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# Facile Enzymatic Synthesis of Ketoses and Chemoenzymatic Reporter Strategy for Probing Complex Glycans

liuqing Wen

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FACILE ENZYMATIC SYNTHESIS OF KETOSES  
&  
CHEMOENZYMATIC REPORTER STRATEGY FOR PROBING COMPLEX GLYCANS

by

LIUQING WEN

Under the Direction of Peng George Wang (PhD)

## ABSTRACT

Of all possible ketoses, only D-fructose occur large scale in nature. Therefore, all the ketoses with the exception of D-fructose are defined as “rare ketose”. Despite their lower accessibility, rare ketoses offer an enormous potential for applications in pharmaceutical, medicine, functional food and synthetic chemistry. However, studies of rare ketoses have been hampered by the lack of efficient preparation methods. Here, a convenient and efficient platform for the facile synthesis of rare ketoses is described. The introduced two-step strategies are based on a “phosphorylation→de-phosphorylation” cascade reaction. Rare ketoses were prepared from readily available starting materials as their ketose-1-phosphate forms in step 1 by one-pot multienzyme reactions, followed by the hydrolysis of the phosphate groups in acidic conditions to produce desired ketoses in step 2. By this strategy, 14 rare ketoses were obtained from readily available starting materials with high yield, high purity, and without having to undergo tedious isomer separation step.

Sialic acids are typically linked  $\alpha$ 2-3 or  $\alpha$ 2-6 to the galactose that located at the non-reducing terminal end of glycans, playing important but distinct roles in a variety of biological and pathological processes. However, details about their respective roles are still largely unknown due to the lack of an effective analytical technique. Lectin and antibody binding have been the primary method to analyze glycans, but lectins and antibodies often suffer from weak binding affinity, limited specificity, and cross-reactivity. To address this issue, we develop a chemoenzymatic reporter strategy for rapid and sensitive detection of *N*-acetylneuraminic acid- $\alpha$ (2-3)-Galactose (Neu5Ac $\alpha$ (2-3)Gal) glycans on cell surface.

INDEX WORDS: Ketoses, phosphorylation, silver nitrate, Chemoenzymatic reporter, Glycans, *N*-acetylneuraminic acid- $\alpha$ (2-3)-Galactose.

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LIUQING WEN

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Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2017

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Liuqing Wen  
2017

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May 2017

**DEDICATION**

To my parents, brother, and sister for their love, understanding, and support.

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To pursue a doctoral career is one of the most important decisions that I have made in my first thirty years. Upon graduation, I want to sincerely acknowledge all those people who helped me with this dissertation.

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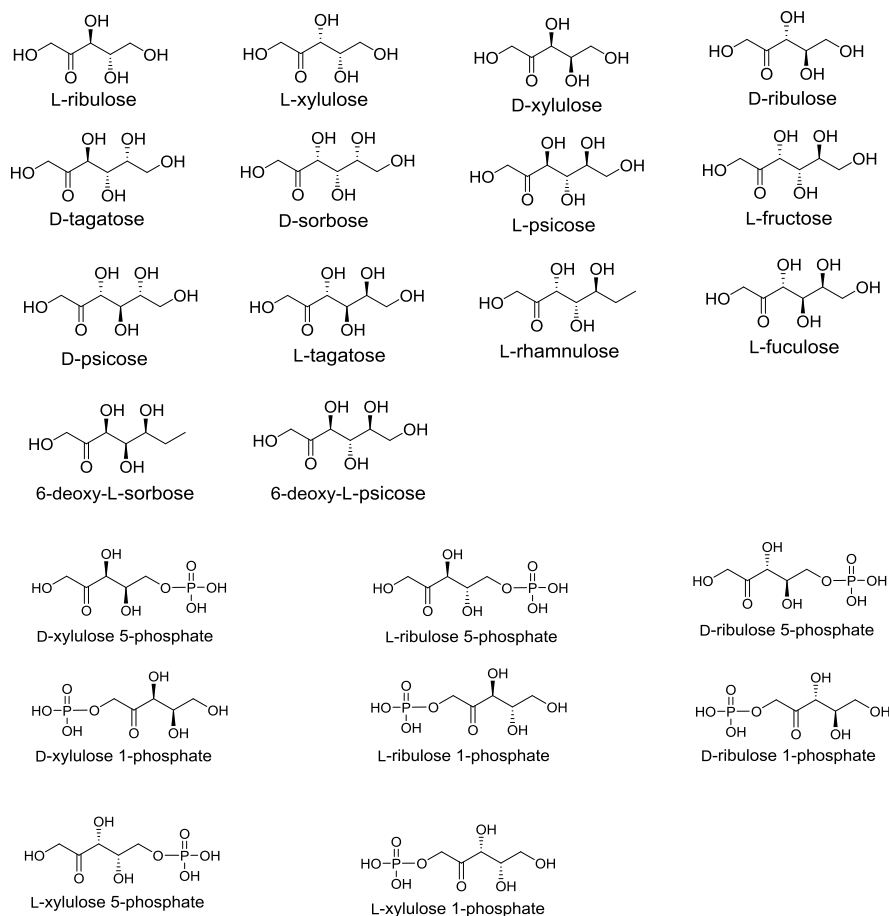
## 1 CHAPTER 1 FACILE ENZYMATIC SYNTHESIS OF KETOSES

### 1.1 Introduction

Rare sugars are defined as monosaccharides that only naturally occur in small amounts.<sup>1</sup> According to the classification by the International Society of Rare Sugars (ISRS), the only seven common monosaccharides that naturally occur in substantial quantities are D-xylose, D-ribose, L-arabinose, D-glucose, D-galactose, D-mannose and D-fructose.<sup>2</sup> Of all possible ketopentoses and ketohexoses, the twelve ketoses can be divided into two types in relation to their C-3 configuration: (3*S*)-ketoses and (3*R*)-ketoses.<sup>3</sup> With the exception of D-fructose, all of these ketoses are defined as “rare sugars”.<sup>3</sup> Despite their lower accessibility, rare ketoses offer an enormous potential for applications in pharmaceutical, medicine, functional food and synthetic chemistry.<sup>4-7</sup> For example, D-psicose has about 70% of the sweetness but only 0.3% of the energy calories of sucrose.<sup>8, 9</sup> It can also inhibit hepatic lipogenous enzyme activity, helping reduce abdominal fat accumulation.<sup>10</sup> L-xylulose was reported to be an inhibitor of glycosidase,<sup>11</sup> while also serving as an indicator for acute or chronic hepatitis and liver cirrhosis.<sup>12</sup> L-rhamnulose is a precursor of furaneol that has been used in the flavor industry for its sweet strawberry aroma.<sup>13</sup>

In addition, phosphorylated ketopentoses are also intermediates in sugar metabolism pathway and synthetic starting materials of many important biomolecular. The ketopentoses can be phosphorylated at the C-1 or C-5 position, resulting in eight phosphorylated ketopentoses. All pentoses including aldopentose and ketopentose that could be utilized by bacteria or other organisms must first be converted to their phosphorylated ketopentose forms to enable metabolic function.<sup>1, 4, 14, 15</sup> In these metabolism pathways, ketopentose 1-phosphates are split into glycolaldehyde and dihydroxyacetone phosphate, which is an intermediate in the glycolytic pathway, by aldolases.<sup>16-18</sup> Ketose 5-phosphates will be epimerized to D-xylulose 5-phosphate or

D-ribulose 5-phosphate, which are key participants of pentose phosphate pathway (PPP), by epimerases.<sup>19-21</sup> PPP is a universal metabolic process present in bacteria, plants and animals,<sup>22</sup> and its main function is to produce reducing power and building blocks for cell growth.<sup>23</sup> Because of their critical position in sugar metabolism pathway, phosphorylated ketopentoses are also the starting materials in synthetic chemistry.<sup>24</sup> For example, D-xylulose 5-phosphate can act as the starting material for the synthesis of heptose in bacteria.<sup>25</sup> Likewise, D-ribulose 5-phosphate can be used for the synthesis of 3-deoxy-D-manno-octulosonic acid (KDO).<sup>26</sup> Therefore, phosphorylated ketopentoses have great potential for applications in investigating the mechanistic and regulatory aspects of sugar metabolism, identification and characterization of new enzymes in nature, and being raw materials in synthetic chemistry.<sup>27-29</sup>



**Figure 1.1** 14 ketoses and 8 phosphorylated ketopentoses synthesized in this work

Izumori and co-workers have established a beautiful strategy termed “Izumoring” for rare sugar synthesis, a method by which most of rare sugars have been produced through isomerization, epimerization, oxidization or reduction reactions.<sup>2</sup> Aldose-ketose isomerization is the most important method for ketose preparation,<sup>30</sup> even though such isomerization is very unfavorable for ketose formation.<sup>31</sup> Great progress has been achieved by using both chemical and enzymatic aldose-ketose isomerization schemes over the last two decades.<sup>32-37</sup> Approaches to improve aldose-ketose conversion, including the addition of borate to break aldose-ketose reaction equilibrium,<sup>38</sup> directed evolution,<sup>39-41</sup> and discovery of novel enzymes in nature<sup>42</sup> have also been suggested. Nevertheless, an extensive isomer separation step is still necessary to obtain a ketose in pure form using these methods.<sup>43, 44</sup> Ion-exchange chromatography (Ca<sup>2+</sup> form) is the main method for sugar isomer separation.<sup>45</sup> Selective degradation of unwanted isomer by bacteria to isolate the desired ketose has also been explored.<sup>46-48</sup> However, both methodologies are time-consuming and suffer from low efficiency. Furthermore, four common aldoses (D-xylulose, D-ribose, L-arabinose and D-galactose) correspond to four rare ketoses (D-xylulose, D-ribulose, L-ribulose and D-tagatose) while D-glucose and D-mannose correspond to D-fructose. Therefore, the synthesis of other rare ketoses (L-xylulose, D-psicose, D-sorbose, L-tagatose, L-sorbose, L-fructose, and L-psicose), all which possess a (3*R*)-configuration with the exception of L-sorbose and L-psicose, has been more challenging. Chemical schemes for the synthesis of these rare ketoses undergoing protecting and de-protecting steps have been explored.<sup>49, 50</sup> Alternatively, enzymatic preparation by epimerizing (3*S*)-ketose to (3*R*)-ketose<sup>51</sup>, oxidizing polyols,<sup>52</sup> or relying on aldol condensation<sup>53</sup> proceeds regio- and stereoselectively without protection. However, these methods suffer from expensive starting materials, low conversion ratios or a complicated isomer separation step. Therefore, despite their commercial availability, most of rare ketoses remain very expensive which in turn

has hindered studies of this fundamental class of carbohydrates. We therefore establish a strategy for a convenient, efficient and cost-effective synthesis of ketoses, by which 14 difficult-to-access ketoses can be obtained from readily available starting materials with high yield and purity and without undergoing a tedious purification steps. Our strategies are based on a “phosphorylation→de-phosphorylation” cascade reaction (Figure 1.1).

## 1.2 Enzymatic synthesis of common rare ketoses

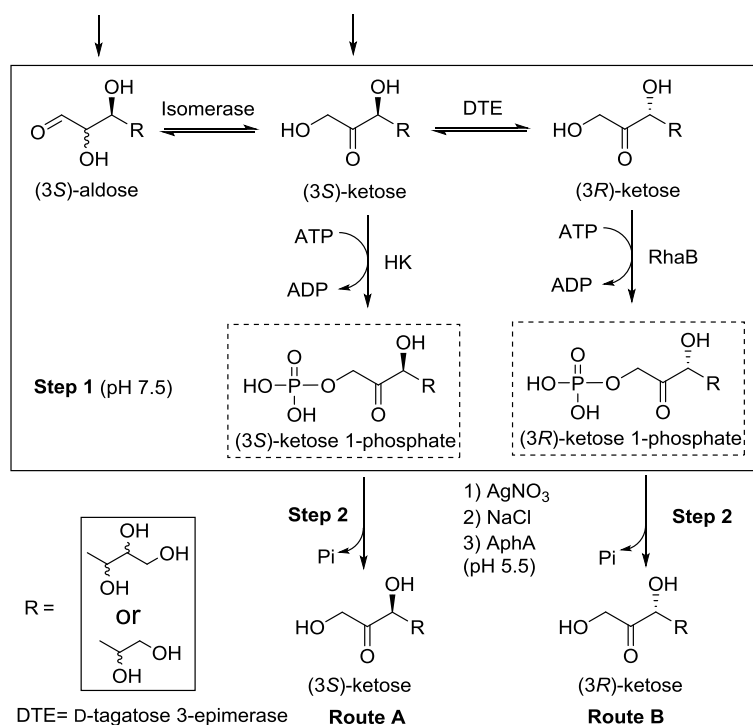
Our strategies are based on a “phosphorylation/de-phosphorylation” cascade reaction. In the first reaction step of Figure 1.2, we combined thermodynamically unfavourable bioconversions of common (3S)-sugars to the desired (3S)-ketoses (route A) or (3R)-ketoses (route B) with phosphorylation reactions by substrate-specific kinases (Fructokinase (HK) from humans in route A, L-rhamnulose kinase (RhaB) from *Thermotoga maritima* MSB8 in route B). In the second reaction step, phosphate adenosines (ATP and ADP) were selectively removed by a convenient method called silver nitrate precipitation. Then, acid phosphatase (AphA) was added to hydrolyze the phosphate groups to produce the desired ketoses.

### 1.2.1 Substrate specificity study of HK and RhaB

To achieve the designed schemes, there are three challenges: 1) the identification of kinases that could phosphorylate the desired ketoses at C-1 position but not starting sugars or intermediates, 2) the availability of a D-tagatose 3-epimerase (DTE) which specifically epimerizes ketoses at C-3 but not ketose 1-phosphates and 3) an efficient method to separate phosphate sugars from phosphate adenosines (ATP and ADP).

The first reaction step of our strategy was carried at a slightly basic condition where all enzymes were quite active. Nevertheless, monosaccharides are unstable in alkaline media,<sup>54</sup> and the isomerases in reaction system may also affect the intermediate of phosphate ketoses to a certain

extent when the kinase that transferred phosphate group to the C-5 (pentose) or C-6 (hexose) position was used. To avoid such unwanted side reactions, kinases that phosphorylate ketoses at the C-1 position were utilized.<sup>55</sup> In route A of Figure 1.2, a kinase that could phosphorylate



**Figure 1.2** one-pot two-step enzymatic synthesis of ketoses

(3S)-ketoses (D-xylulose, L-ribulose, and D-tagatose) but not (3S)-aldoses (D-xylose, L-arabinose and D-galactose) is necessary. In route B, a kinase that specifically recognizes (3R)-ketoses but not (3S)-ketoses and (3S)-aldoses is necessary. By screening the substrate specificity of many kinases (data not shown), we were able to find that fructokinase (HK) from humans<sup>18, 56</sup> accords well with the requirement of route A and L-rhamnulose kinase (RhaB) from *Thermotoga maritima* MSB8 (Table 1.1), a novel enzyme that absolutely requires ketoses with (3R)-configuration, accords well with the requirement of route B. Another requirement for the establishment of route B of Figure 1.2 is that DTE, which catalyzes the interconversion of (3S)- and (3R)-ketoses,<sup>51</sup> does not epimerize ketose 1-phosphates. Otherwise the products obtained finally will be a mixture

containing both (3*S*)-ketoses and (3*R*)-ketoses. DTE does not recognize D-fructose 6-phosphate and D-ribulose 5-phosphate.<sup>51</sup> We, therefore, hypothesized that it would not recognize other phosphate ketoses, such as ketose 1-phosphates.

**Table 1.1** substrate specificity of RhaB and HK

Substrate	C-3 Configuration	RhaB	HK
		Activity (%)	Activity (%)
L-rhamnulose	R	100	NA
L-xylulose	R	91	NA
D-ribulose	R	101	NA
D-sorbose	R	14	NA
L-fructose	R	86	ND
D-psicose	R	34	NA
L-tagatose	R	55	NA
L-ribulose	S	ND	37
D-xylulose	S	0.3	129
D-tagatose	S	ND	13
L-psicose	S	ND	28
D-fructose	S	ND	100
L-sorbose	S	ND	32
L-arabinose	S	ND	ND
D-xylose	S	0.1	ND
D-galactose	S	ND	ND

NA: not assayed. ND: no detectable activity was observed



### 1.2.2 Silver nitrate precipitation

In the second reaction step, acid phosphatase was used to hydrolyze the phosphate group of ketose 1-phosphates to produce ketoses in acidic condition (pH 5.5) where monosaccharides are stable. However, the existence of phosphate adenosines (ATP and ADP) inhibit the hydrolytic activity of the acid phosphatase.[57] To conveniently purify sugar phosphate from ATP and ADP, a method referred to as silver nitrate precipitation was used to remove phosphate adenosines selectively. Silver phosphate is insoluble, and thus silver ions can precipitate ADP and ATP.[58] Interestingly, we noticed that silver ions cannot precipitate monophosphate sugar when the sugars are composed of four or more carbons (Table 1.2). It appears that the binding of the sugar group to phosphate prevents phosphate silver precipitation.

**Table 1.2** precipitation with silver ion

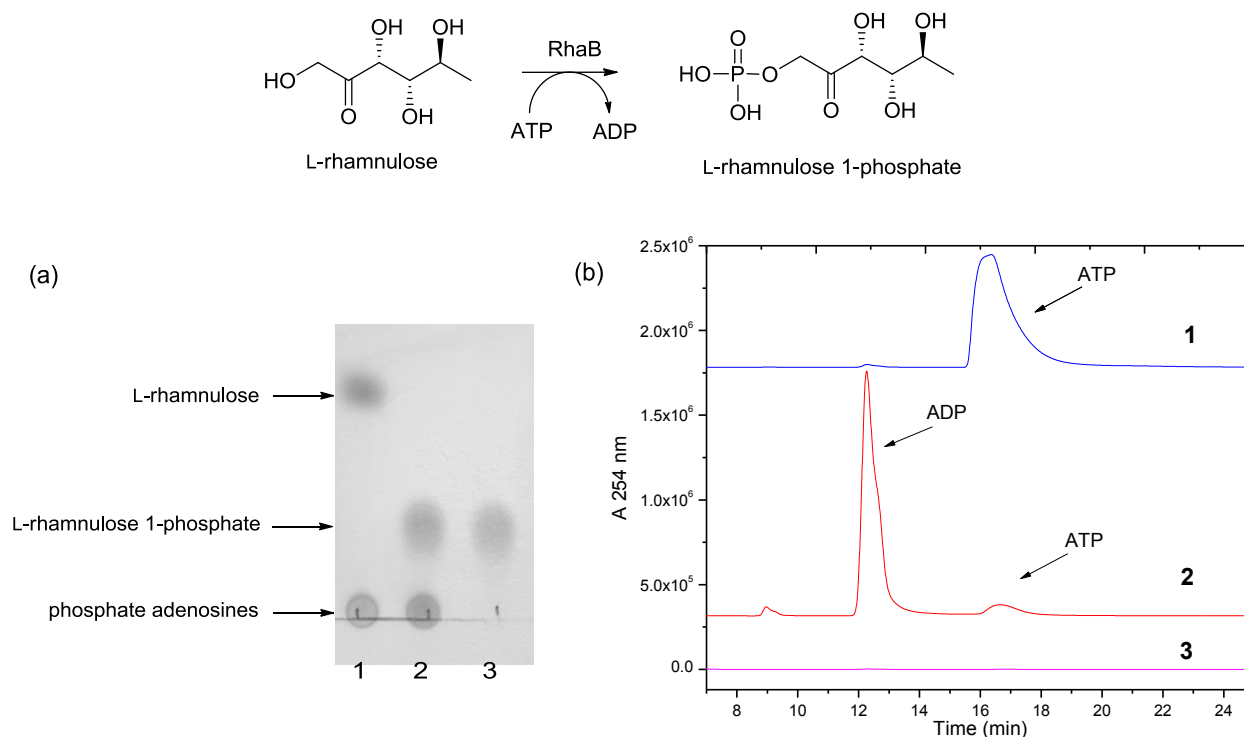
Compound	Precipitation	Compound	Precipitation
PEP	+ <sup>a</sup>	D-erythrose 4-phosphate	- <sup>b</sup>
glycerol 3-phosphate	+	D-ribulose 5-phosphate	-
ATP	+	D-fructose 6-phosphate	-
ADP	+	D-glucose 1-phosphate	-
D-fructose 1,6-bisphosphate	+	AMP	-

a: white precipitation was observed

b: clear solution was observed

Applying this selective precipitation of silver ions, phosphate sugars can be easily and cleanly separated from phosphate adenosines (ATP and ADP), by which more than 99% ATP and ADP were removed (Figure 1.3). Then, excess sodium chloride was added to remove the redundant silver ions. After desalting by using Bio-Gel P-2 column, sugar phosphate can be obtained in pure form. The total separation process can be completed in less than 15 minutes. The precipitate can be re-dissolved in ammonium hydroxide, and the phosphate adenosines (ATP and ADP) or silver ions can be then recycled compared to another phosphate sugar purification method, barium

precipitation, by which phosphate sugar was isolated as barium form,[59] no additional steps to remove toxic ions and no accurate pH control are necessary. Moreover, silver is safer than barium and other metal ions that can be used to precipitate phosphate adenosines such as mercury.[60] These advantages make this method highly attractive for use in rapidly purifying phosphate sugars.



**Figure 1.3** HPLC and TLC analysis of silver nitrate precipitation method

### 1.2.3 The synthesis of *L*-ribulose, *D*-xylulose, *D*-tagatose, *L*-xylulose, *D*-ribulose, *D*-sorbose, *D*-psicose, and *L*-tagatose

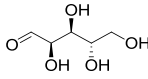
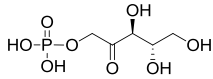
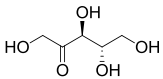
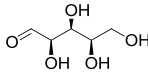
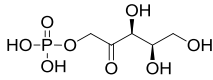
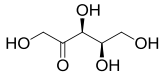
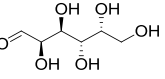
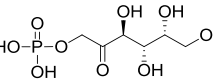
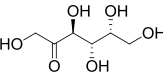
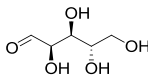
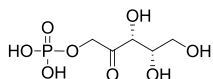
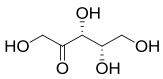
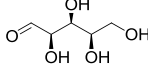
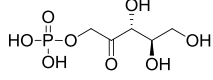
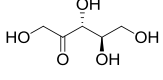
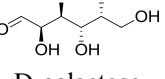
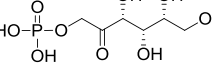
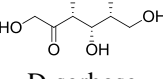
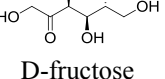
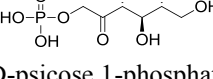
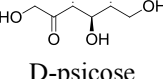
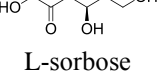
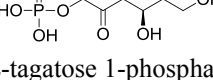
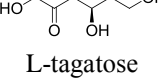
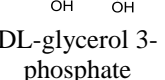
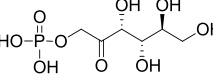
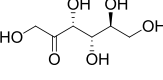
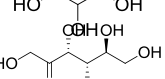
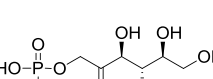
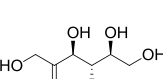
In route A of Figure 1.2, *L*-arabinose, *D*-xylose, and *D*-galactose (entries 1 to 3) were incubated with their corresponding isomerases (Table 1.3) and HK to prepare *L*-ribulose, *D*-xylulose and *D*-tagatose, respectively. The reactions were allowed to proceed until no detectable starting aldoses were found by HPLC (conversion ratios exceeding 99%), making isomer separation unnecessary. Silver nitrate precipitation method was used to remove ATP and ADP.

Proteins were also removed during this process (test by Bradford method). Consequently, two steps of the designed scheme could be carried in one-pot. After the hydrolysis of phosphate groups in step 2, the solution from each reaction was desalting by using Bio-Gel P-2 column. L-ribulose, D-xylulose, and D-tagatose were finally obtained in more than 90% yield (Table 1.3). The products were confirmed by the analysis of NMR, HPLC and MS. HPLC and NMR analyses indicated product purity exceeds 99%.

In route B of Figure 1.2, five (*3R*)-ketoses (L-xylulose, D-ribulose, D-sorbose, D-psicose, and L-tagatose) were prepared from five common (*3S*)-sugars (L-arabinose, D-xylose, D-galactose, D-fructose, and L-sorbose), respectively. The discovery of DTE made it possible to achieve the interconversion between (*3S*)-ketoses and (*3R*)-ketoses and is especially important for the preparation of (*3R*)-ketoses.<sup>51</sup> Nevertheless, ketoses, with the exceptions of two (*3S*)-ketoses (D-fructose and L-sorbose), are not readily available, and the conversions catalyzed by DTE are an equilibrium reaction. For example, the conversion ratio is only 20% for D-fructose to D-psicose and 27% for L-sorbose to L-tagatose.<sup>57</sup> The separation of (*3S*)-ketoses and (*3R*)-ketoses is difficult due to their similar properties. In this work, we combined DTE-catalyzed epimerization with targeted phosphorylation of (*3R*)-ketose by RhaB (entries 7 and 8). To avoid using non-readily available ketoses, enzymatic isomerization when starting with (*3S*)-aldose (entries 4 to 6). All reactions were allowed to proceed until no detectable starting sugars were found by HPLC (conversion ratios exceeding 99%). After the hydrolysis of phosphate groups in step 2, all five (*3R*)-ketoses were obtained in more than 90% yield (Table 1.3). Given the high substrate specificity of RhaB, L-xylulose, D-sorbose, D-psicose and L-tagatose were obtained in more than 99% purity. D-ribulose was obtained in 98.2% purity while 0.6% of D-xylulose and 1.2% of D-

xylose were observed because D-xylose and D-xylulose could be phosphorylated by RhaB to a certain extent (Table 1.3).

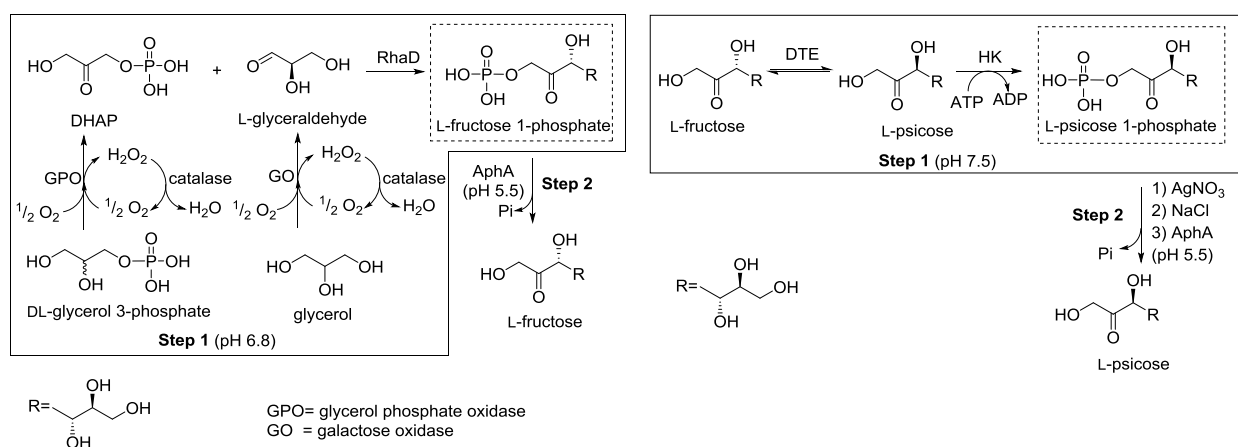
**Table 1.3** Synthesis of rare ketoses using the two-step strategy

Entry	Starting material	Enzyme	Scheme	Intermediate	Ketose	Yield (%) <sup>[a]</sup>	Purity (%) <sup>[b]</sup>
1	 L-arabinose	AraA HK	Route A Figure 1.2	 L-ribulose 1-phosphate	 L-ribulose	93	>99
2	 D-xylose	XylA HK	Route A Figure 1.2	 D-xylulose 1-phosphate	 D-xylulose	92	>99
3	 D-galactose	AraA HK	Route A Figure 1.2	 D-tagatose 1-phosphate	 D-tagatose	92	>99
4	 L-arabinose	AraA DTE RhaB	Route B Figure 1.2	 L-xylulose 1-phosphate	 L-xylulose	91	>99
5	 D-xylose	XylA DTE RhaB	Route B Figure 1.2	 D-ribulose 1-phosphate	 D-ribulose	91	98.2
6	 D-galactose	AraA DTE RhaB	Route B Figure 1.2	 D-sorbose 1-phosphate	 D-sorbose	94	>99
7	 D-fructose	DTE RhaB	Route B Figure 1.2	 D-psicose 1-phosphate	 D-psicose	93	>99
8	 L-sorbose	DTE RhaB	Route B Figure 1.2	 L-tagatose 1-phosphate	 L-tagatose	95	>99
9	 DL-glycerol 3-phosphate	GPO GO catalase RhaD	Figure 1.4	 L-fructose 1-phosphate	 L-fructose	70	99
10	 L-fructose	DTE HK	Figure 1.4	 L-psicose 1-phosphate	 L-psicose	90	>99

[b] Defined as the desired ketose to the sum of all possible sugars.

### 1.2.1 The synthesis of L-fructose and L-psicose

L-fructose was synthesized by using RhaD due to a lack of common corresponding sugars. RhaD exclusively produces L-fructose from dihydroxyacetone phosphate (DHAP) and L-glyceraldehyde.[23] However, DHAP and L-glyceraldehyde are costly and unstable. To increase the practicality of the process, two previously reported strategies[23, 26] were combined allowing the use of inexpensive materials of glycerol and DL-glycerol 3-phosphate to produce L-fructose 1-phosphate in one-pot (Figure 1.4), by which L-glyceraldehyde was oxidized from glycerol by galactose oxidase (GO) and DHAP was oxidized from DL-glycerol 3-phosphate by glycerol phosphate oxidase (GPO). After the hydrolysis of the phosphate group in step 2, L-fructose was finally obtained in 70% yield with a purity of 99%. Then, L-psicose was prepared from L-fructose by DTE. However, the conversion of L-fructose to L-psicose catalyzed by DTE is unfavorable for L-psicose formation, for which the conversion ratio is only 24%. [25] Applying the discovery that HK from humans could efficiently phosphorylate L-psicose but not L-fructose, L-psicose was prepared from L-fructose using the described targeted phosphorylation strategy (Figure 1.4) in 90% yield with a product purity exceeding 99%.



**Figure 1.4** Enzymatic synthesis of L-fructose and L-psicose

### 1.3 Enzymatic synthesis of deoxy rare ketoses

#### 1.3.1 *The synthesis of L-rhamnulose and L-fuculose*

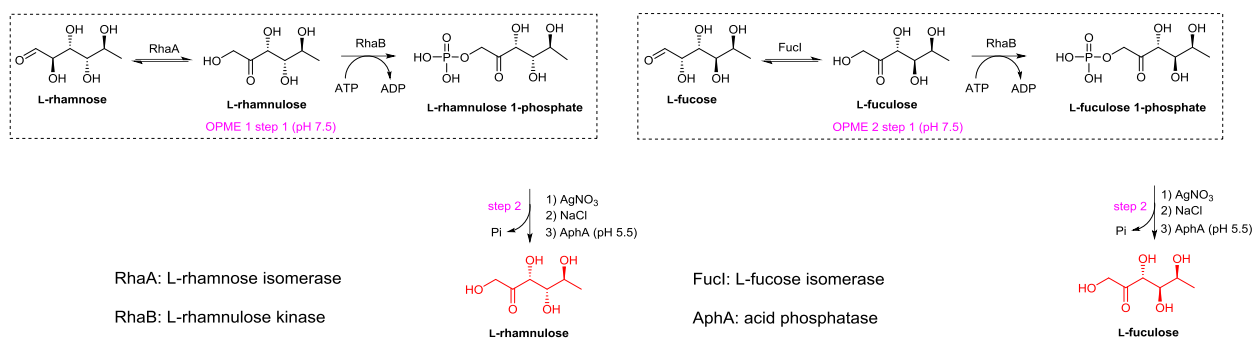
L-rhamnulose and L-fuculose are two crucial rare deoxy ketoses that offer many potential applications. For example, L-rhamnulose is a precursor of furaneol that has been used in the flavor industry for its sweet strawberry aroma.<sup>13</sup> In addition, L-rhamnulose and L-fuculose play important roles in sugar metabolism.<sup>16, 58, 59</sup> In bacteria, L-rhamnose and L-fucose must be converted to their ketose 1-phosphate forms, which are later split into dihydroxyacetone phosphate and L-lactaldehyde by L-rhamnulose 1-phosphate aldolase (RhaD) or L-fuculose 1-phosphate aldolase (FucA) to facilitate further metabolic function.<sup>60</sup> RhaD and FucA are two powerful biocatalysts and have been widely used in synthetic chemistry to produce rare ketoses or their derivatives.<sup>61, 62</sup> Moreover, L-rhamnulose and L-fuculose can also be directly isomerized or epimerized into other rare sugars.<sup>63, 64</sup> Therefore, L-rhamnulose and L-fuculose are not only primary targets for investigating the mechanistic and regulatory aspects of sugar metabolism, but also important starting materials in synthetic chemistry. An efficient system to readily provide both ketoses in considerable amounts is highly attractive in enabling the studies of both deoxy ketoses.

There are two methods that could be used to produce L-rhamnulose and L-fuculose. The most common method is isomerizing L-rhamnose to L-rhamnulose or L-fucose to L-fuculose.<sup>63-65</sup> However, aldose-ketose isomerization mediated by either chemical or enzymatic method (isomerase) is reversible, with reaction equilibrium being very unfavorable for ketose formation.<sup>66</sup> For example, only 11% of L-fucose can be isomerized to L-fuculose by L-fucose isomerase (FucI) in the final reaction equilibrium.<sup>65</sup> Moreover, an extensive isomer separation step is still necessary to obtain a ketose in pure form. Ion-exchange chromatography (Ca<sup>2+</sup> form) is the main method for sugar isomer separation.<sup>45</sup> Nevertheless, it was reported that L-rhamnulose is hard to be separated

from L-rhamnose using ion-exchange chromatography (Ca<sup>2+</sup> form) column.<sup>64</sup> They even can't be separated well by HPLC. Commercially available product only has 80% purity (Sigma-Aldrich). Selective degradation of unwanted isomer by bacteria to isolate the desired ketose has also been explored,<sup>46-48</sup> but this method is time-consuming and suffers from low efficiency. The addition of borate into reaction system has been suggested to improve aldose-ketose isomerization because borate can bind ketose stronger than aldose.<sup>67</sup> Such strategy has been applied on the conversion of L-fucose to L-fuculose, in which a 85% conversion ratio was observed.<sup>65</sup> However, the purification steps require the separation of ketose-borate complex and the splitting of the desired ketoses from ketose-borate complex.<sup>68</sup> These tedious manipulations place a limit on the applications of this strategy. The second method for the production of L-rhamnulose and L-fuculose is based on aldol condensation reaction.<sup>53, 69</sup> In this strategy, RhaD or FucA were used to produce L-rhamnulose 1-phosphate or L-fuculose 1-phosphate from dihydroxyacetone phosphate (DHAP) and L-lactaldehyde, and then the phosphate group was hydrolyzed to afford L-rhamnulose or L-fuculose. However, DHAP and L-glyceraldehyde are costly and unstable, reducing the synthetic practicality. Although DL-glycerol 3-phosphate, an inexpensive starting material, has been used to produce DHAP in a one-pot reaction fashion,<sup>70, 71</sup> ketose production mediated by aldolase still suffers from low yields and tedious purification manipulations.<sup>69</sup> Therefore, while L-rhamnulose and L-fuculose are commercially available, they are cost prohibitive (L-rhamnulose, \$178/10 mg, Sigma-Aldrich; L-fuculose, \$199/10 mg, Carbosynth). The study of L-rhamnulose and L-fuculose has been hindered due to their limited availability. Herein an enzymatic method for the efficient and convenient preparation of rare ketoses L-rhamnulose and L-fuculose from readily available aldoses is reported.

To apply the described scheme on L-rhamnulose and L-fuculose production in this work, the

prerequisite is the availability of a kinase that specifically recognizes L-rhamnulose and L-fuculose but not L-rhamnose or L-fucose. Otherwise, the products obtained finally will be a mixture containing both aldose and ketose. L-rhamnulose kinase is the enzyme that prefers ketoses with (3R)-configuration. Recently, we identified an L-rhamnulose kinase from *Thermotoga maritima* MSB8, which show high substrate specificity towards (3R)-ketoses as compared to (3S)-ketoses or (3S)-aldoses. In this work, the substrate specificity of RhaB towards several deoxy sugars was studied. RhaB failed to recognize L-rhamnose or L-fucose but had high activity towards L-rhamnulose and L-fuculose (Table 1.4), indicating its potential for one-pot multienzyme (OPME) reactions to produce L-rhamnulose and L-fuculose.



**Figure 1.5** Two-step strategy for the enzymatic synthesis of L-rhamnulose and L-fuculose

**Table 1.4** Substrate specificity of RhaB towards several deoxy sugars.

Substrate	RhaB activity (%)
L-rhamnulose	100
L-fuculose	81.3
L-rhamnose	ND
L-fucose	ND

ND: no detectable activity was observed.

Having met the prerequisite, other conversion-related enzymes including L-rhamnose isomerase (RhaA),<sup>72</sup> L-fucose isomerase (FucI),<sup>73</sup> and acid phosphatase (AphA)<sup>74</sup> from *Escherichia coli* were prepared as described in experiment part. To test the potential of RhaB in OPME reactions,

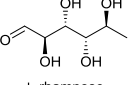
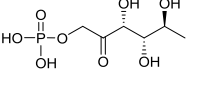
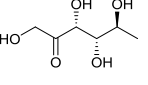
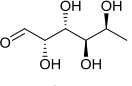
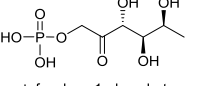
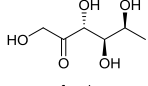


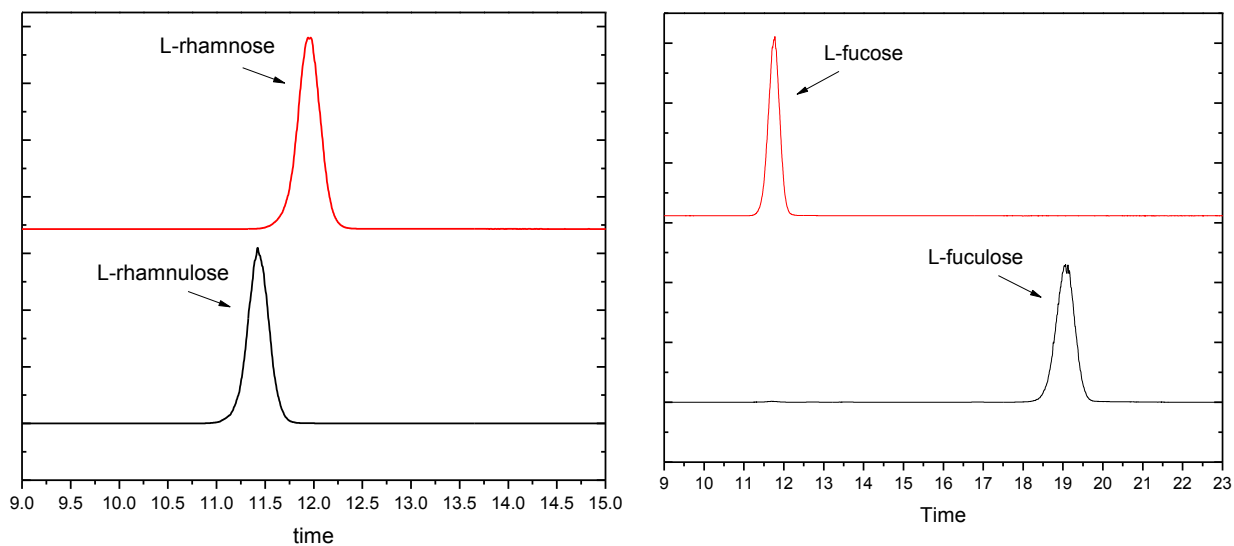
analytical scale reactions (Table 1.5) were performed in one-pot (164 ug scale), and tested by TLC. Reactions without isomerases were done as negative controls. Once the formation of sugar phosphates was observed on TLC while no reactions were observed on the control reactions, preparative reactions (gram scale) were performed.

In the first reaction step, L-rhamnose was incubated with RhaA and RhaB in one-pot in the presence of ATP as the phosphate donor (OPME 1, Figure 1.5). No buffer was used in consideration to purification. The reaction pH was held near 7.5, where all enzymes are quite active, using sodium hydroxide as the reaction occurred. In this one-pot two-enzyme system, RhaA isomerized L-rhamnose to L-rhamnulose, which was immediately phosphorylated by RhaB. It seems that L-rhamnulose was taken out of the reaction balance, and thus the reaction was driven towards the formation of L-rhamnulose in its ketose 1-phosphate form (L-rhamnulose 1-phosphate). The reaction was monitored by TLC and HPLC equipped with ELSD detector (HPX-87H column). Once the reaction finished (conversion ratio exceeding 99%), silver nitrate precipitation was used to precipitate ATP and ADP. In detail, silver nitrate was added into reaction system until no new precipitate formed, and the precipitate was removed by centrifugation. Sodium chloride was added to precipitate the remaining silver ions, and the precipitate was removed by centrifugation. After desalting by using P-2 column, L-rhamnulose 1-phosphate was isolated in 91% yield. The product was analyzed by NMR and MS (see experiment part). The NMR spectra and MS data are well in accord with previously reported data, confirming the isolated product is L-rhamnulose 1-phosphate. In the second reaction step, the phosphate group of L-rhamnulose 1-phosphate was hydrolyzed by AphA in pH 5.5 to produce L-rhamnulose. Once phosphate sugar was no longer observed on TLC, the solution was concentrated and purified by P-2 column to afford final product in 82% yield with regard to L-rhamnose. The product was analyzed by HPLC,

NMR and MS (See experiment part). No obvious peak of L-rhamnose can be found by HPLC (Figure 1.6) and no characteristic peak of aldose can be found in NMR spectra (See experiment part) indicating product purity exceeding 99%.

**Table 1.5** Enzymatic synthesis of L-rhamnulose and L-fuculose from L-rhamnose or L-fucose.

Entry	Starting material	Enzymes	Intermediate	Step 1 yield (%)	Product	Total yield (%)	Scale (mg)	Purity <sup>a</sup> (%)
1	 L-rhamnose	RhaA RhaB AphA	 L-rhamnulose 1-phosphate	91	 L-rhamnulose	82	1080	>99
2	 L-fucose	FucI RhaB AphA	 L-fuculose 1-phosphate	93	 L-fuculose	84	1099	>99



**Figure 1.6** HPLC profiles of L-rhamnulose and L-fuculose compared with starting aldoses.

Similarly, preparative scale synthesis of L-fuculose from L-fucose was also carried in gram scale. In the first reaction step, L-fucose was incubated with FucI and RhaB in the presence of ATP as phosphate donor. Conversion ratio exceeding 99% can be reached. Following the same manipulation as described above, L-fuculose 1-phosphate was isolated in 93% yield. NMR and MS data are well in accord with the previously reported data,<sup>69, 75</sup> confirming the isolated product

is L-fuculose 1-phosphate. After hydrolyzing the phosphate group of L-fuculose 1-phosphate by AphA in step 2, L-fuculose was obtained in 84% yield with regard to L-fucose. The product was analyzed by HPLC, NMR and MS. HPLC and NMR analysis indicates a product purity exceeding 99%.

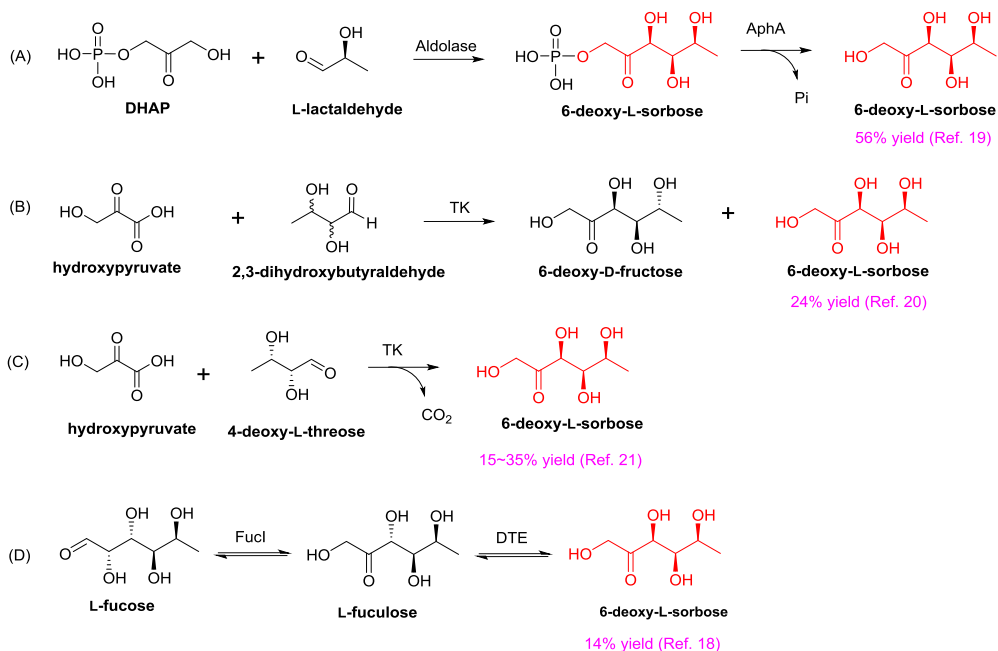
### ***1.3.2 The synthesis of 6-deoxy-L-sorbose***

6-deoxy-L-sorbose can serve as a precursor of furaneol (4-Hydroxy-2,5-dimethyl-3(2H)-furanone), an important compound in the food industry for its caramel-like flavor used in food industry.<sup>76</sup> Moreover, 6-deoxy-L-sorbose is also the starting material for the preparation of 6-deoxy-L-gulose and 6-deoxy-L-idose.<sup>63</sup> The majority of the potential applications of 6-deoxy-L-sorbose have not been fully investigated due to its relative scarcity. Enzymatic synthesis by the condensation of dihydroxyacetone phosphate (DHAP) and L-lactaldehyde employing aldolase has been the primary method for 6-deoxy-L-sorbose preparation (Scheme 1, A), in which 6-deoxy-L-sorbose was obtained in 56% yield.<sup>77</sup> Hecquet and co-workers found that 6-deoxy-L-sorbose could also be prepared from 2,3-dihydroxybutyraldehyde and hydroxypyruvate by using transketolase (TK) (Scheme 1, B).<sup>78</sup> Although a 24 % yield was achieved, up to 26% of an isomer (6-deoxy-D-fructose) was also present in the resulting solution. Subsequently, they found that TK could exclusively produce 6-deoxy-L-sorbose when hydroxypyruvate and 4-deoxy-L-threose were used as starting materials (Scheme 1, C).<sup>79</sup> The process was improved by coupling the isomerization reaction of 4-deoxy-L-threose from 4-deoxy-L-erythrulose giving a yield up to 35%.<sup>79</sup> However, these methods suffer from expensive starting materials, low yields and complicated purification processes. Recently, Shompoosang and co-workers used L-fucose (6-deoxy-L-galactose), a readily available sugar in nature, as starting materials in preparing 6-deoxy-L-sorbose (Scheme, D).<sup>63</sup> By this process, L-fucose was isomerized to L-fuculose (6-deoxy-L-tagatose) by L-fucose isomerase,

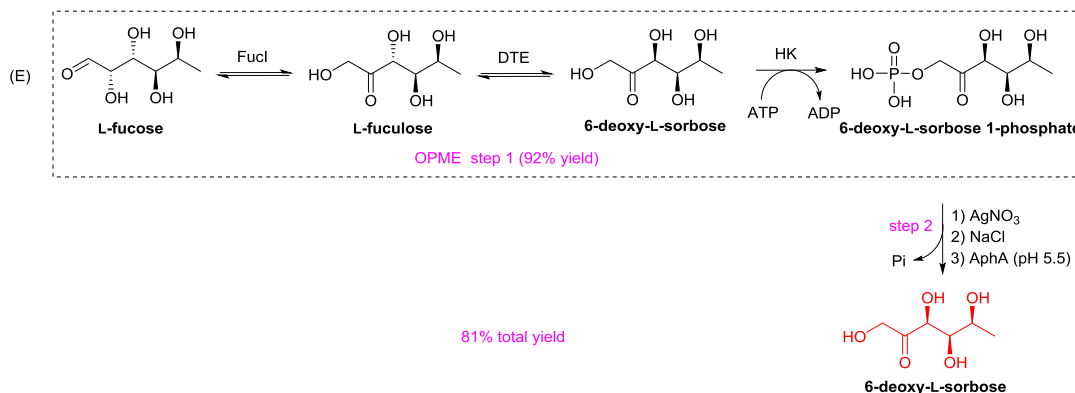
and then 6-deoxy-L-sorbose was epimerized from 6-deoxy-L-tagatose by D-tagatose 3-epimerase (DTE). However, since all aldose-ketose isomerization is very unfavorable for ketose formation, the first step only produce 9% isolated yield. The production was improved by reusing the purified L-fucose several times, but only 27% isolated yield was obtained. The second step produces 50% yield resulting in a final 14% isolated yield of 6-deoxy-L-sorbose with regard to L-fucose. Moreover, this method requires a tedious isomer separation process to obtain 6-deoxy-L-sorbose in pure form.

To establish a facile method for 6-deoxy-L-sorbose preparation, we attempt to apply an “isomerization→epimerization→ phosphorylation→dephosphorylation” cascade reaction to produce 6-deoxy-L-sorbose from L-fucose directly (Figure 1.7). FucI catalyzes the isomerization of L-fucose to L-fuculose.<sup>80</sup> DTE is a novel epimerase that catalyze the epimerization of ketoses at C-3 position resulting (3*S*)- and (3*R*)- interconversion.<sup>51, 81, 82</sup> These two enzymes have been used to produced 6-deoxy-L-sorbose as mentioned above. Although these two reactions can be carried in one-pot to simplify the synthetic process, the purification of 6-deoxy-L-sorbose from the reaction mixture containing L-fucose, L-fuculose and 6-deoxy-L-sorbose is very difficult and makes this strategy impractical. In this work, the isomerization of L-fucose to L-fuculose, and epimerization of L-fuculose to 6-deoxy-L-sorbose were accurately controlled by coupling with a targeted phosphorylation reaction of 6-deoxy-L-sorbose in one-pot fashion. The selective phosphorylation of 6-deoxy-L-sorbose could drive both reversible reactions towards the formation of the 6-deoxy-L-sorbose 1-phosphate in the first reaction step. However, to achieve the designed scheme, the prerequisite is the availability of a kinase that specifically recognizes 6-deoxy-L-sorbose but not L-fucose and L-fuculose. Otherwise, the products obtained finally will be a mixture containing many isomers (L-fucose and L-fuculose), which are difficult to be separate.

