

**Synthetic Saccharides to Study  
Immunological Properties of  
*Streptococcus Pneumoniae* Type 6A and 6B**

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## **Introduction**

### **1.1 Background**

The bacteria *Streptococcus pneumoniae* (SPn) have become one of the most frequent causes of pneumonia and meningitis in the elderly, immunocompromised, and, especially, in young children. The incidence of pneumococcal infection varies geographically, but elevated rates have been observed in both developed and developing countries most likely due to the noted rapid increases in anti microbial drug resistance during last decade.

As a matter of fact, SPn has one of the largest public health and economic impacts amongst all bacterial infectious diseases. In accordance with the World Health Organization (WHO) survey, approximately 1.2 million children die annually worldwide of pneumonia (~ 12,500 in the US); more than half of these deaths are attributed to SPn.

### **1.2 Facts**

It is a known fact that the SPn bacterial cell is surrounded by a polysaccharide capsule, which makes the organism resistant to phagocytosis. In these cases, polysaccharide or saccharide-protein conjugate<sup>1-3</sup> vaccinations are conventional tools against the bacterial invasion; serotype-specific antibodies to the pneumococcal saccharide are formed in response.

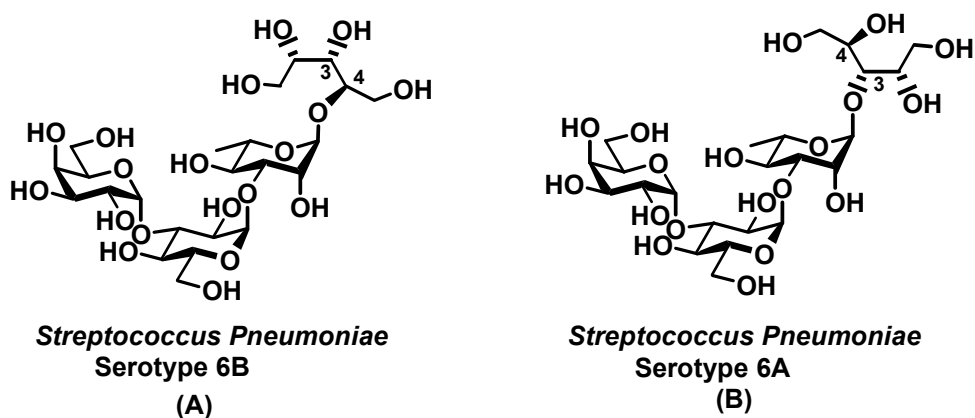
Currently, leading SPn vaccine manufacturers, such as Wyerth, Pasteur, Merck, Aventis, and SmithKline Beecham have marketed various multi-component vaccines whose serotype composition varies geographically. The immunoresponse to polysaccharide vaccines does not develop in infants younger than eighteen months of age. Therefore, the common trend is that the development of future generations of SPn vaccines should focus on structurally related glycoprotein conjugates rather than “plain” polysaccharides.<sup>1-3</sup>

An ultimate goal of the project is to develop a cost-efficient and scalable synthetic strategy for the most effective pneumococcal vaccine.

## Scope & Objective

### 2.1 Overview

Amongst ninety elucidated SPn serotypes, 6A and 6B are equally important causes of bacterial infections. This fact stimulated extensive structural studies and, nowadays, the structures of capsule polysaccharides (PSs) of both types are well established (**Figure 1**).<sup>4,6</sup> It was commonly assumed that due to similarity in their carbohydrate core structures, the elicited antibodies would be cross-reactive against both types.<sup>5, 7, 8,</sup>  
<sup>2</sup>As a result, only hydrolytically more stable and, hence, easier accessible SPn6B was considered for inclusion in vaccines. A number of chemical syntheses of SPn6B, their mimetics and conjugates have also emerged.<sup>9-14</sup>



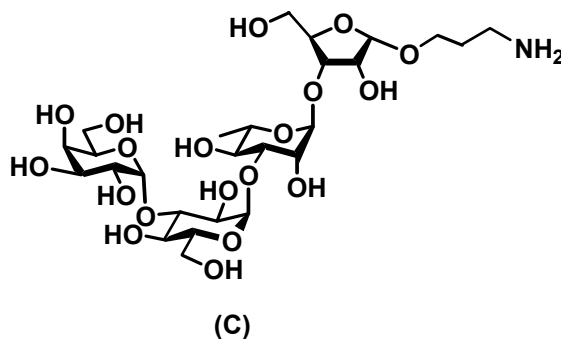
**Figure (1) – Structure of the repeating unit of Capsular polysaccharides of *Streptococcus Pneumoniae* 6A and 6B**

