2.10 $^1$H-NMR and $^{13}$C-NMR analysis of compound 10

$^1$H NMR (400 MHz, D2O) δ 7.98 (d, J = 7.9 Hz, 1H), 6.00 (s, 2H), 5.53 (s, 1H), 4.38 (s, 2H), 4.29 – 4.21 (m, 3H), 4.02 (d, J = 10.2 Hz, 1H), 3.94 (d, J = 10.0 Hz, 1H), 3.85 (m, 3H), 3.56 (t, J = 9.4 Hz, 1H), 2.30 (d, J = 6.4 Hz, 2H), 1.69 (t, J = 7.0 Hz, 2H), 1.04 (s, 3H); $^{13}$C NMR (100 MHz, D2O) δ 175.91, 166.22, 151.79, 141.70, 102.64, 99.82, 94.58, 94.39, 88.54, 83.18, 83.06, 73.78, 70.90, 69.61, 69.58, 69.54, 64.95, 60.30, 53.73, 30.11, 29.81, 26.28, 18.51; $^{31}$P NMR (162 MHz, D2O) δ -11.48 (d, J = 18.6 Hz), -13.24 (d, J = 17.7 Hz).

Figure 3.19 The $^1$H-NMR of Compound 10
3.3 Chemoenzymatic synthesis of UDP-GalNAc derivatives

This reaction is a 15ml reaction system, contains 10mM GalNAc or its derivatives, 15mM UTP, 15 Mm ATP, 10mM Mg\textsuperscript{2+}, 5mM Tris-HCL, using NaOH adjust pH to 8.0, then add 1mL NahK and 1mL AGX1. The reaction progress was determined by Thin-layer Chromatography (EtOAc:MeOH:H\textsubscript{2}O:HOAC=5:2:1.4:0.4).

After overnight reaction at 37°C, and the reaction fully finished (determined by TLC), using NaOH to adjust pH to 9.0, adding 120U of Alkaline phosphatase to hydrolyze by-products UDP and UDP, and the remaining ATP and UTP at 37°C for overnight. Once all those byproducts were hydrolyzed, equal amount of Ethonal was added to precipitate all the remaining enzymes. The mixture was centrifuged at 6000 rpm for 20 mins, the supernatant were collected and concentrated to 5ml.
The concentrated samples were loaded into P2 column for purification. The fractions that contains UV-visible spots are pure product we need. The fractions contains product were collected and concentrated by oil pump. The concentrated product was formed to white powder by lyophilization.

**Table 3.4 Compound Design and reaction yield of UDP-GalNAc Derivatives**

<table>
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<tr>
<th>Entry</th>
<th>Starting material</th>
<th>Enzymes</th>
<th>Product</th>
<th>Yield</th>
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Compound 11-15 were successfully synthesized by NahK and AGX1, but there has no activity through entry b6 to b11. Compound 16 and compound 17 were synthesized by another synthesis pathway which is shown in Figure 3.21.

In summary, 7 UDP-GalNAc derivatives were synthesized successfully, and all the products were then confirmed by NMR spectrum.

3.3.1 ¹H-NMR and ¹³C-NMR analysis of compound 11

¹H NMR (400 MHz, D2O) δ 7.96 (d, J = 8.1 Hz, 1H), 5.98- 5.96 (m, 2H), 5.56 (dd, J = 6.8, 3.1 Hz, 1H), 4.38- 4.34 (m, 2H), 4.31- 4.28 (m, 2H), 4.23- 4.18 (m, 3H), 4.04 (s, 1H), 3.97 (dd, J = 11.0, 2.8 Hz, 1H), 3.81 – 3.73 (m, 3H), 3.61 (t, J = 6.4 Hz, 2H), 2.77 – 2.59 (m, 2H); ¹³C NMR (100 MHz, D2O) δ 174.30, 166.21, 151.78, 141.65, 102.60, 94.68, 88.48, 83.22, 83.13, 73.75, 72.06, 69.62, 68.40, 67.62, 65.00, 61.01, 59.36, 49.78, 49.73, 47.06, 34.84; ³¹P NMR (162 MHz, D2O) δ -11.33 (d, J = 21.1 Hz), -13.00 (d, J = 21.0 Hz).
Figure 3.22 The $^1$H-NMR of Compound 11

Figure 3.23 The $^{13}$C-NMR of Compound 11
3.3.2 $^{1}H$-NMR and $^{13}C$-NMR analysis of compound 12