### Previous strategies for the synthesis of 6-deoxy-L-sorbose



### This work: two step strategy for the efficient and convenient preparation of 6-deoxy-L-sorbose



**Figure 1.7** Enzymatic synthesis of 6-deoxy-L-sorbose

By screening the substrate specificity of many kinases, we recently found that fructose kinase (HK) from human, which phosphorylate ketose to ketose 1-phosphate, preferred ketose with (3*S*)-configuration as its substrate. In this work, substrate specificity of HK towards 6-deoxy-L-sorbose ((3*S*)-configuration), L-fucose ((3*R*)-configuration), and L-fuculose ((3*R*)-configuration) was studied. 6-deoxy-L-sorbose could serve as the substrate of HK while no detectable activity of HK

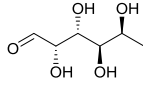
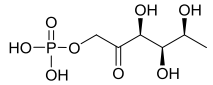
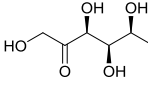
towards either L-fucose or L-fuculose was observed (Table 1.6), indicating its potential for one-pot multienzyme (OPME) reaction to produce 6-deoxy-L-sorbose from L-fucose. Indeed, treatment of 6-deoxy-L-sorbose with HK in the presence of ATP as phosphate donor led to complete conversion to 6-deoxy-L-sorbose 1-phosphate after 2 hour at 37 °C.

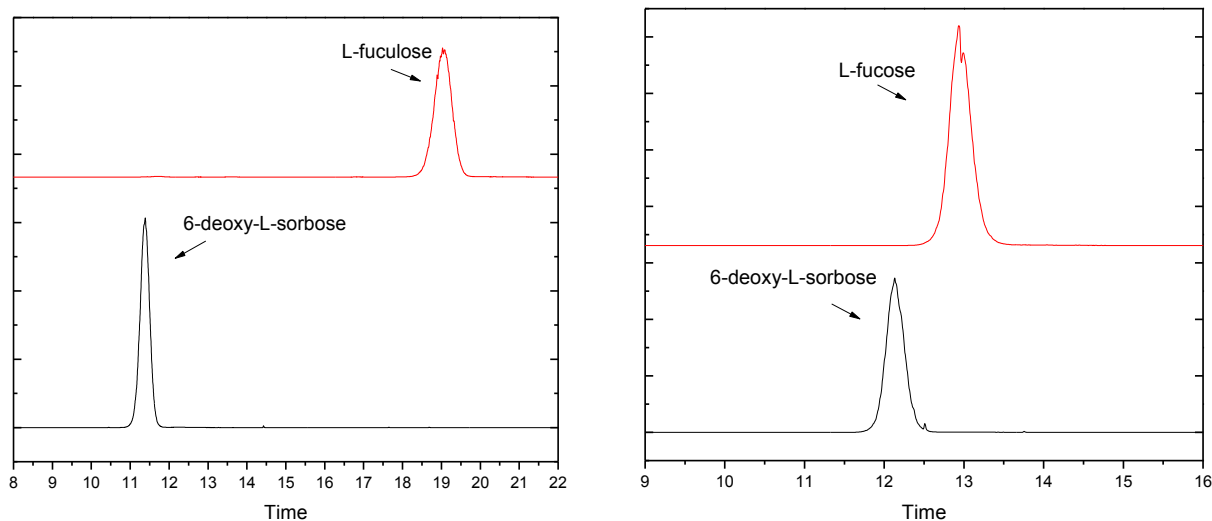
**Table 1.6** Substrate specificity of HK towards several deoxy sugars

Substrate	HK activity (%)	C-3 configuration
L-fucose	ND	<i>R</i>
L-fuculose	ND	<i>R</i>
6-deoxy-L-sorbose	100	<i>S</i>

Having met the prerequisite, other conversion related enzymes including L-fucose isomerase (FucI) from *Escherichia coli*,<sup>73</sup> DTE from *Pseudomonas* Sp, St-24<sup>51</sup>, and AphA from *Escherichia coli*<sup>74</sup> were prepared as described in experiment part. To test the potential of HK in OPME reaction, a small-scale reaction system (50 ul) containing conversion-related enzymes (FucI, DTE and HK) was performed (reaction group). Reactions without FucI (control 1 group) or DTE (control 2 group) were done as negative controls. The reaction was incubated at 37 °C for 1 hour and monitored by TLC (EtOAc/MeOH/H<sub>2</sub>O/HOAc=5:2:1.4:0.4). The formation of 6-deoxy-L-sorbose 1-phosphate was observed in the reaction group and no reaction was observed in either control 1 or control 2, indicating the feasibility of the designed OPME reaction. Preparative reactions were performed in the gram scale. To efficiently convert L-fucose, excess ATP (1.25 equiv) was used. For the convenience of the final purification, no buffer was used. The reaction pH was held near 7.5 using sodium hydroxide as the reaction was ongoing. The reaction was allowed to proceed until no detectable L-fucose was observed by HPLC (conversion ratio exceeding 99%) and thereby making isomer separation step is unnecessary.

**Table 1.7** Enzymatic synthesis of 6-deoxy-L-sorbose from L-fucose

Starting material	Enzymes	Intermediate	Step 1 yield (%)	Product	Total yield (%)	Scale (mg)	Purity <sup>a</sup> (%)
 L-fucose	Fucl DTE HK AphA	 6-deoxy-L-sorbose 1-phosphate	92	 6-deoxy-L-sorbose	81	1059	>99

**Figure 1.8** HPLC profiles of 6-deoxy-L-sorbose

6-deoxy-L-sorbose 1-phosphate was purified by using silver nitrate precipitation method.<sup>83</sup> The principle of this method is that silver ions could precipitate ATP and ADP while monophosphate sugars could not be precipitated when the sugars are composed of four or more carbons. Therefore, by applying this selective precipitating ability of silver ions, sugar phosphate can be cleanly and easily separated from adenosine phosphates. Afterwards, the solution was desalted by Bio-Gel P-2 column to afford 6-deoxy-L-sorbose 1-phosphate in 92% yield with regard to L-fucose. In the second reaction step, the phosphate group of 6-deoxy-L-sorbose 1-phosphate was hydrolyzed by AphA in pH 5.5. Once no detectable sugar phosphate was observed, the reaction was stopped by adding cooled ethanol. The solution was purified by Bio-Rad P-2 column to afford 6-deoxy-L-sorbose in 81 % yield with regard to L-fucose.

### 1.3.3 The synthesis of 6-deoxy-L-psicose

Rare sugars cannot be extracted from natural sources due to their low abundance. Therefore, numerous chemical and enzymatic methods have been explored to synthesize rare sugars from common sugars.<sup>4, 84</sup> Chemical strategies for rare sugars tend to require multiple protection/deprotection and complicated purification steps, suffering from low yield as a result.<sup>85</sup> As an alternative, enzymatic synthesis with mild conditions and high efficiency has distinct advantages in both regio- and stereo-selectivity.<sup>1</sup> Izumori *et al.* have previously shown that the total synthesis of rare sugars by using isomerization, epimerization, oxidization or reduction reactions is achievable.<sup>2, 3</sup> For example, aldose-ketose isomerization catalyzed by a single isomerase has been widely used to produce ketose from aldose as most common sugars are aldoses.<sup>86</sup> Nevertheless, aldose-ketose isomerization is very unfavorable for ketose formation in the final reaction as ketose has a higher energy state than aldose.<sup>66</sup> In most instances, the conversion ratios are no more than 40%.<sup>87-91</sup> Attempts to improve aldose-ketose isomerization include the addition of borate to break the aldose-ketose reaction equilibrium,<sup>38</sup> the discovery of novel enzymes in nature,<sup>42</sup> and directed evolution<sup>92</sup> have also been suggested. Nevertheless, these methods still suffer from low yield, or an isomer separation manipulation, which is a labor-intensive and time-consuming process due to the similar properties of the isomer pair. Ketose-ketose epimerization catalyzed by D-psicose 3-epimerase (DPE) or D-tagatose 3-epimerase (DTE), which was firstly discovered by Izumori and co-workers,<sup>51</sup> is another important reaction for rare sugar synthesis. However, this process also suffers from low yield and isomer separation manipulation. Oxidization or reduction reactions require co-enzyme such as NAD<sup>+</sup>/NADH, which are commercially too expensive for large scale synthesis. Therefore, the study of rare sugars is still hindered due to the lack of an efficient and convenient preparation method.

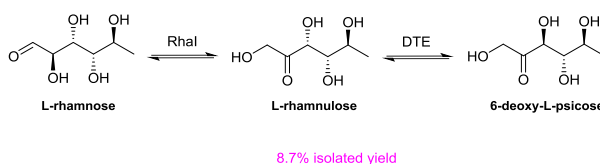


6-deoxy-L-psicose is a 6-deoxy rare sugar and also the C-3 epimer of L-rhamnulose. 6-deoxy sugars are building blocks of a variety of natural products, including antifungals,<sup>93</sup> antibiotics,<sup>94</sup> and anticancer agents.<sup>95, 96</sup> 6-deoxy-L-psicose is the intermediate for the preparation of 6-deoxy-L-allose and 6-deoxy-L-altrose.<sup>64</sup> The more potential applications of 6-deoxy-L-psicose have not been reported, possibly due to its limited availability. Chemical 6-deoxy reaction from corresponding ketoses require complicated protection/deprotection steps, resulting in a low yield.<sup>97, 98</sup> The only enzymatic strategy reported for 6-deoxy-L-psicose synthesis is by Shompoosang *et al*, to the best of our knowledge.<sup>64</sup> They incubated L-rhamnose with L-rhamnose isomerase (RhaI) and D-tagatose 3-epimerase (DTE) to produce 6-deoxy-L-psicose. When the final reaction equilibrium was observed, a mixture containing L-rhamnose, L-rhamnulose and 6-deoxy-L-psicose (55: 35: 15) was obtained. After a tedious purification step, 6-deoxy-L-psicose was obtained in only 8.7% yield with respect to L-rhamnose. Therefore, an efficient and convenient strategy capable of producing 6-deoxy-L-psicose in considerable amount would be of great interest to accelerate the study of 6-deoxy-L-psicose.

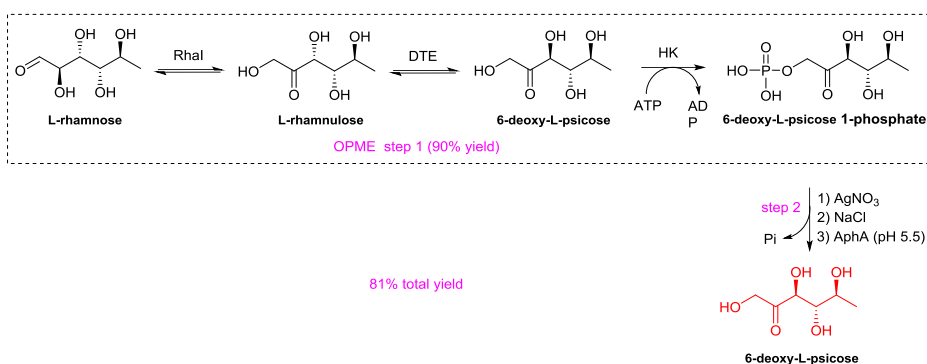
To produce 6-deoxy-L-psicose from L-rhamnose by using the proposed two-step strategy, two prerequisites must first be met: 1) the availability of a D-tagatose 3-epimerase (DTE) to catalyze the interconversion of L-rhamnulose and 6-deoxy-L-psicose, but not the interconversion between 6-deoxy-L-psicose 1-phosphate and L-rhamnulose 1-phosphate; and 2) the availability of a kinase that specifically phosphorylates 6-deoxy-L-psicose but not L-rhamnulose and L-rhamnose. The discovery of DTE, which epimerize ketoses at C-3 position, by Izumori *et al* has made it possible to achieve the interconversion of (3*S*)-sugars and (3*R*)-sugars.<sup>51</sup> They demonstrated that DTE from *Pseudomonas* Sp, St-24 failed to recognize D-fructose 6-phosphate and D-ribulose 5-phosphate.<sup>51</sup> We recently found that DTE from *Pseudomonas* Sp, St-24 also fails to use many ketose phosphates

as the substrate.<sup>99, 100</sup> Therefore, we proposed that it may not recognize 6-deoxy-L-psicose 1-phosphate as substrate in this work. This assumption is supported by the purity analysis of 6-deoxy-L-psicose (Table 1.9). To find a kinase that specifically phosphorylates 6-deoxy-L-psicose but not L-rhamnulose and L-rhamnose, substrate specificity of many kinases was studied in this work (data not shown). Finally, we found that fructose kinase (HK) from human accords well with the requirement of the described Scheme. Substrate specificity study indicated that HK could efficiently phosphorylate 6-deoxy-L-psicose, with only trace activity (<0.1 %) towards L-rhamnulose and no detectable activity towards L-rhamnose (Table 1.8). The high specificity of HK towards 6-deoxy-L-psicose indicated its potential for applications in one-pot multienzyme (OPME) reaction for the preparation of 6-deoxy-L-psicose from L-rhamnose.

**Previous work: enzymatic synthesis of 6-deoxy-L-psicose from L-rhamnose**



**This work: two-step enzymatic synthesis of 6-deoxy-L-psicose from L-rhamnose**



**Figure 1.9** Enzymatic synthesis of 6-deoxy-L-psicose.

To test this potential, analytical scale reaction system (164 ug in 50 ul) containing conversion-related enzymes (RhaI, DTE and HK) was performed. Reactions without RhaI (control 1 group)

or DTE (control 2 group) were done as negative controls. The reaction was incubated at 37 °C for 1 hour and monitored by TLC (EtOAc/MeOH/H<sub>2</sub>O/HOAc=5:2:1.4:0.4). The formation of 6-deoxy-L-psicose 1-phosphate was observed in the reaction group and no reaction was observed in either control 1 or control 2, indicating the feasibility of the designed OPME reaction.

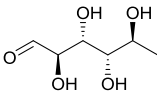
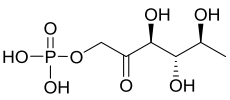
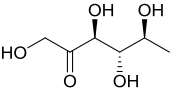
**Table 1.8** Substrate specificity of HK towards several deoxy sugars

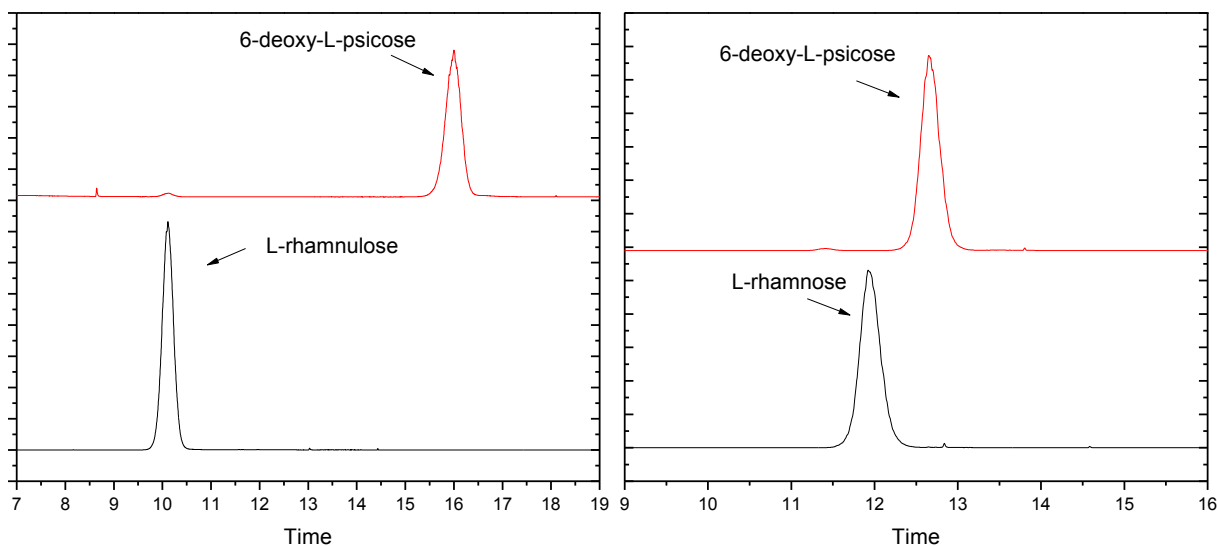
Substrate	HK activity (%)	C-3 configuration
L-rhamnose	ND	<i>R</i>
L-rhamnulose	<0.1	<i>R</i>
6-deoxy-L-psicose	100	<i>S</i>

Preparative scale synthesis was carried in a 400 ml reaction solution containing 20 mM L-rhamnose. As much as possible to consume L-rhamnose, 1.25 equivalent of ATP was added. The reaction pH was held near 7.5 using 1 M of sodium hydroxide as the reaction was ongoing. The reaction was carefully shaken at 37°C for 48 hours to allow the formation of 6-deoxy-L-psicose 1-phosphate. Enzymes were supplemented every 12 hours. Once reaction finished, 95% of L-rhamnose was consumed (as confirmed by HPLC). Afterwards, ATP and ADP was selectively precipitated by using silver nitrate precipitation method,<sup>33</sup> while 6-deoxy-L-psicose 1-phosphate is still in solution. 6-deoxy-L-psicose 1-phosphate was separated from the remaining L-rhamnose by using Bio-Gel P-2 column to afford final product in 90% yield with regard to L-rhamnose. In the second reaction step, 6-deoxy-L-psicose 1-phosphate obtained in first reaction step was dissolved in water and the solution pH was adjusted to 5.5 using 1 M of HCl. Then, acid phosphatase was added to hydrolyze the phosphate group of 6-deoxy-L-psicose 1-phosphate to afford 6-deoxy-L-psicose. After desalting by using Bio-Gel P-2 column, 6-deoxy-L-psicose was obtained in 80% yield with regard to L-rhamnose (Table 1.9).

The obtained product was confirmed by NMR, HRMS and HPLC analysis. The predicted peak ( $[M+Na]^+$  187.0582) was well observed on high resolution mass spectrum. The product purity was analyzed by HPLC using HPX-87H column or Sugar-Pak 1 column equipped with evaporative light scattering detector (ELSD). HPLC analysis using HPX-87H column indicated that no detectable L-rhamnose was found (as also confirmed by  $^1\text{H-NMR}$ ). HPLC analysis using Sugar-Pak 1 column showed that 1.5 % of L-rhamnulose was found in final product (Figure 1.10). This may result from the long reaction time and the trace activity of HK towards L-rhamnulose.

**Table 1.9** Enzymatic synthesis of 6-deoxy-L-psicose from L-rhamnose

Starting material	Enzymes	Intermediate	Product	Total yield (%)	Scale (mg)	Purity <sup>a</sup> (%)
 L-rhamnose	Rhal DTE HK AphA	 6-deoxy-L-psicose 1-phosphate	 6-deoxy-L-psicose	81	1059	98.5



*Figure 1.10 HPLC analysis of 6-deoxy-L-psicose*

#### 1.4 Facile enzymatic synthesis of phosphorylated phosphorylated ketopentoses

Of all possible structures, only 12 pentoses naturally occur comprised of eight aldopentoses (D-xylose, D-lyxose, D-ribose, D-arabinose, L-xylose, L-lyxose, L-ribose, and L-arabinose) and four corresponding ketopentoses (D-xylulose, D-ribulose, L-xylulose, and L-ribulose).<sup>3</sup> The ketopentoses can be phosphorylated at the C-1 or C-5 position, resulting in eight phosphorylated ketopentoses (Figure 1.1). It is now well established that abnormal levels of phosphorylated ketopentoses in mammals are directly correlated to a wide range of diseases such as diabetes, cancer, atherosclerosis, and cystic fibrosis.<sup>101-106</sup> Uyeda and co-workers found that xylulose 5-phosphate acts as a glucose signaling compound, recruiting and activating a specific protein serine/threonine phosphatase (PPase), which is responsible for the activation of transcription of the L-type pyruvate kinase gene and lipogenic enzyme genes.<sup>107-109</sup> Enzymes involved in phosphorylated ketopentoses metabolism are exciting potential targets for therapeutic treatment.<sup>110-113</sup> Additionally, phosphorylated ketopentoses are important intermediates in sugar metabolism pathway. All pentoses including aldopentose and ketopentose that could be utilized by bacteria or other organisms must first be converted to their phosphorylated ketopentose forms to enable metabolic function.<sup>1, 4, 14, 15</sup> In these metabolism pathways, ketopentose 1-phosphates are split into glycolaldehyde and dihydroxyacetone phosphate, which is an intermediate in the glycolytic pathway, by aldolases.<sup>16-18</sup> Ketose 5-phosphates will be epimerized to D-xylulose 5-phosphate or D-ribulose 5-phosphate, which are key participants of pentose phosphate pathway (PPP), by epimerases.<sup>19-21</sup> PPP is a universal metabolic process present in bacteria, plants and animals,<sup>22</sup> and its main function is to produce reducing power and building blocks for cell growth.<sup>23</sup> Because of their critical position in sugar metabolism pathway, phosphorylated ketopentoses are also the starting materials in synthetic chemistry.<sup>24</sup> For example, D-xylulose 5-phosphate can act as the

starting material for the synthesis of heptose in bacteria.<sup>25</sup> Likewise, D-ribulose 5-phosphate can be used for the synthesis of 3-deoxy-D-manno-octulosonic acid (KDO).<sup>26</sup> Therefore, phosphorylated ketopentoses have great potential for applications in investigating the mechanistic and regulatory aspects of sugar metabolism, identification and characterization of new enzymes in nature, and being raw materials in synthetic chemistry.<sup>27-29</sup> A platform for the highly efficient and convenient synthesis of phosphorylated ketopentoses is therefore of considerable interest.

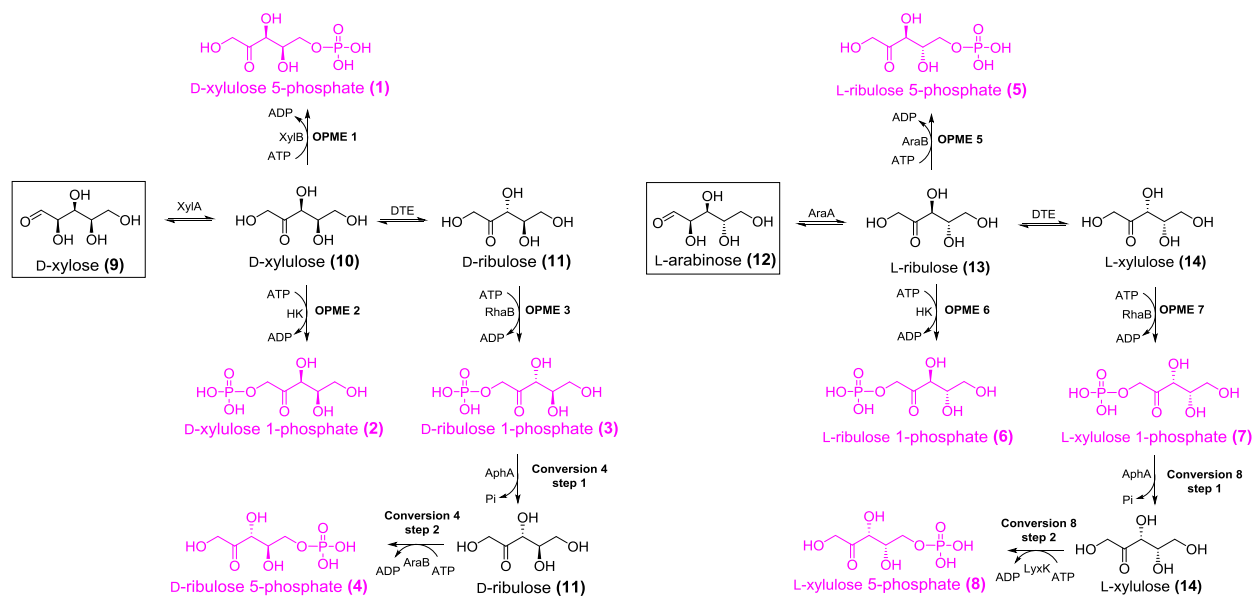
Regarding the biological and synthetic applications of phosphorylated ketopentoses, multiple methods have been developed. The chemical phosphorylation of ketoses involves protection/deprotection steps.<sup>114-116</sup> Alternatively enzymatic catalysis (kinase) with mild reaction conditions and high reaction efficiency is able to proceed regio- and stereoselectively without requiring protection.<sup>117-122</sup> However, ketopentoses are cost prohibitive for preparative scale synthesis. The strategy includes enzymatic isomerization in a one-pot fashion to enable the use of cheaper aldose as starting materials to prepare phosphorylated ketoses,<sup>60, 69, 115, 123</sup> but the availability of substrate-specific kinases is a prerequisite. Moreover, enzymatic phosphorylation requires the separation of sugar phosphate from adenosine phosphates (ATP and ADP) to obtain a sugar phosphate in pure form. The two most common methods used for sugar phosphate purification are ion-exchange chromatography and barium precipitation,<sup>75, 115, 124</sup> but both methods are labor-intensive and time-consuming. Additionally, enzymatic synthesis of phosphorylated ketopentoses employing transketolase,<sup>125-127</sup> aldolase,<sup>60, 75, 128</sup> or oxidase<sup>129</sup> has also been suggested. However, these methods suffer from both expensive starting materials and extensive purification steps. Therefore, while some phosphorylated ketopentoses are commercially available, they are extremely expensive (D-ribulose 5-phosphate, \$1245/25mg, Sigma-Aldrich; D-xylulose 5-phosphate, \$1150/10 mg, Carbosynth). Studies of this fundamental class of carbohydrates have

been hindered by a lack of efficient preparation methods to readily provide substantial amounts of the desired products.

In this work, an efficient and convenient platform for phosphorylated ketose syntheses is described. All eight phosphorylated ketopentoses were produced from D-xylose and L-arabinose utilizing this platform (Figure 1.11). Our strategies are mainly based on “isomerization→phosphorylation” and “isomerization→epimerization→phosphorylation” cascade reactions. We combined the thermodynamically unfavorable bioconversions of D-xylose to D-series of ketopentose (D-xylulose and D-ribulose), or L-arabinose to L-series of ketopentose (L-ribulose and L-xylulose) with phosphorylation reactions by substrate-specific kinases to prepare the desired phosphorylated ketopentoses (**1** to **3**, and **5** to **7**). D-ribulose 5-phosphate (**4**) and L-xylulose 5-phosphate (**8**) were synthesized by a two-step strategy.

D-xylose and L-arabinose are the two common pentoses found in substantial quantities in nature.<sup>2</sup> Although the reactions are reversible, aldose-ketose isomerization and ketose-ketose epimerization has been the main method for ketose preparation.<sup>2</sup> Nevertheless, separation of the desired ketose from its isomeric mixture is difficult. To avoid such separation, we combined these reversible reactions with targeted phosphorylation reactions catalyzed by kinases in a one-pot fashion to prepare phosphorylated ketopentoses instead of directly phosphorylating the ketoses. These reactions are known as one-pot multienzyme reaction (OPME).<sup>130</sup> The phosphorylation reaction coupled with the reversible conversions (isomerization or epimerization) drives the reactions (isomerization or epimerization) towards the formation of ketoses in their phosphorylated forms until a high conversion ratio was reached.

In order to establish the basis of OPME reactions for the preparation of D-xylulose 5-phosphate from D-xylose, and L-ribulose 5-phosphate from L-arabinose, the substrate specificity of



**Figure 1.11** Total synthesis of phosphorylated ketopentoses from D-xylose and L-arabinose

D-xylulose kinase (XylB) and L-ribulose kinase (AraB) from *E.coli*,<sup>120, 122</sup> were tested (see experiment part). No detectable activity of XylB towards D-xylulose, or AraB towards L-ribulose was found (Table 1.10), indicating their potential for OPME reactions. To test the practicability of the designed OPME reactions, analytical reactions were firstly carried in 50  $\mu$ l system containing conversion-related enzymes (Table 1.11), starting materials and ATP. Reactions without isomerase were performed as negative controls. The reactions were tested by TLC (EtOAc/MeOH/H<sub>2</sub>O/HOAc=5:2:1.4:0.4). After observing sugar phosphates formation by TLC and no reactions in control samples, preparative scale syntheses were performed.

Preparative scale syntheses were routinely performed in gram scale (Table 1.11). D-xylose was incubated with D-xylose isomerase (XylA) from *E.coli*<sup>131</sup> and XylB in the presence of ATP (1.25 molar equiv) as phosphate donor to prepare D-xylulose 5-phosphate (OPME 1, Figure 1.11). L-arabinose was incubated with ATP (1.25 molar equiv), L-arabinose isomerase (AraA) from *Bacillus subtilis*,<sup>132</sup> and AraB to prepare L-ribulose 5-phosphate (OPME 5, Figure 1.11). No buffer was used in consideration to purification. The reaction pH was held near 7.5, where all enzymes



are quite active, using sodium hydroxide as the reaction occurred. Both reactions were allowed to proceed until a conversion ratio exceeding 99% was reached (as confirmed by HPLC).

**Table 1.10** Substrate specificity of kinases<sup>a</sup>

Substrate	XylB	AraB	LyxK	RhaB <sup>b</sup>	HK <sup>b</sup>
	Activity (%)	Activity (%)	Activity	Activity	Activity
L-arabinose	ND	ND	ND	ND	ND
D-xylose	ND	ND	ND	0.1	ND
L-xylulose	7.3	1.3	100	100	NA
D-ribulose	4.9	139	2.5	111	NA
L-ribulose	16.9	100	2.5	ND	100
D-xylulose	100	3.4	1.6	0.3	349

a: Substrate specificity was studied by the reactions that were performed at 37°C for 10 minutes in 50 ul reaction mixture containing a Tris-HCl buffer (100 mM, pH 7.5), 20 mM of sugar standards, 20mM of ATP, 5 mM of Mg<sup>2+</sup>, and 10 ug of enzymes. NA: not assayed. ND: no detectable activity was observed.

Afterwards, silver nitrate precipitation method<sup>99</sup> was used to purify D-xylulose 5-phosphate and L-ribulose 5-phosphate. In detail, silver nitrate was added to the solution to precipitate ATP and ADP selectively until no new precipitation was observed. Precipitates were removed by centrifugation. Sodium chloride was added to precipitate residual silver ions and silver chloride precipitate was removed by centrifugation. The entire separation process can be finished in less than 15 minutes. After desalting by a P-2 column, D-xylulose 5-phosphate and L-ribulose 5-phosphate were isolated in more than 90% yield (Table 1.11) comparable to the yield for ion-exchange purification methods. Compared to barium precipitation method, by which sugar phosphate was isolated as a barium salt in the presence of barium and ethanol,<sup>115, 124</sup> no additional steps to remove toxic ions and no accurate pH control are necessary. Moreover, silver is safer than

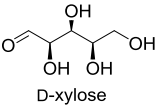
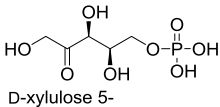
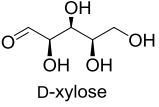
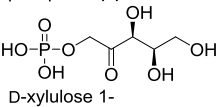
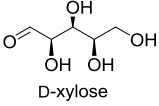
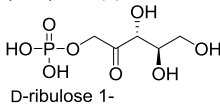
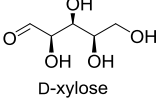
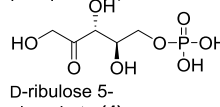
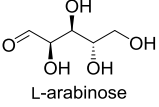
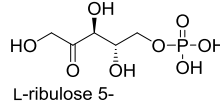
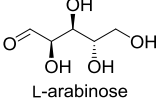
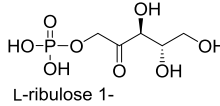
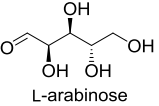
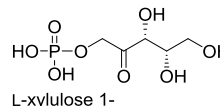
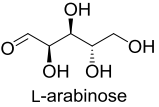
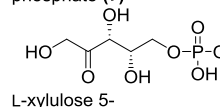
barium. The isolated products were confirmed by NMR and MS analysis (see experiment part). Compared to the previous synthesis,<sup>115, 123, 127</sup> not only a higher isolated yield was obtained, but also the tedious purification step was avoided.

To assess product purity, the phosphate groups on both phosphorylated sugars were hydrolyzed by acid phosphatase. The resultant monosaccharides were analyzed by HPLC (see experiment part). A product distribution of D-xylulose to D-xylose (98.4: 1.6) was observed indicating the isolated product of D-xylulose 5-phosphate contains 1.6% of D-xylose 5-phosphate (as also observed by NMR). Similarly, a product distribution of L-ribulose to L-arabinose (99.5: 0.5) was observed indicating the isolated product of L-ribulose 5-phosphate contains 0.5% of L-arabinose 5-phosphate (as also observed by NMR). The existence of aldoses may be because XylA and AraA can incorrectly isomerize D-xylulose 5-phosphate and L-ribulose 5-phosphate to a certain extent.

To prepare D-xylulose 1-phosphate and L-ribulose 1-phosphate from D-xylose and L-arabinose using OPME reactions, an enzyme that could phosphorylate D-xylulose and L-ribulose at the C-1 position but not D-xylose and L-arabinose is required. Human fructosekinase (HK) catalyzes the phosphorylation of ketoses to ketose 1-phosphates.<sup>56</sup> Recently, we found HK could specifically recognize D-xylulose and L-ribulose but not D-xylose and L-arabinose.<sup>99</sup> Analytical reactions containing conversion-related enzymes (Table 1.11) were performed and tested as described above. Once the formation of sugar phosphates were observed by TLC and no further reactions in control samples seen, preparative scale syntheses were performed. D-xylose was incubated with ATP (1.25 molar equiv), XylA, and HK to prepare D-xylulose 1-phosphate (OPME 2, Figure 1.11). L-arabinose was incubated with ATP (1.25 molar equiv), AraA, and HK to prepare L-ribulose 1-phosphate (OPME 6, Figure 1.11). Both reactions were monitored by TLC and HPLC.

Once no detectable amounts of the starting aldoses were observed (conversion ratio exceeding

**Table 1.11** Total synthesis of phosphorylated ketopentoses from D-xylose and L-arabinose using the strategy shown in Scheme 1<sup>a</sup>

Entry	Starting material	Enzymes	Scheme	Product	Isolated yield (%)	Scale (mg)	Purity <sup>a</sup> (%)
1	 D-xylose	XylA XylB	OPME 1	 D-xylulose 5-phosphate ( <b>1</b> )	91	1143	98.4
2	 D-xylose	XylA HK	OPME 2	 D-xylulose 1-phosphate ( <b>2</b> )	92	1159	>99
3	 D-xylose	XylA DTE RhaB	OPME 3	 D-ribulose 1-phosphate ( <b>3</b> )	94	1183	98.2
4	 D-xylose	XylA DTE RhaB AphA AraB	Conversion 4	 D-ribulose 5-phosphate ( <b>4</b> )	85	1291	99
5	 L-arabinose	AraA AraB	OPME 5	 L-ribulose 5-phosphate ( <b>5</b> )	92	1164	99.5
6	 L-arabinose	AraA HK	OPME 6	 L-ribulose 1-phosphate ( <b>6</b> )	93	1170	>99
7	 L-arabinose	AraA DTE RhaB	OPME 7	 L-xylulose 1-phosphate ( <b>7</b> )	96	1204	>99
8	 L-arabinose	AraA DTE RhaB AphA LyxK	Conversion 8	 L-xylulose 5-phosphate ( <b>8</b> )	84	1265	99

a: Defined as the percentage of desired phosphorylated ketose out of the sum of all possible isomers (as confirmed by HPLC).

99%), silver nitrate precipitation was used to purify the sugar phosphates. After desalting by a P-2 column, D-xylulose 1-phosphate and L-ribulose 1-phosphate were isolated in more than 90% yield (Table 1.11). The products were confirmed by NMR and MS analysis (see experiment part). The purity was analyzed in the same manner mentioned above (see experiment part). Since the C-

1 position was blocked by the phosphate group, the side reactions were avoided to result in a product purity exceeding 99% (Table 1.11).

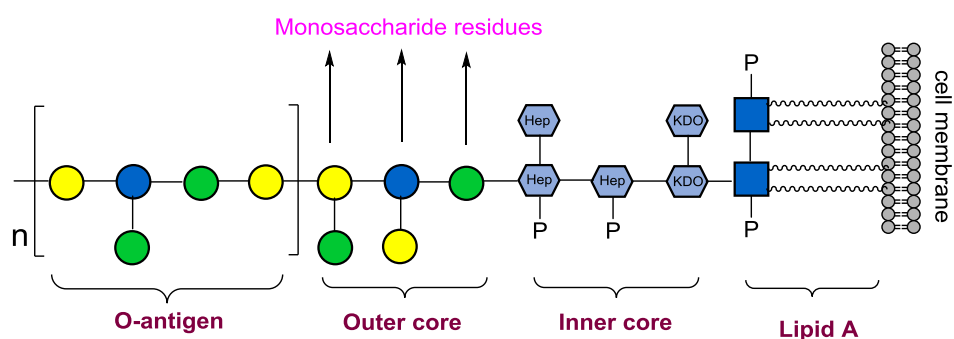
D-ribulose 1-phosphate and L-xylulose 1-phosphate were prepared from D-xylose and L-arabinose using OPME 3 and OPME 7 (Figure 1.11). Although D-ribulose 1-phosphate and L-xylulose 1-phosphate have great potential applications, they have been difficult to prepare in substantial quantities. For example, it has been reported that D-ribulose 1-phosphate can be isomerized from D-ribose 1-phosphate, which result from the phosphorylation of D-ribose, by MTR 1-P isomerase.<sup>133</sup> However, this process is impractical, requiring not only a sugar phosphate purification step but also a protracted isomer separation. D-ribulose and L-xylulose have a (3*R*)-configuration which is different from D-xylose and L-arabinose. To prepare D-ribulose 1-phosphate from D-xylose, and L-xylulose 1-phosphate from L-arabinose, isomerization catalyzed by isomerase (XylA or AraA) and a C-3 epimerization catalyzed by D-tagatose 3-epimerase (DTE) from *Pseudomonas* Sp, ST-24<sup>51</sup> were combined in one-pot. These two reversible conversions were coupled with L-rhamnulose kinase (RhaB) from *Thermotoga maritima* MSB8, a novel enzyme that requires ketoses with (3*R*)-configuration,<sup>99</sup> resulting in the formation of (3*R*)-ketose 1-phosphates. Once the starting aldoses were no longer detected, D-ribulose 1-phosphate and L-xylulose 1-phosphate were purified by silver nitrate precipitation in more than 90% yield (Table 1.11) and confirmed by NMR and MS analysis (see experiment part). A product distribution of D-ribulose, D-xylulose and D-xylose (98.2: 1.2: 0.6) was observed indicating the purity of D-ribulose 1-phosphate is 98.2%. For L-xylulose 1-phosphate analysis, no detectable L-arabinose or L-ribulose was found indicating L-xylulose 1-phosphate has a purity exceeding 99%. Such conversions are important because most of sugars that naturally occur in large amounts are (3*S*)-aldoses.<sup>2</sup> Therefore,

the reactions described herein (OPME 3 and OPME 7) represent a novel strategy for the preparation of (3*R*)-ketoses from (3*S*)-aldoses directly.

D-ribulose 5-phosphate and L-xylulose 5-phosphate also have been difficult to prepare in quantity. The methods known in the literature for the synthesis of D-ribulose 5-phosphate are the phosphorylation of D-ribulose,<sup>115</sup> the isomerization of D-ribose 5-phosphate, and the oxidization of D-gluconate 6-phosphate.<sup>129</sup> L-xylulose 5-phosphate could be prepared by phosphorylating L-xylulose.<sup>119</sup> Nevertheless, these methods also suffer from expensive starting materials or a tedious purification step. In this work, D-ribulose 5-phosphate and L-xylulose 5-phosphate were prepared from D-xylose and L-arabinose by a two-step strategy (conversion 4 and conversion 8, Figure 1.11) due to an inability to identify kinases that could specifically phosphorylate D-ribulose and L-xylulose at the C-5 position. In the first conversion step, D-ribulose and L-xylulose were obtained by hydrolyzing the phosphate groups of D-ribulose 1-phosphate and L-xylulose 1-phosphate, both of which were prepared from D-xylose and L-arabinose using OPME 3 and OPME 7, by acid phosphatase (AphA) from *E.coli*,<sup>74</sup>. AraB is well known as L-ribulose kinase,<sup>122</sup> but it also displays high activity towards D-ribulose (Table 1.10). Thus, in the second conversion step, D-ribulose was incubated with AraB to prepare D-ribulose 5-phosphate. L-xylulose was incubated with L-xylulose kinase (LyxK) from *E.coli*<sup>119</sup> to prepare L-xylulose 5-phosphate. The products were purified by using silver nitrate precipitation. After desalting by using a P-2 column, the products were isolated in more than 84% yield with regard to D-xylose and L-arabinose (Table 1.11), respectively. Purity analysis indicated both D-ribulose 5-phosphate and L-xylulose 5-phosphate have a purity of 99%.

## 1.5 Enzymatic synthesis of 3-deoxy-D-mano-octulosonic acid (KDO) and its application for LPS assembly.

Lipopolysaccharides (LPS), also known as endotoxins, are large molecules that anchored in the outer membrane of Gram-negative bacteria by lipid A, to which a nonrepeating core oligosaccharide and a distal polysaccharide termed as O-antigen are attached (Figure 1).<sup>134</sup> Nonrepeating core oligosaccharide part contains 3-deoxy-D-manno-octulosonic acid (KDO) and heptose and is highly conserved in different bacteria.<sup>135</sup> KDO is the only sugar that found in all known core structures, although in some cases a derivative, D-glycero-D-talo-2-octulosonic acid (KO), is also present.<sup>135</sup> KDO was also found in capsular polysaccharides of many bacteria. For example, the repeating unit of *Neisseria meningitidis* serogroup E capsule consists of alternating D-galactosamine and KDO residues.<sup>136</sup> *Escherichia coli* K12 capsule contains rhamnose and KDO residues.<sup>137</sup> Besides, KDO was found in plant and green algae.<sup>138-141</sup> Concerning the importance of KDO in kinds of biological processes, enzymes that involved in KDO biosynthetic pathway are exciting targets for the development of new classes of antibiotics.<sup>142, 143</sup> Core polysaccharides of LPS are also the potential vaccines against bacterial infection. Many KDO-containing



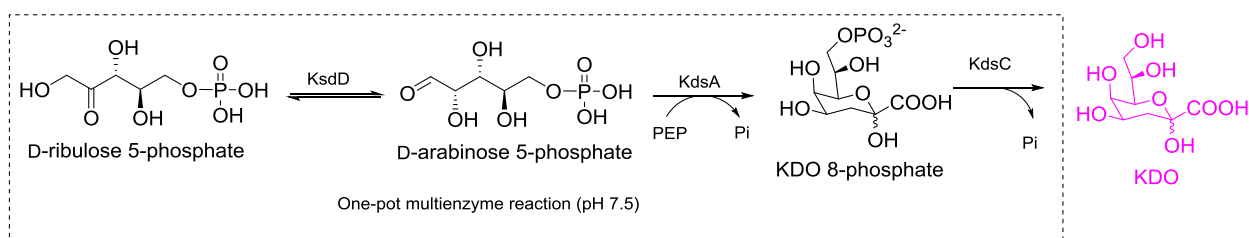
**Figure 1.12** The structure of *E. coli* LPS

polysaccharides have been synthesized and evaluated in recent years.<sup>144-147</sup> The fact that immunizations with many of these polysaccharides lead to antibody responses provides an impetus

to explore further KDO-containing polysaccharides as a vaccine candidate.<sup>147</sup> Nevertheless, such studies have been hampered by the lack of efficient and convenient preparation methods for KDO preparation.

Chemical methods for KDO synthesis have been developed over the past decades,<sup>148-154</sup> but the tedious protection/de-protection steps can be complicated and suffer from low yield. Alternatively, enzymatic syntheses employing KDO aldolase,<sup>155</sup> sialic acid aldolase,<sup>156, 157</sup> KDO phosphate synthetase<sup>158</sup> proceed regio- and stereoselectively without protection. KDO aldolase and sialic acid aldolase could condense arabinose and pyruvate into KDO directly, but both enzymes suffer from low specific activity,<sup>155, 156</sup> making these processes impractical for the scalable synthesis of KDO. In contrast with both aldolases, KDO 8-phosphate synthetase showed significantly higher specific activity,<sup>158-160</sup> and more than 120 mg of protein could be obtained from one liter of LB culture medium by using pET protein expression system (data in this work). KDO 8-phosphate synthetase catalyzes the aldol condensation of D-arabinose 5-phosphate and phosphoenolpyruvate (PEP), resulting in KDO 8-phosphate, which can be hydrolyzed to afford KDO by phosphatase.<sup>158</sup> The only block of this process for large scale synthesis is the low accessibility of D-arabinose 5-phosphate. Commercially available D-arabinose 5-phosphate is extremely expensive (\$643/25mg, Sigma-Aldrich) for preparative scale synthesis. Moreover, D-arabinose 5-phosphate has been difficult to prepare in quantity because there is a lack of kinase that could efficiently phosphorylate D-arabinose at C-5 position directly. Bednarski and co-workers used hexokinase and ATP-regeneration system to produce D-arabinose 5-phosphate for KDO synthesis.<sup>158</sup> Nevertheless, the low specific activity of hexokinase towards D-arabinose requires a large amount of hexokinase. To avoid using expensive starting materials, Pohl and co-workers have developed a biological “living factory”, by which KDO was produced from glucose through cell fermentation.<sup>26</sup> Although

hundreds milligram of KDO could be produced in one liter of medium, the purification of the final product from fermentation broth can be complicated. Therefore, an efficient and convenient method to readily provide KDO in considerable amounts is highly attractive in enabling the studies of KDO.



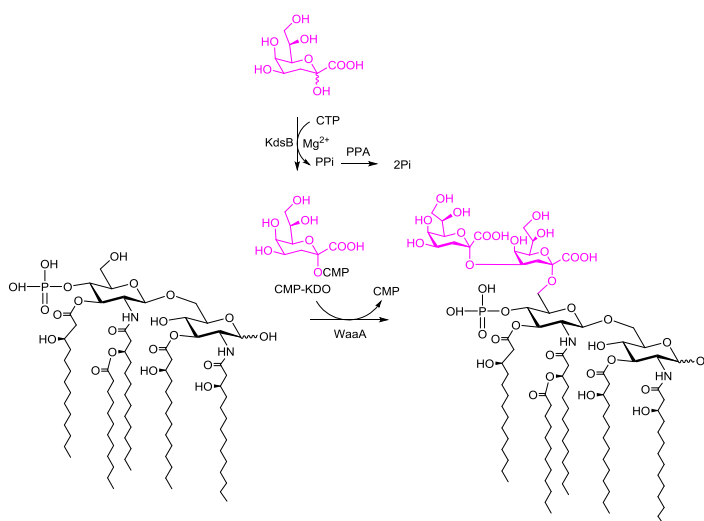
**Figure 1.13** One-pot multienzyme system for the production of KDO

Herein, an efficient enzymatic strategy for the facile synthesis of KDO from easy-to-get starting materials is described (Figure 1.13). In the first stage, D-ribulose 5-phosphate was prepared from D-xylose in multi-gram scale. In the second stage, D-ribulose 5-phosphate was incubated with D-arabinose 5-phosphate isomerase (KdsD), KDO 8-phosphate synthetase (KdsA), and KDO 8-phosphate phosphatase (KdsC) in one-pot fashion to produce KDO. The obtained KDO was further transferred into lipid A by KDO transferase from *E.coli* (WaaA) (Figure 1.14).

D-arabinose 5-phosphate is a rare sugar phosphate, and there is a lack of kinase that could efficiently phosphorylate D-arabinose directly. Therefore, D-arabinose 5-phosphate has been difficult to prepare in quantity, and the commercially available product is extremely expensive. Meanwhile, many synthetic methods have been explored for the synthesis of D-ribulose 5-phosphate, which is a key intermediate in pentose phosphate pathway (PPP) and widely exists in bacteria, plants, and animals.<sup>22</sup> The reported methods for the synthesis of D-ribulose 5-phosphate relies on the isomerization of D-ribose 5-phosphate,<sup>161</sup> the phosphorylation of D-ribulose,<sup>133</sup> and the oxidization of D-gluconate 6-phosphate.<sup>162</sup> Although scalable product could be produced by using these methods, these processes still suffer from expensive starting materials, low yields, or



a complicated purification step. As a consequence, commercially available D-ribulose 5-phosphate is also extremely expensive (\$1245/25mg, Sigma-Aldrich). Recently, we have developed an efficient and convenient platform for the facile synthesis of phosphorylated ketopentoses,<sup>83</sup> in which the synthesis of D-ribulose 5-phosphate was also included. In this strategy, D-ribulose was prepared from D-xylose by a one-pot two-enzyme system in first reaction stage,<sup>163</sup> and then D-ribulose was phosphorylated by using L-ribulose kinase at C-5 position. The product was purified by using silver nitrate precipitation.<sup>83</sup> Having got a considerable amount of D-ribulose 5-phosphate in hand in this work (multi-gram), we try to use a sequential one-pot three-enzyme (OP3E) system containing KdsD, KdsA, and KdsC to synthesize KDO (Figure 1.13).