However, recent cross-reactivity studies challenged this hypothesis as it was clearly demonstrated that SPn6B-based vaccines produce 6B-specific antibodies that cross-react with SPn6A at a much lower rate (up to 1/20).<sup>14-17</sup> Hence, the importance of including the SPn6A carbohydrate conjugates in the future generations of multi-serotype anti-SPn vaccines has been acknowledged.<sup>18</sup>

This achievement is challenging for a number of reasons, major of which is lower hydrolytic stability and, consequently, low availability of sufficiently pure SPn6A isolates.<sup>19</sup> A possible solution for this would be the chemical synthesis of the related sugar derivatives and their application as vaccine components.

## 2.2 Research Objective

Structure shown in Figures 1(B) and 1(C) are related and differ only in the sense that the ribitol unit in the original structure 1(B) is replaced by a spacer containing cyclic ribofuranoside 1(C). The spacer moiety is essential for subsequent conjugation with a carrier protein (or polymer), the approach that may lead to highly immunogenic vaccine candidates.



**Figure 1(c) – Structure of glycomimetic of SPn6A**

A major difficulty associated with the chemical synthesis of 1(C) is the necessity to stereoselectively introduce two challenging 1,2-cis linkages. Also, it would be desirable to perform the oligosaccharide assembly in a convergent manner with minimal number of synthetic steps.

Our goal is to perform efficient chemical synthesis of the natural oligosaccharide repeating unit of SPn6A and structurally related glycoconjugates that will help to generate substantial quantities of pure samples for immunological studies and subsequent vaccine development. For this purpose we will develop a highly convergent strategy to access compounds of this level of complexity.

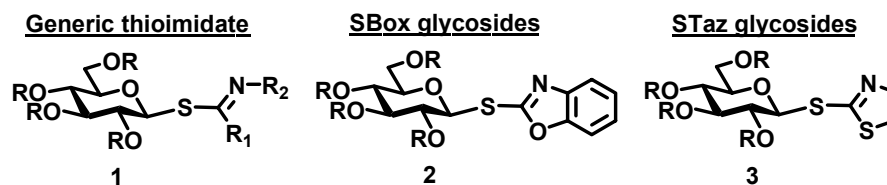
The methods and strategies developed will significantly facilitate further steps toward the vaccine development including preparation of a range of compounds structurally related to serotype 6A, elucidation of their structural properties, and determination of the immunological properties of synthetic conjugates.

## Preliminary Results

### 3.1 Analysis

The previous work in our lab on the development of glycosylation methods has resulted in the discovery of novel glycosyl donors with a generic thioimidoyl leaving group ( $\text{SCR}_1=\text{NR}_2$ , **1**, **Figure 2**). Our lab has already reported that S-benzoxazolyl (**2**, SBox)<sup>20, 21</sup> and, especially, S-thiazolyl (**3**, STaz)<sup>22</sup> moieties are stable towards a broad spectrum of reaction conditions. Conversely, they can be activated for glycosylation in the presence of mild electrophilic promoters.

The high stability along with accessibility and excellent stereoselectivity make these derivatives attractive and very promising targets for future studies as building blocks for sophisticated oligosaccharide syntheses.