$^{1}H$ NMR (400 MHz, D2O) $\delta$ 7.95 (d, $J = 8.1$ Hz, 1H), 5.97 – 5.95 (m, 2H), 5.53 (dd, $J = 6.8$, 3.3 Hz, 1H), 4.37 – 4.33 (m, 2H), 4.29 – 4.26 (m, 2H), 4.22– 4.17 (dd, $J = 12.6$, 7.0 Hz, 2H), 4.03 (d, $J = 2.4$ Hz, 1H), 3.96 (dd, $J = 11.0$, 2.9 Hz, 1H), 3.79 – 3.70 (m, 2H), 2.57 (dd, $J = 13.4$, 6.1 Hz, 1H), 2.50 -2.47 (m, 2H), 2.35- 2.34 (m, 1H); $^{13}C$ NMR (100 MHz, D2O) $\delta$ 175.36, 166.20, 151.76, 141.64, 102.61, 94.79, 88.47, 83.61, 83.20, 83.11, 73.76, 72.02, 69.95, 69.59, 68.38, 67.56, 64.98, 64.95, 60.98, 49.69, 49.67, 34.28, 14.30; $^{31}P$ NMR (162 MHz, D2O) $\delta$ -11.37 (d, $J = 20.7$ Hz), -13.08 (d, $J = 20.9$ Hz).

*Figure 3.24 The $^{1}H$-NMR of Compound 12*
3.3.3 $^1$H-NMR and $^{13}$C-NMR analysis of compound 13

$^1$H NMR (400 MHz, D2O) $\delta$ 7.97 (d, $J = 8.0$ Hz, 1H), 5.98- 5.95 (m, 2H), 5.59 (dd, $J = 6.5$, 2.6 Hz, 1H), 4.68 (s, 2H), 4.37 - 4.34 (m, 2H), 4.268- 4.23 (m, 2H), 4.19 – 4.16 (m, 2H), 4.04 (s, 1H), 3.95 (dd, $J = 29.0$, 10.7 Hz, 2H), 3.82 – 3.69 (m, 2H); $^{13}$C NMR (100 MHz, D2O) $\delta$ 166.20, 157.69, 151.78, 141.64, 102.64, 95.02, 88.40, 83.26, 83.17, 78.47, 75.71, 73.82, 72.04, 69.65, 68.38, 67.85, 64.88, 61.00, 52.96, 51.58; $^{31}$P NMR (162 MHz, D2O) $\delta$ -11.32 (d, $J = 20.7$ Hz), -12.96 (d, $J = 20.7$ Hz).
Figure 3.26 The $^1$H-NMR of Compound 13

Figure 3.27 The $^{13}$C-NMR of Compound 13
3.3.4 \textit{\textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR analysis of compound 14}

\textsuperscript{1}H NMR (400 MHz, D2O) $\delta$ 7.96 (d, J = 8.1 Hz, 1H), 6.00 – 5.90 (m, 3H), 5.59 (dd, J = 6.9, 3.2 Hz, 1H), 5.34 (dd, J = 17.3, 1.5 Hz, 1H), 5.24 (dd, J = 10.6, 1.3 Hz, 1H), 4.58 -4.56 (m, 2H), 4.37 – 4.33 (m, 2H), 4.28 – 4.23 (m, 2H), 4.20 – 4.17 (m, 2H), 4.01 -3.97 (m, 1H), 3.98 (s, 1H), 3.92 (dd, J = 10.9, 3.0 Hz, 1H), 3.77 – 3.72 (m, 2H); \textsuperscript{13}C NMR (100 MHz, D2O) $\delta$ 166.21, 158.50, 151.78, 141.66, 132.70, 117.37, 102.63, 95.14, 95.08, 88.46, 83.24, 73.82, 72.03, 69.64, 68.43, 67.86, 66.07, 64.93, 61.02, 51.52; \textsuperscript{31}P NMR (162 MHz, D2O) $\delta$ -11.27 (d, J = 19.8 Hz), -12.84 (d, J = 19.8 Hz).