**Figure 1.14** One-pot multienzyme system for the synthesis of Re-LPS.

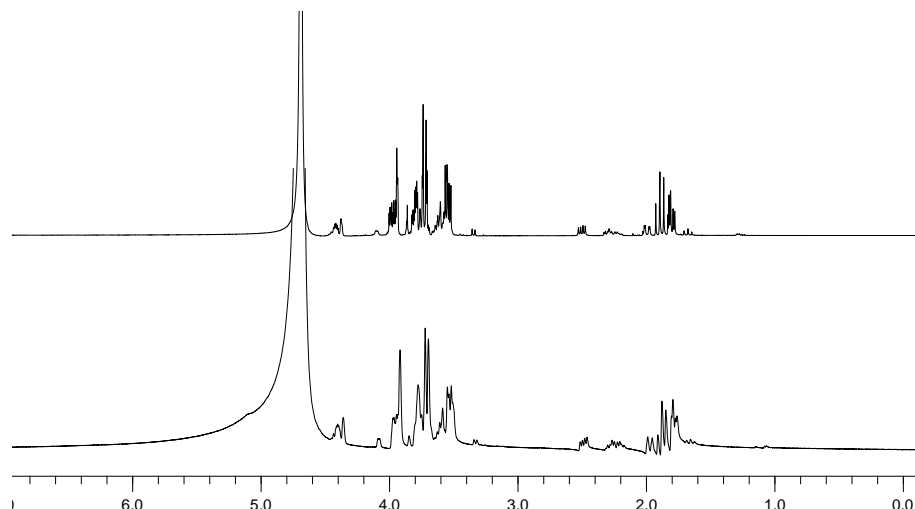
The requirement of several enzyme-catalyzed reactions being carried in one-pot is that the enzymes must explicitly recognize their individual substrate. Otherwise, the cross-reactions will result in unpredictable by-products and increase the purification difficulties. KDO 8-phosphate synthetase could specifically recognize D-arabinose 5-phosphate but not D-ribulose 5-phosphate,<sup>160</sup> making our design (Figure 1.13) reasonable. KdsC is highly active to hydrolyze the phosphate group of KDO 8-phosphate, while only trace activity towards D-arabinose 5-phosphate

and PEP was observed (thousands of times lower than KDO 8-phosphate),<sup>164</sup> indicates its potential applications in OPME reaction. However, its substrate specificity towards D-ribulose 5-phosphate is unknown. To test the substrate specificity of KdsC towards D-ribulose 5-phosphate, D-ribulose 5-phosphate was incubated with KdsC in excess amount for three hours, while D-ribulose 5-phosphate was incubated with alkaline phosphatase as a control. No observable D-ribulose was found on TLC, indicating D-ribulose 5-phosphate can't serve as the substrate of KdsC.

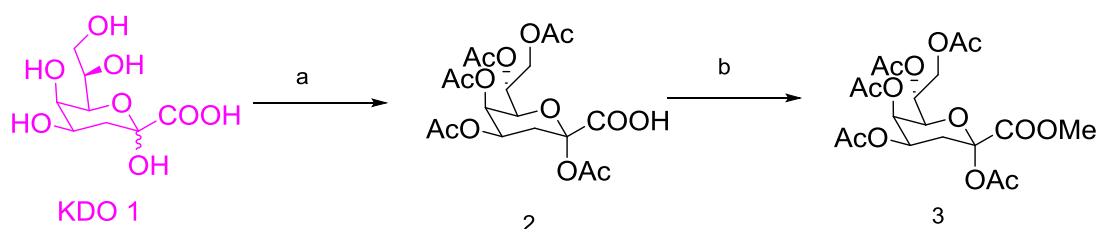
To test the practicability of the designed OPME reaction for the production of KDO, analytical scale reaction was carried in a 50 ul system containing D-ribulose 5-phosphate KdsD, KdsA, and KdsC. The reactions were monitored by TLC while employing authentic KDO as a control. After observing the formation of KDO on TLC, preparative scale synthesis was carried in 300 ml system (gram scale). To efficiently convert D-ribulose 5-phosphate, excess PEP (2.5 equiv) was used. For the convenience of the final purification, no buffer was used. The reaction pH was held near 7.5 using sodium hydroxide as the reaction was ongoing. Once reaction no longer moves forward, KDO was purified by using DEAE column ( $\text{HCO}_3^-$  form). After desalting by Bio-Gel P-2 column, the product was isolated in 72% yield concerning D-ribulose 5-phosphate. The product was confirmed by NMR and HRMS analysis.  $^1\text{H-NMR}$  of the obtained KDO is well accord with the authentic standard (Figure 1.15). A single peak (237.0561, M-H) on high resolution mass spectrum was observed as well.

To further confirm the structure of the obtained KDO, the product was converted to the known pentaacetate methyl ester of KDO **3** (Figure 1.15). **3** was characterized by NMR and HRMS (see experiment part). The proton and carbon NMR spectra of the **3** were good accordance with the previously reported data.<sup>141, 155</sup>

Having got a considerable amount of KDO in hand, we further try to install it on lipid A to



**Figure 1.15**  $^1\text{H-NMR}$  spectra of the obtained KDO (upper graph) compared to the authentic KDO (bottom graph)



**Figure 1.16** Synthesis of the pentaacetate methyl ester of KDO. (a)  $\text{Ac}_2\text{O}$ , DMAP, pyridine, rt; (b)  $\text{TMSCHN}_2$ ,  $\text{DCM}/\text{MeOH}$

synthesize Re-type LPS (lipid A linked with KDO residues)<sup>165</sup> by using KDO transferase. The synthesis of Re-type LPS is the key step to synthesize lipopolysaccharide (lipid A linked with polysaccharide) to develop vaccine candidate against Gram-negative bacteria infection. Lipid A can serve as an adjuvant to enhance the immunogenicity of polysaccharide portion.<sup>166, 167</sup> Although many efforts have been made to install polysaccharide on lipid A, only Re-type LPS has been synthesized successfully by using chemical strategy to the best of our knowledge.<sup>168</sup> Nevertheless, the process undergoing multi protection/deprotection steps can be complicated and suffer from

very low yield. The synthesis of more complex lipopolysaccharides is still challenged. Compared to chemical method, enzymatic synthesis of oligosaccharides has distinct advantages in regio- and stereo-selectivity. In this work, we tried to develop an enzymatic system to prepare Re-LPS for further synthesis of complex lipopolysaccharides (Figure 1.14).

KDO transferase from *E.coli* (WaaA) can transfer two KDO residues onto lipid A.<sup>169, 170</sup> Before its incorporation into LPS or CPS, KDO is activated to CMP-KDO, which serves as the substrate for KDO transferase, by CMP-KDO synthase.<sup>171-173</sup> However, CMP-KDO is very unstable under physiological conditions. It has been reported that the half-life-time of CMP-KDO is only 34 min at 25°C.<sup>174</sup> Therefore, a one-pot reaction system was used to transfer KDO on lipid A (Scheme 3),<sup>170</sup> in which KDO, CTP, CMP-KDO synthase from *E.coli* (KdsB), inorganic pyrophosphatase (PPA) from *Pasteurella multocida* and WaaA were included. KdsB catalyzes the formation of CMP-KDO from KDO and CTP. PPA was added to improve the whole reaction by hydrolyzing the by-product of PPi. The produced CMP-KDO could serve as the substrate of WaaA. Although we successfully observed the formation of the product on the high resolution mass spectrum, a large-scale synthesis to obtain enough products for NMR analysis and vaccine evaluation was not achieved. The difficulties lie in the extremely poor solubility of lipid A in water. Kinds of detergents have been tested to improve the reaction, but a practical scale synthesis is still unsuccessful. Tylor and co-workers recently found that heptose transferases can recognize lipid A without intact lipid tails,<sup>175, 176</sup> which solubility is better than the normal lipid A. Inspired by this result, a circuitous strategy is re-designed to try to synthesize Re-LPS in large scale in our lab.

## 1.6 Conclusions

In summary, we have successfully established of a novel platform for the facile synthesis of ketoses, which relied on substrate-specific kinases and the improved aldol condensation reaction,

makes it possible to use one-pot multienzyme (OPME)<sup>130</sup> reactions to prepare 14 non-readily available ketopentoses and ketohexoses from common and inexpensive materials. The described two-step strategy not only provides unprecedentedly high yields but also avoids the need to undergo a complicated isomer separation step. ATP is commercially cheap due to its increased industrial production over the past decade and ATP-regeneration system has also been suggested,<sup>177</sup> making the transformation reaction described herein of particular interest for large-scale production. This study represents a highly convenient and efficient strategy for ketose syntheses. We anticipate that this platform will accelerate an understanding of both biological roles and synthetic applications of rare ketoses, and advance the further synthesis of rare aldoses because the aldose-ketose isomerization reaction is very favorable for aldose formation. Future studies will enable the identification of new kinases to be used in more sugar syntheses, providing a powerful set of tools for carbohydrate research.

In addition, a novel method for the efficient and convenient synthesis of phosphorylated ketoses was established. This method relies on substrate-specific kinases and a convenient sugar phosphates purification method (silver nitrate precipitation). Starting from two common and inexpensive aldoses (D-xylose and L-arabinose), all phosphorylated ketopentoses were produced utilizing this strategy with high yield and purity without a tedious sugar phosphate separation step. ATP is also commercially affordable due to the increased industrial production over the past decades,<sup>177, 178</sup> making the transformation reaction described herein of particular interest for large-scale preparation. The precipitate (silver-adenosine phosphates complex) produced during purification process can be redissolved with ammonium hydroxide, and the adenosine phosphates (ATP and ADP) or silver ions can be then recycled, reducing the preparation cost when this method was applied on large scale purification. Moreover, based on the availability of D-xylulose 5-

phosphate and D-ribulose 5-phosphate in considerable quantities, we have further established a biosynthetic strategy for the efficient synthesis of KDO, which are the building block of the lipopolysaccharide and have been difficult to obtain. This strategy will accelerate an understanding of both biological roles and synthetic applications of phosphorylated ketopentoses. Future studies will enable the identification of new substrate-specific kinases to be used in more phosphorylated sugar syntheses, providing a powerful set of tools for carbohydrate research.

In addition, a practical system for the facile synthesis of KDO in large scale is described. We demonstrate herein that KDO could be efficiently and conveniently prepared from D-ribulose 5-phosphate by using a one-pot multienzyme (OPME) system. The advantages of this strategy are that all the materials used in this strategy are easy-to-get and all enzymes involved in the synthetic process are highly active. Moreover, an attempt for the installation of KDO to lipid A is also made. Although two KDO residues can be easily transferred to lipid A by a single KDO transferase, a practical reaction system that could produce enough products is still necessary. We anticipate this work will accelerate an understanding of both biological roles and synthetic applications of KDO.

## 2 CHEMOENZYMATIC REPORTER STRATEGY FOR PROBING COMPLEX GLYCANS

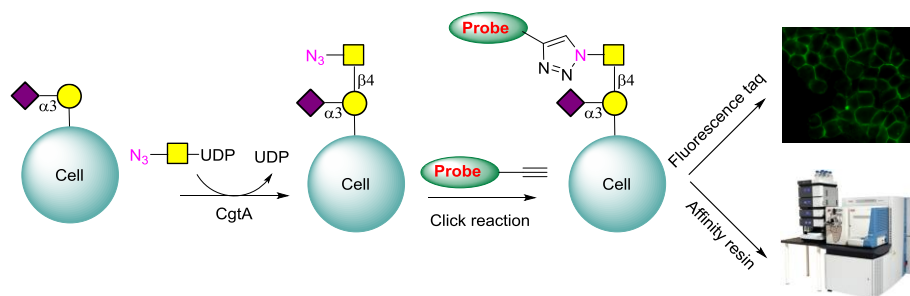
### 2.1 Introduction

*N*-acetylneuraminic acid (Neu5Ac) is the most widespread form of sialic acid and almost the only form found in humans.<sup>179</sup> *N*-glycolylneuraminic acid (Neu5Gc) and ketodeoxynonulosonic acid (Kdn) are common in other vertebrates but rarely present in humans.<sup>179</sup> Neu5Ac is present essentially in all human tissues and always attaches to the galactose residue at the nonreducing terminal end of glycans through  $\alpha$ 2-3 or  $\alpha$ 2-6 linkage.<sup>180</sup> It is well established that Neu5Ac $\alpha$ (2-3)Gal and Neu5Ac $\alpha$ (2-6)Gal glycans play crucial but distinctive roles in diverse biological and pathological processes including immune responses, cell–cell and cell–pathogen interactions.<sup>181-184</sup> However, studies are hindered due to the lack of an effective method to analyze such glycans or glycoproteins.

The Neu5Ac $\alpha$ (2-3)Gal epitope localized on cell surface is well known to be the receptor of many infectious microbes such as the influenza virus.<sup>180</sup> Abnormal Neu5Ac $\alpha$ (2-3)Gal expression has frequently been observed in many carcinomas.<sup>182</sup> Traditionally, lectin binding using *Maackia amurensis* leucoagglutinin (MAL I) and hemagglutinin (MAH or MAL II) is the main method for Neu5Ac $\alpha$ (2-3)Gal detection. However, MAL I only bind terminal Neu5Ac $\alpha$ (2-3)Gal $\beta$ (1-4)GlcNAc trisaccharide in *N*-glycans.<sup>185, 186</sup> MAH binds preferentially trisaccharide Neu5Ac $\alpha$ (2-3)Gal $\beta$ (1-3)GalNAc in *O*-glycans.<sup>187</sup> They also bind some nonsialylated structures such as SO<sub>4</sub><sup>3-</sup>-Gal $\beta$ (1-3)GalNAc.<sup>188</sup> Moreover, it was reported that *Maackia amurensis* lectins require a high minimum agglutinating concentration (up to 125 ug to 500 ug),<sup>189</sup> and therefore a long incubation time (typically overnight) is necessary for glycoprotein detection.<sup>190, 191</sup> Thus, the development of

an simple, rapid and sensitive method for detecting Neu5Ac $\alpha$ (2-3)Gal glycans remains an unmet need.

Lectin binding has been the primary method to analyze sialylated glycans,<sup>192-194</sup> but lectins often suffer from weak binding affinity, limited specificity, and cross-reactivity. In recent years, the development of bioorthogonal chemistry provides a powerful tool for probing glycans, proteins and lipids.<sup>195-198</sup> Bioorthogonal functional groups (azide and alkyne) carried by N-acetylmannosamine or Neu5Ac analogues were metabolically incorporated into glycans, allowing the covalent conjugation by click chemistry reaction with either fluorescent tags for visualization, or affinity probes for enrichment of sialylated glycans and glycoproteins.<sup>199-204</sup> Chemical approach to tag sialylated glycans has also been suggested.<sup>205</sup> Nevertheless, these methods suffer from low detection sensitivity and efficiency, toxicity of labeling reagents, and the inability to detect complicated glycan structures.



**Figure 2.1** Chemoenzymatic reporter strategy for probing Neu5Ac $\alpha$ (2,3)Gal glycans

As a complementary strategy to remodel glycans with non-natural functionalities, chemoenzymatic labeling glycans relies on the substrate-specific glycosyltransferases, which transfer non-natural sugars that contain bioorthogonal functional groups onto target glycan *in vitro*.<sup>206-210</sup> Chemoenzymatic method does not rely on cell's biosynthetic machinery and therefore can be employed in any desired biological contexts where feeding cells with non-natural sugar analogs is not possible, such as human tissue extracts.<sup>207</sup> As glycosyltransferase-mediated reaction



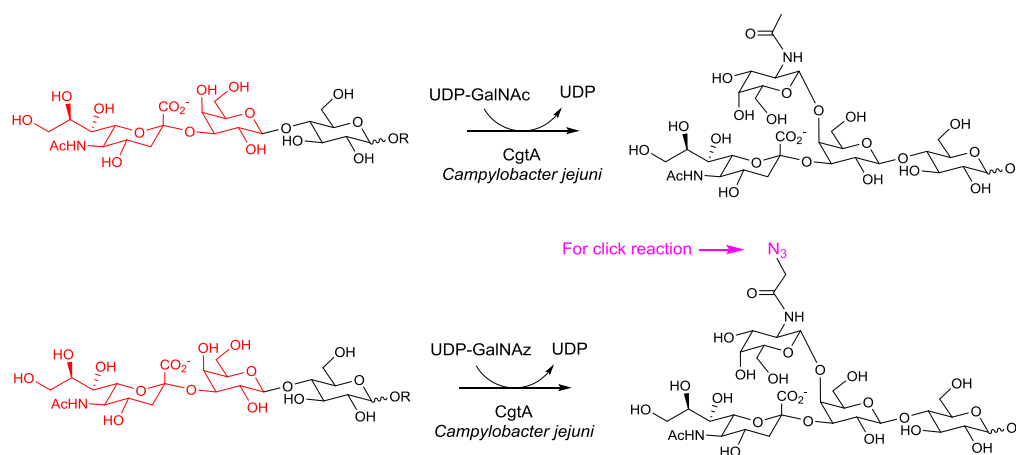
and click chemistry reaction proceed high specificity and efficiency, chemoenzymatic labeling provides a higher sensitivity and selectivity compared to other analytical methods such as antibodies, lectins, and metabolic labeling. In this communication, we report a two-step chemoenzymatic method that takes advantage of the substrate promiscuity of a  $\beta$ -(1,4)-N-acetylgalactosaminyltransferase from *Campylobacter jejuni* (CgtA) and click chemistry reaction to rapidly and sensitively detect Neu5Ac $\alpha$ (2-3)Gal glycans (Figure 2.1).

## 2.2 Donor specificity study of CgtA

GalNAz was prepared from galactosamine hydrochloride as described previously with minor changes.<sup>211</sup> In detail, galactosamine hydrochloride (1290 mg, 6 mmol) was dissolved in MeOH (50 mL) in which an equivalent amount of sodium methoxide was added. The reaction mixture was carried at 25°C for 1 hour and then 1 ml of TEA (6.7 mmol) and 3.06 g of chloroacetic anhydride were added. The reaction was stirred overnight to allow the formation of intermediate chloro-intermediate. Chloro-intermediate was partially purified by flash chromatography with MeOH: CH<sub>2</sub>Cl<sub>2</sub> gradually from 10:1 to 6:1. The resulting chloro-intermediate was dissolved in DMF (50 ml), in which 3.9 g of sodium azide (60 mmol) and 200  $\mu$ l 15-crown-5 were added. The reaction mixture was stirred at 60°C overnight to allow the formation of GalNAz. The insoluble substance was removed by filtration. The flow through containing GalNAz was concentrated under reduced pressure and purified by flash chromatography with MeOH: CH<sub>2</sub>Cl<sub>2</sub> gradually from 10:1 to 6:1. The obtained GalNAz was further purified by using Bio-Gel P-2 column to provide 938 mg of GalNAz (60% yield).

UDP-GalNAz was prepared by the reaction (100 ml) containing 50 mM Tris-HCl buffer (pH 7.5), 20 mM of GalNAz (2 mmol), 5 mM of Mg<sup>2+</sup>, 10 mg of NaH<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 10 mg of AGX1 and 1 mg of PPA.<sup>212</sup> PPA was added to improve the total conversion ratio by hydrolyzing the newly

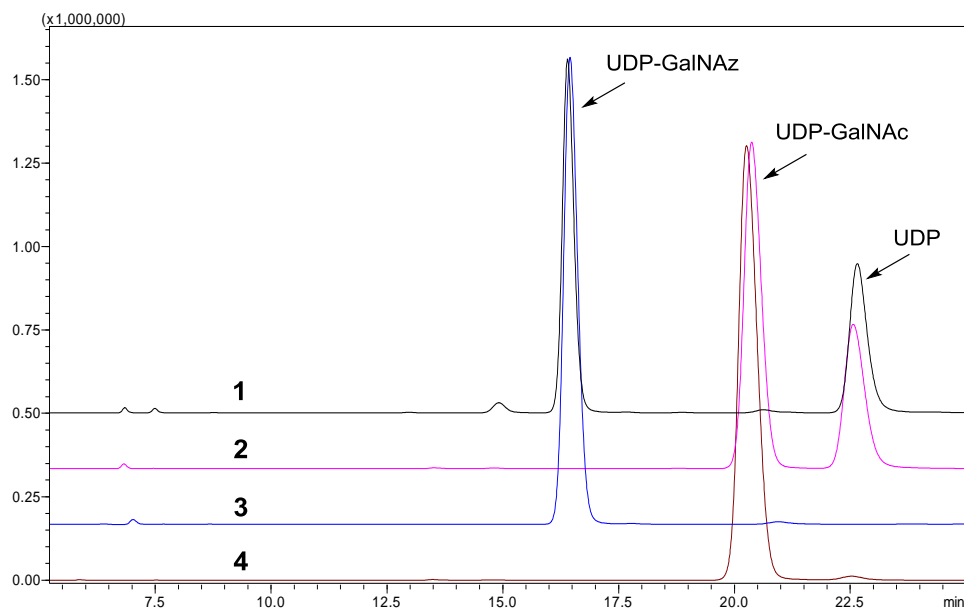
formed inorganic pyrophosphate. The reaction was carefully shaken at 37°C overnight to allow the formation of UDP-GalNAz. The reaction was monitored by TLC. Once the reaction finished, equal volume of cooled ethanol was added to precipitate proteins. The precipitate was removed by centrifugation and the supernatant containing UDP-GalNAz was concentrated under reduced pressure. UDP-GalNAz was purified by DEAE (HCO<sub>3</sub><sup>-</sup>) column. The column was eluted with a 2 L linear gradient of NH<sub>4</sub>HCO<sub>3</sub> (from 0 to 0.5 M). The fractions containing UDP-GalNAz was collected and desalted by using Bio-Gel P-2 column to afford final product in 62% yield (820 mg, ammonium form).



**Figure 2.2** CgtA recognize Neu5Aca(2,3)Gal structure with UDP-GalNAc or UDP-GalNAz

Neu5Aca(2-3)Gal disaccharide is the outer core component of many *Campylobacter jejuni* strains.<sup>213</sup> CgtA is responsible for the extension of Neu5Aca(2-3)Gal with GalNAc residue (Figure 2.2).<sup>213</sup> We reasoned that CgtA might tolerate substitution at the C-2 position of GalNAc, allowing for the introduction of an azide group for further click chemistry reaction (Figure 2.2). Substrate specificity study using Ganglio-oligosaccharide GM3 (entry 1, Table 2.1) shows that both UDP-GalNAz and UDP-GalNAc are efficient substrate of CgtA. Kinetic analysis revealed a  $k_{cat}/K_m$  value of 1.34 nM<sup>-1</sup>min<sup>-1</sup> for UDP-GalNAc, and 1.51 nM<sup>-1</sup>min<sup>-1</sup> for UDP-GalNAz. Indeed,

treatment of GM3 with CgtA and UDP-GalNAz overnight led to a complete conversion of GM3. The product was confirmed by MALDI-TOF-MS and NMR.



**Figure 2.3** HPLC profile of the CgtA-catalyzed reactions. 1, Neu5Ac $\alpha$ (2-3)Gal $\beta$ (1-4)Glc- $\beta$ -Me was incubated with UDP-GalNAz. 2, Neu5Ac $\alpha$ (2-3)Gal $\beta$ (1-4)Glc- $\beta$ -Me was incubated with UDP-GalNAc. 3, Neu5Ac $\alpha$ (2-6)Gal $\beta$ (1-4)Glc- $\beta$ -Me was incubated with UDP-GalNAz. 4, Neu5Ac $\alpha$ (2-6)Gal $\beta$ (1-4)Glc- $\beta$ -Me was incubated with UDP-GalNAc.

### 2.3 Acceptor specificity study of CgtA

Having demonstrated that CgtA accepts UDP-GalNAz as substrate, we next tested its substrate specificity towards sialylated oligosaccharides with UDP-GalNAz. Many sialylated oligosaccharides containing  $\alpha$ 2-3-,  $\alpha$ 2-6-, or  $\alpha$ 2-8-linked sialic acid (Table 2.1 and Table S1) were synthesized as previously reported.<sup>99, 214-216</sup> We found that CgtA require only linear disaccharide structure of Neu5Ac $\alpha$ (2-3)Gal (Table 2.1, entries 1 to 5) or Neu5Gc $\alpha$ (2-3)Gal (Table 2.1, entry 2) when using UDP-GalNAz as donor. Meanwhile, only very low relative activity towards the structure containing  $\alpha$ 2-6-linked sialic acid or without sialic acid was detected (Table 1, Entries 6 to 10; Table 2.1, entries 3 to 5). Indeed, no observable product or by-product (UDP) could be found




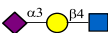





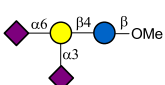
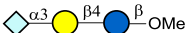



on TLC after the incubation of these compounds with CgtA and UDP-GalNAz overnight. These findings indicated the potential of CgtA for application in selective labeling Neu5Ac $\alpha$ (2-3)Gal glycans.

#### **2.4 Chemoenzymatic labeling Neu5Ac $\alpha$ (2-3)Gal glycans in fetuin**

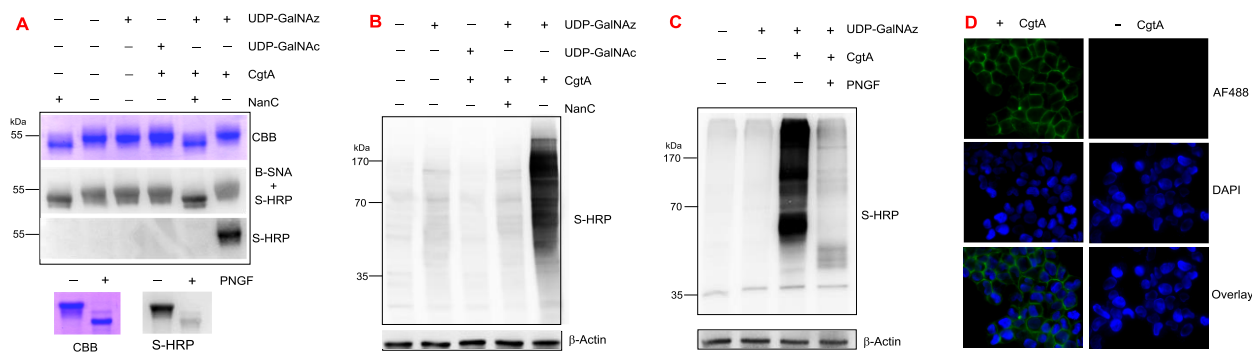
To test the practicality of the described strategy on protein labeling, we used fetal bovine fetuin as an example. Fetal bovine fetuin is a well-studied model protein for sialylated glycans analysis and commercially available. It contains three *N*-glycosylation and three *o*-glycosylation sites, on which Neu5Ac attached to the terminal galactose residues through  $\alpha$ 2-3- or  $\alpha$ 2-6-linkage.<sup>217</sup> To perform a control, fetal bovine fetuin was treated with a sialidase (NanC), which specifically hydrolyzes  $\alpha$ 2-3-linked Neu5Ac.<sup>218</sup> After the treatment of NanC, a slight migration change compare to native fetuin was observed on SDS-PAGE gel (Figure 2.4). The  $\alpha$ 2-6-linked Neu5Ac was confirmed by biotinylated SNA (Figure 2.4). Native fetuin or NanC-treated fetuin was labeled by CgtA with UDP-GalNAz at 37°C for 1 hour, while other control groups were performed in parallel. Following copper-free click reaction (DIBO-alkyne, 10  $\mu$ M), the proteins were analyzed by western blot using streptavidin-linked horseradish peroxidase (S-HRP). Strong fluorescence in native fetuin was observed, while all the control groups failed to be labeled (Figure 2.4), demonstrating that the designed scheme could be used to specifically label Neu5Ac $\alpha$ (2-3)Gal glycans on glycoprotein. The labeled fetuin was further treated with peptide *N*-glycosidase F (PNGF), which remove *N*-Glycans from glycoprotein, and detected by S-HRP. Although there is a significant fluorescence reduction, fluorescence labeling in the PNGF-treated sample still can be observed (Figure 2.4, bottom graph), indicating that both *N*-glycans and *o*-glycans were labeled. Meanwhile, the probe of same amount of fetuin with biotinylated MAL II is unsuccessful. Thus, our chemoenzymatic approach provides a more credible detection strategy for Neu5Ac $\alpha$ (2-3)Gal

glycans and enable the highly sensitive detection of glycoproteins.

**Table 2.1** Substrate specificity of CgtA with UDP-GalNAz

Entry	Substrate	RA (%) <sup>a</sup>
1		100
2		104
3		101
4		113
5		97
6		<1
7		<1
8		<1
9		<1
10		<1
11		110
12		<1
13		<1
14		<1

<sup>a</sup>RA: relative activity. See experiment part for details.

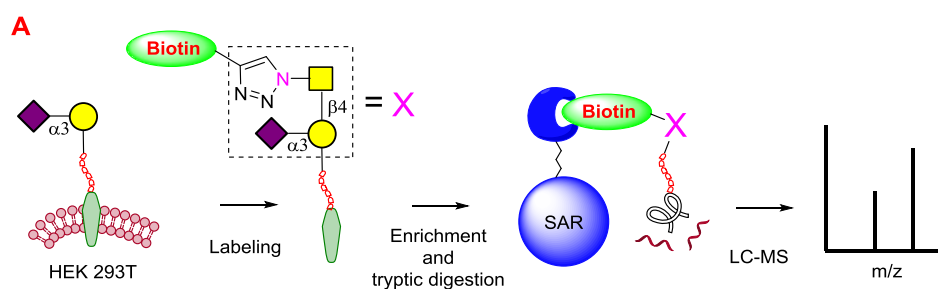


**Figure 2.4** Chemoenzymatic detection Neu5Ac $\alpha$ (2-3)Gal glycoproteins. CBB: Coomassie brilliant blue staining. S-HRP: streptavidin-linked horseradish peroxidase. B-SNA: biotinylated SNA. NanC: The sample was treated with NanC before performing labeling reaction. PNGF: the labeled fetuin (rightmost) was further treated with PNGF (bottom graph). (B) Chemoenzymatic detection Neu5Ac $\alpha$ (2-3)Gal glycoproteins from cell lysates of HEK293T cells. (C) Chemoenzymatic detection Neu5Ac $\alpha$ (2-3)Gal glycoproteins on cell surface of HEK 293T. (D) The imaging of Neu5Ac $\alpha$ (2-3)Gal glycoproteins on live HeLa cells (Green) using fluorescence microscopy. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue).

## 2.5 Chemoenzymatic labeling Neu5Ac $\alpha$ (2-3)Gal glycoproteins in complex glycoproteins

We next determined whether the approach could be used to track Neu5Ac $\alpha$ (2-3)Gal glycoproteins in complex samples. Cell lysates from human embryonic kidney 293 (HEK293T) cells was incubated with CgtA and UDP-GalNAz at 37°C for 1 hour, while control groups were performed parallel. Following the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction with diazo-biotin-alkyne, biotinylated cell lysates was detected by S-HRP (Figure 2.4). Strong fluorescence labeling of cell lysates was observed, while only background nonspecific labeling in NanC-treated group and other control groups was observed (Figure 2.4), highlighting further the specificity of the designed strategy towards Neu5Ac $\alpha$ (2-3)Gal glycoproteins. Cell surface Neu5Ac $\alpha$ (2-3)Gal glycoproteins were selectively labeled by incubating suspension HEK293T living cells with CgtA

and UDP-GalNAz at 37°C for 30 min. Following the biotinylation by CuAAC reaction, strong fluorescence labeling was observed compared to control groups in western blot detection (Figure 2.4). Then, several other randomly selected human cancer cell lines including A549, HeLa, and HepG2 were chemoenzymatically labeled using the same strategy (see experiment part). The labeled samples were further treated with PNGF, resulting in significant fluorescence reduction, which indicates that Neu5Ac $\alpha$ (2-3)Gal mainly attach to N-glycans in these cell lines.



**Figure 2.5** Global identification of cell surface Neu5Ac $\alpha$ (2-3)Gal glycoproteins .

We next investigated the potential application of the described strategy for Neu5Ac $\alpha$ (2-3)Gal glycans imaging and quantification. The determination of the expression level of Neu5Ac $\alpha$ (2-3)Gal glycans is very important to understand sialic-acid-related microbe infection and carcinogenesis.<sup>219, 220</sup> Adherent HeLa cells were labeled by CgtA and UDP-GalNAz at 37°C for 30 min. After copper-free click reaction (DIBO-biotin, 30  $\mu$ M), a fluorescent reporter was subsequently installed by incubation with streptavidin-linked Alexa Fluor 488 (10  $\mu$ g/ml). Membrane-associated fluorescence was observed for cells treated with both CgtA and UDP-GalNAz, whereas no fluorescence labeling was detected for control cells labeled in the absence of CgtA, confirming the specificity of the in situ chemoenzymatic reaction (Figure 2.4). The fluorescence intensity, which reflects the expression level of Neu5Ac $\alpha$ (2-3)Gal glycans proportionally, was determined by flow cytometry.

## 2.6 Conclusions

In conclusion, on the basis of a glycosyltransferase that could specifically recognize Neu5Ac $\alpha$ (2-3)Gal with UDP-GalNAz and site-specific click chemistry reaction, we have developed the first strategy for the rapid and sensitive detecting Neu5Ac $\alpha$ (2-3)Gal glycans. This method is far superior to the traditional lectin-based methods to detect Neu5Ac $\alpha$ (2-3)Gal, which are limited by their inherent disadvantages. This method also allows that the global analysis of Neu5Ac $\alpha$ (2-3)Gal glycoproteins is achievable, providing a powerful tools for sialic-acid-related research. Moreover, substrate specificity study indicated that the described strategy can be also used to probe Neu5Gc $\alpha$ (2-3)Gal glycans, which are currently detected by polyclonal monospecific antibody.<sup>180</sup> Future studies will enable the exploration of new glycosyltransferase for use in more glycan detecting.



### 3 EXPERIMENTAL PROCEDURES

#### 3.1 Experiment procedures for facile enzymatic synthesis of rare sugars

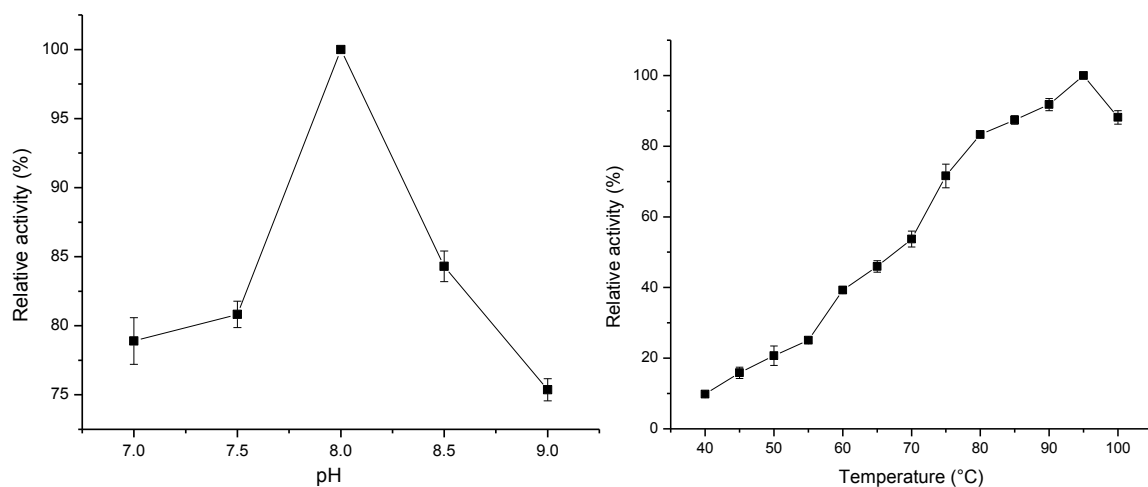
##### 3.1.1 General methods

$^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$  and  $^{31}\text{P-NMR}$  spectra were recorded on a Bruker 400-MHz NMR spectrometer ( $\text{D}_2\text{O}$  as the solvent). High resolution electrospray ionization (ESI) mass spectra were obtained using Thermo HPLC-Orbitrap Elite. Thin-layer chromatography (TLC) was performed on silica gel 60 F254 plates (Merck, MA) using *p*-anisaldehyde sugar stain. High performance liquid chromatography (HPLC) was performed on a Shimadzu SPD-20A equipped with ultraviolet (UV) detector and evaporative light scattering detector (ELSD). The HPLC columns used in this work are ZIC®-CHILIC (Merck, Darmstadt, Germany), HPX-87H (Bio-Rad, Hercules, CA), Sugar-Pak 1 column (Waters Corp., Milford, MA), YMC Polyamine II column (YMC Corp., Kyoto, Japan), and XBridge Amide column (Waters Corp., Milford, MA). Gel filtration chromatography was performed using a column (100 cm  $\times$  2.5 cm) packed with Bio-Gel P-2 fine resins (45–90 $\mu\text{m}$ ) (Bio-Rad, Hercules, CA).

Platinum® *Pfx* DNA Polymerase, Subcloning Efficiency™ DH5 $\alpha$ ™ competent cells and *E. coli* BL21 (DE3) chemically competent cells were from Invitrogen (Grand Island, NY). Plasmid pET-28a was from Novagen (Madison, WI). Restriction enzymes and T4 DNA ligase were from NEB (Beverly, MA). Sugar standards were bought from Carbosynth (San Diego, CA). *Escherichia coli* genomic DNA used for PCR in this work was extracted from *Escherichia coli* BL21 (DE3) cells. *Thermotoga maritima* MSB8 genomic DNA were purchased from American Type Culture Collection (Rockville, MD). Gene synthesis service was provided by GenScript (Piscataway, NJ). All other chemicals or enzymes unless otherwise stated were purchased from Sigma without further purification.

### 3.1.2 Temperature effect on RhaB.

The effect of temperature was determined by the reactions that were performed in a 50  $\mu$ L reaction mixture containing a Tris-HCl buffer (100 mM, pH 7.5), L-rhamnulose (20 mM), ATP (20 mM),  $Mg^{2+}$  (5 mM) and RhaB (15 ng). Reactions were allowed to proceed for 10 minutes at different temperatures (from 45°C to 100°C), and were stopped by diluting ten times using a buffer of acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60% acetonitrile). The reactions were quantified by analyzing the formation of ADP by HPLC equipped with UV detector at 254 nm using ZIC®-cHILIC column. The column was eluted at 30°C with acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60% acetonitrile) at a flow rate of 0.6 ml/min (Figure 3.1).



**Figure 3.1** Temperature effect and pH profile of RhaB.

### 3.1.3 pH profiles study of RhaB

The effect of pH was determined by the reactions that were performed in 50  $\mu$ L reaction mixture containing a Tris-HCl buffer(100 mM) with a pH in the range of 7.0- 9.0, L-rhamnulose (20 mM), ATP (20 mM),  $Mg^{2+}$  (5 mM) and RhaB (15 ng). Reactions were allowed to proceed for 10 minutes at 45°C and were stopped by diluting ten times using a buffer of acetonitrile/100

mM aqueous ammonium acetate pH 4.5 (60% acetonitrile). Samples were analyzed by HPLC using ZIC®-cHILIC column as what described above (Figure 3.1).

### ***3.1.4 Substrate specificity study of RhaB***

Substrate specificity of RhaB was studied by the reactions that were performed in 50 ul reaction mixture containing a Tris-HCl buffer (100 mM, pH 7.5), 20 mM of sugar standards (Table 1.3), 5 mM of Mg<sup>2+</sup>, and 15 ng of RhaB. The reactions were carried at 45°C for 10 minutes and were stopped by diluting ten times using a buffer of acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60% acetonitrile). Samples were analyzed by HPLC using ZIC®-cHILIC column as what described above.

### ***3.1.5 Substrate specificity of HK***

Substrate specificity of HK was studied by the reactions that were performed in 50 ul reaction mixture containing a Tris-HCl buffer (100 mM, pH 7.5), 20 mM of sugar standards (Table 1.3), 5 mM of Mg<sup>2+</sup>, and 15 ug of HK. The reactions were carried at 37°C for 10 minutes and were stopped by diluting ten times using a buffer of acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60% acetonitrile). Samples were analyzed by HPLC using ZIC®-cHILIC column as what described above.

### ***3.1.6 Preparative scale synthesis of L-ribulose, D-xylulose, and D-tagatose***

Reactions were carried in a final volume of 150 ml reaction system containing 25 mM of ATP, 3 mM of Mg<sup>2+</sup>, 3 mM of Mn<sup>2+</sup>, 20 mM starting sugars and conversion-related enzymes (Table 1.3). L-arabinose (3 mmol, entry 1), D-xylose (3 mmol, entry 2) and D-galactose (3 mmol, entry 3) were incubated with 10 to 30 mg of their corresponding isomerases and 10 to 20 mg of HK, respectively. The reactions were carefully shaken at pH near 7.5 for 12 to 36 hours at 37°C to allow the formation of ketose 1-phosphates. The reactions were monitored by TLC

(EtOAc/MeOH/H<sub>2</sub>O/HOAc=5:2:1.4:0.4) and HPLC equipped ELSD using a HPX-87H column with pure water as the mobile phase. All the reactions were allowed to proceed until no detectable starting sugars were found. Silver nitrate was added to precipitate ATP and ADP until no more precipitate formed (~2.2 g silver nitrate was used for each reaction). The precipitate was removed by centrifugation (14000 g, 1 min) and washed twice using distilled water. Sodium chloride was added to a final concentration of 200 mM to remove the remnant silver ions. Silver chloride was removed by centrifugation (14000 g, 1 min). The pH was then adjusted to 5.5 and 5 mg of AphA was added. The mixture was shaken at 37°C to produce ketoses. Until no ketose 1-phosphates were found by TLC, the solution from each reaction was concentrated under reduced pressure and purified by using Bio-Gel P-2 column to afford final products in more than 90% yield (Table 1). HPLC retention times (Sugar-Pak 1 column) and NMR spectra are consistent with authentic samples, indicating the isolated products are the desired ketoses.[7] The possible existence of starting aldoses was analyzed by NMR and HPLC equipped ELSD using Sugar-Pak 1 column with pure water as the mobile phase.

**L-ribulose.** 417 mg; yield, 93%; white syrup. HRMS (ESI) m/z calcd for C<sub>5</sub>H<sub>10</sub>O<sub>5</sub> Na [M+Na]<sup>+</sup> 173.0426, found 173.0413.

**D-xylulose.** 415 mg; yield, 92%; white syrup. HRMS (ESI) m/z calcd for C<sub>5</sub>H<sub>10</sub>O<sub>5</sub> Na [M+Na]<sup>+</sup> 173.0426, found 173.0413.

**D-tagatose.** 495 mg; yield, 92%; white foam. HRMS (ESI) m/z calcd for C<sub>6</sub>H<sub>12</sub>O<sub>6</sub> Na [M+Na]<sup>+</sup> 203.0532, found 203.0640.

### ***3.1.7 Preparative scale synthesis of L-xylulose, D-ribulose, D-sorbose, D-psicose, and L-tagatose.***

Reactions were carried in a final volume of 150 ml reaction system containing 25 mM of ATP, 3 mM of Mg<sup>2+</sup>, 3 mM of Mn<sup>2+</sup>, 20 mM starting sugars and conversion-related enzymes (Table 1.3). L-arabinose (3 mmol, entry 4), D-xylose (3 mmol, entry 5) and D-galactose (3 mmol, entry 6) were incubated with 10 to 30 mg of their corresponding isomerases, 10 mg of DTE and 5 to 10 mg of RhaB, respectively. D-fructose (3 mmol, entry 7) and L-sorbose (3 mmol, entry 8) were incubated with 3 mg or 15 mg of DTE and 5 mg of RhaB, respectively. The reactions were carefully shaken at pH near 7.5 for 12 to 24 hours at 45°C to allow the formation of ketose 1-phosphates. The reactions were monitored by TLC and HPLC equipped ELSD using HPX-87H column. All the reactions were allowed to proceed until no detectable starting sugars were found. Followed the same manipulation described above, the products were isolated in 91 to 95% yield. HPLC retention times (Sugar-Pak 1 column) and NMR spectra of the isolated products are in good agreement with the authentic samples.[7] The possible existence of starting aldoses was analyzed by NMR and HPLC equipped ELSD using YMC Polyamine II column (L-arabinose detection) with acetonitrile/water (75:25) as the mobile phase, XBridge Amide column (D-galactose detection) with acetonitrile/water (75:25) as mobile phase or Sugar-Pak 1 column (D-xylose detection). The possible existence of (3S)-ketoses was analyzed by HPLC equipped ELSD using Sugar-Pak 1 column.

**L-xylulose.** 408 mg; yield, 91%; white syrup. HRMS (ESI) m/z calcd for C<sub>5</sub>H<sub>10</sub>O<sub>5</sub> Na [M+Na]<sup>+</sup> 173.0426, found 173.0421.

**D-ribulose.** 410 mg; yield, 91%; white syrup. HRMS (ESI) m/z calcd for C<sub>5</sub>H<sub>10</sub>O<sub>5</sub> Na [M+Na]<sup>+</sup> 173.0426, found 173.0422.

**D-sorbose.** 508 mg; yield, 94%; white foam. HRMS (ESI) m/z calcd for C<sub>6</sub>H<sub>12</sub>O<sub>6</sub> Na [M+Na]<sup>+</sup> 203.0532, found 203.0525.

**D-psicose.** 502 mg; yield, 93%; white foam. HRMS (ESI) m/z calcd for C<sub>6</sub>H<sub>12</sub>O<sub>6</sub> Na [M+Na]<sup>+</sup> 203.0532, found 203.0519.

**L-tagatose.** 513 mg; yield, 95%; white foam. HRMS (ESI) m/z calcd for C<sub>6</sub>H<sub>12</sub>O<sub>6</sub> Na [M+Na]<sup>+</sup> 203.0532, found 203.0519.

### ***3.1.8 Preparative scale synthesis of L-fructose and L-psicose***

1 M of glycerol (200 ml) was incubated with galactose oxidase and catalase as previously reported. Then, 1944 mg of DL-glycerol 3-phosphate corresponding to 5.0 mmol of L-glycerol 3-phosphate, 20 mg of RhaD, glycerol 3-phosphate oxidase (600 units) and catalase (3000 units) were added. The reaction mixture was carefully shaken at room temperature while vented to the atmosphere for 48 hours to allow the formation of L-fructose 1-phosphate. 1 volume of cooled ethanol was added to remove proteins. The solution was concentrated under reduced pressure and purified by Bio-Gel P-2 column to remove most of the impurities. The fractions contain L-fructose 1-phosphate were collected and concentrated under reduced pressure. The pH was adjusted to 5.5, and 10 mg of AphA was added to produce L-fructose. Product was purified as previous reported and finally obtained in 70% yield with regard to DL-glycerol 3-phosphate. L-psicose was prepared by a reaction mixture (150 ml, pH 7.5) containing L-fructose (20 mM, 3.0 mmol), 25 mM of ATP, 3 mM of Mg<sup>2+</sup>, 3 mM of Mn<sup>2+</sup>, 6 mg of DTE and 10 mg of HK. The reaction was shaken at 37°C and monitored by TLC and HPLC. Until no L-fructose was detected, silver nitrate precipitation method was used to remove ATP and ADP. The supernatant was concentrated and purified by Bio-Gel P-2 column to afford L-psicose 1-phosphate. Fractions containing L-psicose 1-phosphate were collected and concentrated in vacuo. The solution was adjusted to pH 5.5 and 3 mg of AphA

was added to produce L-psicose. Product was finally obtained in 90% yield with regard to L-fructose. HPLC retention times (Sugar-Pak 1 column) and NMR spectra are consistent with authentic samples.[7] The product distributions were analyzed by HPLC equipped ELSD using Sugar-Pak 1 column.

**L-fructose.** 628 mg; yield, 70%; white foam. HRMS (ESI)  $m/z$  calcd for  $C_6H_{12}O_6 Na$   $[M+Na]^+$  203.0532, found 203.0519.

**L-psicose.** 487 mg; yield, 90%; white foam. HRMS (ESI)  $m/z$  calcd for  $C_6H_{12}O_6 Na$   $[M+Na]^+$  203.0532, found 203.0519.

### 3.1.9 *FucI* and *RhaI* preparation

L-fucose isomerase (*FucI*),<sup>73</sup> L-rhamnose isomerase (*RhaA*)<sup>72</sup> were cloned into pET-28a vector and the recombinant plasmids were confirmed by restriction mapping and sequencing. The confirmed constructs were subsequently transformed into *E.coli* BL21 (DE3) for protein expression. After being induced by IPTG at 16 °C overnight, bacteria cells were harvested by centrifugation and re-suspended in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imadozle; pH 8.0). Cells were disrupted by a microfluidizer and the lysate was removed by centrifugation (12,000 g, 25 min). The supernatant was loaded onto a Ni-NTA agarose column equilibrated with the lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imadozle; pH 8.0). The column was washed with 2 column volumes of the lysis buffer and 2 column volumes of the wash buffer (50 mM Tris-HCl, 300 mM NaCl, 30 mM imadozle; pH 8.0). The proteins were finally eluted with elution buffer (50 mM Tris-HCl, 300 mM NaCl, 300 mM imadozle; pH 8.0). The purified proteins were desalted by filtration (Millipore, 30,000 MWCO). Each protein could be obtained more than 80 mg from 1 liter culture medium.

L-rhamnuloase kinase (*RhaB*) from *Thermotoga maritima* MSB8, and acid phosphatase (*AphA*) from *Escherichia coli* were prepared as previously reported.<sup>99</sup> The protein concentration

was determined by the Bradford method with bovine serum as a standard and the purity was confirmed by SDS-PAGE.

### ***3.1.10 Substrate specificity of RhaB towards deoxy sugars***

Substrate specificity of RhaB was studied by the reactions that were performed in 50  $\mu$ l reaction mixture containing a Tris-HCl buffer (100 mM, pH 7.5), 20 mM of sugar standards (Table 1.4), 20mM of ATP, 5 mM of  $Mg^{2+}$ , and 100 ng of enzymes. The reactions were carried at 45°C for 10 minutes and were stopped by diluting ten times using a cold buffer of acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60% acetonitrile). The reactions were quantified by analyzing the formation of ADP by HPLC equipped with UV detector at 254 nm using ZIC®-cHILIC column. The column was eluted at 30°C with acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60% acetonitrile) at a flow rate of 0.6 ml/min.

### ***3.1.11 Preparative synthesis of L-rhamnulose and L-fuculose***

In the first step, reactions were carried in a final volume of 400 ml reaction system containing 25 mM of ATP, 3 mM of  $Mg^{2+}$ , 3 mM of  $Mn^{2+}$ , 20 mM starting sugars and conversion-related enzymes (Table 1.5). L-fucose (8.0 mmol) was incubated with 30 mg of FucI and 10 mg of RhaB. L-rhamnose (8.0 mmol) was incubated with 30 mg of RhaA and 10 mg of RhaB. The reactions were carefully shaken at pH near 7.5 at 45°C to allow the formation of ketose 1-phosphates. The reactions were monitored by TLC, and HPLC equipped ELSD using HPX-87H column. Once the reactions no longer move forward, silver nitrate was added to precipitate ATP and ADP until no new precipitate formed. The precipitate was removed by centrifugation (14000 g, 1 min) and washed twice using distilled water. Sodium chloride was added to a final concentration of 200 mM to remove the remnant silver ions. Silver chloride was removed by centrifugation (14000 g, 1 min). The solution from each reaction was concentrated under reduced



pressure and purified by using Bio-Gel P-2 column to afford final products. The products were confirmed by NMR and MS analysis.