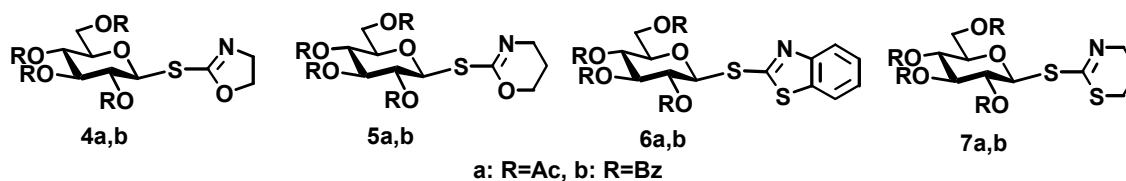


**Figure (2) – Glycosyl donors with a generic thioimidoyl leaving group**

### 3.2 Methods Employed

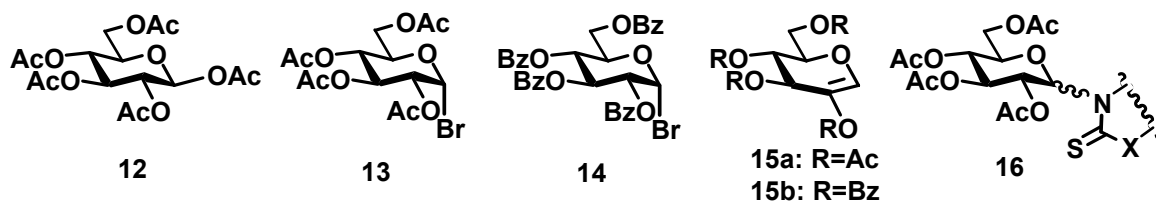
#### 3.2.1 Research

Although previously developed thioimidates were found to be suitable building blocks for oligosaccharide synthesis, it seemed to be essential to extend these studies to a range of other five and six-membered heterocyclic moieties, structurally related to the SBox and STaz glycosides. For this purpose we synthesized novel per-acylated S-oxazolyl (**4**), S-oxaziny (**5**), S-benzothiazolyl (**6**), and S-thiaziny (**7**) derivatives. (**Figure 3**)



**Figure (3) – Peracylated thioimidoyl derivatives**

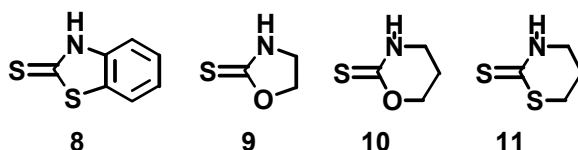
Table 1. Synthesis of Glycosyl Thioimidates 4-7.



Entry	SM	Aglycone	Conditions	Product	Yield	Side products
1	12	8	TMSOTf, CH <sub>2</sub> Cl <sub>2</sub> , rt	6a	85 %	
2	13	8, K salt	acetone, rt	6a	99 %	
3	14	8, K salt	18-c-6, acetone, rt	6b	88 %	
4	12	9	TMSOTf, CH <sub>2</sub> Cl <sub>2</sub> , rt	4a	0 %	16 (80%)
5	13	9, K salt	MeCN, rt	4a	34 %	15a (36%), 16 (24%)
6	14	9, K salt	18-c-6, MeCN, rt	4b	23 %	15b (49%)
7	12	10	TMSOTf, CH <sub>2</sub> Cl <sub>2</sub> , rt	5a	0 %	Complex mixture
8	13	10, K salt	MeCN, rt	5a	0 %	15a (58%), 16(27%)
9	14	10, K salt	18-c-6, MeCN, rt	5b	12 %	15b (70%)
10	12	11	TMSOTf, CH <sub>2</sub> Cl <sub>2</sub> , rt	7a	84 %	
11	13	11, K salt	MeCN, rt	7a	22 %	15a (54%), 16 (18%)
12	14	11, K salt	18-c-6, MeCN, rt	7b	18 %	15b (55%)
13	7a	-	1. MeONa, 2. BzCl/C <sub>5</sub> H <sub>5</sub> N	7b	72 %	

**SM** – starting material

A general procedure to introduce the anomeric moiety (aglycone) involved the use of anomeric acetates or bromides as suitable starting compounds for these syntheses. In this context, aglycones **8**, **9** (Figure 4) for the synthesis of **6** and **4**, respectively, are readily available from commercial sources, while tetrahydro-1,3-oxazine-2-thione (**10**)<sup>23</sup> and tetrahydro-1,3-thiazine-2-thione (**11**)<sup>24</sup> needed to be synthesized. (Figure 4)



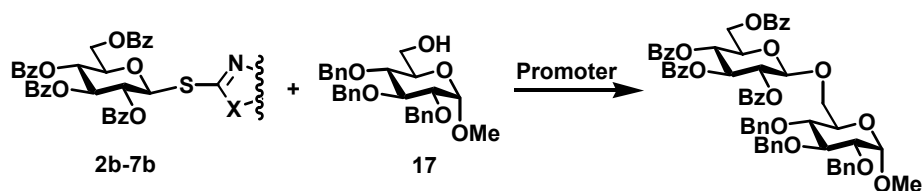
**Figure (4) – Aglycones used for glycosyl donor synthesis**

The per-acetylated thioimidoyl derivatives **4-7** were obtained from glucose pentaacetate **12** in the presence of TMSOTf at room temperature (**Table 1**). The synthesis of acetylated thioimidates was also accomplished from acetobromoglucose **13**<sup>25</sup> by the reaction of a potassium salt of **8-11** in acetone or MeCN at room temperature. Similarly, benzobromoglucose **14**<sup>25</sup> was employed in the syntheses of per-benzoylated thioimidates. The syntheses of benzoylated thioimidates were accomplished in the presence of 18-crown-6. The use of the crown ether was not only found to be essential for the efficient conversion but also very influential for the synthesis of the desired S-linked derivatives, as opposed to their N-linked counterparts.