\textbf{Figure 3.28 The \textsuperscript{1}H-NMR of Compound 14}
3.3.5 $^1$H-NMR and $^{13}$C-NMR analysis of compound 15

$^1$H NMR (400 MHz, D2O) $\delta$ 7.91 (d, $J = 8.0$ Hz, 1H), 5.93-5.91 (m, 2H), 5.51 (dd, $J = 6.9$, 3.1 Hz, 1H), 4.34 – 4.29 (m, 2H), 4.27– 4.19(m, 3H), 4.17 – 4.13 (m, 2H), 3.99 (s, 1H), 3.92 (dd, $J = 10.9$, 2.5 Hz, 1H), 3.76 – 3.68 (m, 2H), 2.78 – 2.67 (m, 4H); $^{13}$C NMR (100 MHz, D2O) $\delta$ 173.31, 166.20, 151.73, 141.58, 120.72, 102.54, 94.61, 88.47, 83.10, 83.01, 73.71, 72.05, 69.52, 68.31, 67.49, 64.97, 64.92, 60.98, 49.74, 30.41, 12.64; 31P NMR (162 MHz, D2O) $\delta$ -11.34 (d, $J = 21.0$ Hz), -12.94 (d, $J = 21.0$ Hz).
Figure 3.30 The $^1$H-NMR of Compound 15

Figure 3.31 The $^{13}$C-NMR of Compound 15
3.3.6 $^1$H-NMR and $^{13}$C-NMR analysis of compound 16

$^1$H NMR (400 MHz, D2O) $\delta$ 7.96 (d, J = 8.1 Hz, 1H), 6.00 – 5.90 (m, 3H), 5.59 (dd, J = 6.9, 3.2 Hz, 1H), 5.34 (dd, J = 17.3, 1.5 Hz, 1H), 5.24 (dd, J = 10.6, 1.3 Hz, 1H), 4.58 – 4.56 (m, 2H), 4.37 – 4.33 (m, 2H), 4.28 – 4.23 (m, 2H), 4.20 – 4.17 (m, 2H), 4.01 – 3.97 (m, 1H), 3.98 (s, 1H), 3.92 (dd, J = 10.9, 3.0 Hz, 1H), 3.77 – 3.72 (m, 2H); $^{13}$C NMR (100 MHz, D2O) $\delta$ 166.21, 158.50, 151.78, 141.66, 132.70, 117.37, 102.63, 95.14, 95.08, 88.46, 83.24, 73.82, 72.03, 69.64, 68.43, 67.86, 66.07, 64.93, 61.02, 51.52; $^{31}$P NMR (162 MHz, D2O) $\delta$ -11.27 (d, J = 19.8 Hz), -12.84 (d, J = 19.8 Hz).

![Figure 3.32 The $^1$H-NMR of Compound 16](image)
Figure 3.33 The $^{13}$C-NMR of Compound 16

3.3.7 $^1$H-NMR and $^{13}$C-NMR analysis of compound 17

$^1$H NMR (400 MHz, D2O) $\delta$ 7.91 (d, $J$ = 8.0 Hz, 1H), 5.93–5.91 (m, 2H), 5.51 (dd, $J$ = 6.9, 3.1 Hz, 1H), 4.34–4.29 (m, 2H), 4.27–4.19(m, 3H), 4.17–4.13 (m, 2H), 3.99 (s, 1H), 3.92 (dd, $J$ = 10.9, 2.5 Hz, 1H), 3.76–3.68 (m, 2H), 2.78–2.67 (m, 4H); $^{13}$C NMR (100 MHz, D2O) $\delta$ 173.31, 166.20, 151.73, 141.58, 120.72, 102.54, 94.61, 88.47, 83.10, 83.01, 73.71, 72.05, 69.52, 68.31, 67.49, 64.97, 64.92, 60.98, 49.74, 30.41, 12.64; $^{31}$P NMR (162 MHz, D2O) $\delta$ -11.34 (d, $J$ = 21.0 Hz), -12.94 (d, $J$ = 21.0 Hz).
Figure 3.34 The $^1$H-NMR of Compound 17

Figure 3.35 The $^{13}$C-NMR of Compound 17
4 CONCLUSIONS

In this study, I have successfully synthesized 17 UDP-GlcNAc derivatives and UDP-GalNAc derivatives in total by using chemoenzymatic synthesis method. Enzymes NahK, AGX1, GlmU and PpA were used. All the UDP-GlcNAc derivatives and UDP-GalNAc derivatives contain bioorthogonal groups. More significantly, those sugar nucleotides derivatives are potential new probes for further chemoenzymatic labeling of complex glycans. We anticipate this work will accelerate the development and application of chemoenzymatic labeling.
REFERENCES