**L-rhamnulose 1-phosphate** (1929 mg, Yield 91%).  $\alpha:\beta=1:3$ .  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz):  $\delta$  4.02-4.09 (m, 1 H), 3.72-3.85 (m, 4 H), 1.29 (d, 3 H,  $J = 5.3$  Hz, H-6);  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ , 100 Hz):  $\delta$  102.5 (d, C-1 $\alpha$ ,  $J = 8.4$  Hz), 99.1 (d, C-1 $\beta$ ,  $J = 9.5$  Hz), 81.2, 79.78.1, 76.4, 75.2, 74.6, 17.8, 16.6;  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ , 133 Hz):  $\delta$  1.85 ( $\alpha$ ), 1.21 ( $\beta$ ). HRMS (ESI)  $m/z$  calcd for  $[\text{C}_6\text{H}_{13}\text{O}_8\text{P-H}]^-$  243.0348, found 243.0259.

**L-fuculose 1-phosphate** (1975 mg, Yield 93%).  $\alpha:\beta=1:3$ .  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz):  $\delta$  4.31-4.37 (m, 1 H, H-5 $\alpha$ ), 4.27 (d, 1 H,  $J = 4.3$  Hz, H-3 $\beta$ ), 4.21-4.24 (m, 2 H, H-3 $\alpha$ , H-4 $\alpha$ ), 4.08-4.16 (m, 2 H, H-4 $\beta$ , H-5 $\beta$ ), 3.92-3.96 (dd, 1 H,  $J = 11.2, 6.6$  Hz, H-1 $\alpha\alpha$ ), 3.70-3.83 (m, H-1 $\alpha\beta$ , H-2 $\beta\alpha$ , H-2 $\beta\beta$ ), 1.26 (d, 3 H,  $J = 6.1$  Hz, H-6 $\beta$ ), 1.21 (d, 3 H,  $J = 6.5$  Hz, H-6);  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ , 100 Hz):  $\delta$  102.7 (C-1 $\alpha$ ), 100.2 (C-1 $\beta$ ), 77.0 (C-5 $\alpha$ ), 75.4 (C-5 $\beta$ ), 74.6 (C-4 $\alpha$ ), 71.4 (C-3 $\alpha$ ), 71.3 (C-3 $\alpha$ , C-4 $\beta$ ), 70.6 (C-2 $\beta$ ), 65.1 (C-2 $\alpha$ ), 13.4 (C-6 $\beta$ ), 12.8 (C-6 $\alpha$ );  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ , 133 Hz):  $\delta$  2.48 ( $\alpha$ ), 1.76 ( $\beta$ ). HRMS (ESI)  $m/z$  calcd for  $[\text{C}_6\text{H}_{13}\text{O}_8\text{P-H}]^-$  243.0348, found 243.0259.

In the second reaction step, ketose 1-phosphates were dissolved in water, the pH was adjusted to 5.5 using 1 M of HCl. Then, 3 mM of  $\text{Mg}^{2+}$ , 0.2 mM of  $\text{Zn}^{2+}$ , and 5 mg of AphA was added. The reactions were carried at 37°C to allow the hydrolysis of phosphate groups. Once no sugar phosphates were observed on TLC, the solution from each reaction was concentrated under reduced pressure and purified by using Bio-Gel P-2 column to afford final products. The product was analyzed by NMR, MS and HPLC. L-rhamnulose was analyzed by HPLC equipped with evaporative light scattering detector (ELSD) using HPX-87H column with pure water as mobile phase. L-fuculose was analyzed by using Sugar-Pak 1 column with pure water as mobile phase.

**L-rhamnulose** (1080 mg, Yield 82%).  $\alpha:\beta=1:3$ .  $^1\text{H NMR}$  ( $\text{D}_2\text{O}$ , 400 MHz):  $\delta$  3.91-3.94 (m, 1 H), 3.75 (t, 0.75 H,  $J = 8.0$  Hz), 3.67 (dd, 0.75 H,  $J = 12.4, 6.2$  Hz), 3.59 (t, 0.25 H,  $J = 5.8$  Hz), 3.49 (t, 0.25 H,  $J = 12.0$  Hz), 3.45 (t, 0.25 H,  $J = 6.8$  Hz), 3.35-3.43 (m, 1.75 H), 1.19 (d, 2.25 H,  $J = 6.2$  Hz), 1.16 (d, 0.75 H,  $J = 6.3$  Hz);  $^{13}\text{C NMR}$  ( $\text{D}_2\text{O}$ , 100 Hz):  $\delta$  102.8, 100.0, 81.4, 80.1, 78.3, 75.4, 75.3, 74.1, 61.8, 17.9, 16.4. HRMS (ESI)  $m/z$  calcd for  $\text{C}_6\text{H}_{12}\text{O}_5 \text{Na}$   $[\text{M}+\text{Na}]^+$  187.0582, found 187.0528.

**L-fuculose** (1099 mg, Yield 84%).  $\alpha:\beta=1:3$ .  $^1\text{H NMR}$  ( $\text{D}_2\text{O}$ , 400 MHz):  $\delta$  4.17 (d, 0.25 H,  $J = 5.0$  Hz), 4.11 (d, 0.75 H,  $J = 4.5$  Hz), 3.95-4.01 (m, 2 H), 3.36-3.05 (m, 2 H), 1.15 (d, 2.25 H,  $J = 6.2$  Hz), 1.08 (d, 0.75 H,  $J = 6.5$  Hz);  $^{13}\text{C NMR}$  ( $\text{D}_2\text{O}$ , 100 Hz):  $\delta$  104.5, 102.1, 78.4, 76.4, 75.4, 72.8, 72.4, 71.0, 62.7, 62.4, 14.5, 13.6. HRMS (ESI)  $m/z$  calcd for  $\text{C}_6\text{H}_{12}\text{O}_5 \text{Na}$   $[\text{M}+\text{Na}]^+$  187.0582, found 187.0528.

### ***3.1.12 Preparative scale synthesis of 6-deoxy-L-sorbose***

In the first step, reactions were carried in a final volume of 400 ml reaction system containing 25 mM of ATP, 3 mM of  $\text{Mg}^{2+}$ , 3 mM of  $\text{Mn}^{2+}$ , 20 mM L-fucose (8.0 mmol), 30 mg of FucI, 35 mg of DTE and 30 mg of HK. The reactions were carefully shaken at pH near 7.5 at  $37^\circ\text{C}$  to allow the formation of 6-deoxy-L-sorbose 1-phosphates. The reactions were monitored by TLC, and HPLC equipped ELSD using HPX-87H column. The column was eluted at  $60^\circ\text{C}$  with pure water as mobile phase at a flow rate of 0.6 ml/min. Once the L-fucose was no longer detected, silver nitrate was added to precipitate ATP and ADP until no new precipitate formed. The precipitate was removed by centrifugation (14000 g, 1 min) and washed twice using distilled water. Sodium chloride was added to a final concentration of 200 mM to remove the remnant silver ions. Silver chloride was removed by centrifugation (14000 g, 1 min). The supernatant was concentrated

under reduced pressure and purified by using Bio-Gel P-2 column to afford 6-deoxy-L-sorbose 1-phosphate.

In the second reaction step, 6-deoxy-L-sorbose 1-phosphate was dissolved in water, and the pH was adjusted to 5.5 using 1 M of HCl. Then, 3 mM of  $Mg^{2+}$ , 0.2 mM of  $Zn^{2+}$ , and 10 mg of AphA was added. The reactions were carried at 37°C to allow the hydrolysis of phosphate group. Once no sugar phosphate was observed on TLC, equal volume of cold ethanol was added to stop reaction. The solution was concentrated under reduced pressure and purified by using Bio-Gel P-2 column to afford 6-deoxy-L-sorbose.

**6-deoxy-L-sorbose (1059 mg, 81% yield).**  $^1H$  NMR ( $D_2O$ , 400 MHz):  $\delta$  4.32-4.37 (m, 1 H), 4.13-4.16 (m, 1 H), 4.01-4.02 (m, 1 H), 3.48-3.56 (m, 2 H), 1.13 (d,  $J = 6.4$  Hz, 3 H,  $-CH_3$ );  $^{13}C$  NMR ( $D_2O$ , 100 Hz):  $\delta$  212.8, 105.1, 101.7, 80.2, 78.1, 76.7, 76.5, 76.4, 75.8, 75.7, 74.8, 68.0, 65.9, 63.4, 62.5, 18.1, 14.8, 14.0. HRMS (ESI)  $m/z$  calcd for  $C_6H_{12}O_5 Na [M+Na]^+$  187.0582, found 187.0582.

### *3.1.13 Substrate specificity of HK towards deoxy sugars*

Substrate specificity of HK towards L-rhamnose, L-rhamnulose and 6-deoxy-L-psicose was studied by the reactions that were performed in 50  $\mu$ l reaction mixture containing a Tris-HCl buffer (100 mM, pH 7.5), 20 mM of sugar standards (Table 1.8), 20 mM of ATP, 5 mM of  $Mg^{2+}$ , and 10  $\mu$ g of enzymes. The reactions were performed at 37°C for 10 minutes and were stopped by diluting ten times using a cold buffer of acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60% acetonitrile). The solutions were centrifuged 5 minutes (12000 g) before HPLC injection. The reactions were quantified by analyzing the formation of ADP by HPLC equipped with UV detector at 254 nm using ZIC®-cHILIC column. The column was eluted at room temperature with

acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60% acetonitrile) at a flow rate of 0.6 ml/min.

### ***3.1.14 Preparative scale synthesis of 6-deoxy-L-psicose***

To a 400 ml reaction solution, 20 mM of L-rhamnose (8.0 mmol), 25 mM of ATP, 3 mM of  $Mg^{2+}$ , 3 mM of  $Mn^{2+}$ , 20 mg of FucI, 25 mg of DTE and 20 mg of HK were added. The solution pH was adjusted to 8.0 using sodium hydroxide and was carefully shaken at 37°C to allow the formation of 6-deoxy-L-psicose 1-phosphate. The reaction was monitored by TLC, and HPLC (HPX-87H column). 5 mg of FucI, 5 mg of DTE and 5 mg of HK were supplemented every 12 hours. After the reaction was performed 48 hours, 95% conversion ratio was reached (as confirmed by HPLC). A longer reaction time makes no contribution to the improvement of conversion ratio. Afterwards, silver nitrate was added to precipitate adenosine phosphates (ATP and ADP) until no new precipitate formed. The precipitate was removed by centrifugation (14000 g, 1 min) and washed twice using distilled water. Sodium chloride was added to a final concentration of 200 mM to remove the remnant silver ions. Silver chloride was removed by centrifugation (14000 g, 1 min). The supernatant was concentrated under reduced pressure and purified by using Bio-Gel P-2 column to afford 6-deoxy-L-psicose 1-phosphate.

In the second reaction step, the fractions that containing 6-deoxy-L-psicose 1-phosphate were collected and dissolved in water. The solution pH was adjusted to 5.5 using 1 M of HCl. Then, 3 mM of  $Mg^{2+}$ , 0.2 mM of  $Zn^{2+}$ , and 10 mg of AphA was added. The reaction was carried at 37°C to allow the hydrolysis of phosphate group. Once no sugar phosphate was observed on TLC, equal volume of cold ethanol was added to precipitate proteins. The solution was concentrated under reduced pressure and purified by using Bio-Gel P-2 column to afford 6-deoxy-L-psicose.

**6-deoxy-L-psicose (1059 mg, 81% yield).**  $^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$  4.04 (t,  $J = 6.6$  Hz, 2H), 3.76 (t,  $J = 5.7$  Hz, 1H), 3.67 (d,  $J = 14.5$  Hz, 1H), 3.46 (q,  $J = 12.0$  Hz, 2H), 1.26 (d,  $J = 6.2$  Hz, 1H), 1.17 (d,  $J = 6.3$  Hz, 2H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ )  $\delta$  105.24, 102.92, 78.50, 78.06, 75.95, 75.11, 70.28, 63.47, 62.49, 59.35, 19.35, 17.75. HRMS (ESI)  $m/z$  calcd for  $\text{C}_6\text{H}_{12}\text{O}_5 \text{Na} [\text{M}+\text{Na}]^+$  187.0582, found 187.0579.

### 3.1.15 Preparative scale synthesis of *D*-xylulose 5-phosphate and *L*-ribulose 5-phosphate.

Reactions were carried in a final volume of 250 ml reaction system containing 25 mM of ATP, 3 mM of  $\text{Mg}^{2+}$ , 3 mM of  $\text{Mn}^{2+}$ , 20 mM starting sugars and conversion-related enzymes (Table 1). *D*-xylose (5.0 mmol) was incubated with 20 mg of XylA and 20 mg of XylB. *L*-arabinose (5.0 mmol) was incubated with 15 mg of AraA and 20 mg of AraB. The reactions were carefully shaken at pH near 7.5 at  $37^\circ\text{C}$  to allow the formation of ketose 5-phosphates. The reactions were monitored by TLC, and HPLC equipped ELSD using HPX-87H column with pure water as mobile phase. Once no detectable starting sugars were found, silver nitrate (1 M) was added to precipitate ATP and ADP until no new precipitate formed. The precipitate was removed by centrifugation (14000 g, 1 min) and washed twice using distilled water. Sodium chloride was added to a final concentration of 200 mM to remove the remnant silver ions. Silver chloride was removed by centrifugation (14000 g, 1 min). The solution from each reaction was concentrated under reduced pressure and purified by using Bio-Gel P-2 column to afford final products.

***D*-xylulose 5-phosphate (1).** (1143 mg, Yield 91%);  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz):  $\delta$  4.64 (d, 1 H,  $J = 19.4$  Hz, H-1a), 4.52 (d, 1 H,  $J = 19.4$  Hz, H-1b), 4.50 (d, 1 H,  $J = 1.8$  Hz, H-3), 4.18-4.22 (m, 1 H, H-4), 3.85-3.89 (m, 2 H, H-5);  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ , 100 Hz):  $\delta$  211.9, 73.9, 69.7, 65.0, 63.6;  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ , 133 Hz):  $\delta$  2.23. HRMS (ESI)  $m/z$  calculated for  $[\text{C}_5\text{H}_{11}\text{O}_8\text{P} - \text{H}]^-$  229.0119, found 229.0122.

**L-ribulose 5-phosphate (5).** (1164 mg, Yield 92%);  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz):  $\delta$  4.61 (d, 1 H,  $J = 19.4$  Hz, H-1a), 4.54 (d, 1 H,  $J = 19.4$  Hz, H-1b), 4.40 (d, 1 H,  $J = 5.8$  Hz, H-3), 4.02 (dd,  $J = 10.6, 5.2$  Hz, 1 H, H-5a), 3.83-3.91 (m, 2 H, H-4, H-5b);  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ , 100 Hz):  $\delta$  212.6, 74.9, 71.4 (d,  $J = 7.0$  Hz), 66.3, 63.7;  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ , 133 Hz):  $\delta$  4.38. HRMS (ESI)  $m/z$  calculated for  $[\text{C}_5\text{H}_{11}\text{O}_8\text{P} - \text{H}]^-$  229.0119, found 229.0122.

### ***3.1.16 Preparative scale synthesis of D-xylulose 1-phosphate and L-ribulose 1-phosphate.***

Reactions were carried in a final volume of 250 ml reaction system containing 25 mM of ATP, 3 mM of  $\text{Mg}^{2+}$ , 3 mM of  $\text{Mn}^{2+}$ , 20 mM starting sugars and conversion-related enzymes (Table 1.11). D-xylulose (5.0 mmol) was incubated with 20 mg of XylA and 15 mg of HK. L-arabinose (5.0 mmol) was incubated with 15 mg of AraA and 25 mg of HK. The reactions were carefully shaken at pH near 7.5 at  $37^\circ\text{C}$  to allow the formation of ketose 1-phosphates. All the reactions were allowed to proceed until no detectable starting sugars were found. Silver nitrate precipitation method (as described above) was used to purify D-xylulose 1-phosphate and L-ribulose 1-phosphate. The solution from each reaction was concentrated under reduced pressure and purified by using Bio-Gel P-2 column to afford final products.

**D-xylulose 1-phosphate (2).** 1159 mg; Yield 92%;  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz):  $\delta$  4.29-4.33 (m, 0.7 H), 4.22-4.26 (m, 0.49 H), 4.19-4.23 (m, 0.25 H), 4.12-4.16 (m, 0.80 H), 4.02-4.05 (m, 1.01 H), 3.93 (dd, 1 H,  $J = 11.3, 8.6$  Hz, 0.33 H), 3.87 (dd,  $J = 9.4, 0.33$  Hz), 3.72-3.78 (m, 1.67 H), 3.59-3.63 (m, 0.94 H);  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ , 100 Hz):  $\delta$  105.9 (d,  $J_{\text{C-P}} = 6.1$  Hz,  $\beta_{\text{C-1}}$ ), 102.3 (d,  $J_{\text{C-P}} = 7.4$  Hz,  $\alpha_{\text{C-1}}$ ), 80.1, 76.9, 75.3, 74.6, 72.7, 69.7, 65.9 (d,  $J_{\text{C-P}} = 3.1$  Hz), 64.4 (d,  $J_{\text{C-P}} = 4.1$  Hz);  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ , 133 Hz):  $\delta$  5.11 ( $\alpha$ ), 4.24 ( $\beta$ );  $\alpha:\beta=1:2$ . HRMS (ESI)  $m/z$  calculated for  $[\text{C}_5\text{H}_{11}\text{O}_8\text{P} - \text{H}]^-$  229.0119, found 229.0123.

**L-ribulose 1-phosphate (5).** 1170 mg; Yield 93%;  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz):  $\delta$  4.58 (dd, 0.33 H,  $J = 12.5, 6.4$  Hz), 4.31 (brs, 0.57 H), 4.11-4.17 (m, 0.85 H), 3.99-4.04 (m, 1.12 H), 3.88-3.90 (m, 0.59 H), 3.69-3.75 (m, 1.67 H);  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ , 100 Hz):  $\delta$  105.9 (d,  $J_{\text{C-P}} = 6.2$  Hz,  $\beta_{\text{C-1}}$ ), 101.9 (d,  $J_{\text{C-P}} = 8.5$  Hz,  $\alpha_{\text{C-1}}$ ), 74.9, 71.5, 71.1, 70.5, 70.0, 69.9, 65.9 (d,  $J_{\text{C-P}} = 3.3$  Hz), 64.7 (d,  $J_{\text{C-P}} = 4.4$  Hz);  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ , 133 Hz):  $\delta$  5.18 ( $\alpha$ ), 4.40 ( $\beta$ );  $\alpha:\beta = 1:2$ . HRMS (ESI)  $m/z$  calculated for  $[\text{C}_5\text{H}_{11}\text{O}_8\text{P} - \text{H}]^-$  229.0119, found 229.0123.

### *3.1.17 Preparative scale synthesis of D-ribulose 1-phosphate and L-xylulose 1-phosphate.*

Reactions were carried in a final volume of 250 ml reaction system containing 25 mM of ATP, 3 mM of  $\text{Mg}^{2+}$ , 3 mM of  $\text{Mn}^{2+}$ , 20 mM starting sugars and conversion-related enzymes (Table 1.11). D-xylose (5.0 mmol) was incubated with 20 mg of XylA, 35 mg of DTE and 13 mg of RhaB. L-arabinose (5.0 mmol) was incubated with 15 mg of AraA, 35 mg of DTE and 13 mg of RhaB. The reactions were carefully shaken at pH near 7.5 at  $45^\circ\text{C}$  to allow the formation of ketose 1-phosphates. Once no detectable starting sugars were found, silver nitrate precipitation method (as described above) was used to purify D-ribulose 1-phosphate and L-xylulose 1-phosphate. The solution from each reaction was concentrated under reduced pressure and desalted by using Bio-Gel P-2 column to afford final products.

**D-ribulose-1-phosphate (3).** 1183 mg; Yield 94%;  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz):  $\delta$  4.31 (s, 1 H), 4.14 (s, 1 H), 4.00-4.02 (m, 1 H), 3.88-3.90 (m, 1 H), 3.69-3.78 (m, 2 H);  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ , 100 Hz):  $\delta$  105.5 (d,  $J_{\text{C-P}} = 7.0$  Hz,  $\beta_{\text{C-1}}$ ), 101.7 (d,  $J_{\text{C-P}} = 8.0$  Hz,  $\alpha_{\text{C-1}}$ ), 74.9, 71.2, 70.9, 70.5, 70.0 (2 C), 65.9 (2 C), 65.9 (d,  $J_{\text{C-P}} = 3.0$  Hz), 65.1 (d,  $J_{\text{C-P}} = 5.0$  Hz);  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ , 133 Hz):  $\delta$  3.60 ( $\alpha$ ), 2.60 ( $\beta$ );  $\alpha:\beta = 1:2$ . HRMS (ESI)  $m/z$  calculated for  $[\text{C}_5\text{H}_{11}\text{O}_8\text{P} - \text{H}]^-$  229.0119, found 229.0123.

**L-xylulose-1-phosphate (7).** 1204 mg; Yield, 96%;  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz):  $\delta$  4.32 (brs, 1 H), 4.22-4.26 (m, 1 H), 4.14-4.16 (m, 1 H), 4.04-4.06 (m, 1 H), 3.79 (brs, 1 H), 3.63 (brs, 1 H);  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ , 100 Hz):  $\delta$  105.9 (d,  $J_{\text{C-P}} = 6.0$  Hz,  $\beta_{\text{C-1}}$ ), 102.4 (d,  $J_{\text{C-P}} = 8.0$  Hz,  $\alpha_{\text{C-1}}$ ), 80.3, 76.9, 75.3, 74.7, 72.7, 69.7, 66.0 (d,  $J_{\text{C-P}} = 5.0$  Hz), 64.4 (d,  $J_{\text{C-P}} = 4.0$  Hz);  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ , 133 Hz):  $\delta$  5.28 ( $\alpha$ ), 4.44 ( $\beta$ );  $\alpha:\beta = 1:2$ . HRMS (ESI)  $m/z$  calculated for  $[\text{C}_5\text{H}_{11}\text{O}_8\text{P} - \text{H}]^-$  229.0119, found 229.0121.

### 3.1.18 Preparative scale synthesis of D-ribulose 5-phosphate and L-xylulose 5-phosphate

In the first reaction step, D-ribulose 1-phosphate and L-xylulose 1-phosphate were prepared from 6 mmol of D-xylose or L-arabinose as described above. To prepare D-ribulose and L-xylulose, a reaction mixture in total volume of 100 ml containing ketose 1-phosphates, 5 mM of  $\text{Mg}^{2+}$ , 0.1 mM  $\text{Zn}^{2+}$  and 10 mg of AphA. The reactions were carefully shaken at  $37^\circ\text{C}$  to allow the formation of ketoses. Once no detectable ketose 1-phosphates were found by TLC, The solution from each reaction was concentrated under reduced pressure and desalted by using Bio-Gel P-2 column to afford ketose 1-phosphates. In the second reaction step, reactions were carried in a final volume of 300 ml reaction system containing 25 mM of ATP, 3 mM of  $\text{Mg}^{2+}$ , 3 mM of  $\text{Mn}^{2+}$ ,  $\sim 20$  mM of ketoses and kinases (Table 1.11). D-ribulose was incubated with 15 mg of AraB. L-xylulose was incubated with 20 mg of LyxK. The reactions were carefully shaken at pH near 7.5 at  $37^\circ\text{C}$  to allow the formation of ketose 5-phosphates. Once no detectable ketoses were found, silver nitrate precipitation method (as described above) was used to purify D-ribulose 5-phosphate and L-xylulose 5-phosphate. The solution from each reaction was concentrated under reduced pressure and desalted by using Bio-Gel P-2 column to afford final products.

**D-ribulose-5-phosphate (4).** 1291 mg; Yield, 85%;  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz):  $\delta$  4.61 (d, 1 H,  $J = 19.4$  Hz, H-1a), 4.55 (d, 1 H,  $J = 19.4$  Hz, H-1b), 4.41 (d,  $J = 5.8$ , H-3), 4.01 (dd,  $J = 10.2$ ,



4.9 Hz, H-5a), 3.82-3.92 (m, 2 H, H-4, H-5b);  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ , 100 Hz):  $\delta$  211.4, 73.8, 70.2, 65.4, 63.4;  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ , 133 Hz):  $\delta$  4.24. HRMS (ESI)  $m/z$  calculated for  $[\text{C}_5\text{H}_{11}\text{O}_8\text{P} - \text{H}]^-$  229.0119, found 229.0099.

**L-xylulose-5-phosphate (8).** 1265 mg; Yield, 84%;  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz):  $\delta$  4.64 (d, 1 H,  $J = 19.4$  Hz, H-1a), 4.52 (d, 1 H,  $J = 19.4$  Hz, H-1b), 4.51 (d, 1 H,  $J = 1.5$  Hz, H-3), 4.17 (dd, 1 H,  $J = 5.8, 4.4$  Hz, H-5a), 3.80-3.83 (m, 2 H, H-4, H-5b);  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ , 100 Hz):  $\delta$  213.1, 75.2, 71.2 (d,  $J = 6.9$  Hz), 66.0, 63.8 (d,  $J = 4.6$  Hz);  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ , 133 Hz):  $\delta$  4.35. HRMS (ESI)  $m/z$  calculated for  $[\text{C}_5\text{H}_{11}\text{O}_8\text{P} - \text{H}]^-$  229.0119, found 229.0122.

### 3.1.19 Purity analysis

To analyze the purity of phosphorylated ketoses, the phosphate group of each sugar phosphate was hydrolyzed by a reaction system (pH 5.5) containing 20 mM of phosphorylated sugars, 3 mM of  $\text{Mg}^{2+}$  and 10 U of acid phosphatase. The reactions were incubated at  $37^\circ\text{C}$  until no sugar phosphates were observed by TLC (EtOAc/MeOH/ $\text{H}_2\text{O}$ /HOAc=5:2:1.4:0.4). The mixture was then analyzed by HPLC employing authentic sugar standards as controls as previously reported.<sup>99</sup>

### 3.1.20 Enzyme preparation for KDO synthesis

KdsA, KdsC, KdsD, KdsB, and WaaA were amplified from *Escherichia coli* genomic DNA using the primers listed below.

KdsA (F): GGAATTCCATATGAAACAAAAGTGGTTAGC (*NdeI*)

KdsA (R): CCCAAGCTTTTACTTGCTGGTATCCAGTTC (*HindIII*)

KdsC (F): GGAATTCCATATGAGCAAAGCAGGTGCGT (*NdeI*)

KdsC (R): CCCAAGCTTTCATATCGATTGCCCTTTGG (*HindIII*)

KdsD (F): CATGCCATGGATGTCGCACGTAGAGTTACAA (*NcoI*)

KdsD (R): CCCAAGCTTCACTACGCCTGCACGCAG (*HindIII*)

WaaA(F): GGAATTCCATATGCTCGAATTGCTTTACA (*NdeI*)

WaaA(R): CGCGGATCCTCAATGCGTTTTTCGGTG(*BamHI*)

KdsB(F): GGAATTCCATATGAGTTTTGTGGTCATTATTC(*NdeI*)

KdsB (R): CCCAAGCTTTTAGCGCATTTCAGCGCGAA (*HindIII*)

All genes were cloned into pET-28a vectors and the recombinant plasmids were confirmed by restriction mapping and sequencing. The confirmed constructs were subsequently transformed into *E.coli* BL21 (DE3) for protein expression. After being induced by IPTG at 16 °C overnight, bacteria cells were harvested by centrifugation and re-suspended in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole; pH 8.0). Cells were disrupted by a microfluidizer and the lysate was removed by centrifugation (12,000 g, 25 min). The supernatant was loaded onto a Ni-NTA agarose column equilibrated with the lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole; pH 8.0). The column was washed with 2 column volumes of the lysis buffer and 2 column volumes of the wash buffer (50 mM Tris-HCl, 300 mM NaCl, 30 mM imidazole; pH 8.0). The proteins were finally eluted with elution buffer (50 mM Tris-HCl, 300 mM NaCl, 300 mM imidazole; pH 8.0). The purified proteins were desalted by filtration (Millipore, 30,000 MWCO) for further use. The protein concentration was determined by the Bradford method with bovine serum albumin as a standard and the purity was confirmed by SDS-PAGE.

### **3.1.21 Substrate specificity of KdsC**

Substrate specificity of KdsC toward D-ribulose 5-phosphate was studied by the reaction that was performed in 50 ul reaction mixture containing a Tris-HCl buffer (100 mM, pH 7.5), 20

mM of D-ribulose 5-phosphate, 5 mM of  $Mg^{2+}$ , and 100 ng of KdsC. Meanwhile, reaction contains 5 units of alkaline phosphatase (without KdsC) was performed as a control. The reactions were carried at 37°C for 3 hours and analyzed by TLC (EtOAc/MeOH/H<sub>2</sub>O/HOAc=5:2:1.4:0.4) employing authentic D-ribulose 5-phosphate and D-ribulose as controls.

### **3.1.22 Preparative scale synthesis of KDO**

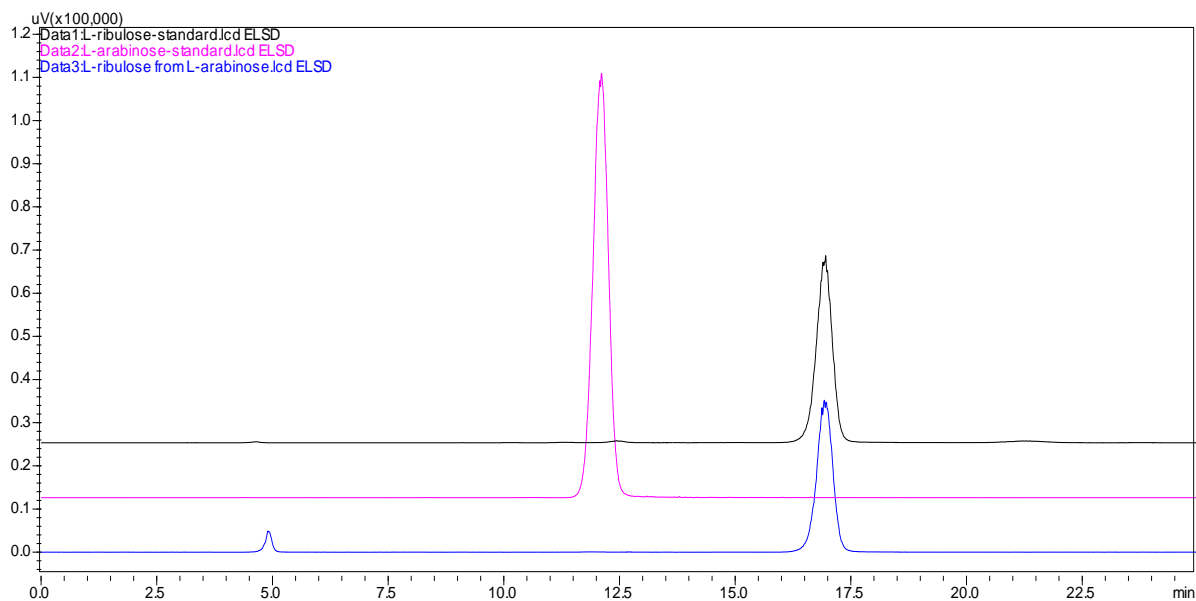
Reaction was carried in a final volume of 300 ml reaction system containing 20 mM of D-ribulose 5-phosphate (6 mmol), 40 mM of PEP (12 mmol), 3 mM of  $Mg^{2+}$ , 3 mM of  $Mn^{2+}$ , 15 mg of KdsD, 30 mg of KdsA and 25 mg of KdsC. The reactions were carefully shaken at pH near 7.5 at 37°C to allow the formation of KDO. The reactions were monitored by TLC(EtOAc/MeOH/H<sub>2</sub>O/HOAc=5:2:1.4:0.4). Once the reactions no longer move forward, the equal volume of ethanol was added to stop the reaction. The solution was concentrated under reduced pressure. KDO was purified using FPLC equipped with a DEAE column ( $HCO_3^-$  form). The column was eluted with a 2 L linear gradient of  $NH_4HCO_3$  (from 0 to 0.5 M). The column fractions were monitored using TLC. The fractions containing KDO was collected and desalted by using Bio-Gel P-2 column to afford final product in 72% yield (1109 mg, ammonium form) with regard to D-ribulose 5-phosphate. The product was analyzed by MS and NMR.

### **3.1.23 Acetylated KDO methyl**

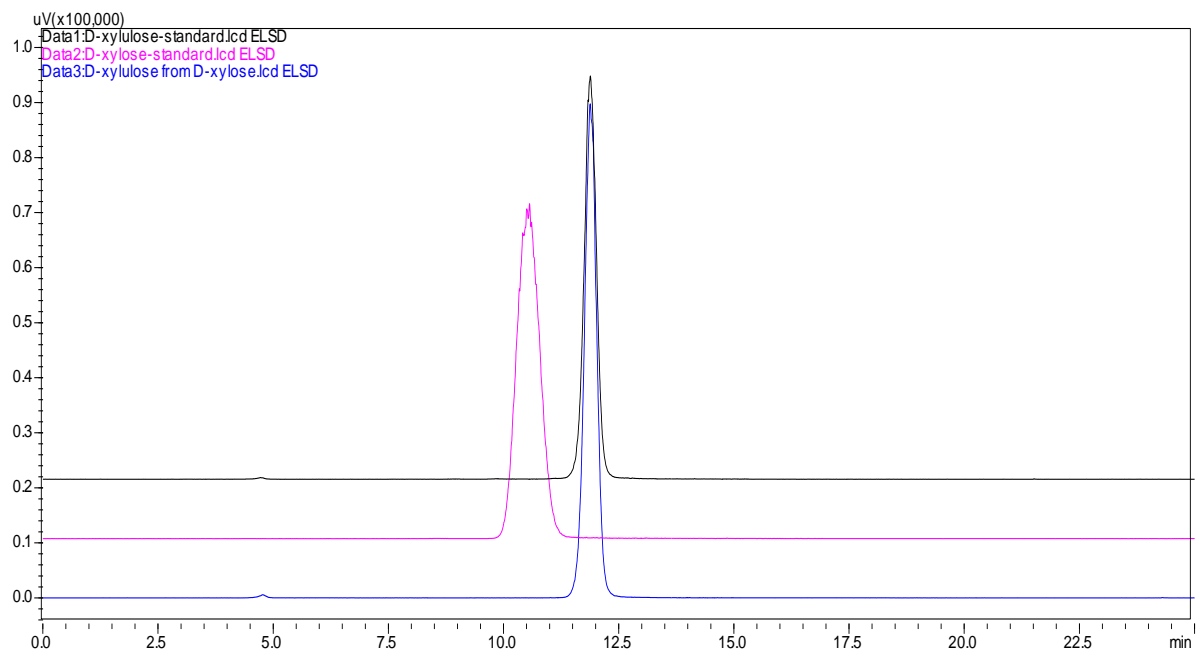
Acetylated KDO methyl ester was prepared from KDO as previously reported.<sup>141, 155</sup> In detail, 50 mg of KDO was added to a solution containing 1.5 ml of acetic anhydride, 3 ml of pyridine, and the catalytic amount of 4-Dimethylamino pyridine and the mixture was stirred under argon atmosphere at room temperature overnight. The reaction mixture was concentrated *in vacuo*. The resulting residue was diluted with DCM (5 mL), washed with 1 M HCl aqueous solution (10 mL), saturated aqueous solution of  $NaHCO_3$  (10 mL) and brine (10 mL). The organic layer was dried

over anhydrous Na<sub>2</sub>SO<sub>4</sub> and filtered. The filtrate was concentrated *in vacuo* to afford **2**. Then, **2** was dissolved in 4 mL of CH<sub>2</sub>Cl<sub>2</sub> and 4 mL of methanol, and then trimethylsilyldiazomethane (1mL, 2 M in diethyl ether) was slowly added. The above solution was stirred under argon atmosphere at room temperature overnight. The reaction mixture was concentrated *in vacuo*, and the resulting residue was purified by silica gel chromatography (hexane/ethyl acetate, 1:1) to afford a white solid **3** (28 mg). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz): δ 1.98 (s, 3H, COCH<sub>3</sub>), 1.99 (s, 3H, COCH<sub>3</sub>), 2.03 (s, 3H, COCH<sub>3</sub>), 2.10 (s, 3H, COCH<sub>3</sub>), 2.13 (s, 3H, COCH<sub>3</sub>), 2.19-2.26 (m, 2H, H-3a, H-3b), 3.79 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 4.10 (dd, 1H, J = 4.0, 12.4 Hz, H-8b), 4.16 (d, 1H, J = 10.0 Hz, H-4), 4.46 (dd, 1H, J = 2.0, 12.0 Hz, H-8a), 5.19-5.23 (ddd, 1H, J = 2.0, 3.6, 12.0 Hz, H-7), 5.29-5.34 (ddd, 1H, J = 2.8, 5.6, 11.2 Hz, H-6), 5.37 (s, 1H, H-5); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): δ 20.60 (4x COCH<sub>3</sub>), 20.68 (COCH<sub>3</sub>), 30.90 (C3), 53.14 (CO<sub>2</sub>CH<sub>3</sub>), 62.09 (C8), 63.91, 65.88, 67.28, 69.70, 97.43 (C2), 166.63 (C1), 167.92 (COCH<sub>3</sub>), 169.50 (COCH<sub>3</sub>), 169.94 (COCH<sub>3</sub>), 170.23 (COCH<sub>3</sub>), 170.35 (COCH<sub>3</sub>); ESI HRMS: m/z calcd for C<sub>19</sub>H<sub>26</sub>NaO<sub>13</sub> [M +Na]<sup>+</sup> 485.1271, found 485.1276.

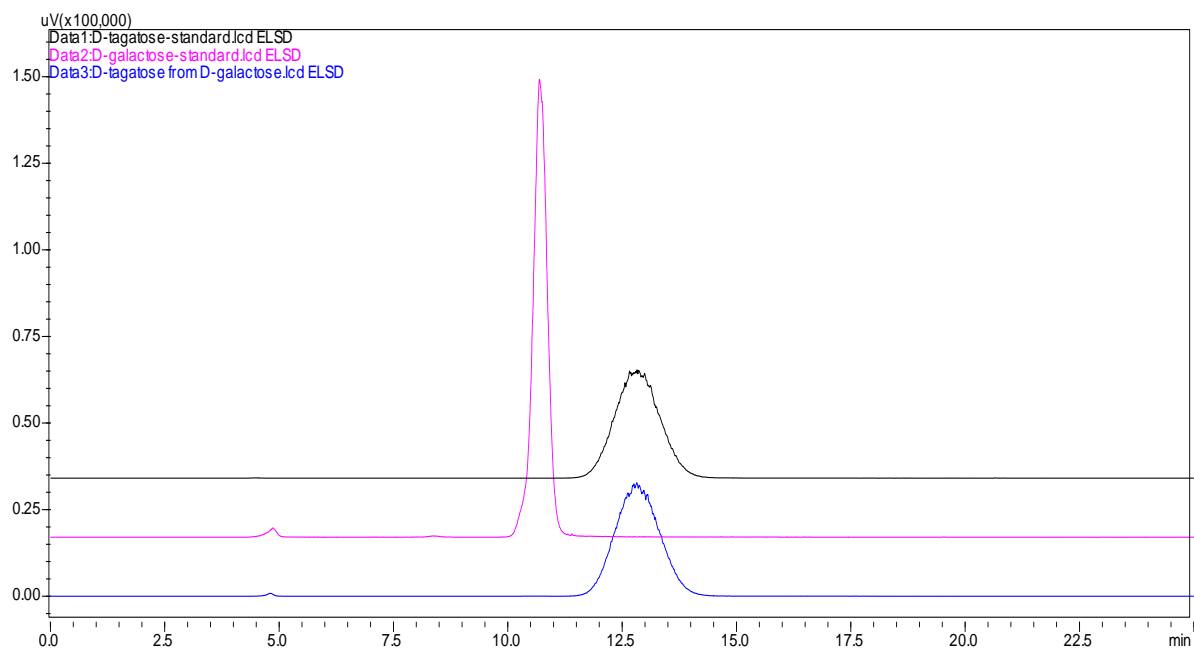
### 3.1.24 HEPLC profile of ketoses



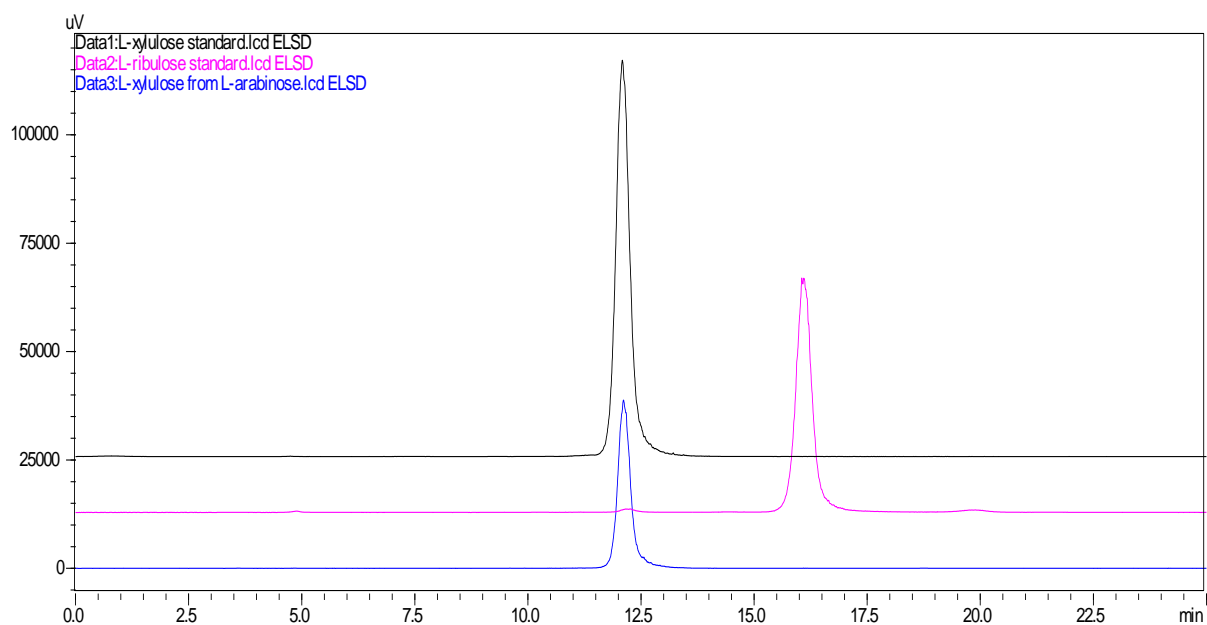
**Figure 3.2** HPLC profiles of L-ribulose compared with authentic samples (Sugar-Pak 1 column at 70 °C with pure water as the mobile phase at a flow rate of 0.6 ml/min).



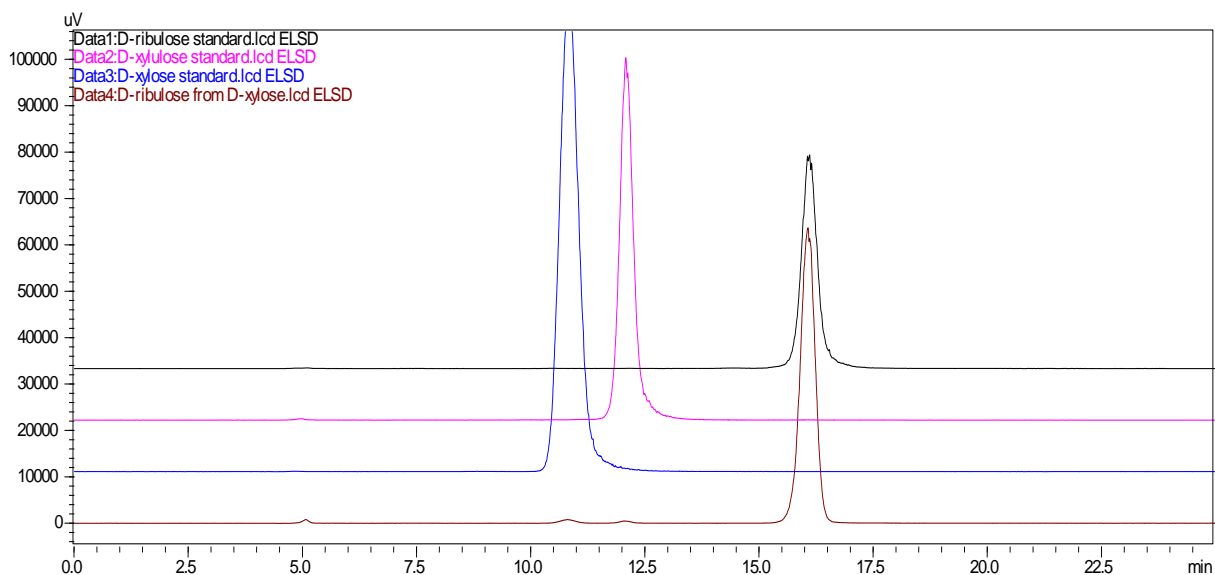
**Figure 3.3** HPLC profiles of D-xylulose compared with authentic samples (Sugar-Pak 1 column at 70 °C with pure water as the mobile phase at a flow rate of 0.6 ml/min).



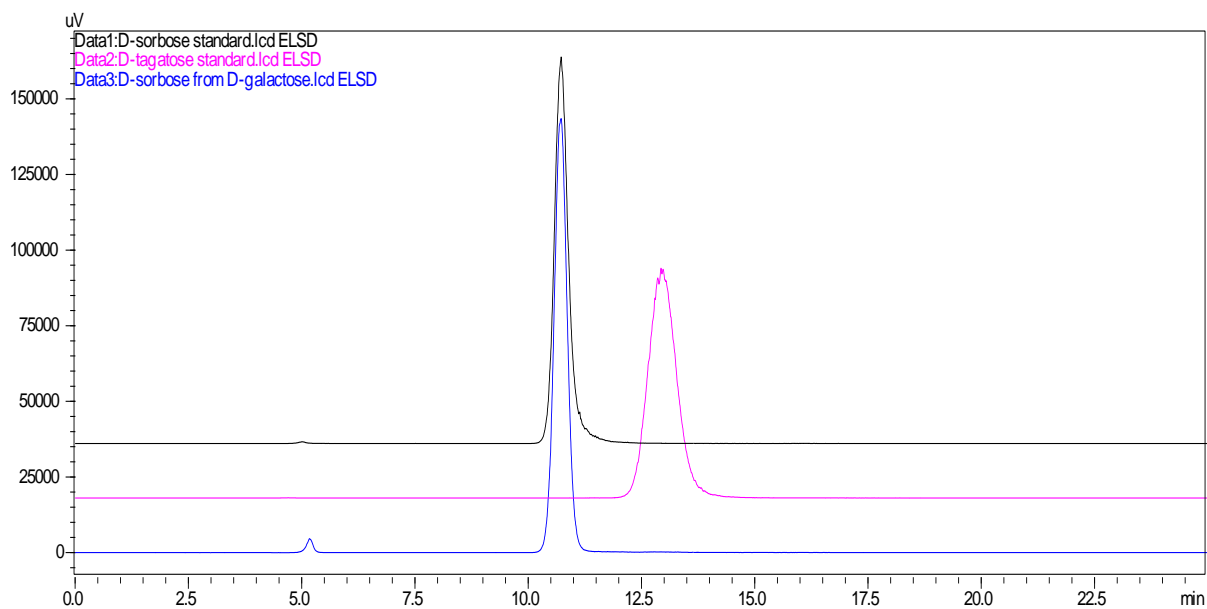
**Figure 3.4** HPLC profiles of *D*-tagatose compared with authentic samples (Sugar-Pak 1 column at 70 °C with pure water as the mobile phase at a flow rate of 0.6 ml/min).



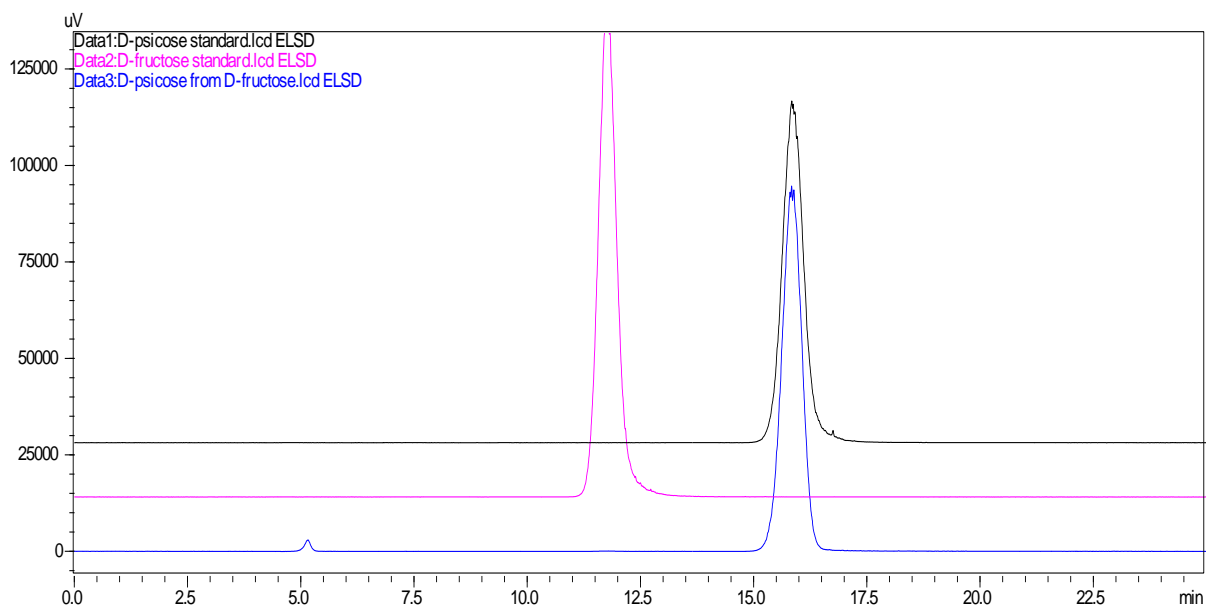
**Figure 3.5** HPLC profiles of *L*-xylulose compared with authentic samples (Sugar-Pak 1 column at 70 °C with pure water as the mobile phase at a flow rate of 0.6 ml/min).