### 3.2.2 Glycosidation

Having obtained the above thioimidates, a common glycosyl acceptor (**17**) was chosen for glycosylation experiments. These reactions were conducted in the presence of MeOTf and AgOTf as promoters; and dichloroethane as a solvent, common conditions for thioimidate glycosylation. As a matter of fact, the glycosidation of perbenzoylated thioimidates **4b**, **6b**, and **7b** were studied under essentially the same reaction conditions as previously investigated for the glycosidations of SBox (**2b**) and STaz (**3b**) glycosides.<sup>20-22</sup> Herein, also presented comparative results obtained with **2b** and **4b** (**entries 1-4, Table 2**).

As seen from entry **9 (Table 1)**, the product **5b** was obtained in extremely low yield and hence its use for glycosidation was found to be impractical. The use of per-acetylated glycosyl donors **4a**, **6a** and **7a** was found to be compromised due to a competing process of the 2-O-acetyl moiety migration from a glycosyl donor to the 6-OH of the glycosyl acceptor **17**.<sup>26, 27</sup>

Table 2. Glycosylation of **17** with per-Benzoylated Glycosyl Donors **2-7**:

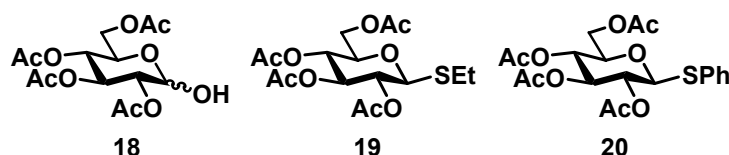
Entry	Donor	Promoter	Time	Yield	Ref
1	2b	AgOTf	5 min	94 %	20
2	2b	MeOTf	1 h	95 %	20
3	3b	AgOTf	16 h	97 %	22
4	3b	MeOTf	2 h	97 %	22
5	4b	AgOTf	5 min	60 %	
6	4b	MeOTf	3.5 h	81 %	
7	6b	AgOTf	16 h	79 %	
8	6b	MeOTf	2 h	67 %	
9	7b	AgOTf	30 min	65 %	
10	7b	MeOTf	16 h	51 %	

### 3.2.3 Hydrolytic Stability Studies

These studies were planned as a part of the investigation of novel leaving groups in order to evaluate their relative stability in comparison to common thioglycosides **19** and **20**. The rationale for these studies is to check a possibility for selective activation of one leaving group over another. Upon success of these studies, we expect to have achieved a valuable information for subsequent oligosaccharide synthesis in a highly convergent manner. Hydrolytic stability studies were carried out under acidic conditions in the presence of thiophilic reagents.

Per-acetylated derivatives **2a**, **3a**, **4a**, **6a**, and **7a** were studied herein (synthesis of **18**, **Figure 5**). Reaction conditions selected for this purpose were essentially the same as those previously reported for the hydrolysis of S-ethyl or S-phenyl glycosides (**19**<sup>28</sup> or

**20**<sup>29</sup>, respectively, **Figure 5**). Hydrolysis was carried out in the presence of N-iodosuccinimide (NIS, 2 equiv) and TfOH (0.1-0.2 equiv) in wet CH<sub>2</sub>Cl<sub>2</sub>.<sup>30</sup> Under these reaction conditions **2a**, **19**, and **20** were rapidly converted into the hemiacetal **18**; these reactions required 15, 2, and 20 min, respectively, whereas the hydrolysis of **3a**, **4a**, **6a**, and **7a** was significantly slower and required 16, 2, 16, and 48 h, respectively.



**Figure (5)**

The second procedure involved hydrolysis in the presence of N-bromosuccinimide (2 equiv) in aqueous acetone.<sup>31</sup> Under these reaction conditions **19** was hydrolyzed in 5 min, **3a**, **7a**, and **20** were hydrolyzed in 3-5 h, while **2a** and **6a** were significantly more resistant: their hydrolysis was sluggish and the reaction was not completed even in 48 h.

### 3.3 Interpretations

We investigated a number of novel thioimidoyl glycosides, which can be obtained from anomeric acetates and halides. In case of glycosidation of somewhat more basic oxygen containing heterocyclic aglycones, the isolated yields were compromised due to competing side processes, namely  $\beta$ -elimination and N-glycosylation. The glycosyl donor properties of these derivatives were studied in comparison to other glycosyl donors of this class, SBox and STaz glycosides. Hydrolytic stability studies have clearly demonstrated that the glycosyl thioimidates are more stable compounds overall than their S-ethyl and S-phenyl counterparts.