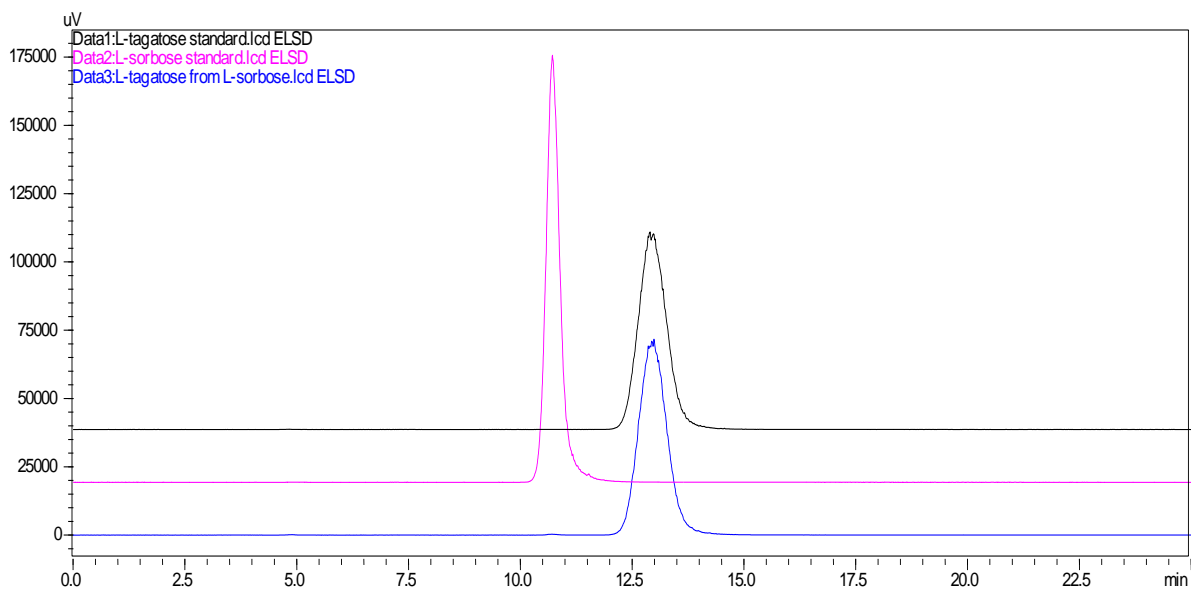
**Figure 3.6** HPLC profiles of D-ribulose compared with authentic samples (Sugar-Pak 1 column at 70 °C with pure water as the mobile phase at a flow rate of 0.6 ml/min).



**Figure 3.7** HPLC profiles of D-sorbose compared with authentic samples (Sugar-Pak 1 column at 70 °C with pure water as the mobile phase at a flow rate of 0.6 ml/min).

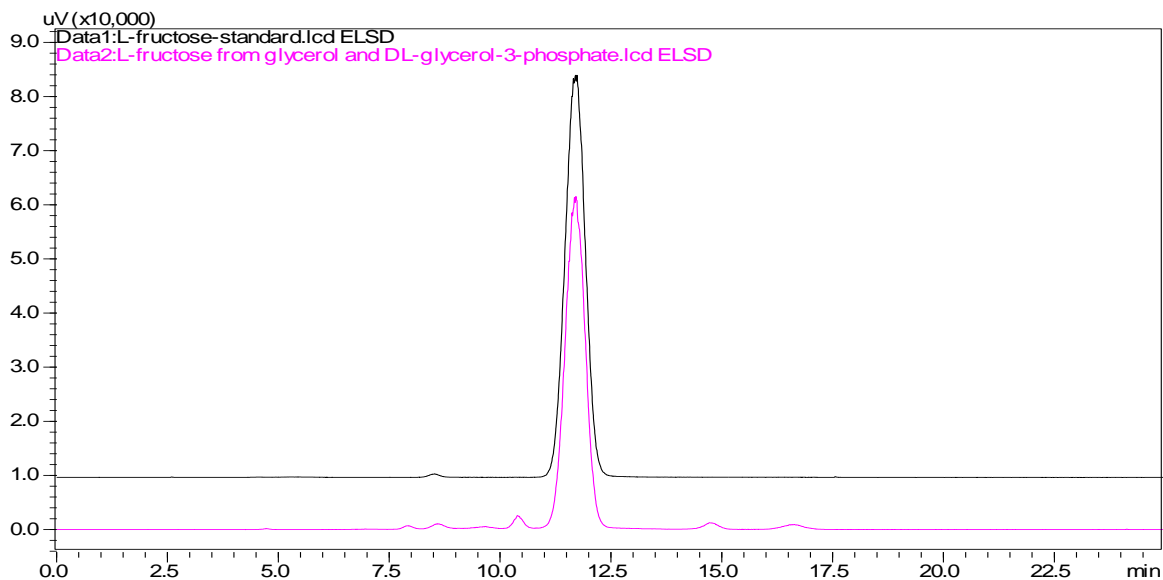


**Figure 3.8** HPLC profiles of *D*-psicose compared with authentic samples (Sugar-Pak 1 column at 70 °C with pure water as the mobile phase at a flow rate of 0.6 ml/min).

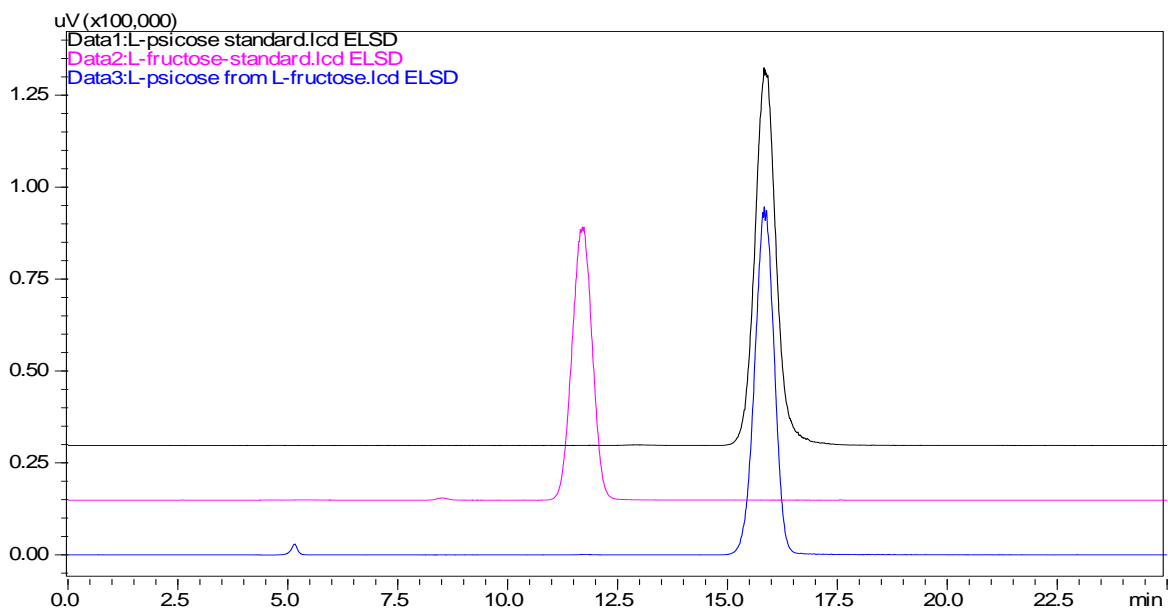


**Figure 3.9** HPLC profiles of *L*-tagatose compared with authentic samples (Sugar-Pak 1 column at 70 °C with pure water as the mobile phase at a flow rate of 0.6 ml/min).





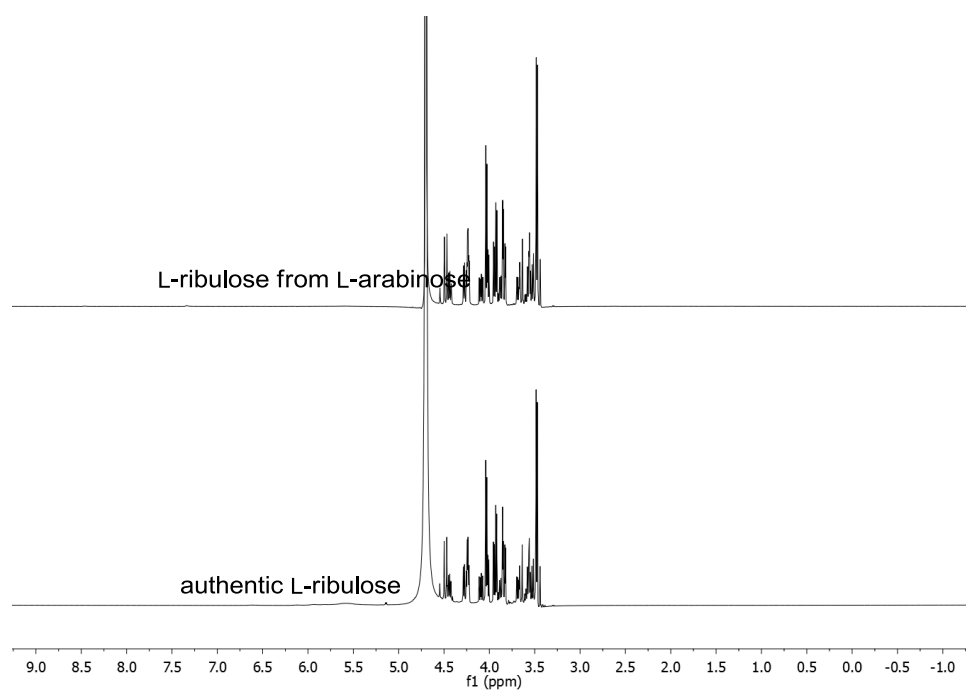
**Figure 3.10** HPLC profiles of L-fructose compared with authentic samples (Sugar-Pak 1 column at 70 °C with pure water as the mobile phase at a flow rate of 0.6 ml/min).



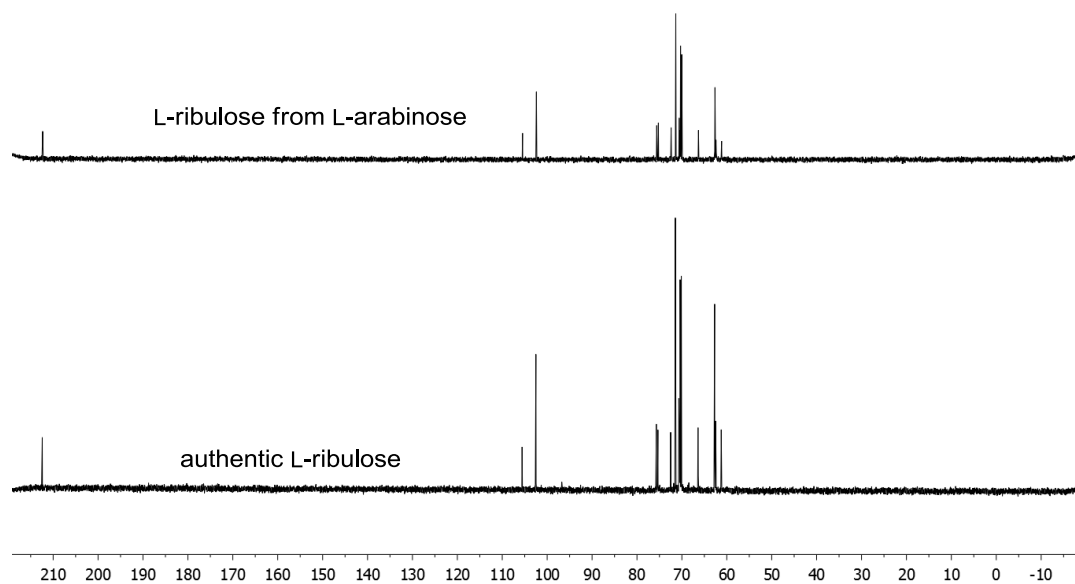
**Figure 3.11** HPLC profiles of L-psicose compared with authentic samples (Sugar-Pak 1 column at 70 °C with pure water as the mobile phase at a flow rate of 0.6 ml/min).

### 3.1.25 NMR spectra

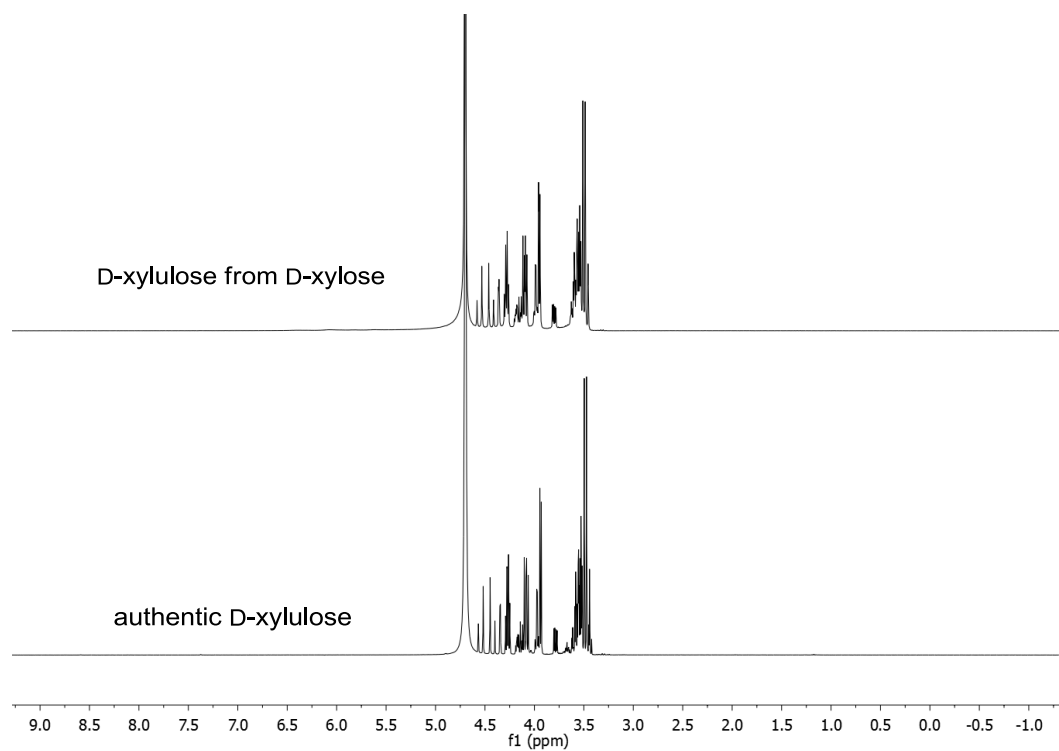
#### $^1\text{H}$ -NMR of L-ribulose



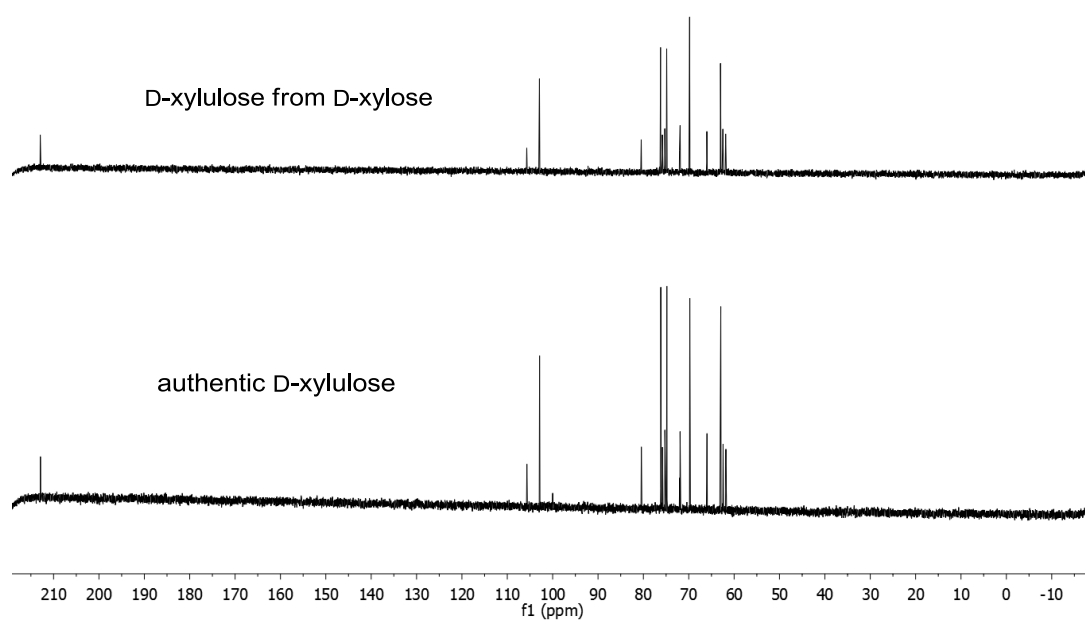
#### $^{13}\text{C}$ -NMR of L-ribulose



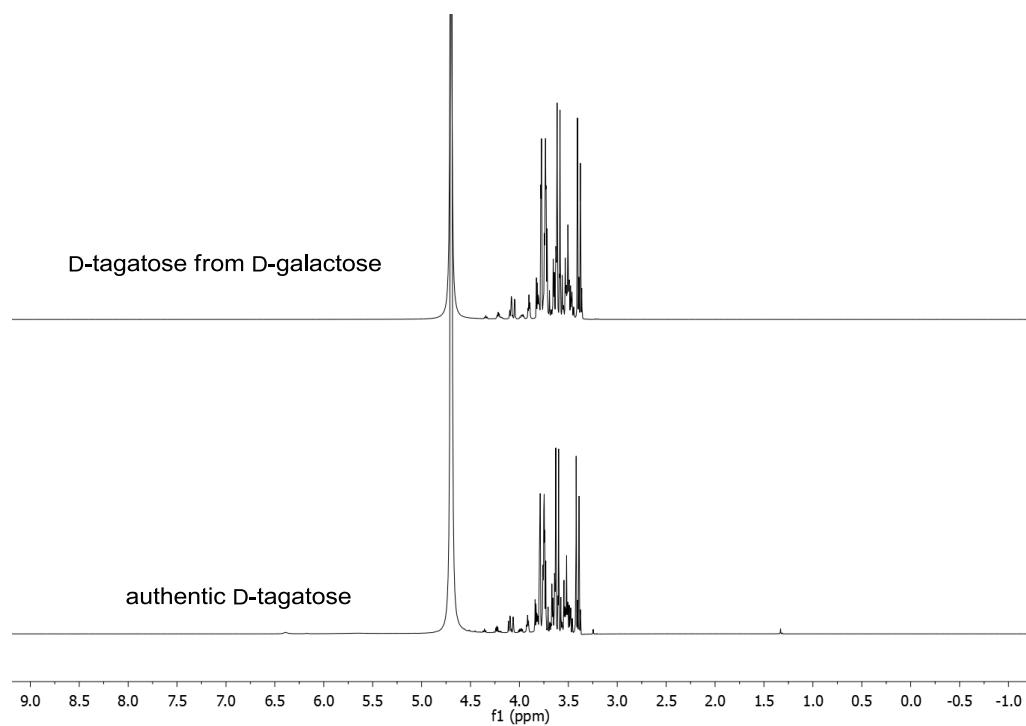
### $^1\text{H-NMR}$ of D-xylulose



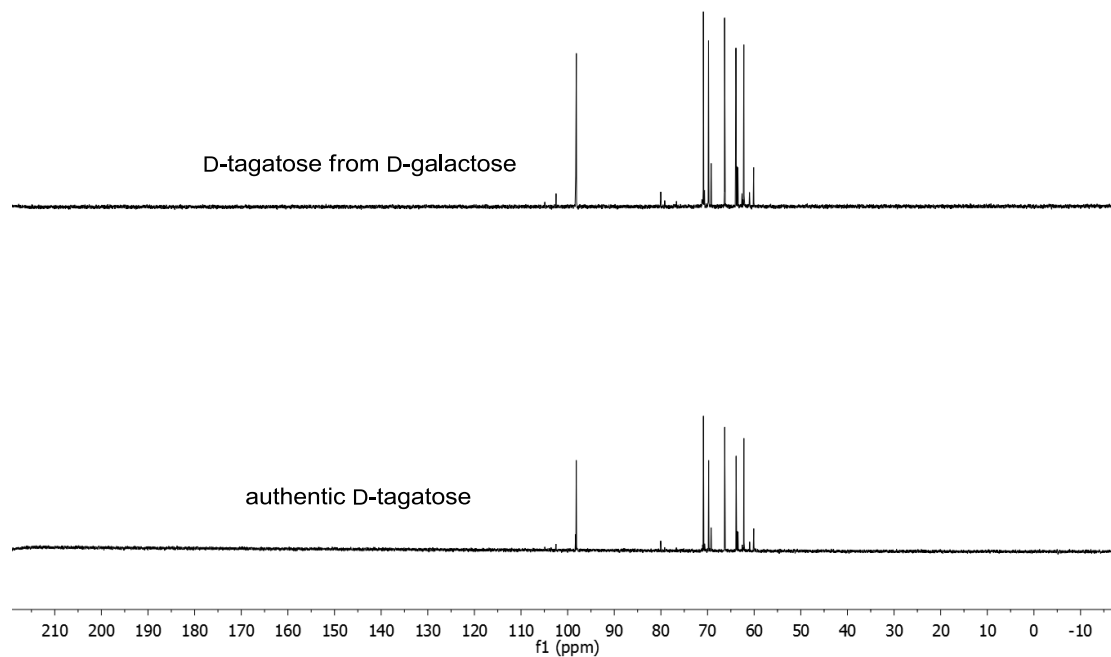
### $^{13}\text{C-NMR}$ of D-xylulose



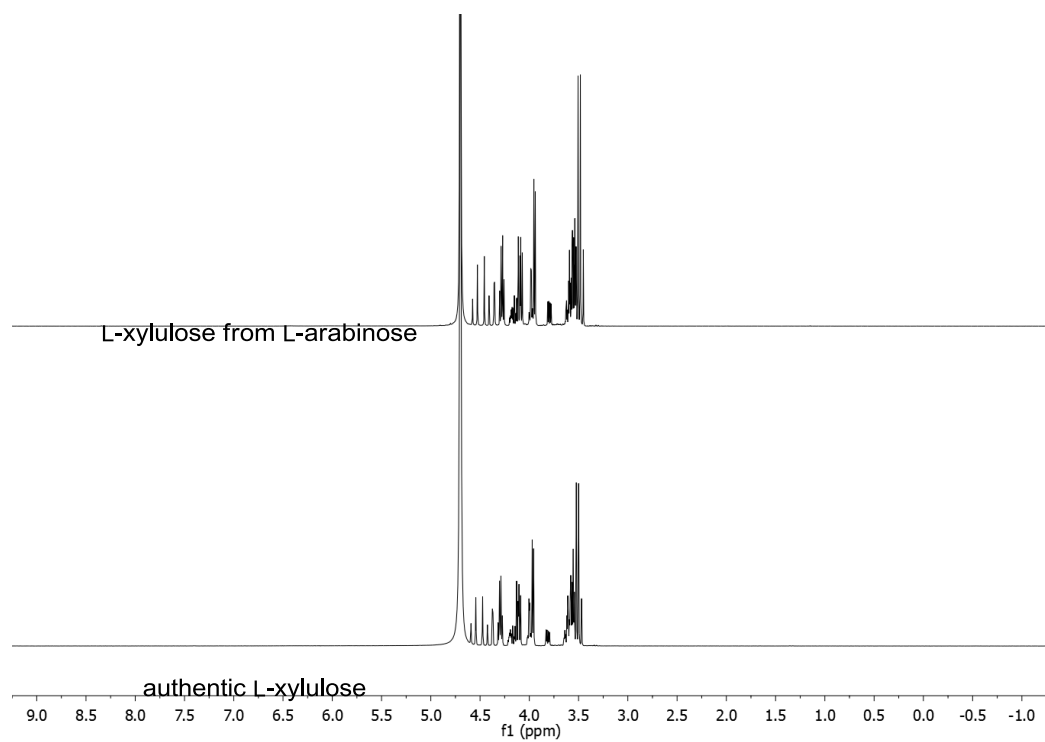
### $^1\text{H-NMR}$ of D-tagatose



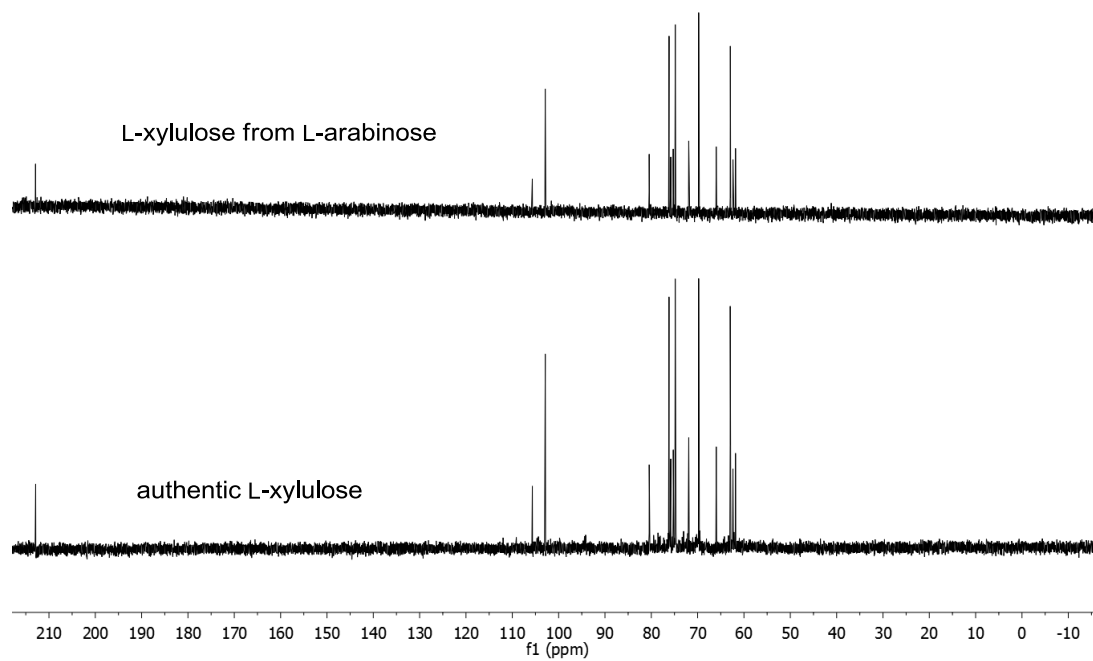
### $^{13}\text{C-NMR}$ of D-tagatose



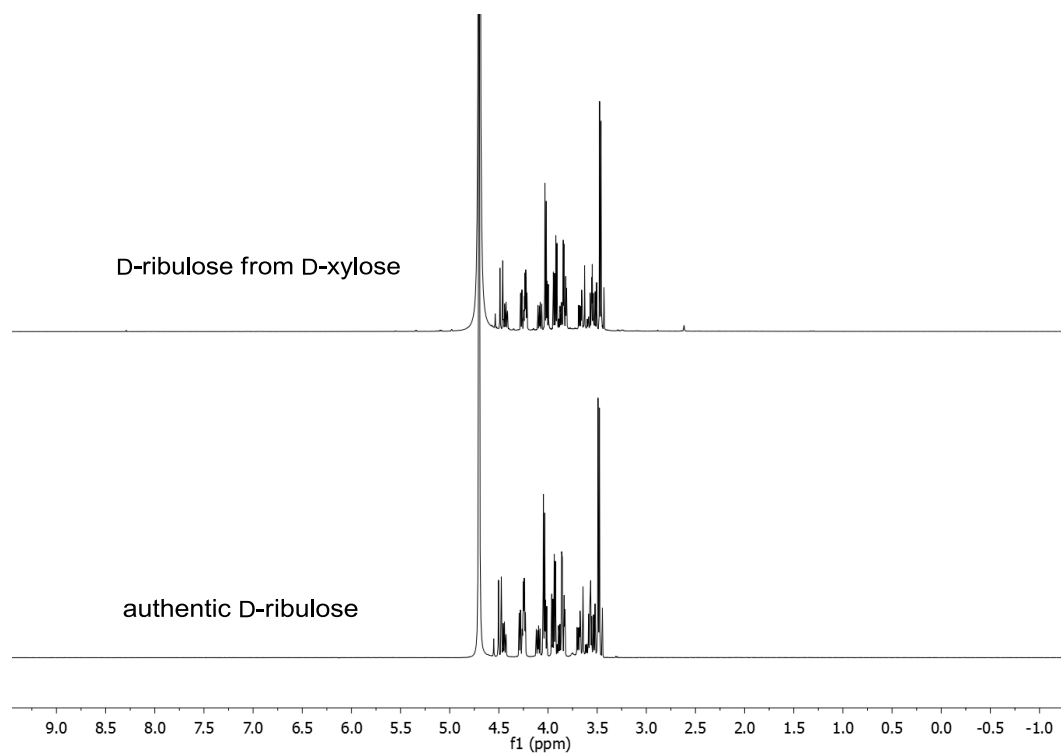
### $^1\text{H-NMR}$ of L-xylulose



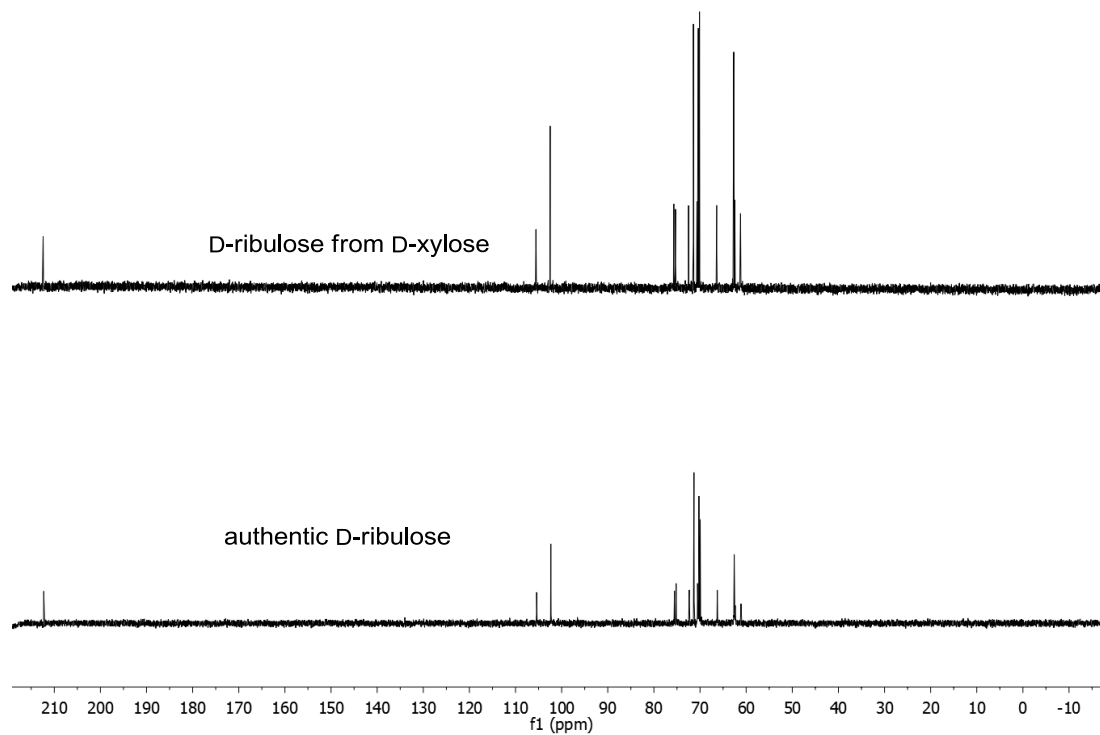
### $^{13}\text{C-NMR}$ of L-xylulose

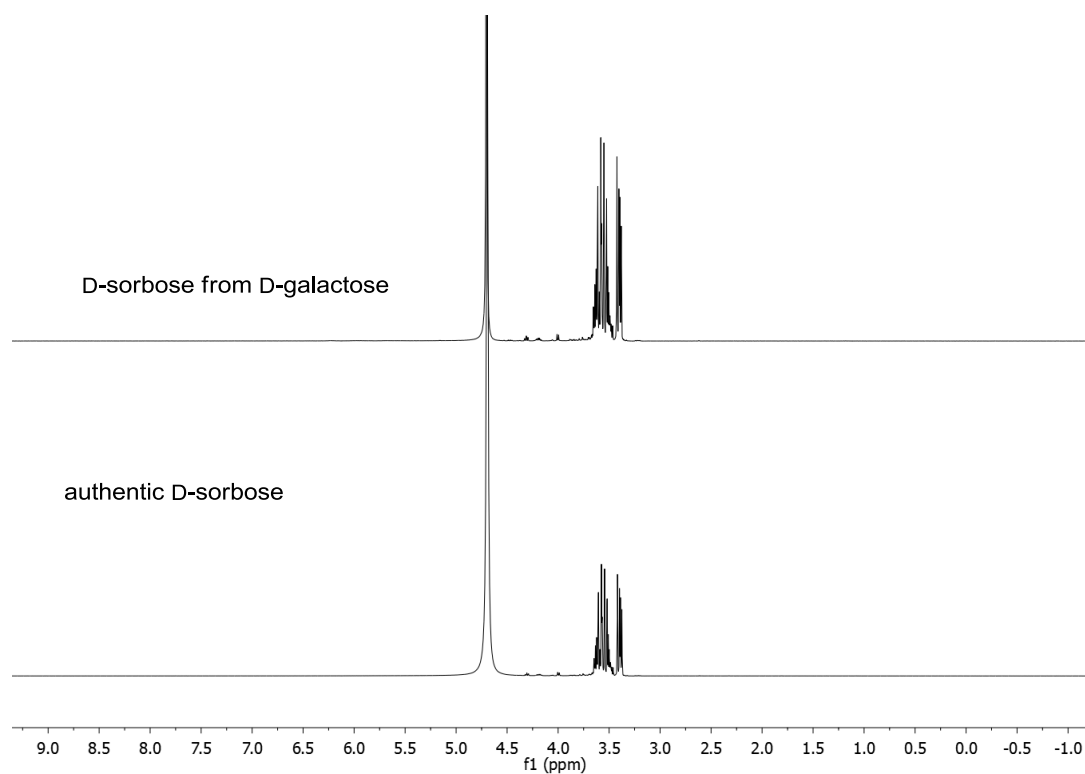
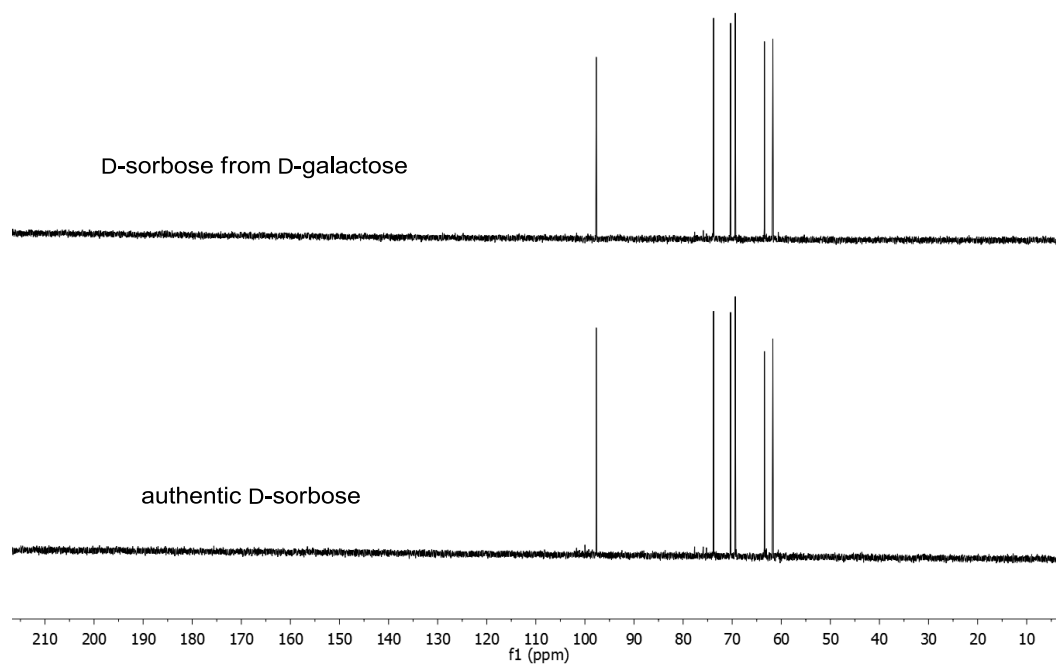


### $^1\text{H}$ -NMR of D-ribulose

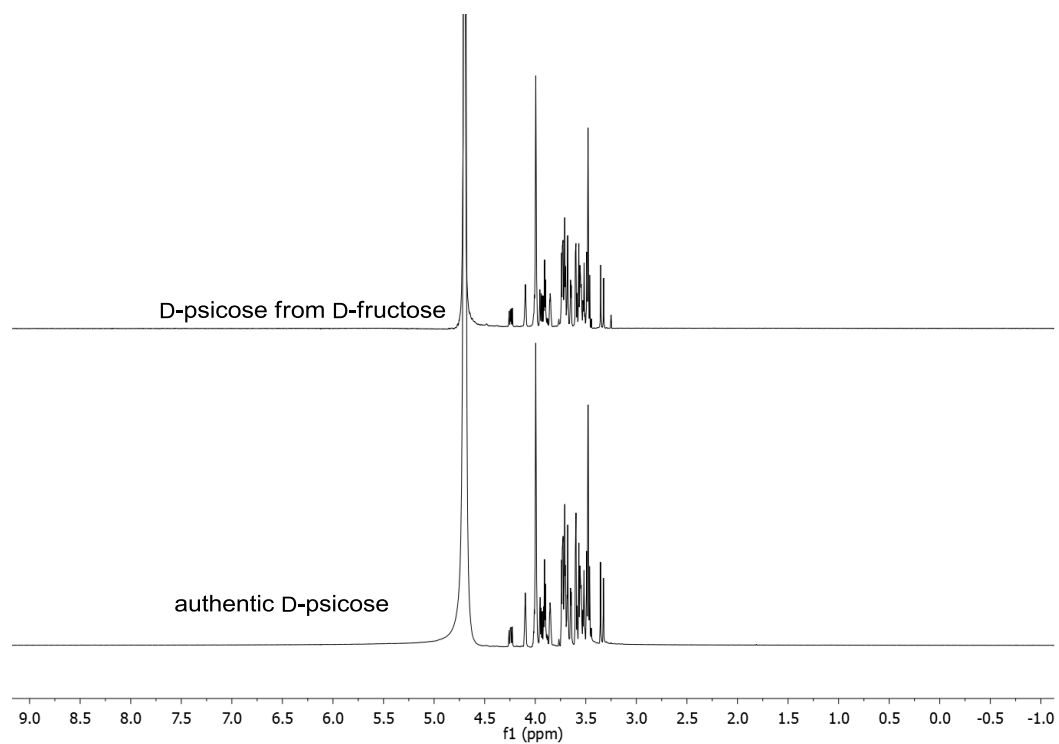


### $^{13}\text{C}$ -NMR of D-ribulose

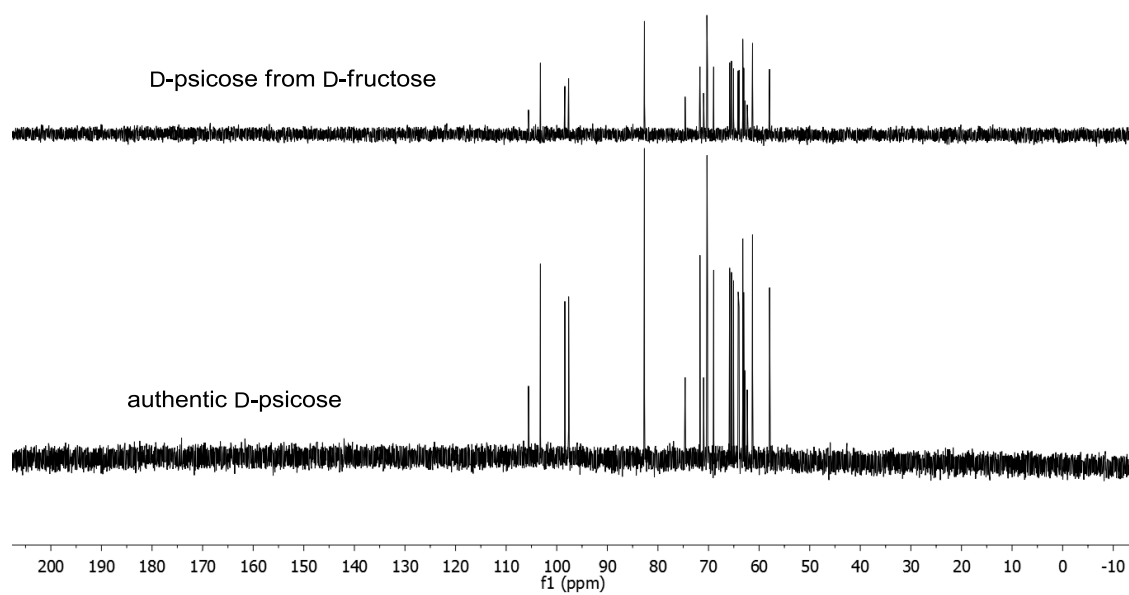


$^1\text{H}$ -NMR of D-sorbose $^{13}\text{C}$ -NMR of D-sorbose

### $^1\text{H-NMR}$ of D-psicose

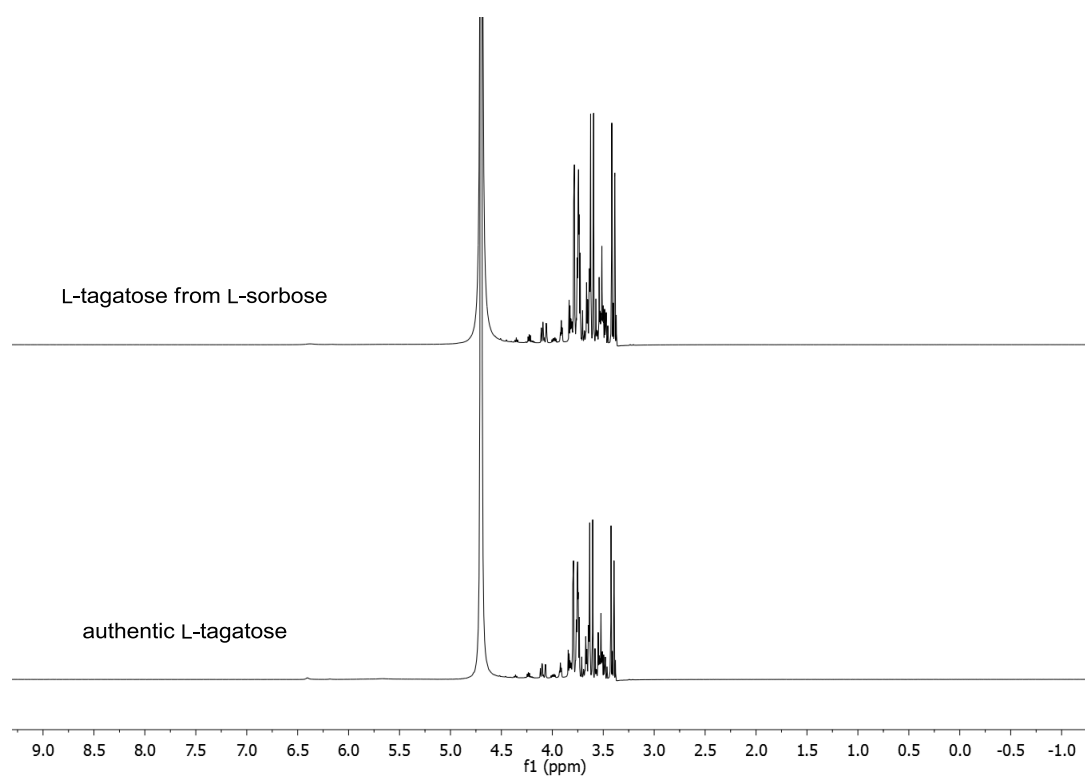


### $^{13}\text{C-NMR}$ of D-psicos

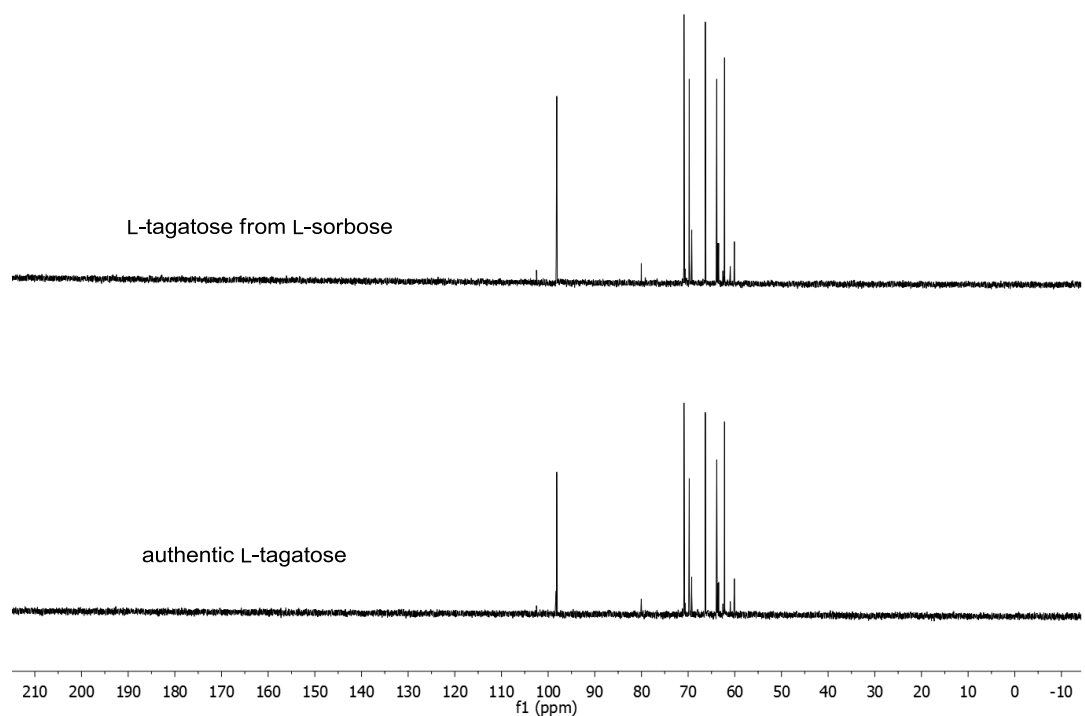




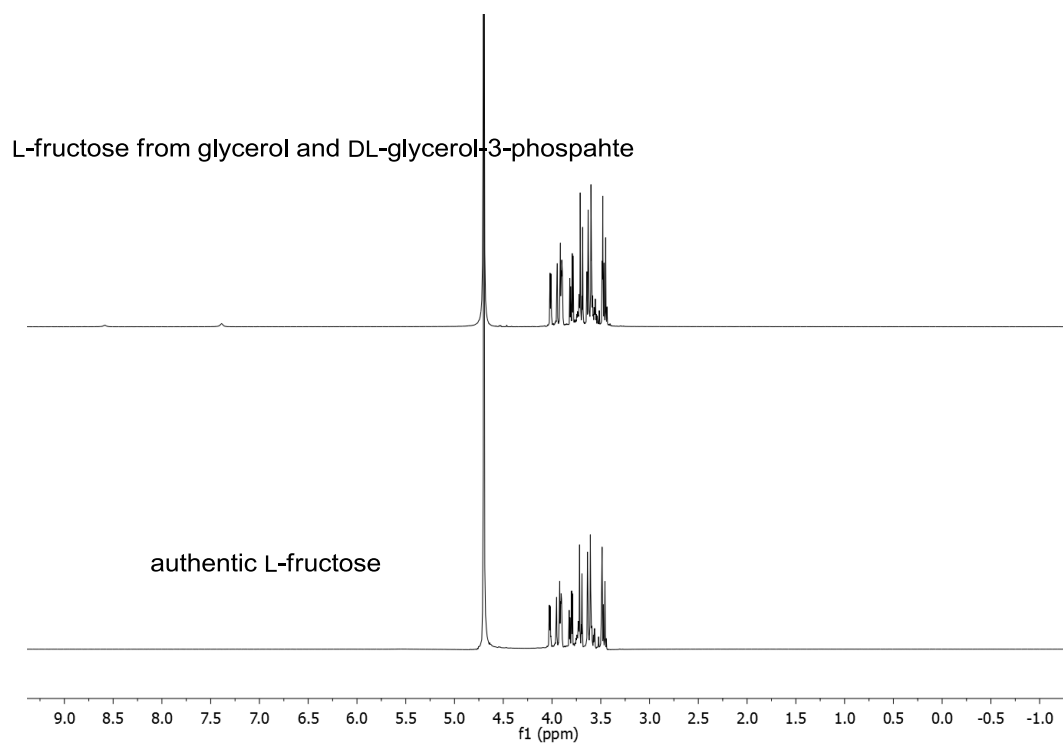
### $^1\text{H-NMR}$ of L-tagatose



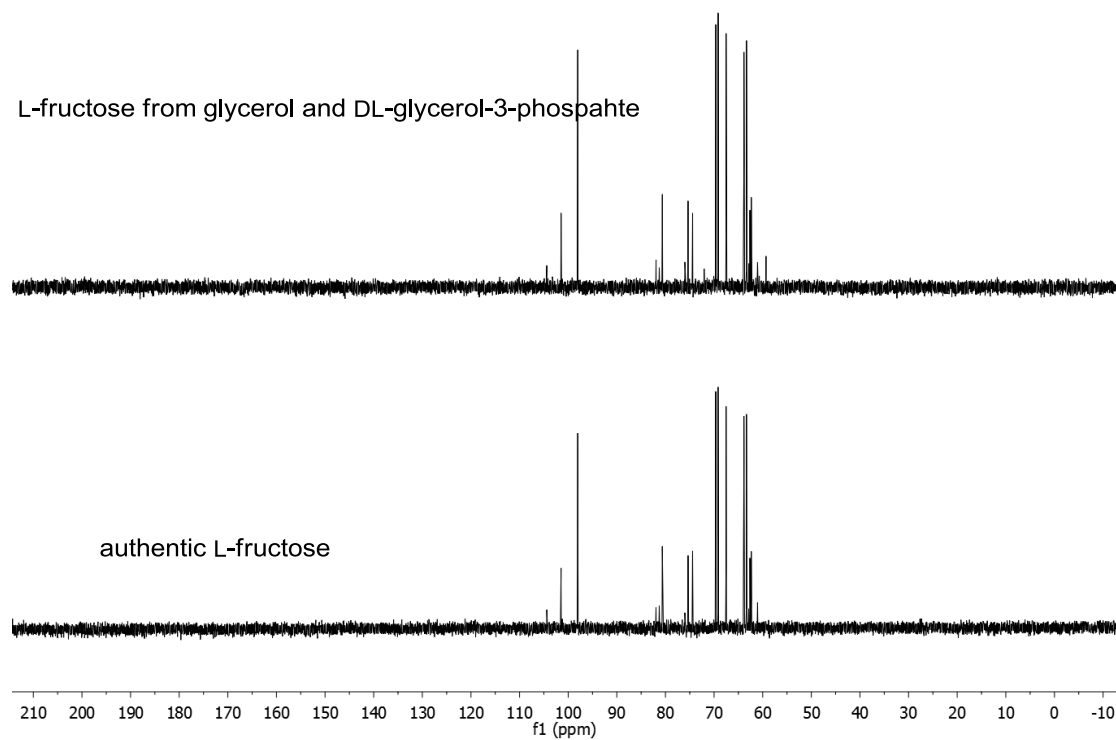
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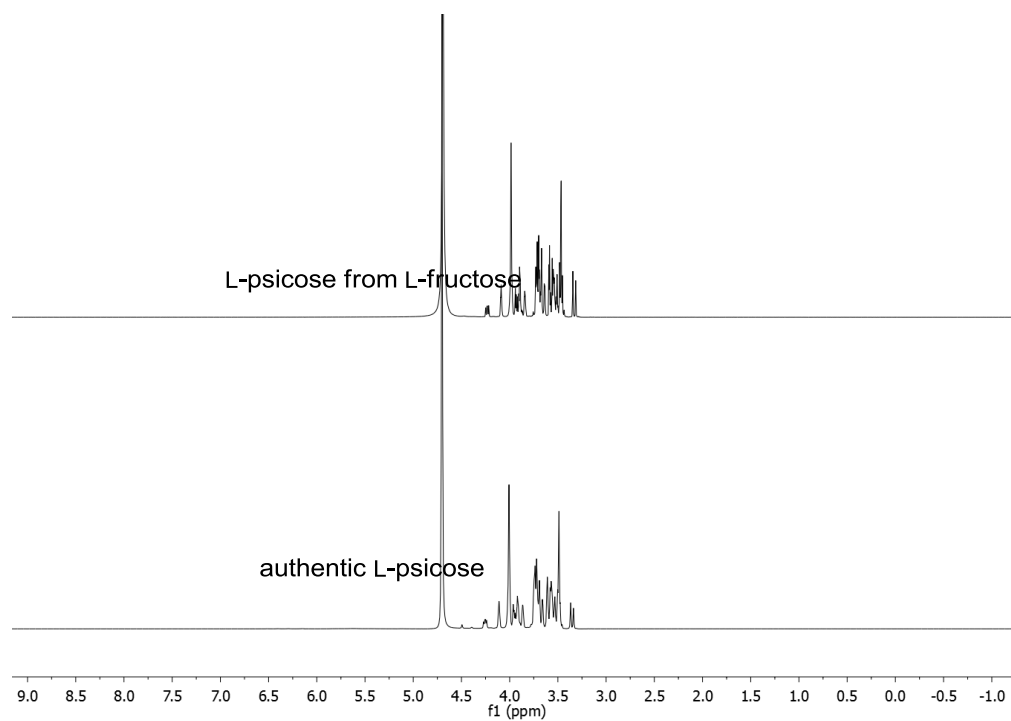
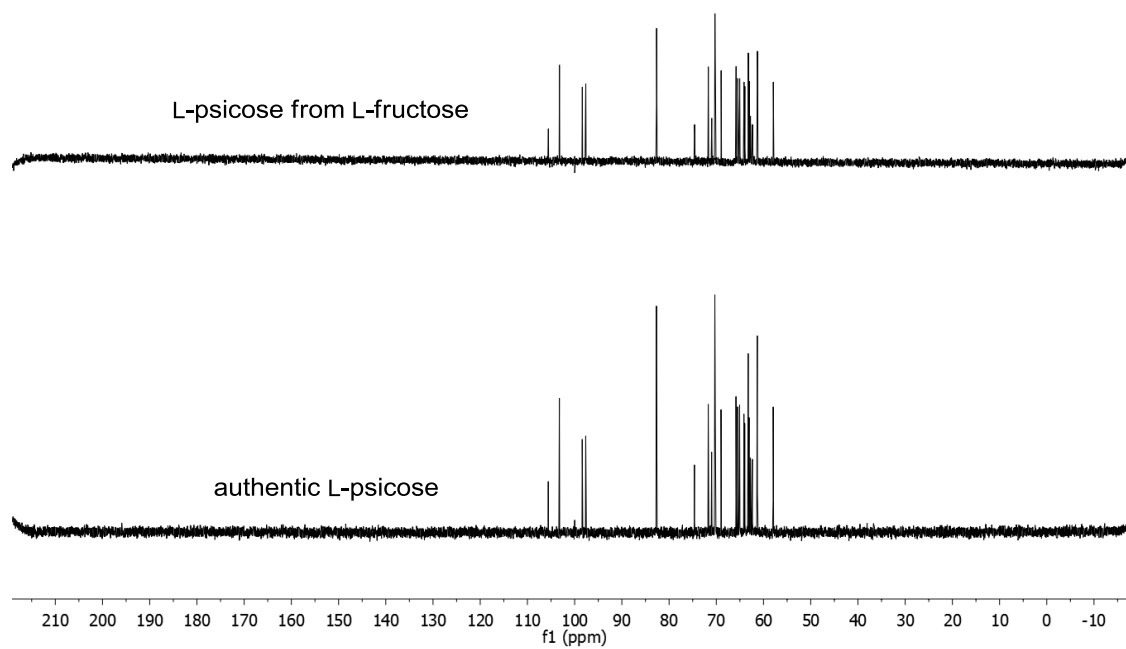


# $^1\text{H-NMR}$ of L-fructose

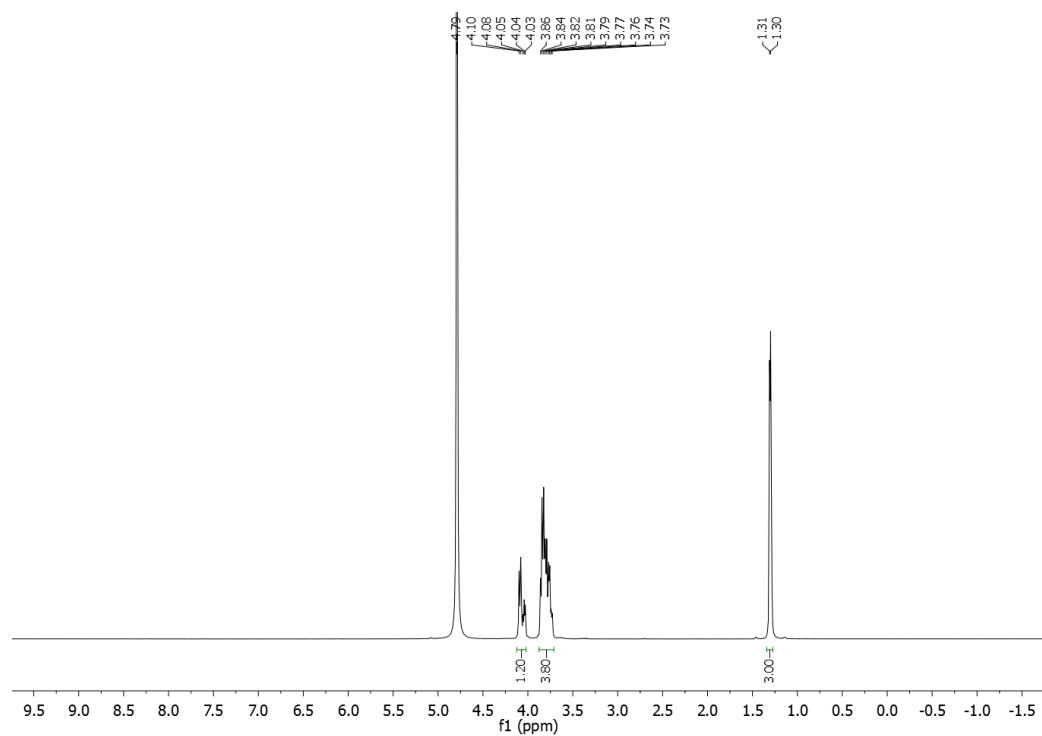


# $^{13}\text{C-NMR}$ of L-fruct

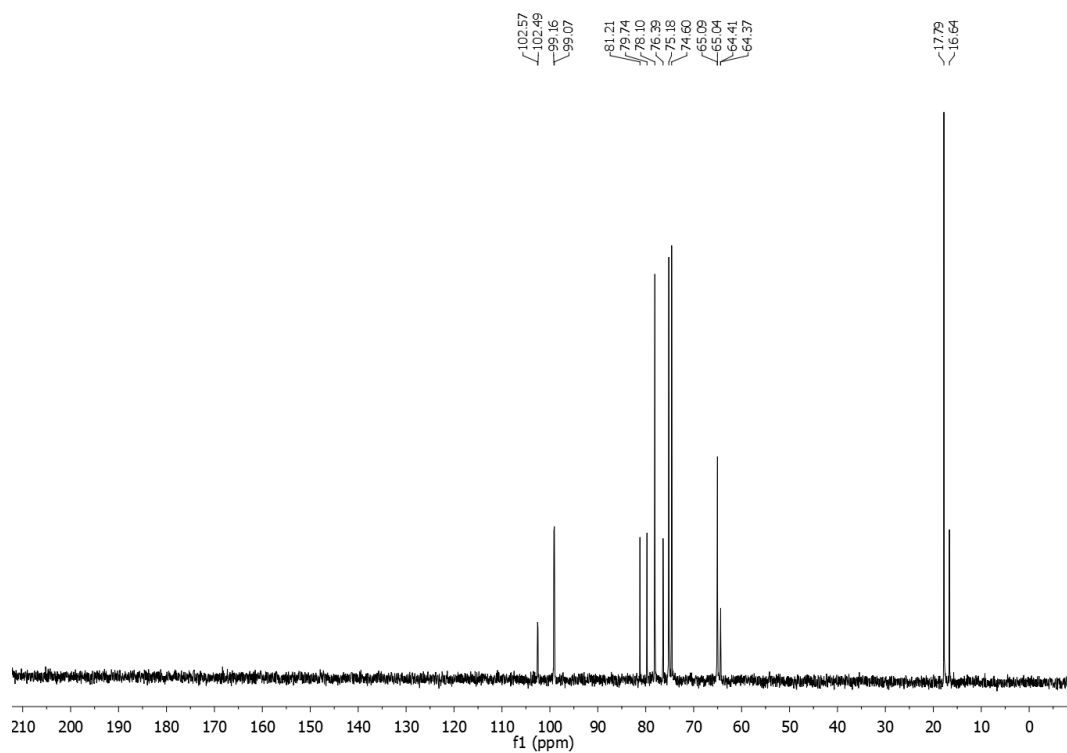


$^1\text{H-NMR}$  of L-psicose $^{13}\text{C-NMR}$  of L-psicose

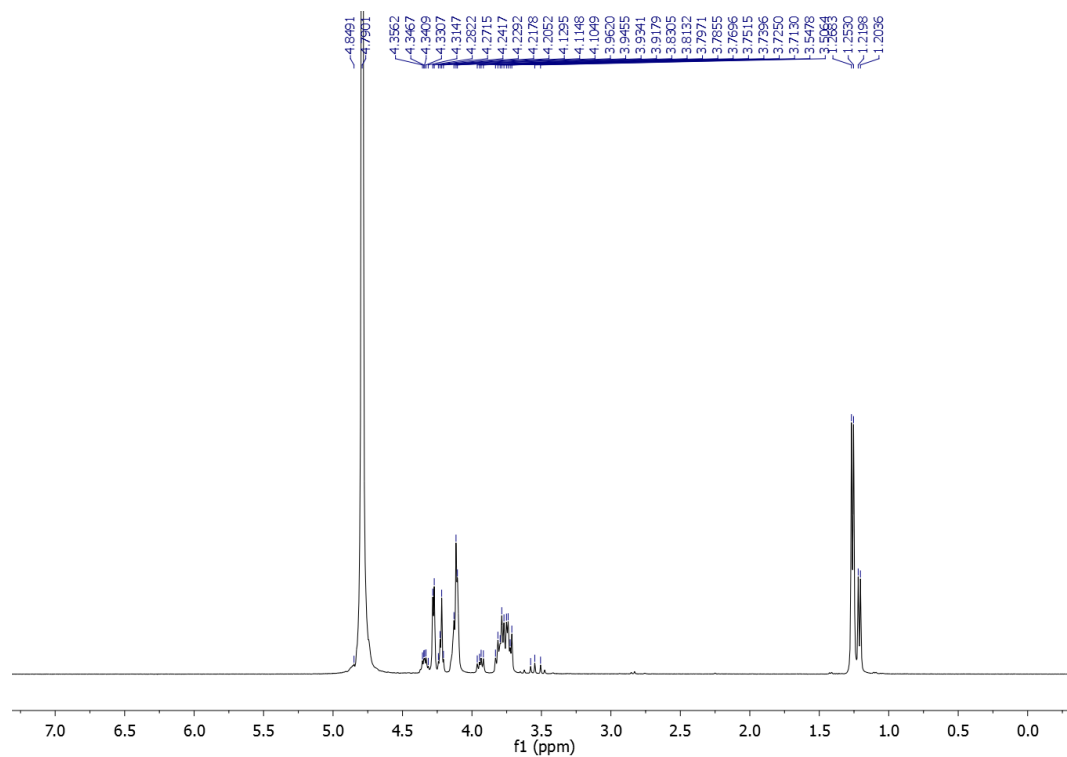
### $^1\text{H-NMR}$ of L-rhamnulose 1-phosphate



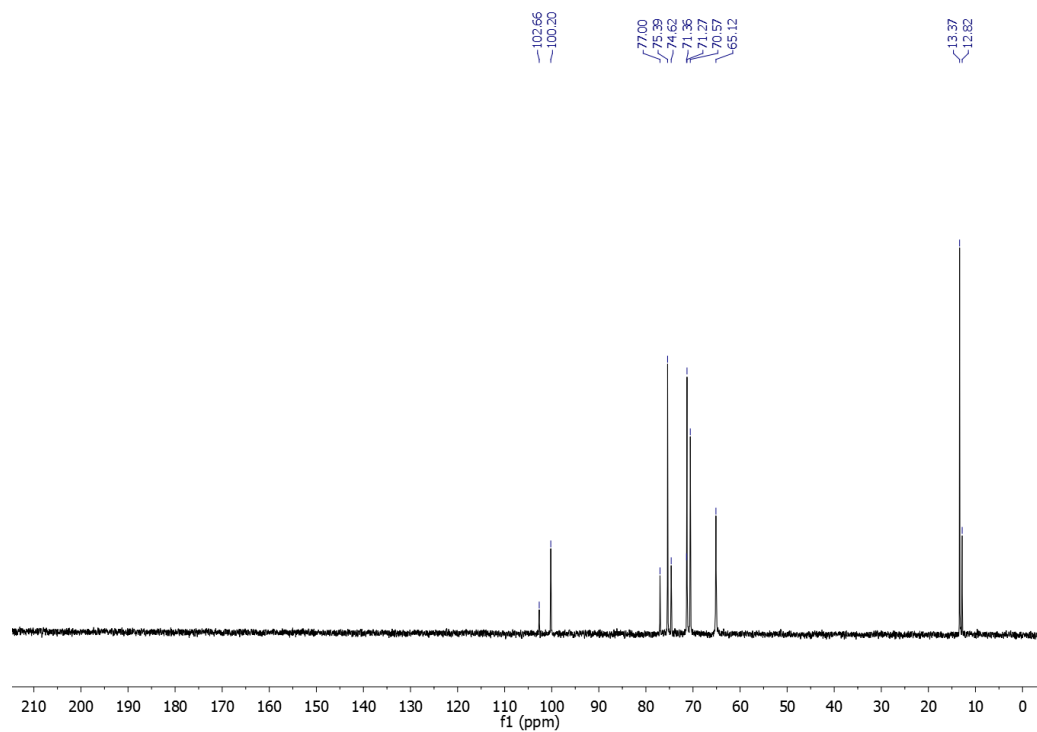
### $^{13}\text{C-NMR}$ of L-rhamnulose 1-phosphate



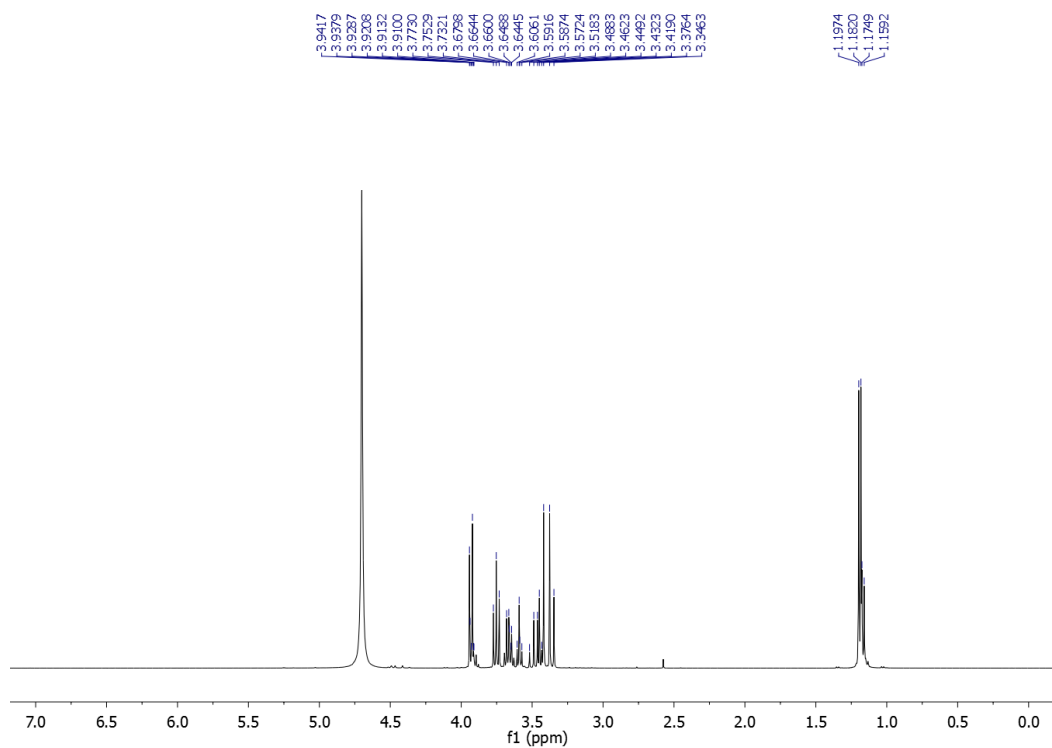
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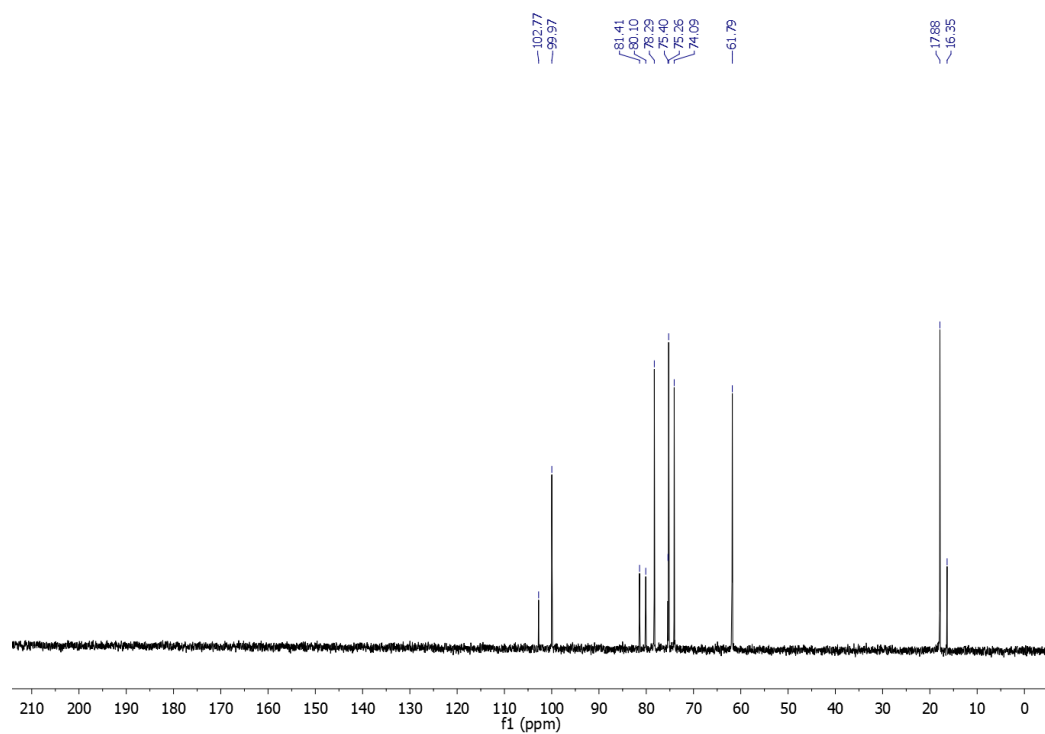
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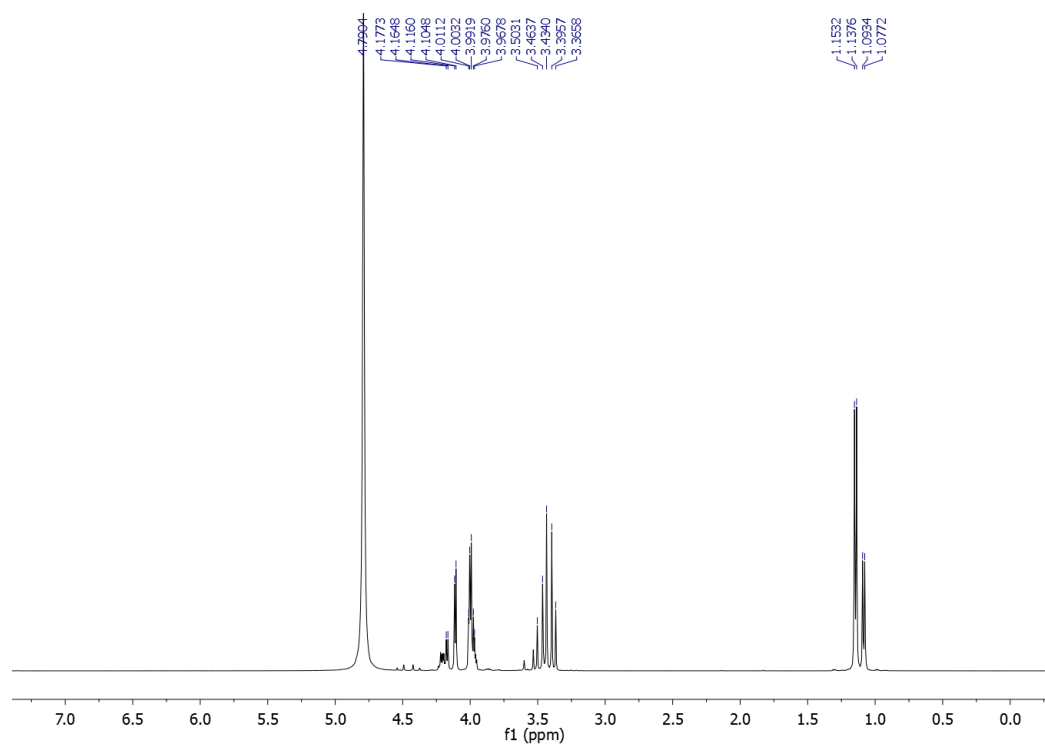
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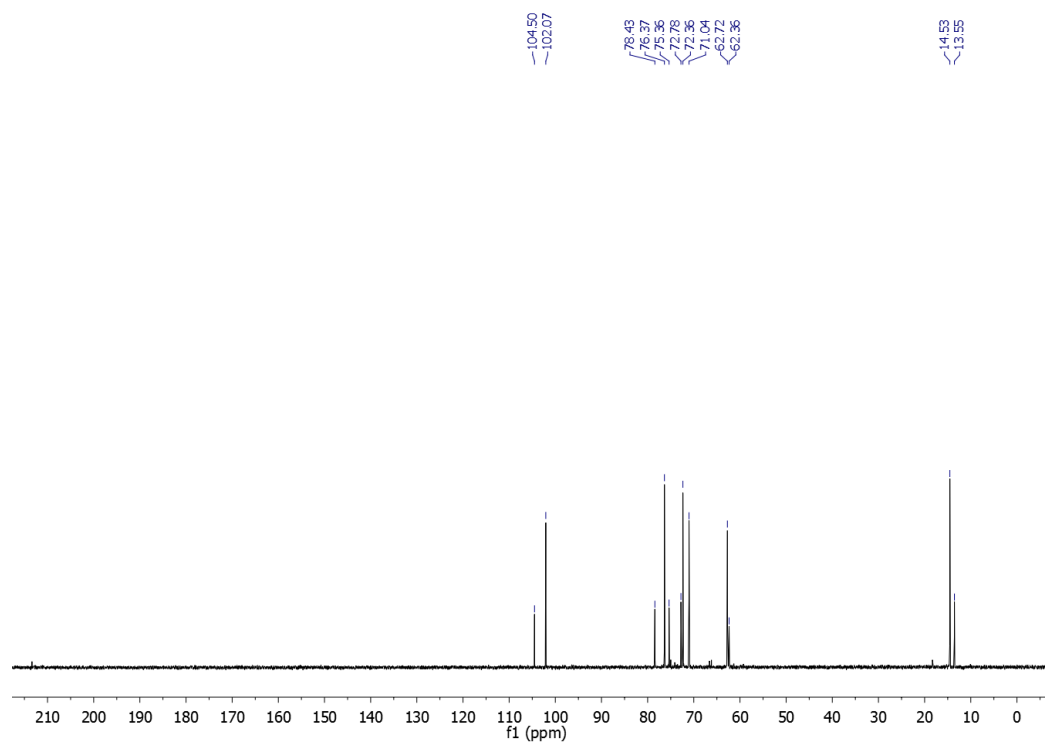
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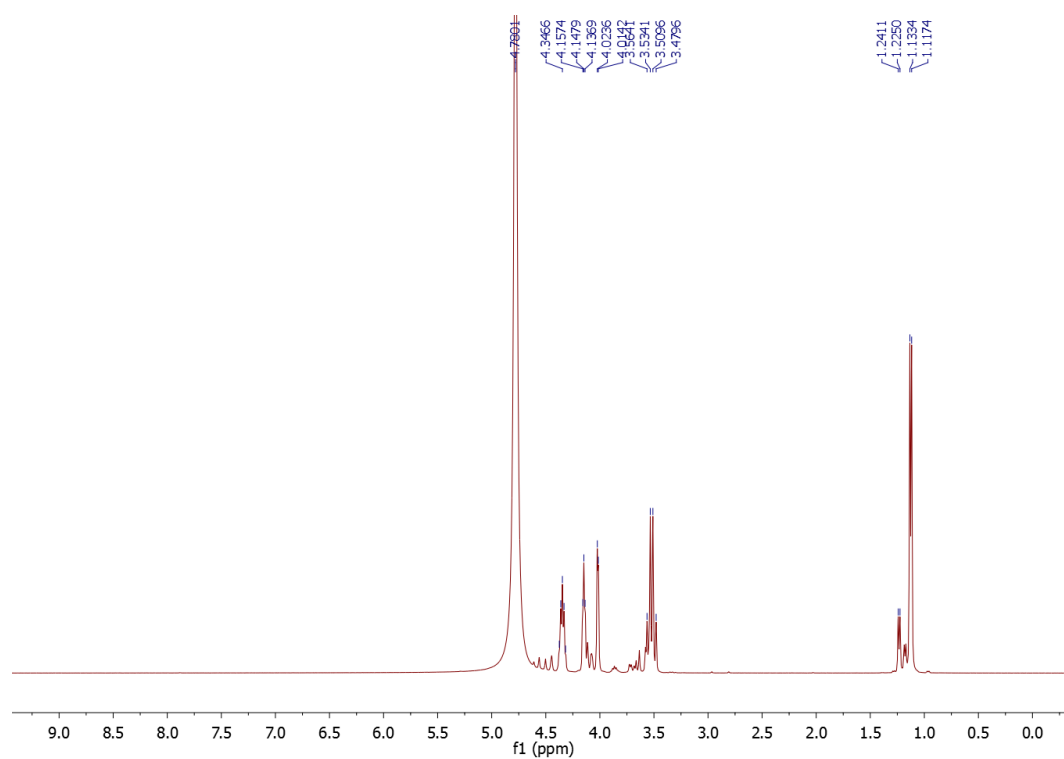
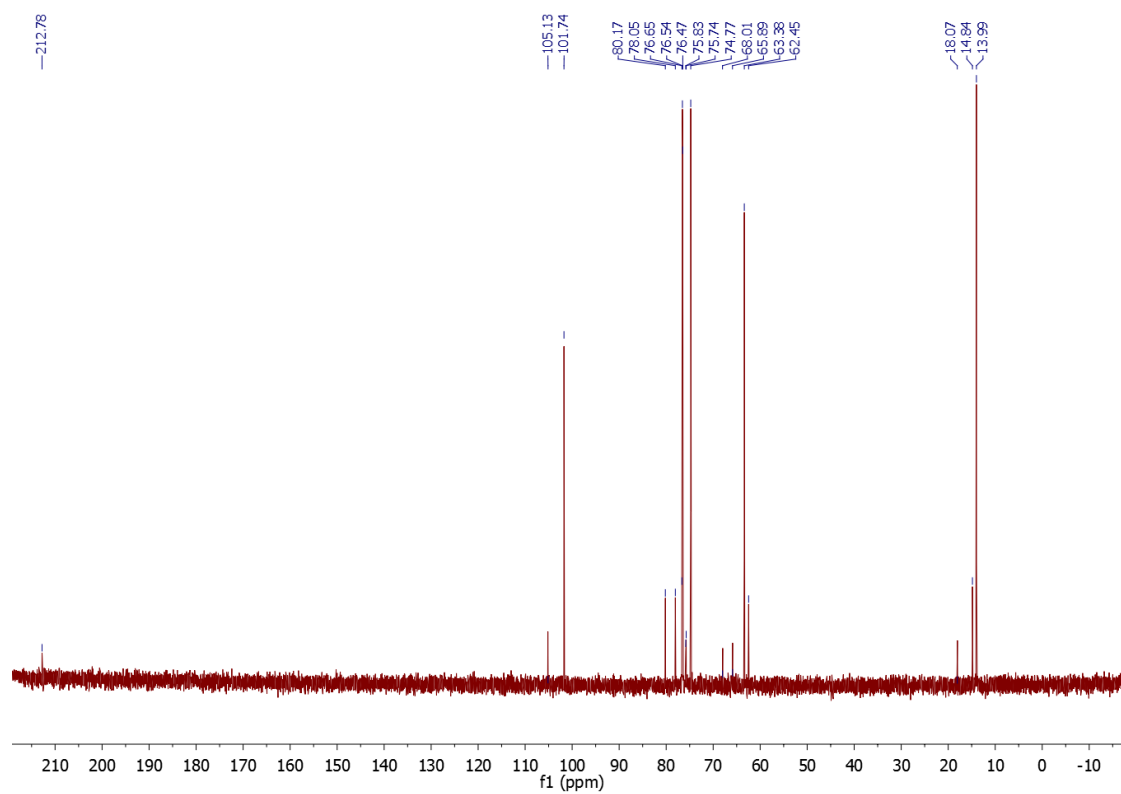


# $^1\text{H}$ -NMR of L-fuculose

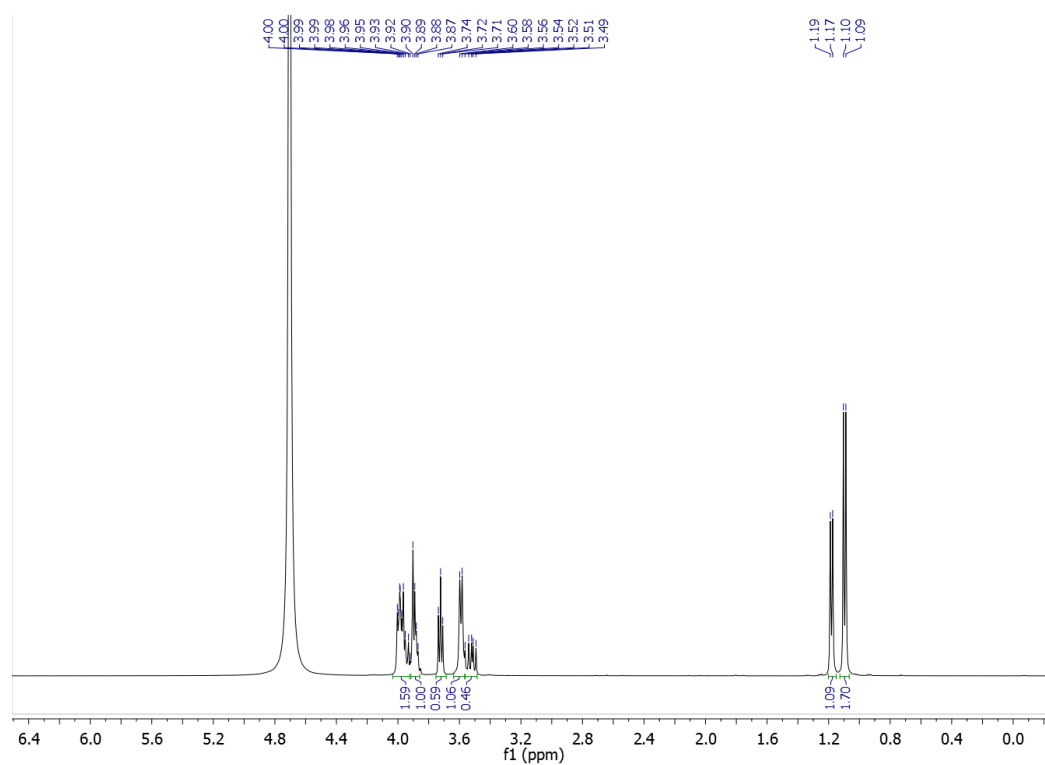
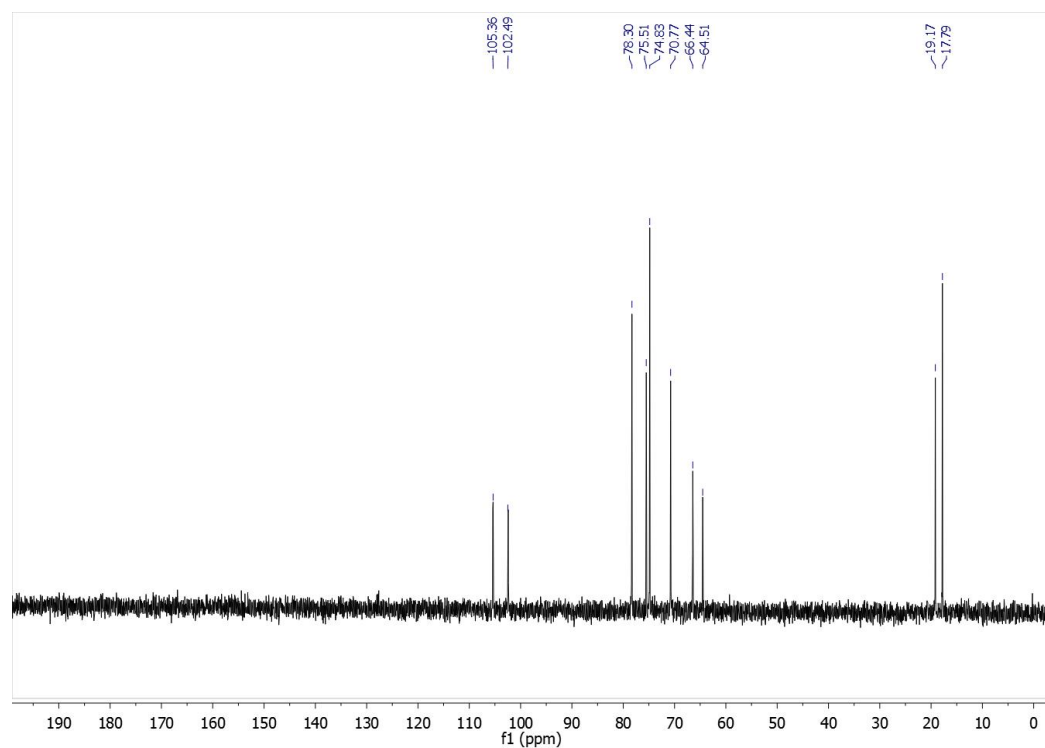


# $^{13}\text{C}$ -NMR of L-fuculose

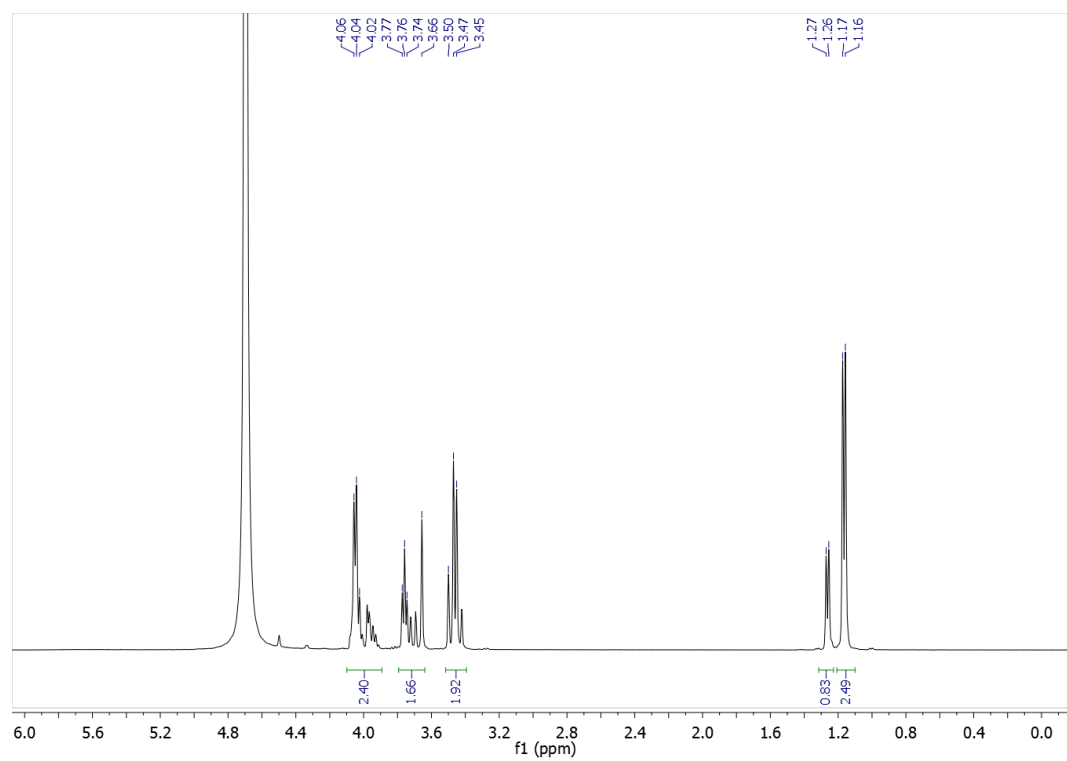


$^1\text{H-NMR}$  of 6-deoxy-L-sorbose $^{13}\text{C-NMR}$  of 6-deoxy-L-sorbose

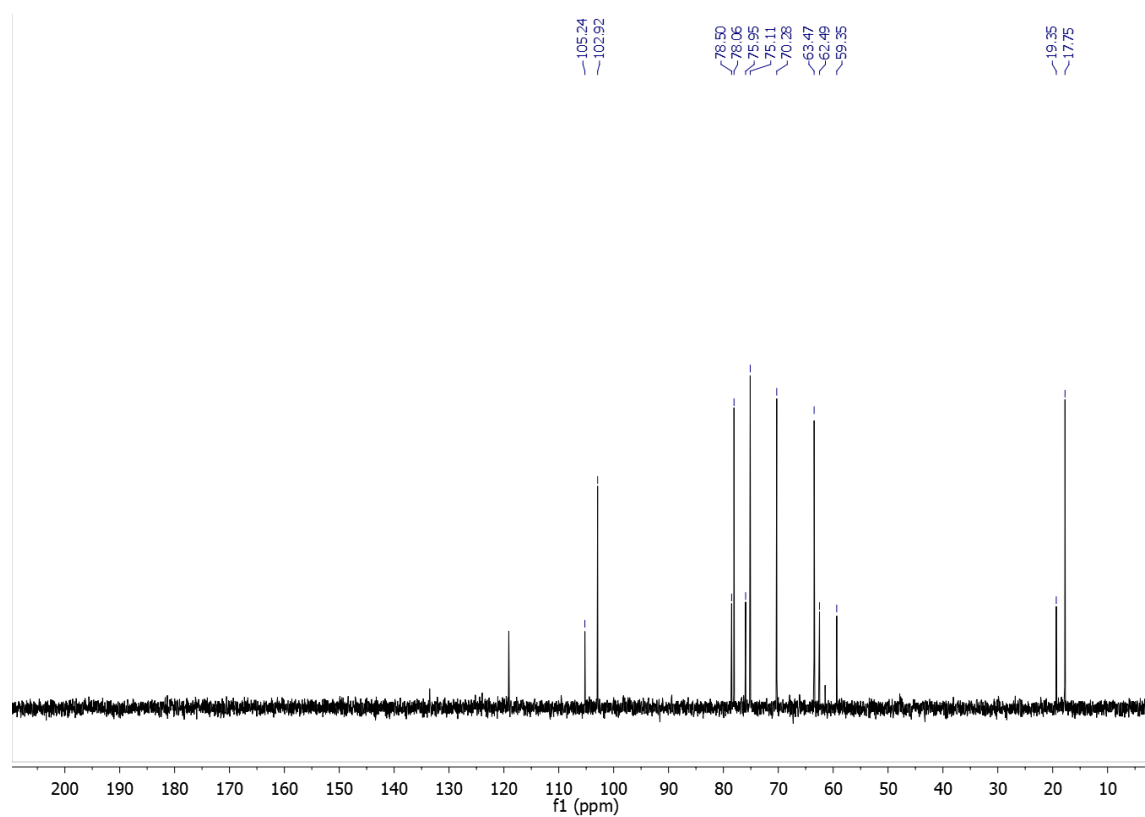


$^1\text{H-NMR}$  of 6-deoxy-L-psicose-1-phosphate $^{13}\text{C-NMR}$  of 6-deoxy-L-psicose-1-phosphate

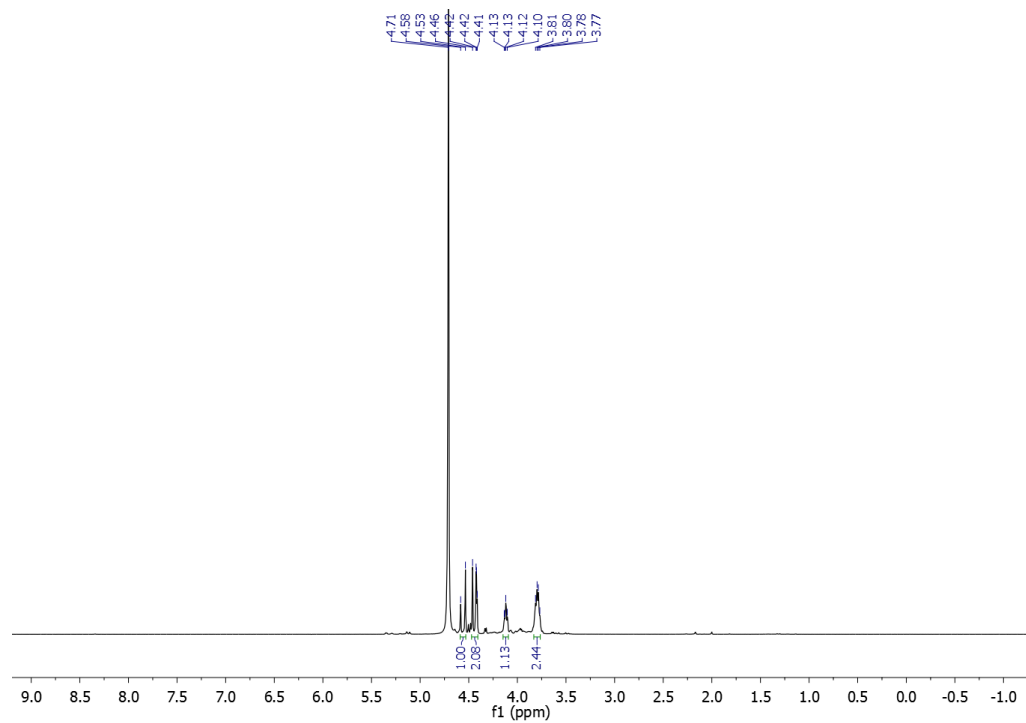
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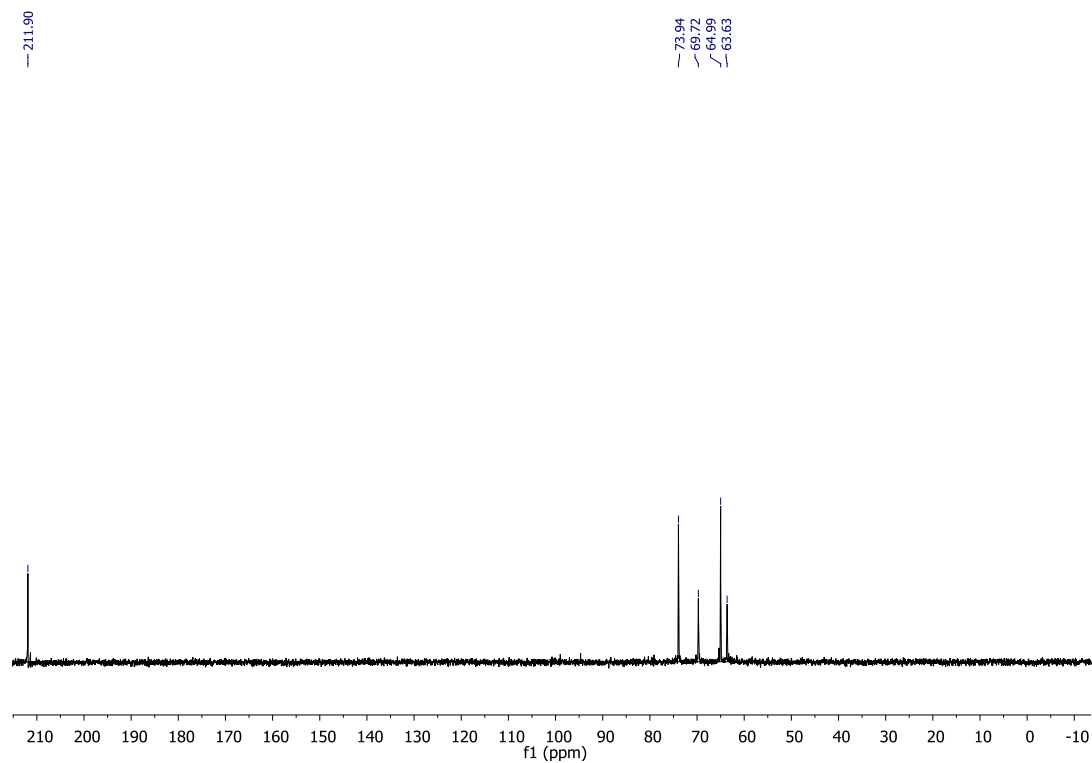
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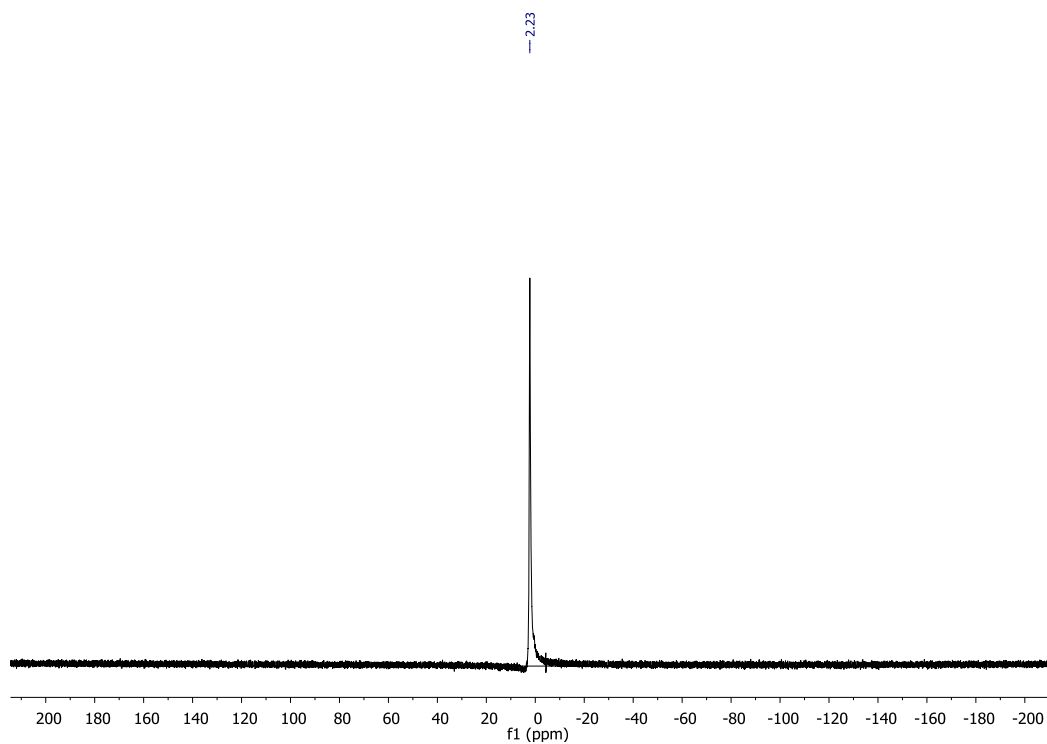
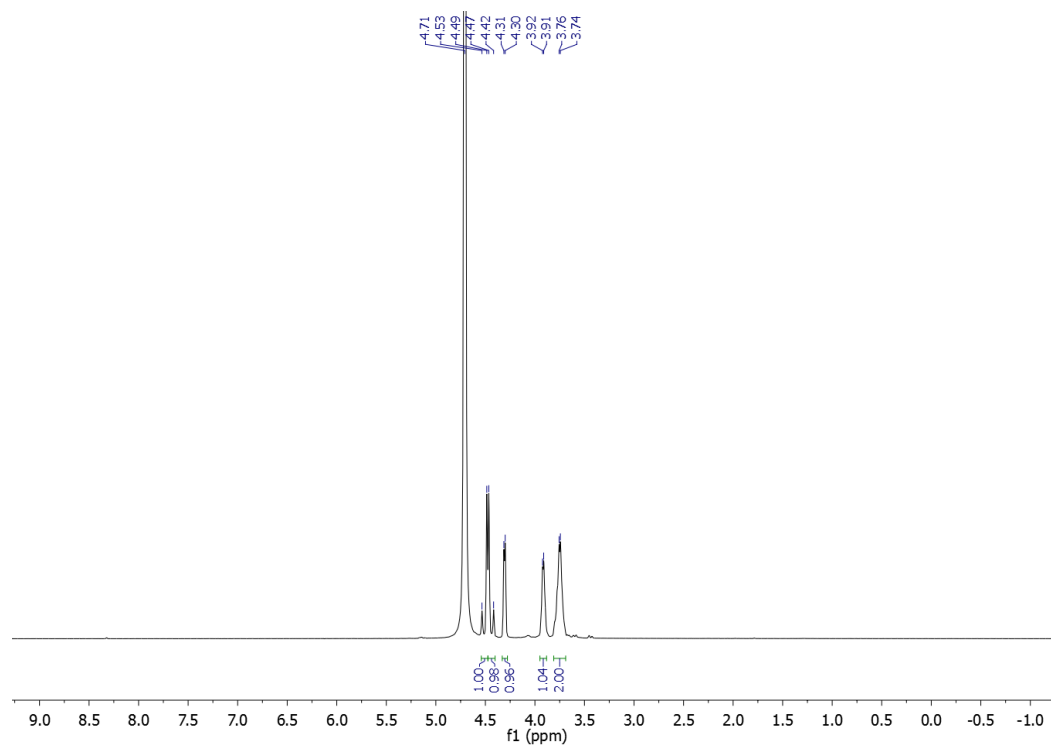


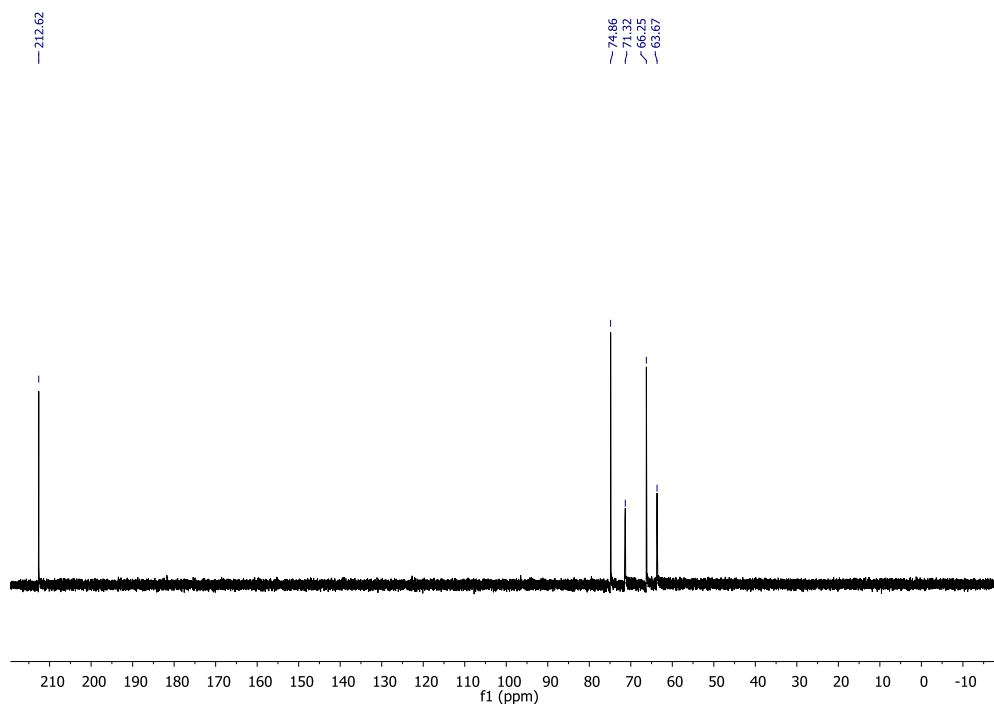
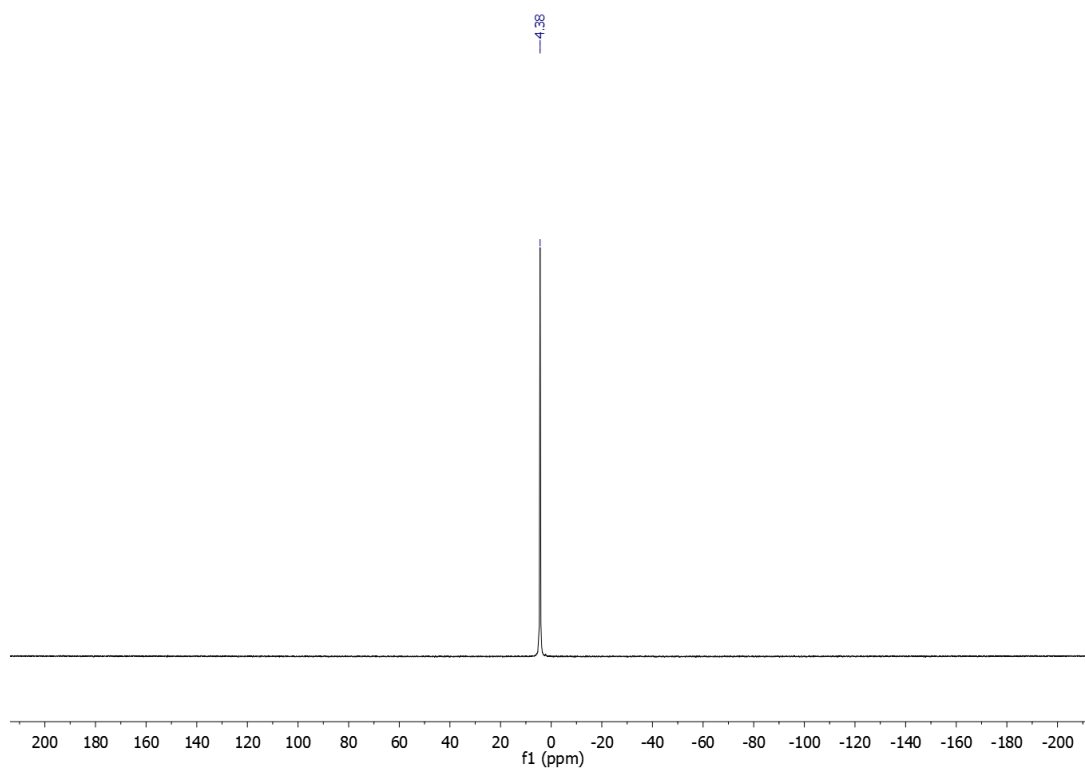
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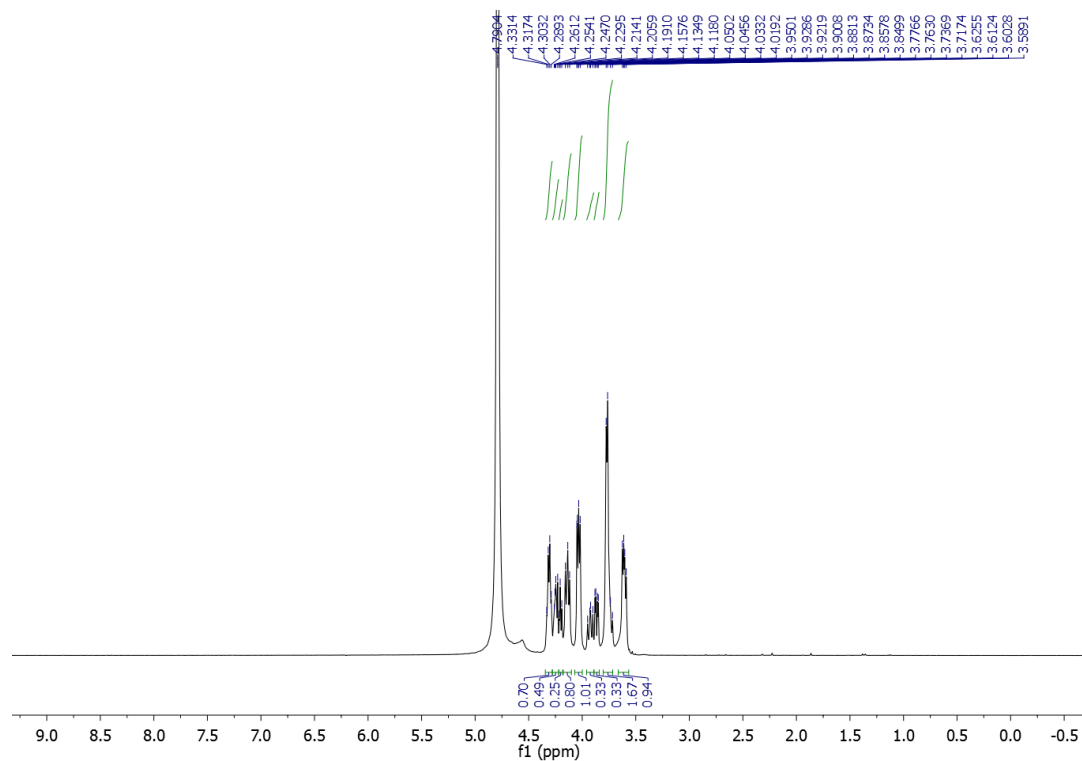
### $^{13}\text{C-NMR}$ of D-xylulose 5-phosphate



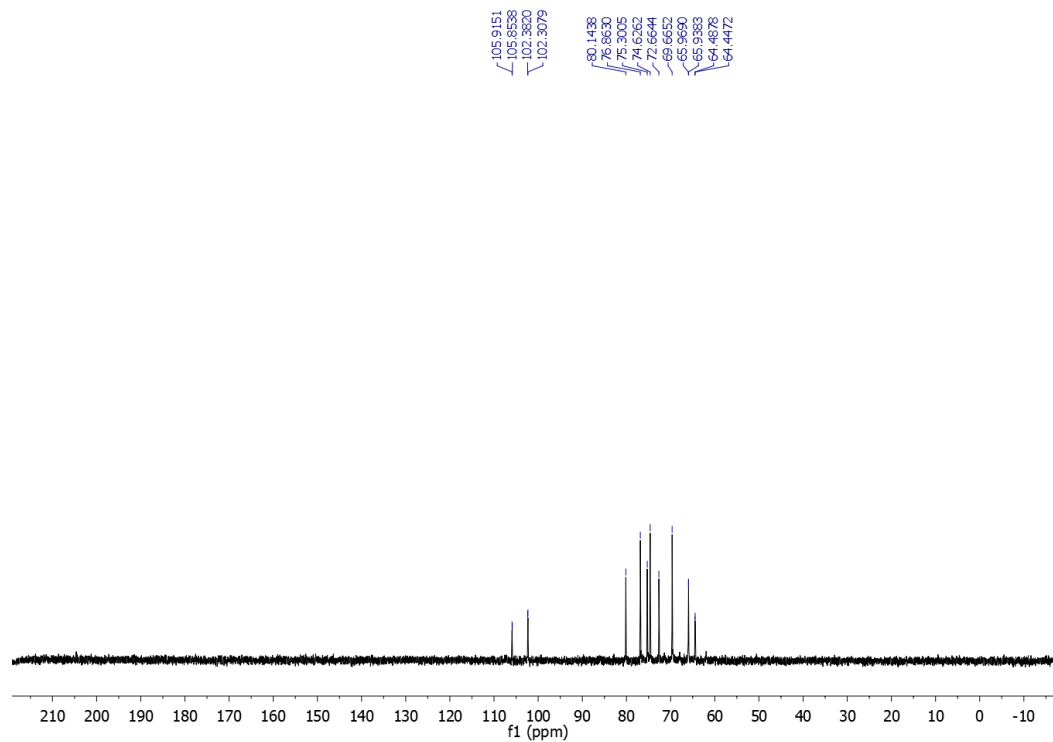
$^{31}\text{P}$ -NMR of D-xylulose 5-phosphate $^1\text{H}$ -NMR of L-ribulose 5-phosphate

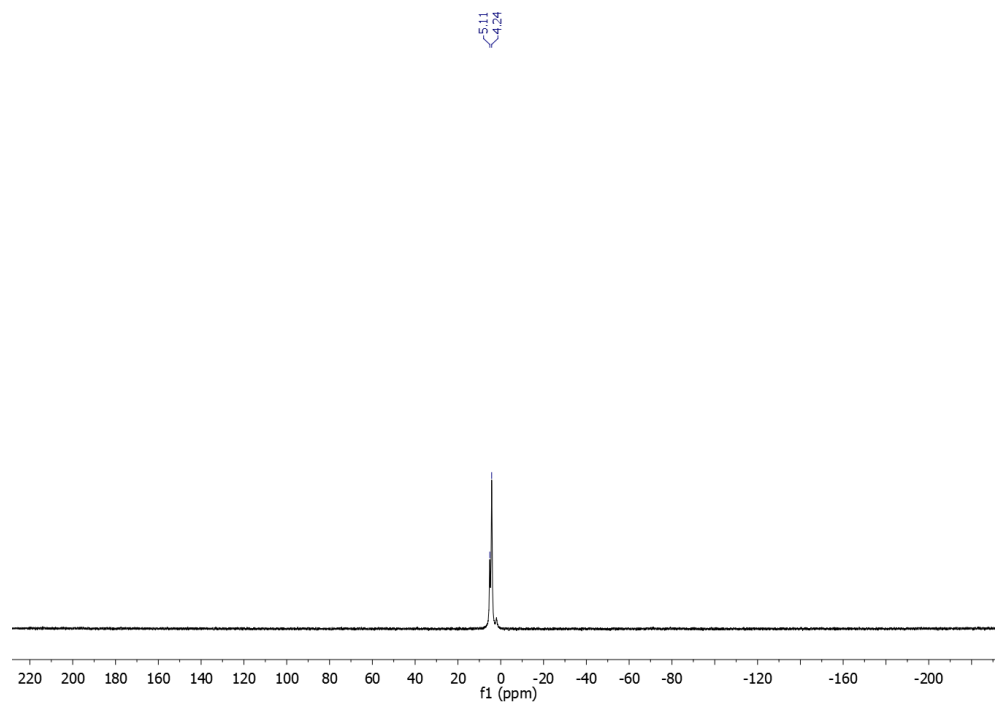
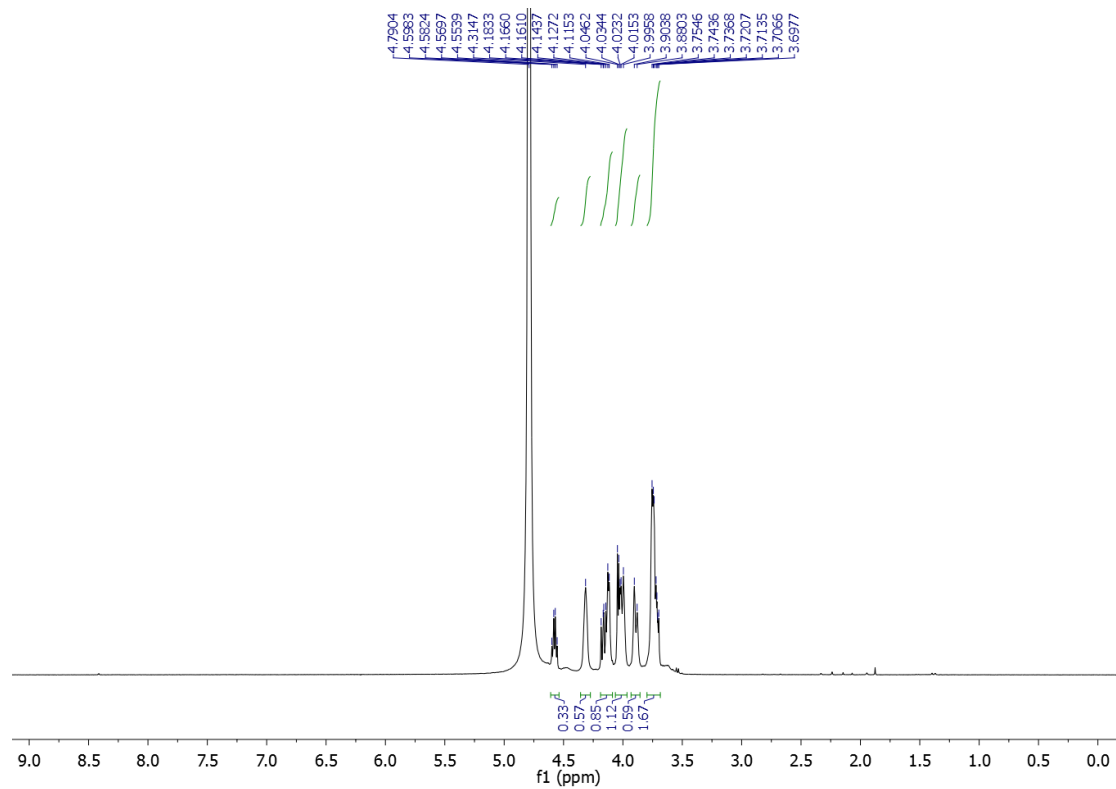
$^{13}\text{C}$ -NMR of L-ribulose 5-phosphate $^{31}\text{P}$ -NMR of L-ribulose 5-phosphate

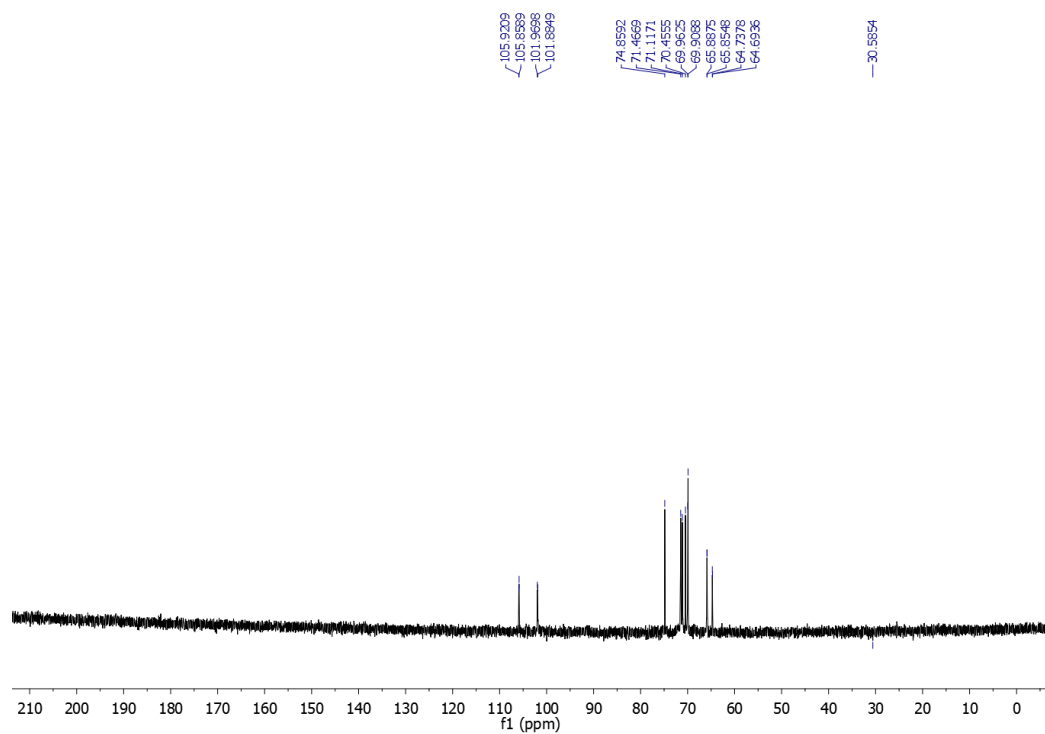
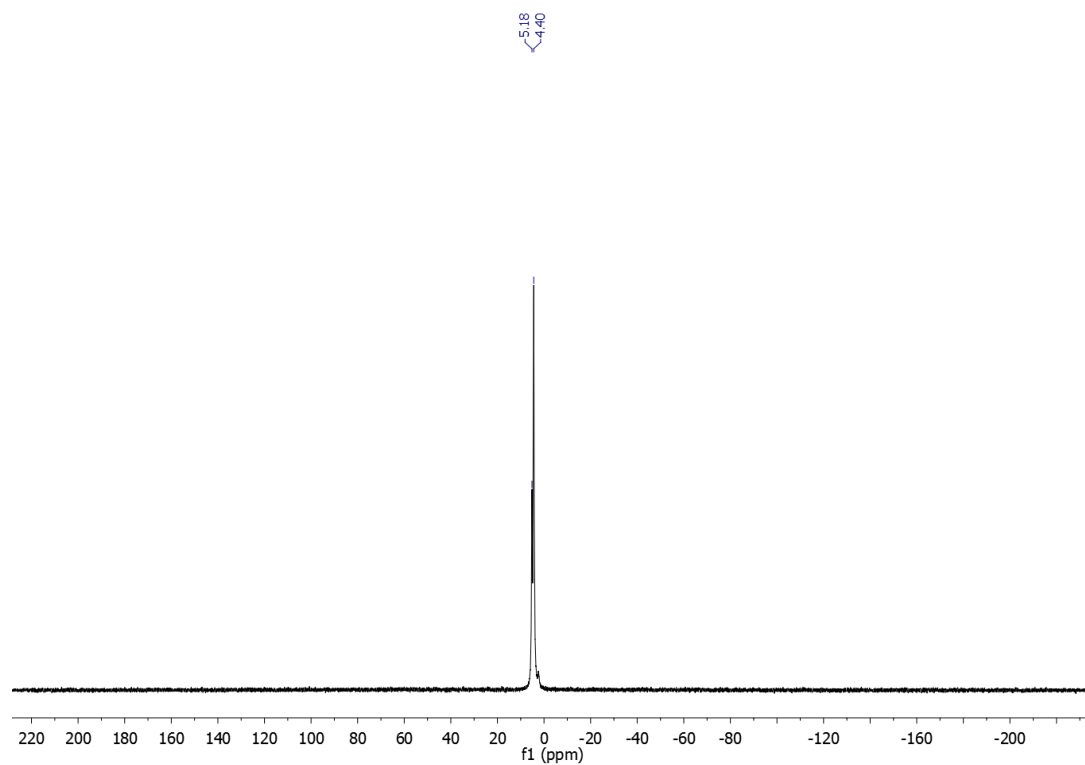
# $^1\text{H-NMR}$ of D-xylulose 1-phosphate



# $^{13}\text{C-NMR}$ of D-xylulose 1-phosphate

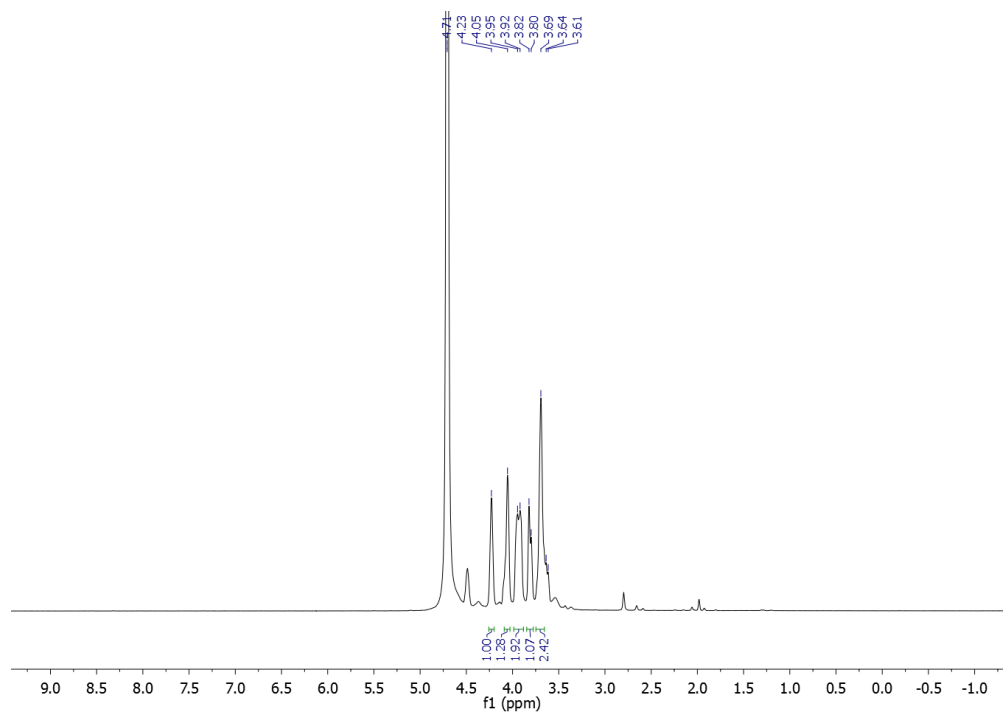


$^{31}\text{P}$ -NMR of D-xylulose 1-phosphate $^1\text{H}$ -NMR of L-ribulose 1-phosphate

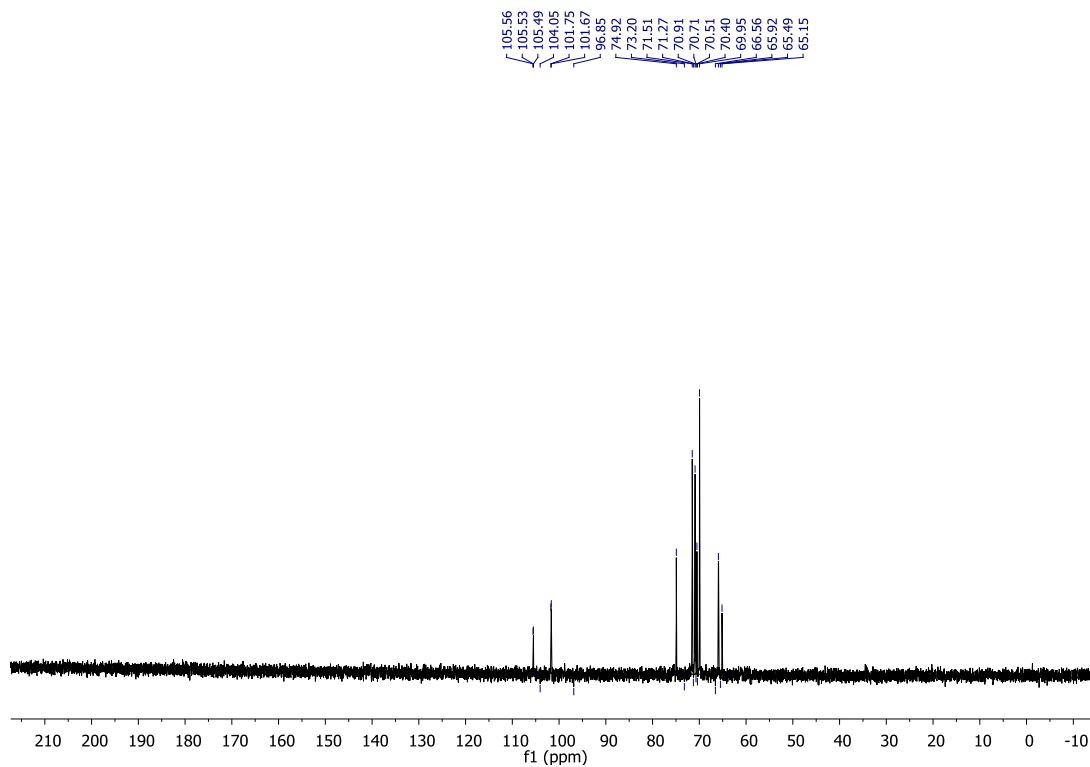
$^{13}\text{C}$ -NMR of L-ribulose 1-phosphate $^{31}\text{P}$ -NMR of L-ribulose 1-phosphate

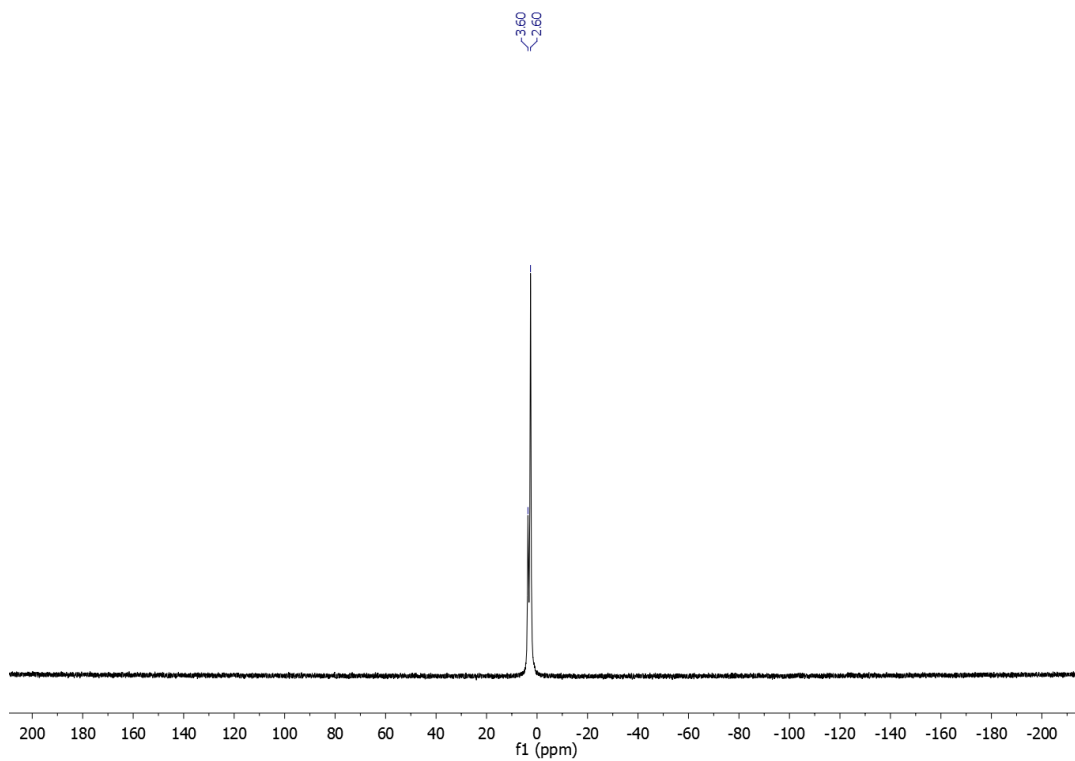
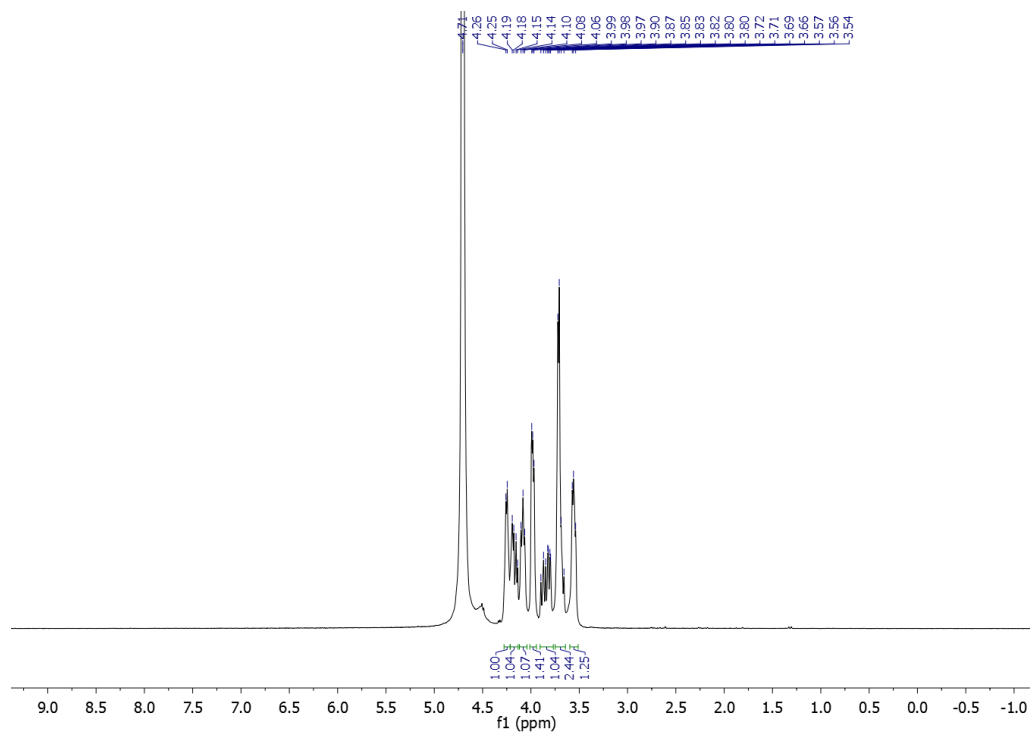


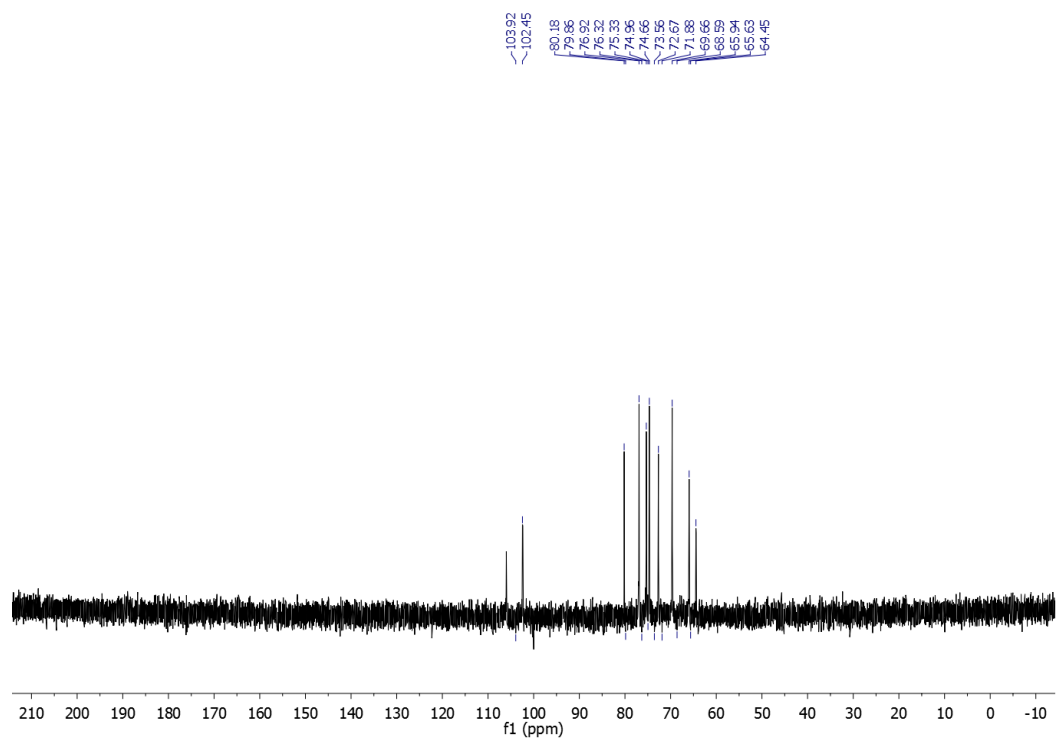
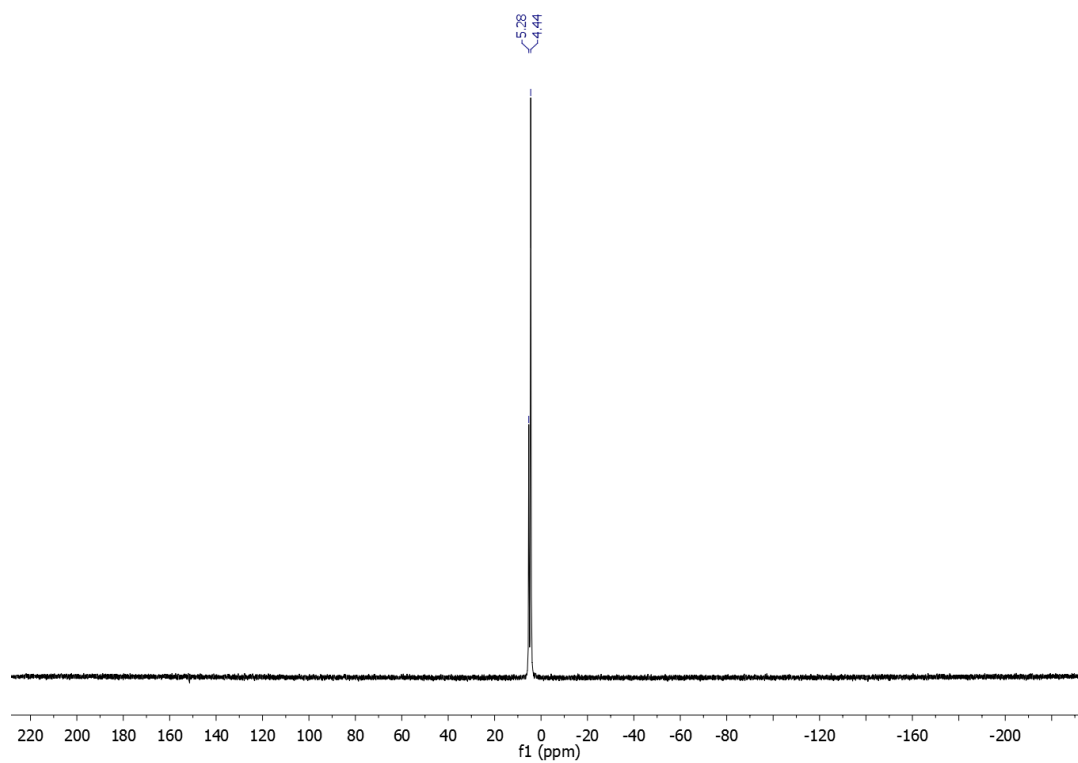
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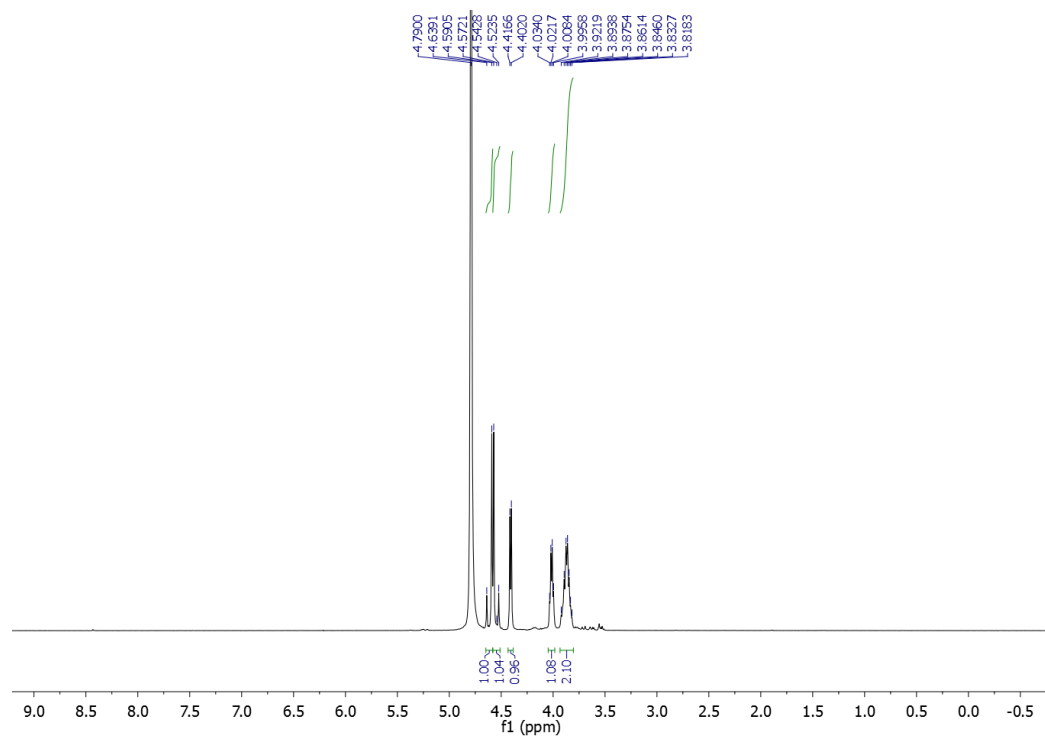
# $^{13}\text{C}$ -NMR of D-ribulose 1-phosphate



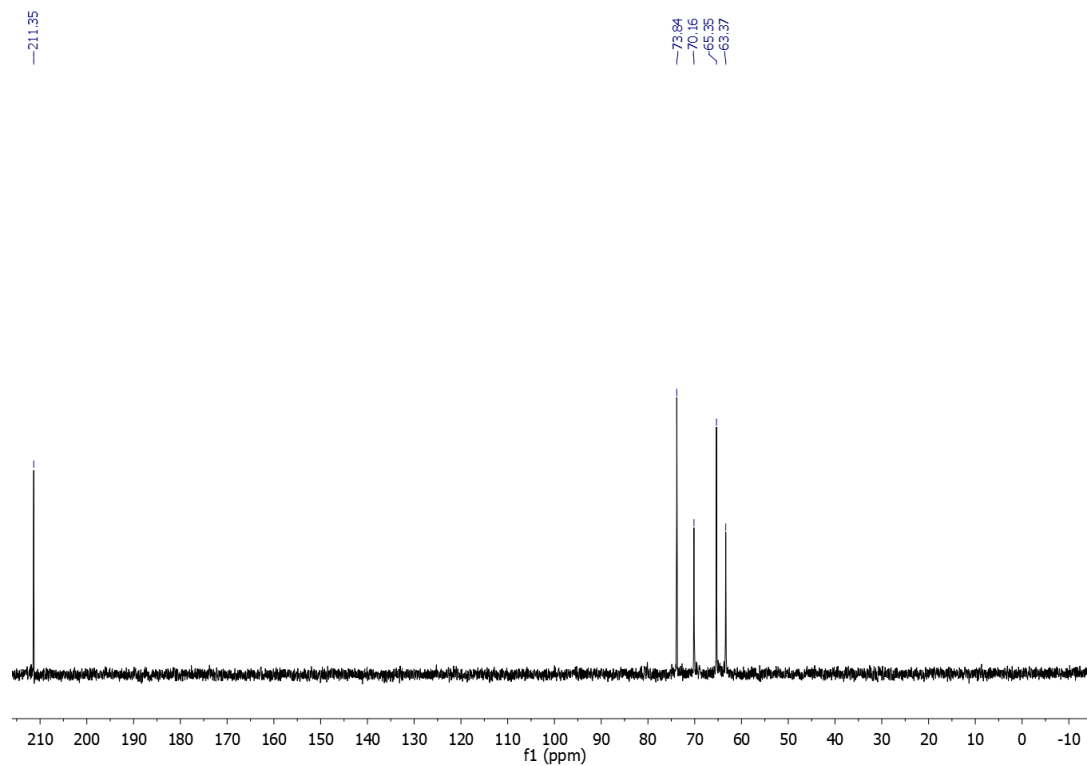
$^{31}\text{P}$ -NMR of D-ribose 1-phosphate $^1\text{H}$ -NMR of L-xylulose 1-phosphate

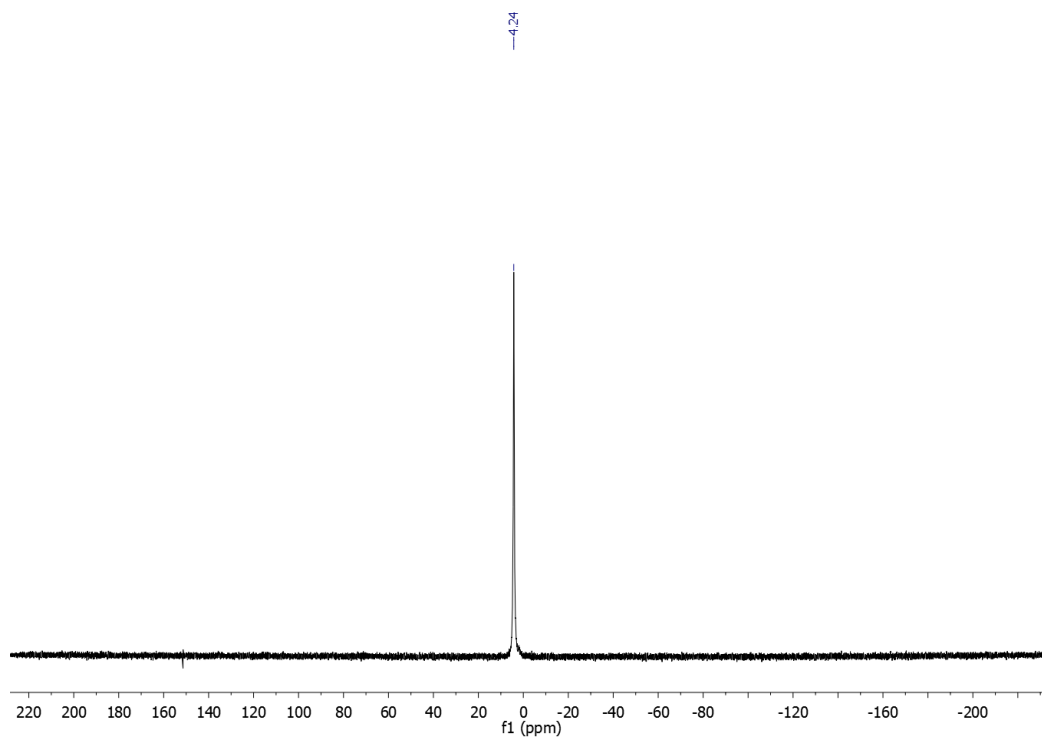
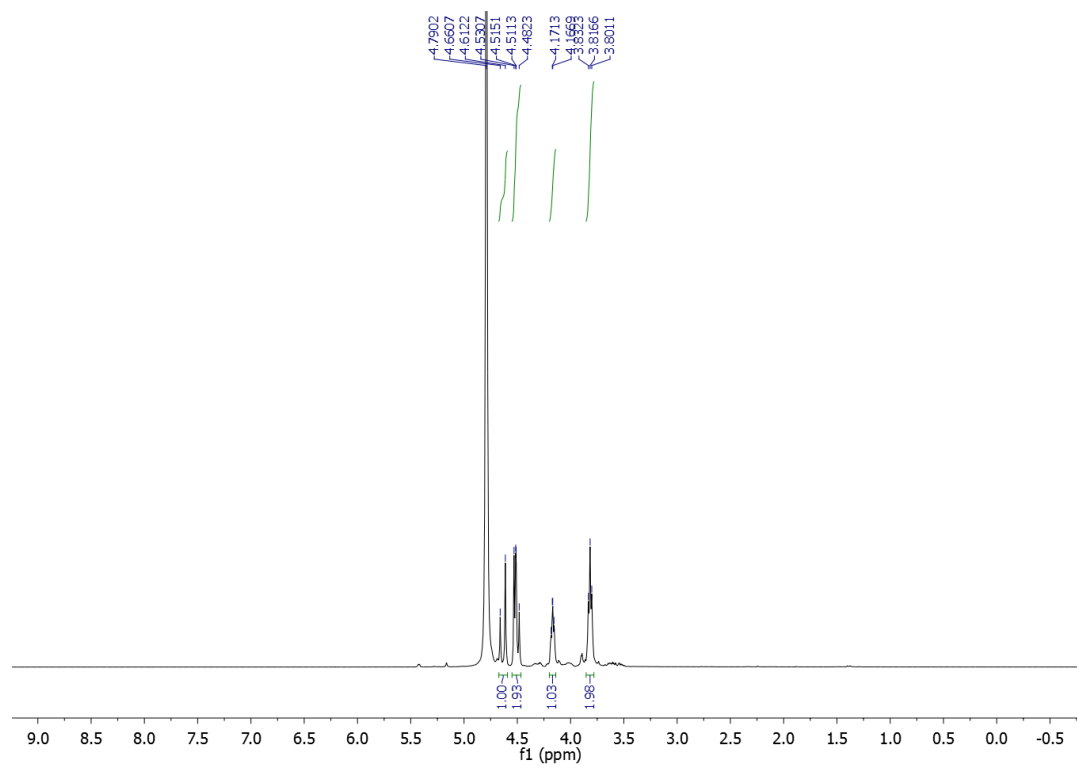
$^{13}\text{C}$ -NMR of L-xylulose 1-phosphate $^{31}\text{P}$ -NMR of L-xylulose 1-phosphate

### $^1\text{H-NMR}$ of D-ribulose 5-phosphate

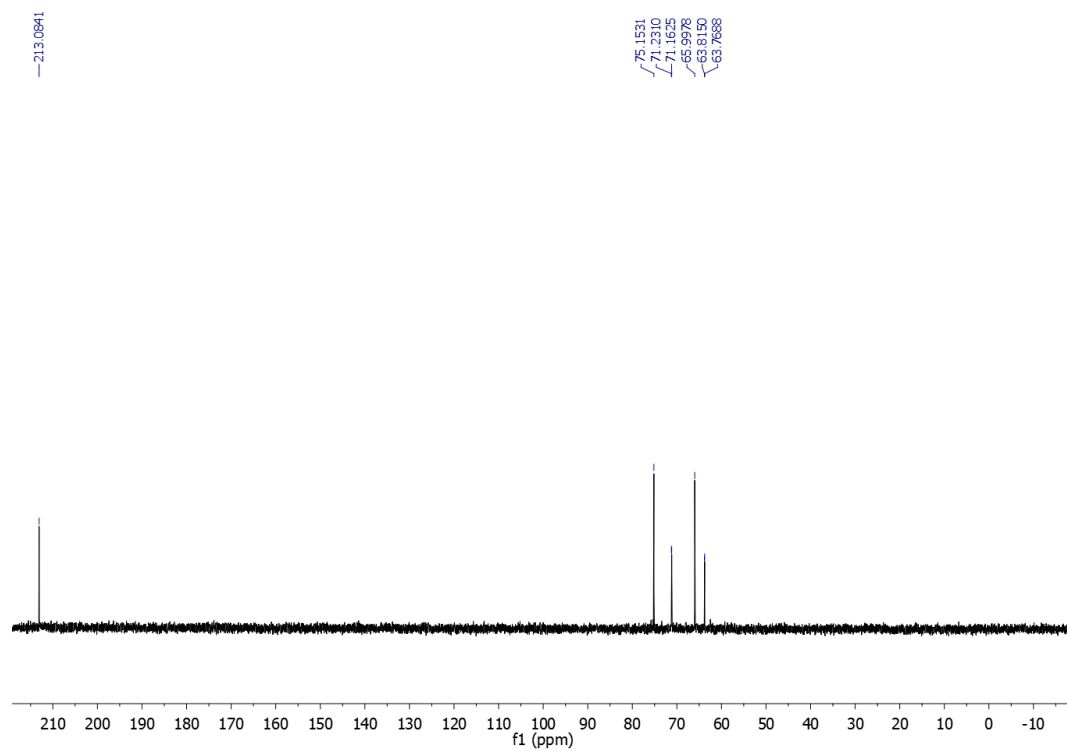


### $^{13}\text{C-NMR}$ of D-ribulose 5-phosphate

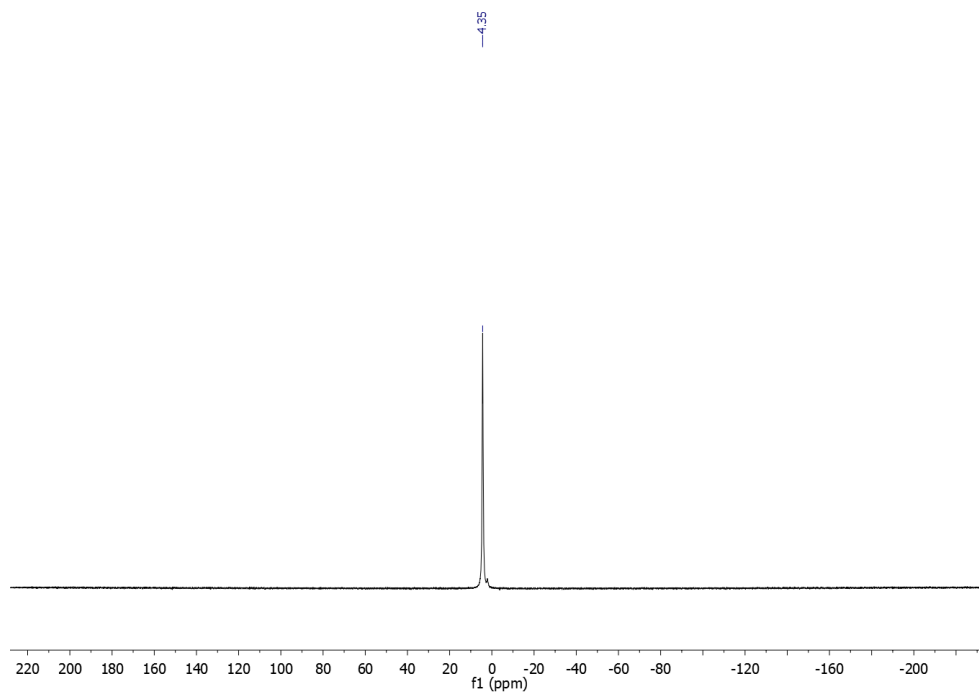


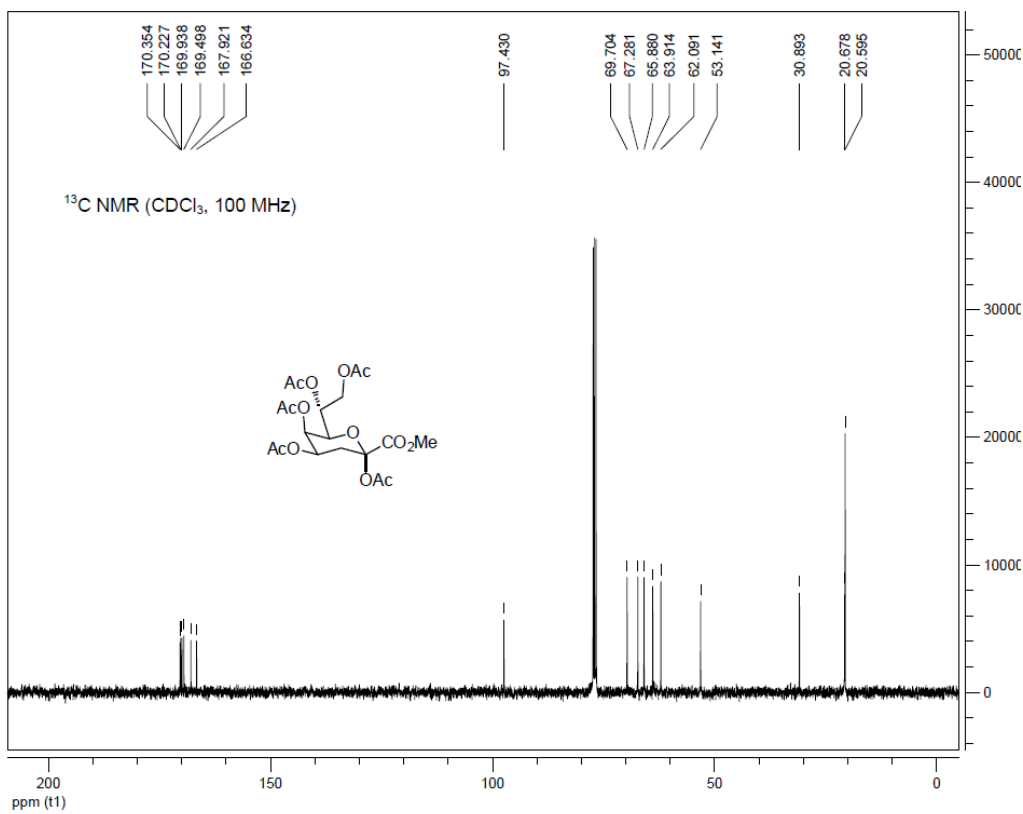
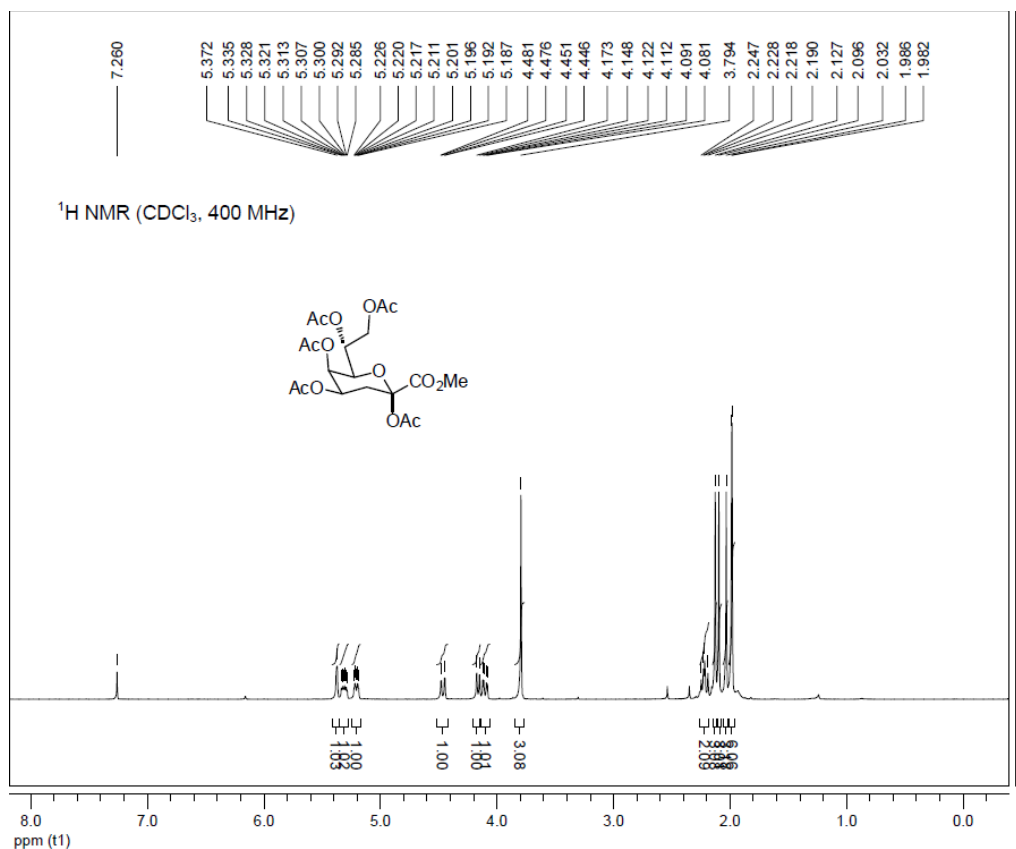
$^{31}\text{P}$ -NMR of D-ribulose 5-phosphate $^1\text{H}$ -NMR of L-xylulose 5-phosphate

### $^{13}\text{C}$ -NMR of L-xylulose 5-phosphate



### $^{31}\text{P}$ -NMR of L-xylulose 5-phosphate





### 3.2 Experiment procedures for chemoenzymatic reporter strategy for probing complex glycans on cell surface.

#### 3.2.1 Enzyme preparation

Plasmid encoding CgtA from *Campylobacter jejuni* was kindly provided by Prof. Xi Chen (University of California, Davis). *Neisseria meningitides* CMP-Sia synthetase (NmCSS), *Pasteurella multocida* inorganic pyrophosphatase (PPA), *Pasteurella multocida*  $\alpha$  2,3-sialyltransferase (PmST1) and *E.coli* aldolase were prepared as previously reported.<sup>214</sup> Pd2,6ST ( $\alpha$  2,6-sialyltransferase) from *Photobacterium damsela*,<sup>221</sup> CstII ( $\alpha$  2,8-sialyltransferase) from *Campylobacter jejuni*<sup>215</sup> and  $\beta$ 1–4-galactosyltransferases (LgtB) from *Neisseria meningitidis*<sup>222</sup> were prepared as previously reported. D-galactosyl- $\beta$ 1–3-N-acetyl-D-hexosamine phosphorylase (BiGalHexNAcP) and galactose kinase (GalK) from *Bifidobacterium infantis* was prepared as previously reported.<sup>223</sup> Sialidase (NanH) from *Bifidobacterium longum* subsp. *Infantis*(ATCC 15697) was prepared as previously reported.<sup>224</sup> Sialidase (NanC) from *Streptococcus pneumoniae*<sup>218</sup> was cloned into pET-28a and overexpressed in *E.coli* BL21 (DE3). The purified proteins were concentrated and desalted with 10 kDa molecular weight cut-off (Millipore, MWCO) spin filters for further use. Protein concentration was determined by BCA Protein Assay Kit.

#### 3.2.2 General protocol for the synthesis of oligosaccharides

Lactose- $\beta$ -Me was chemically synthesized starting from lactose as previously reported.<sup>214</sup> LacNAc was enzymatically synthesized by incubating D-GlcNAc with UDP-Gal and LgtB and purified by Bio-Gel P-2 column.<sup>225</sup> Gal- $\beta$ 1,3-GalNAc was enzymatically synthesized by incubating D-GalNAc with BiGalHexNAcP and Gal-1-phosphate, which was prepared from galactose by using GalK and purified by using silver nitrate precipitation method.<sup>83, 99</sup>



Oligosaccharides that containing  $\alpha$ 2-3-linked Neu5Ac were synthesized from appropriate acceptor (Lactose- $\beta$ -Me, LacNAc, Gal- $\beta$ 1,3-GalNAc or Galactose) in hundreds milligram scale using the one-pot two-enzyme system containing Tris-HCl (pH 7.5),  $Mg^{2+}$ , PmST1, NmCSS, CTP (1.5 equiv.) and Neu5Ac (1.5 equiv.).<sup>214</sup> Oligosaccharides that containing  $\alpha$ 2,3-linked Neu5Gc and KDN were synthesized from appropriate acceptor in hundreds milligram scale using the one-pot three-enzyme (OPME) system containing Tris-HCl (pH 7.5),  $Mg^{2+}$ , PmST1, NmCSS, aldolase, ManNAc or Mannose (1.5 equiv.), sodium pyruvate (5 equiv.), CTP (1.5 equiv.) and Neu5Ac (1.5 equiv.).<sup>214</sup>

Oligosaccharides that containing  $\alpha$ 2-6-linked sialic acids were synthesized from appropriate acceptor (Lactose- $\beta$ -Me, LacNAc, or Gal- $\beta$ 1,3-GalNAc) using the similar one-pot multienzyme system as described above, in which  $\alpha$ 2-6 sialic acid transferase Pd2,6ST instead of PmsT1 was employed.<sup>216</sup>

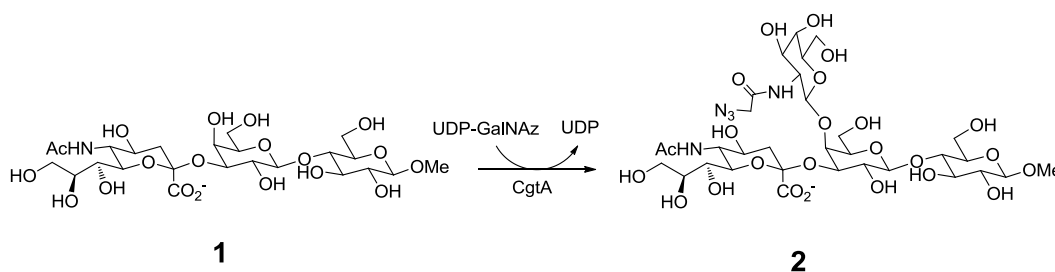
Oligosaccharides that containing  $\alpha$ 2-8-linked Neu5Ac was prepared from the appropriate acceptor obtained from above using one-pot two-enzyme system containing Tris-HCl (pH 7.5),  $Mg^{2+}$ , CstII, NmCSS, CTP (1.5 equiv.) and Neu5Ac (1.5 equiv.).<sup>215</sup>

### **3.2.3 Substrate specificity study of CgtA with UDP-GalNAc and UDP-GalNAz**

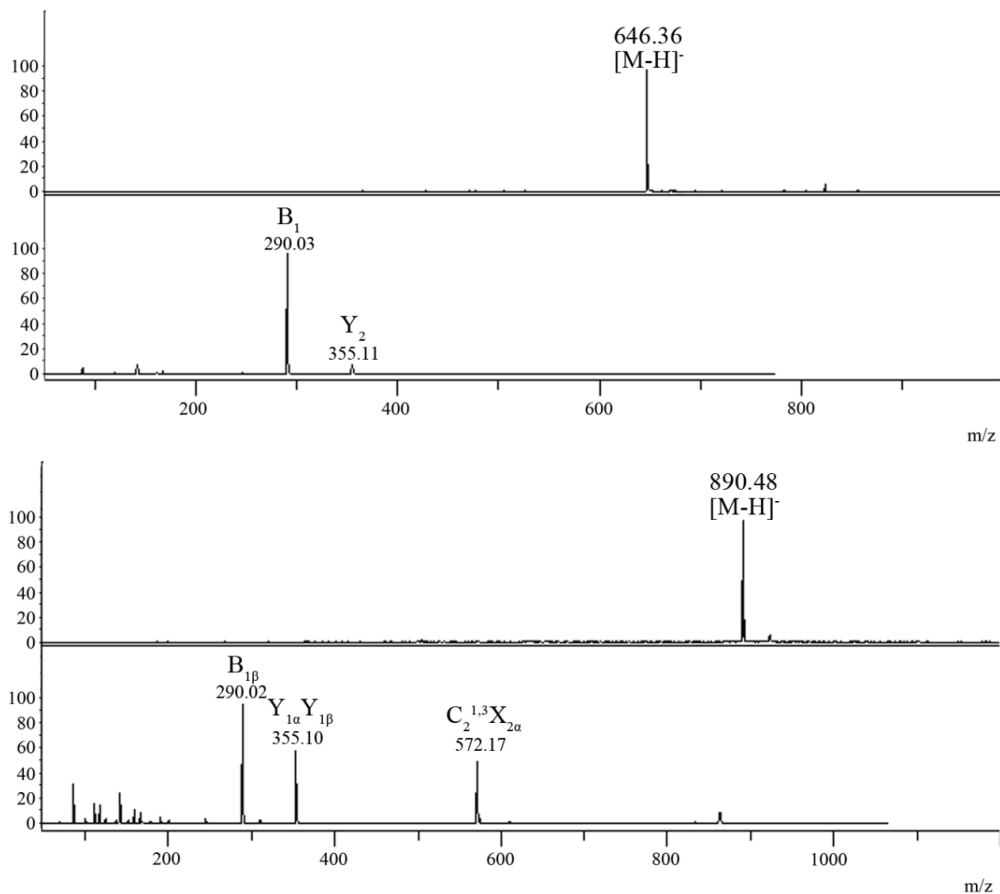
A 20  $\mu$ l mixture containing 4 mM of Neu5Ac $\alpha$ (2-3)Gal $\beta$ (1-4)Glc-Me (Entry 1 in Table 2.1) or Neu5Ac $\alpha$ (2-6)Gal $\beta$ (1-4)Glc-Me (Entry 7 in Table 2.1), 0.8  $\mu$ g of CgtA, 50 mM of Tris-HCl (pH 7.5), 5 mM of  $MgCl_2$ , 4 mM of UDP-GalNAc or UDP-GalNAz was incubated at 37°C for 20 minutes. The reaction was stopped by diluting the reaction with equal volume of cooled buffer acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60% acetonitrile). The diluted solution was analyzed by HPLC equipped with UV detector at 254 nm using ZIC®-CHILIC

column. The column was eluted at 30°C with acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60% acetonitrile) at a flow rate of 0.6 ml/min. The quantitation of the formation of UDP showed that UDP-GalNAz (112%) has a higher relative activity than UDP-GalNAc (100%). After incubation of Neu5Ac $\alpha$ (2-6)Gal $\beta$ (1-4)Glc-Me with UDP-GalNAz, only trace amount of UDP could be detected.

### 3.2.4 Enzymatic synthesis of **2** from **1** using CgtA and UDP-GalNAz



Reaction was carried in a 10 ml mixture containing 10 mM of **1**, 50 mM of Tris-HCl (pH 7.5), 5 mM of MgCl<sub>2</sub> and 0.5 mg of CgtA. 20 units of alkaline phosphatase were added to hydrolyze the newly formed UDP to improve the conversion. The reaction was carefully shaken at 37°C overnight to allow the formation **2**. **2** was purified by using Bio-Gel P-2 column. The fractions that containing **2** was collected and lyophilized to afford 82 mg of final product (92% yield with regard to **1**). The product was confirmed by MALDI TOF and NMR analysis. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O)  $\delta$  4.74 (d,  $J$  = 8.6 Hz, 2H), 4.44 (d,  $J$  = 7.9 Hz, 1H), 4.32 (d,  $J$  = 8.0 Hz, 1H), 4.06 (dd,  $J$  = 13.1, 3.2 Hz, 2H), 3.96 – 3.82 (m, 5H), 3.78 (d,  $J$  = 11.9 Hz, 1H), 3.75 – 3.60 (m, 11H), 3.54 (m, 4H), 3.49 (s, 4H), 3.38 (d,  $J$  = 8.3 Hz, 1H), 3.24 (dt,  $J$  = 16.9, 8.7 Hz, 2H), 2.63 – 2.52 (m, 1H), 1.95 (s, 3H). <sup>13</sup>C NMR (100 MHz, D<sub>2</sub>O)  $\delta$  175.00, 173.95, 170.96, 103.05, 102.58, 102.41, 101.58, 78.51, 76.95, 74.70, 74.37, 74.01, 73.01, 72.70, 72.18, 71.03, 70.01, 68.67, 67.98, 67.78, 62.82, 61.13, 60.55, 60.09, 57.20, 52.41, 51.79, 51.60, 37.09, 22.05.



**Figure 3.12** MS and MS/MS analysis of 1 and 2 (negative scanning mode). The MS spectrum for each compound is shown on top; the MS/MS spectrum for the most abundant ion is shown on the bottom. The corresponding fragmentation products and probable cleavage sites are denoted in the respective structures.

### 3.2.5 Kinetic analysis of CgtA with UDP-GalNAc and UDP-GalNAz

Reactions were performed in three repetitions with 50 mM Tris-HCl (pH 7.5), 100  $\mu$ M acceptor substrate **1**, 5 mM of Mg<sup>2+</sup>, 0.4  $\mu$ g of CgtA, and varying concentrations of UDP-GalNAz or UDP-GalNAc (50 to 800  $\mu$ M) in a total volume of 20  $\mu$ L. The reactions were carried at 37°C for 5 minutes and were stopped by adding equal volume of a cold buffer containing acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60% acetonitrile). The reactions were analyzed by quantifying the formation of UDP by HPLC equipped with UV detector at 254 nm using ZIC®-cHILIC column. The column was eluted at 30°C with acetonitrile/100 mM aqueous

ammonium acetate pH 4.5 (60% acetonitrile) at a flow rate of 0.6 ml/min. The kinetic parameters,  $K_m$ ,  $V_{max}$ , and  $k_{cat}$ , were obtained by linear regression analysis of initial velocity vs. donor substrate concentration using software KaleidaGraph (Table 3.1).

**Table 3.1** Kinetic parameters for UDP-GalNAz and UDP-GalNAc by CgtA

Substrate	$V_{max}$ (nmol <sup>-1</sup> min <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$K_{cat}/K_m$ (nM <sup>-1</sup> min <sup>-1</sup> )
UDP-GalNAc	0.254	16.887	1.34
UDP-GalNAz	0.249	14.725	1.51

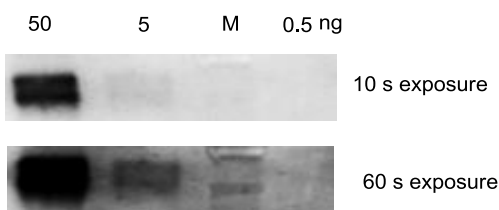
### 3.2.6 Substrate specificity study of CgtA with sialylated oligosaccharides

Substrate specificity of CgtA with UDP-GalNAz was studied by the reactions that were performed in 20  $\mu$ l reaction mixture containing a Tris-HCl buffer (50 mM, pH 7.5), 4 mM of oligosaccharides (prepared as described above), 5 mM of Mg<sup>2+</sup>, and 0.4  $\mu$ g of CgtA. The reactions were carried at 37°C for 20 minutes, and were stopped by adding equal volume of cold buffer containing acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60% acetonitrile). The reactions were quantified by analyzing the formation of UDP by HPLC equipped with UV detector at 254 nm using ZIC®-cHILIC column. The column was eluted at 30°C with acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60% acetonitrile) at a flow rate of 0.6 ml/min. Relative specificity towards oligosaccharides was shown in Table 2.1.

### 3.2.7 Chemoenzymatic detection Neu5Aca(2,3)Gal glycans on bovine fetuin using CgtA and UDP-GalNAz.

Fetal bovine fetuin was dissolved in water to a final concentration 2 mg/ml. To perform a control reaction,  $\alpha$ 2-3-linked sialic acid that attached on the terminal of glycans was hydrolyzed by NanC. Briefly, a 200  $\mu$ l solution containing 2 mg/ml of fetuin, 5 mM of Mg<sup>2+</sup>, and 10  $\mu$ g of

NanC was carefully shaken at 37°C for 1 hour. After the treatment of NanC, a slight migration change compare to native fetuin was observed on SDS-PAGE gel, indicating the remove of  $\alpha$ 2-3-linked Neu5Ac (Figure 2.4 A). To perform labeling reaction, a 200  $\mu$ l of above-mentioned solution containing 20 mM Tris-HCl buffer (pH 7.5), 5 mM of  $Mg^{2+}$ , 10  $\mu$ g of CgtA, and 100  $\mu$ M of UDP-GalNAz was carefully shaken at 37°C for 1 hour to allow the introduction of GalNAz onto Neu5Ac $\alpha$ (2-3)Gal. Meanwhile, the controls with the absence of either enzyme or substrate were performed parallel. Afterwards, protein was precipitated using methanol/chloroform/water. Briefly, 600  $\mu$ L of MeOH, 200  $\mu$ L of  $CHCl_3$  and 450  $\mu$ L  $H_2O$  were added sequentially. The resulted solutions were centrifuged at 23,000 g for 10 min. The upper and lower solutions were carefully removed by pipette. Precipitated protein that existed in intermediate layer was washed twice with 1ml of cooled MeOH and centrifuged at 23,000 g for 10 min. After the protein pellet was allowed to dry briefly, the precipitated protein was redissolved in PBS containing 1% SDS. The solution

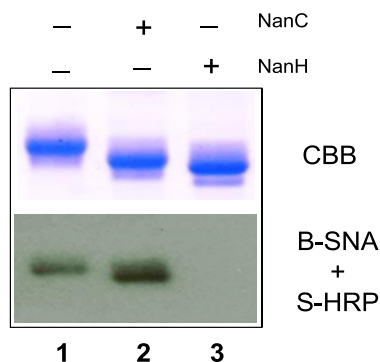


**Figure 3.13** Chemoenzymatic detection fetuin Neu5Ac $\alpha$ (2-3)Gal glycans. After labeling of fetuin with CgtA and UDP-GalNAz, different concentration (0.5, 5, and 50 ng) were loaded for western blot detection. M: protein marker.

was diluted to 1 mg/ml using PBS. The resuspended protein was subsequently reacted DIBO-biotin (10  $\mu$ M) at room temperature for 1 hour in dark. Then, protein was precipitated using chloroform/methanol/water as described above and washed by 1 ml of MeOH twice. The protein was finally redissolved in 2% SDS and the concentrations were determined as described above. 3  $\mu$ g of protein was loaded for coomassie brilliant blue staining. 0.5  $\mu$ g of protein was loaded for

western blot analysis using S-HRP. For sensitivity experiment, 0.5, 5 and 50 ng of labeled fetuin was loaded for western blot analysis using S-HRP (Figure 3.13).

### 3.2.8 Lectin binding experiments.



**Figure 3.14** B-SNA detection of  $\alpha$ 2-6-linked Neu5Ac on fetal bovine fetuin to double check that NanC not affect  $\alpha$ 2-6-linked sialic acids on glycoproteins. NanH: a sialidase that could hydrolyze both  $\alpha$ 2-3- and  $\alpha$ 2-6-linked sialic acid. 1, native fetal bovine fetuin. 2, native fetal bovine fetuin was treated with NanC to hydrolyze  $\alpha$ 2-3-linked Neu5Ac. 3, native fetal bovine fetuin was treated with NanH to hydrolyze both  $\alpha$ 2-3- and  $\alpha$ 2-6-linked Neu5Ac.

Biotinylated *Sambucus Nigra* Lectin (B-SNA) and biotinylated *Maackia Amurensis* Lectin II (B-MAL II), which binds  $\alpha$ 2-6-linked sialic acids or  $\alpha$ 2-3-linked sialic acids in *O*-glycans, were from Vector Laboratories. Fetal bovine fetuin (3  $\mu$ g) prepared as described above were separated on SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were blocked in blocking buffer (10 mM HEPES (pH 7.5), 150 mM NaCl, 0.02% Tween 20, 0.08% NaN<sub>3</sub>, 2% BSA) at room temperature for 2 hour. The membranes were probed overnight with 10 mL of 10  $\mu$ g/mL lectin in probing buffer (10 mM HEPES (pH 7.5), 150 mM NaCl, 0.02% Tween 20, 0.08% NaN<sub>3</sub>, 2% BSA, 1mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>). The membranes were washed three times for 10 min with 10 mL of binding buffer (10 mM HEPES (pH 7.5), 150 mM NaCl, 0.02% Tween 20, 0.08% NaN<sub>3</sub>) and then probed with Streptavidin HRP (1: 20000) in blocking buffer at room temperature for 2 hour. The membranes were washed three

times for 10 min and imaged with ECL reagents. After probing by SNA, strong fluorescence could be observed in native bovine fetuin and NanC-treated fetuin, demonstrating that NanC selectively hydrolyze  $\alpha$ 2-3-linked sialic acids on protein but not affect  $\alpha$ 2-3-linked sialic acids (Figure 2.4). There is no fluorescence could be observed when bovine fetuin was treated with NanH, which hydrolyze both  $\alpha$ 2-3- and  $\alpha$ 2-6-linked sialic acids (Figure 3.14).

### **3.2.9 Chemoenzymatic detection Neu5Aca(2,3)Gal glycans from cell lysates**

Cells were cultured as described above and harvested by centrifugation (1000 g, 5 min). After two washes using labeling buffer (3% FBS in PBS), the cell was disrupted in lysis buffer (50 mM Tri-HCl, 8.0; 150 mM NaCl; 1% NP 40; 5% glycerol) supplemented with protease inhibitor cocktail at 4°C for 15 minutes. Cell debris was removed by centrifugation (12000 g, 5 min). The supernatant was diluted to 1 mg/ml using lysis buffer. To perform a control reaction, in a 200 ul of above mentioned solution, 5 mM of Mg<sup>2+</sup>, and 10 ug of NanC was added. The reaction was carefully shaken at 37°C for 1 hour to hydrolyze the  $\alpha$ 2-3-linked Neu5Ac. To perform labeling reaction, 5 mM of Mg<sup>2+</sup>, 100 uM of UDP-GalNAz, and 30 ug of CgtA were added into 200 ul of above mentioned solutions. The reactions were shaken carefully at 37°C for 1 hour, while the reactions with the absence of either substrate or CgtA were performed as parallel. Afterwards, proteins were precipitated using methanol/chloroform/water as described above and washed three times using 1 ml of MeOH. After the protein pellet was allowed to dry briefly at room temperature, the precipitated proteins were redissolved in PBS containing 1% SDS and diluted to 1 mg/ml using PBS. To perform Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction, 1 mM of CuSO<sub>4</sub>, 3 mM of THPTA, 4 mM of sodium ascorbate and 50 uM of Diazo Biotin-Alkyne were added. After the reaction was carried at room temperature for 1 hour in dark, the proteins were precipitated using methanol/chloroform/water as described above and washed twice using 1ml of MeOH. The

proteins were redissolved in 2% SDS. About 20 ug of protein was loaded for SDS-PAGE separation and western blot analysis.

### ***3.2.10 Chemoenzymatic detection cell surface Neu5Aca(2,3)Gal glycoproteins***

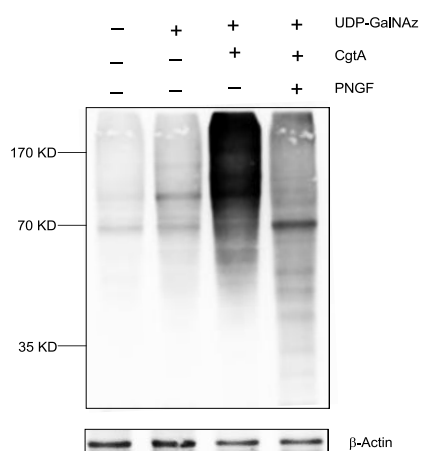
Cells were cultured as described above and harvested by centrifugation (1000 g, 5 min). After wash three times using labeling buffer (PBS containing 3% FBS), the cell was redissolved in labeling buffer. To perform labeling reaction, 500 uM of UDP-GalNAz, 10 mM of Mg<sup>2+</sup> and 100 ug/ml CgtA were added. The reaction was carefully at 37°C for 30 min, while the reactions with the absence of either substrate or CgtA were performed as parallel. Afterwards, the reactions were stopped by precipitating cells by centrifugation (1000 g, 5 min). After three washes by labeling buffer, the cells were disrupted by RIPA buffer supplemented with protease inhibitor cocktail. Cell debris was removed by centrifugation (12000 g, 5 min), and the supernatant solution was diluted to 1 mg/ml using lysis buffer. To perform labeling reaction, 1 mM of CuSO<sub>4</sub>, 3 mM of THPTA, 4 mM of sodium ascorbate and 50 uM of Diazo Biotin-Alkyne were added. After the reaction was carried at room temperature for 1 hour in dark, the proteins were precipitated using methanol/chloroform/water as described above and washed twice using MeOH. The proteins were redissolved in 2% SDS. About 15 ug of protein was loaded for SDS-PAGE separation and western blot analysis.

### ***3.2.11 Western blotting***

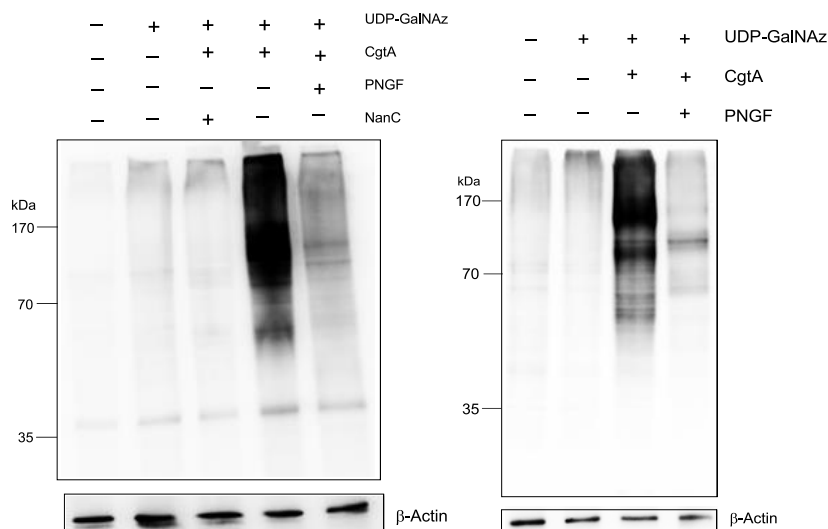
The purified, labeled sample from above was separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked in 3% BSA in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4) at room temperature for 2 hours. For biotinylated protein analysis, the membrane was incubated streptavidin-linked horseradish peroxidase (1:20000) at 4°C overnight. The membrane was washed three times with



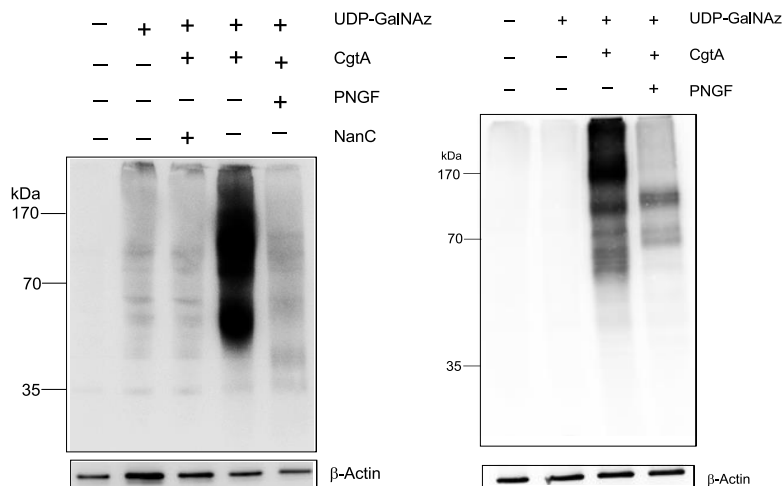
TBST for 10 min, and the blots were developed using ECL reagents and ImageQuant LAS 4000 mini imager (GE Healthcare). For actin western blot analysis, the membrane was incubated with anti-actin (1: 20000 dilution) in TBST containing 3% BSA at 4°C overnight. The membrane was washed three times and incubated with second antibody (Goat anti-rabbit, 1: 3000) as room temperature for 1hour. The membrane was washed three times with TBST for 10 min, and the blots were developed using ECL reagents and ImageQuant LAS 4000 mini imager (GE Healthcare).



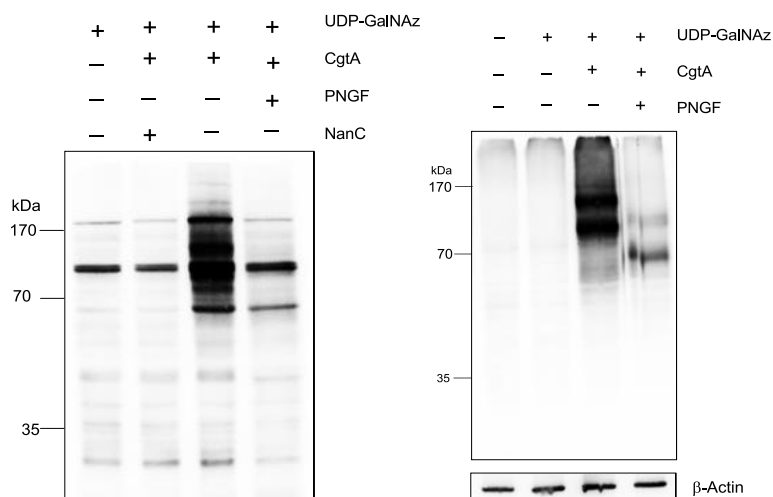
**Figure 3.15** Chemoenzymatic detection *Neu5Ac*  $\alpha(2-3)$ Gal glycoproteins from HEK293T cell lysates. PNGF: the labeled sample was further treated with PNGF to remove N-glycans.



**Figure 3.16** Chemoenzymatic detection *Neu5Ac*  $\alpha(2-3)$ Gal glycoproteins from HeLa cell lysates (left) and cell surface (right). *NanC*: The sample was treated with *NanC* to hydrolyze  $\alpha(2-3)$ -linked *Neu5Ac* before performing labeling reaction. *PNGF*: the labeled sample was further treated with *PNGF* to remove *N*-glycans.



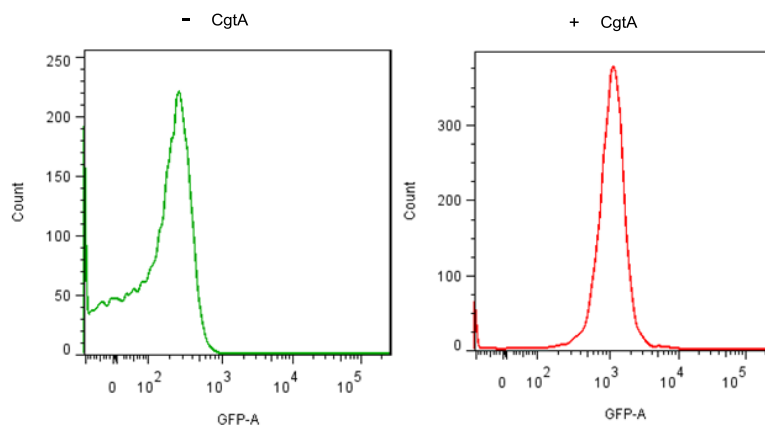
**Figure 3.17** Chemoenzymatic detection *Neu5Ac*  $\alpha(2-3)$ Gal glycoproteins from HepG2 cell lysates (left) and cell surface (right). *NanC*: The sample was treated with *NanC* to hydrolyze  $\alpha(2-3)$ -linked *Neu5Ac* before performing labeling reaction. *PNGF*: the labeled sample was further treated with *PNGF* to remove *N*-glycans.



**Figure 3.18** Chemoenzymatic detection *Neu5Ac*  $\alpha(2-3)$ Gal glycoproteins from A549 cell lysates (left) and cell surface (right). *NanC*: The sample was treated with *NanC* to hydrolyze  $\alpha(2-3)$ -linked *Neu5Ac* before performing labeling reaction. *PNGF*: the labeled sample was further treated with *PNGF* to remove *N*-glycans.

### 3.2.12 Fluorescence microscopy and flow cytometry analysis of Neu5Ac $\alpha$ (2,3)Gal glycan on living cells.

HeLA cells cultured overnight to allow adhesion. Monolayers were washed three times using labeling buffer (PBS containing 3% FBS), and were enzymatically labeled in labeling buffer containing CgtA (25ug/ml), UDP-GalNAz (50 uM) and Mg<sup>2+</sup> (10 mM) at 37°C for 1 hour. After three washes with cooled labeling buffer, the cells were incubated with DIBO-biotin (30 uM) at RT for 1 hour. Next, the cells were incubated with streptavidin–Alexa Fluor 488 (10 ug/ml) in labeling buffer at 4 °C for 30 min in dark. Cells were washed twice with labeling buffer and fixed with formaldehyde (3.7% in PBS) at RT for 15 min. The nucleus was labeled with DAPI before imaging by fluorescence microscope (Olympus BX 63). To analyze the expression level of Neu5Ac $\alpha$ (2,3)Gal glycans by flow cytometry, HeLA cells were lifted off the plate and washed



**Figure 3.19** Flow cytometry analysis of the expression level of Neu5Ac $\alpha$ (2-3)Gal glycans on the cell surface of HeLA cells. Cells were chemoenzymatically labeled in the presence (right) or absence (left) of CgtA. 10,000 live cells were analyzed in each experiment.

three times using labeling buffer. One million cells were enzymatically labeled in labeling buffer containing CgtA (25ug/ml), UDP-GalNAz (50 uM) and Mg<sup>2+</sup> (10 mM) at 37°C for 1 hour. Once reaction finished, the cells were precipitated by centrifugation (1500g, 5 min) and washed three times using labeling buffer. The cells were incubated with DIBO-biotin (30 uM) at RT for 1 hour

and then washed three times. The fluorescence reporter was installed by incubation with streptavidin–Alexa Fluor 488 (10 ug/ml) in labeling buffer at 4 °C for 30 min in dark. After three washes using labeling buffer, the cell was resuspended in PBS containing CMF HBSS for flow cytometry analysis. For each experiment, 10,000 live cells were analyzed, and data analysis was performed on FlowJo. Robust fluorescent labeling was detected in cells subjected to enzymatic treatments (Figure 3.19).

### ***3.2.13 Chemoenzymatic probing Nue5Ac $\alpha$ (2,3)Gal Glycoproteins***

The SDS-solubilized, biotin labelled or control sample (10 mg total protein per sample) was diluted with PBS (100 mM phosphate, 150 mM sodium chloride; pH 7.4) into a final concentration of 0.2% SDS. The solution was then incubated with 150  $\mu$ L of streptavidin-agarose beads (300  $\mu$ L of a 50% slurry per sample, Pierce) for 2 h at room temperature. The beads were washed with 10 ml 0.2% SDS/PBS, 3  $\times$  10 mL PBS and 3  $\times$  10 ml H<sub>2</sub>O. Centrifugation (1300 $\times$ g, 2 min) of beads was carried out between washes. The pelleted beads were suspended in freshly prepared 6 M urea/PBS and 10 mM DTT (500  $\mu$ L) and placed in 37 °C for 2 h. Iodoacetamide (20 mM, from 25  $\times$  stock in H<sub>2</sub>O) was then added to the solution and incubated for 30 min at room temperature (25 °C). Following reduction and alkylation, the beads were washed with 5  $\times$  1 mL of 6 M urea/PBS, 5  $\times$  1 mL of 2 M urea/PBS, pelleted by centrifugation (1300 $\times$ g, 2 min) and resuspended in 200  $\mu$ L of 2 M urea/PBS, 1 mM CaCl<sub>2</sub> (10  $\times$  stock in H<sub>2</sub>O), and trypsin (2  $\mu$ g). The digestion was allowed to proceed overnight at 37 °C. The tryptic solution and beads were then transferred into a spin column (Pierce) and the eluates were collected by centrifugation. The beads were washed twice with 200  $\mu$ L of 2 M urea/PBS. The eluates and washes were combined and loaded onto a C18 SepPak column (Waters) pre-conditioned with 3  $\times$  1 mL of 80% ACN containing 0.1% TFA and 3  $\times$  1 mL of 5% ACN containing 0.1% TFA and washed with 3  $\times$  1 mL

of 5% ACN containing 0.1% TFA. The tryptic peptides were eluted with  $2 \times 0.5$  mL of 80% ACN containing 0.1% TFA and concentrated.

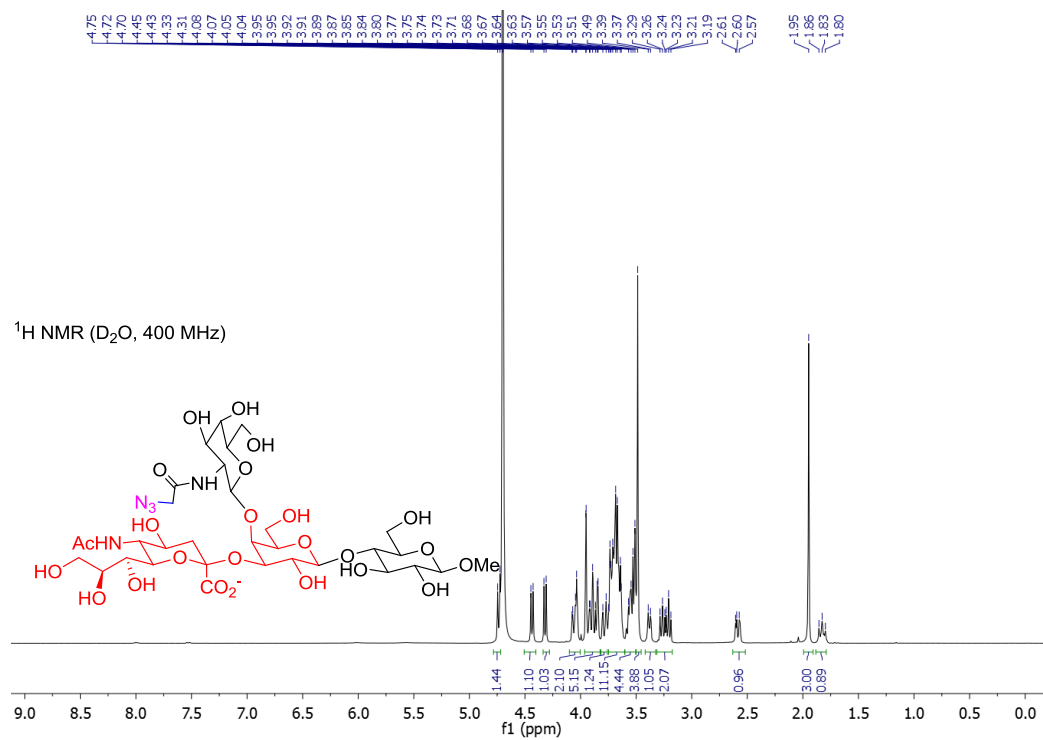
### ***3.2.14 LC-MS and data analysis***

Peptides were resolubilized in 0.1% formic acid and analyzed by a LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher) equipped EASY-spray source and nano-LC UltiMate 3000 high-performance liquid chromatography system (Thermo Fisher). EASY-Spray PepMap C18 Columns ( $75 \mu\text{m} \times 15 \text{ cm}$ ,  $3 \mu\text{m}$ , Thermo Fisher) were used for separation. Separation was achieved with a linear gradient from 3% to 40% solvent B for 120 min at a flow rate of 300 nL/min (mobile phase A, 2% ACN, 98% H<sub>2</sub>O, 0.1% FA; mobile phase B, 80% ACN, 20% H<sub>2</sub>O, 0.1% FA). The LTQ-Orbitrap Elite mass spectrometer was operated in the data-dependent mode. A full-scan survey MS experiment ( $m/z$  range from 400 to 1600; automatic gain control target, 1,000,000 ions; resolution at  $m/z$  400, 60,000; maximum ion accumulation time, 50 ms) was acquired by the Orbitrap mass spectrometer, and the 10 most intense ions were fragmented by collision-induced dissociation (CID). The MS/MS scan model was set as the centroid. The other conditions used were temperature of 200 °C, collision energy of 35 eV.

The raw MS data were searched against the human protein database (UniProt, Feb. 2016) using the SEQUEST (Proteome Discoverer 1.4, Thermo Fisher Scientific) with full MS peptide tolerance of 20 ppm and MS2 peptide fragment tolerance of 0.5 Da, and a false discovery rate (FDR) of 1% was applied to all data sets at the peptide level. Three replicate experiments of enzymatic labeling and their negative controls (no CgtA added) were performed for proteomics analysis. Labeled proteins must have been identified by at least 1 unique peptide in each of the three data sets, and proteins detected in negative controls (data not shown) were excluded from the final lists of proteins. Biological functions and pathways enriched in the generated proteomic data were

evaluated using IPA (Ingenuity Systems, [www.ingenuity.com](http://www.ingenuity.com)). Cellular location was identified using IPA and UniProt. Protein functional classification is available on the PANTHER website (<http://pantherdb.org>).

## 3.2.15 NMR spectra



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7. **Wen, L. Q.**; Huang, K.; Zheng, Y.; Liu, Y. P.; Zhu, H.; Wang, P. G. A two-step strategy for the preparation of 6-deoxy-l-sorbose. *Bioorg. Med. Chem. Lett.* **2016**, 26, 218.
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5. **Wen, L. Q.**; Huang, K.; Liu, Y. P.; Wang, P. G. Facile Enzymatic Synthesis of Phosphorylated Ketopentoses. *ACS Catal.* **2016**, 6, 1649.
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3. **Wen, L. Q.**; Huang, K.; Wei, M. H.; Meisner, J.; Liu, Y. P.; Garner, K.; Zang, L. L.; Wang, X.; Li, X.; Fang, J. Q.; Zhang, H. C.; Wang, P. G. Facile Enzymatic Synthesis of Ketoses. *Angew. Chem. Int. Ed.* **2015**, 54, 12654.
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