*These preliminary results (Pages 6-10) were published in the Journal of Carbohydrate Chemistry, 2005, vol. 24, pp. 649-663.*<sup>32</sup>

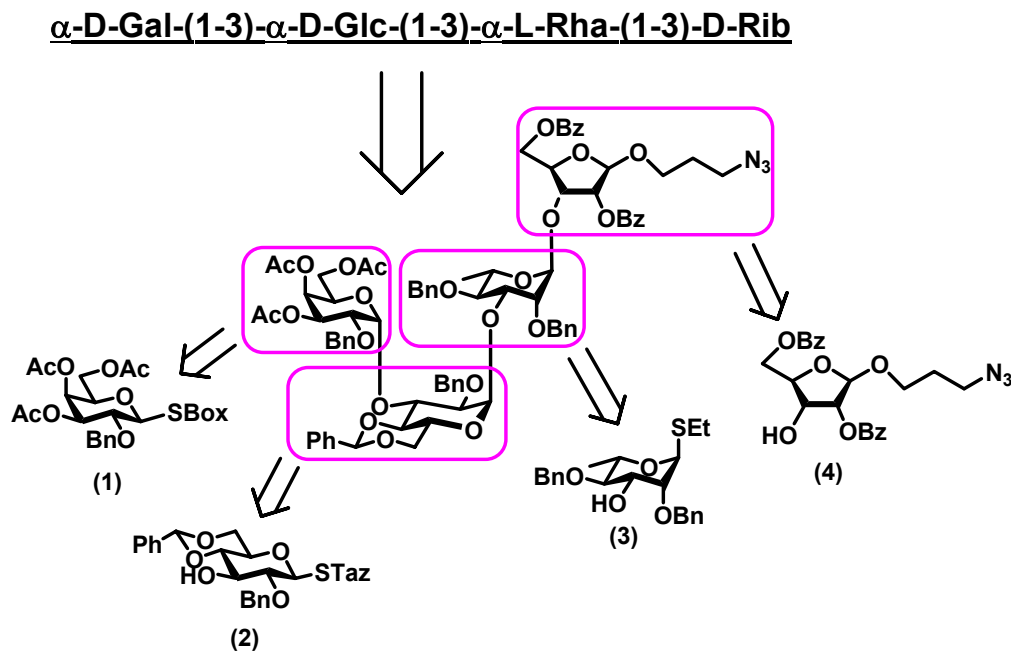
### 3.4 Retrosynthetic Analysis

Based on the above results, the retro synthesis of repeating unit of SPn6A is proposed. This will be achieved by application of a new thioimide glycosylation methodology using conventional thioglycoside SEt, along with the SBox and STaz moieties due to their promising results in comparison with the other thioimides of the same class.

As seen from the structure, SPn6A is a complex tetrasaccharide consisting of a D-galactosyl residue at the terminal end, which is  $\alpha$ -glycosidically linked (1,2-cis) to C-3 of a D-glucose unit. The latter is linked via 1,2-cis-glycosidic bond to C-3 of L-rhamnose, which is connected via  $\alpha$ -glycosidic linkage (1,2-trans) to C-3 of D-ribose.

This tetrasaccharide assembly can be achieved from four monosaccharide building blocks (**Scheme 1**) D-galactose (1), D-glucose (2), L-rhamnose (3) and D-ribose (4). Glycosylation steps using a suitable promoter will involve the activation of one leaving group over another. It should be noted that a non-participating group (-Bn) is needed at C-2 to provide cis- selectivity in the introduction of the galactose and glucose units.

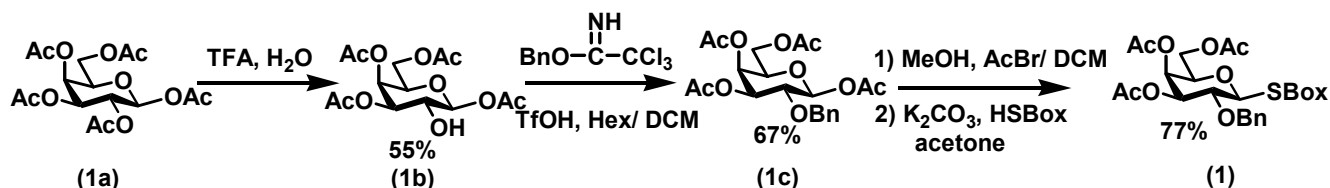
**Scheme 1**



### 3.5 Building Block Synthesis

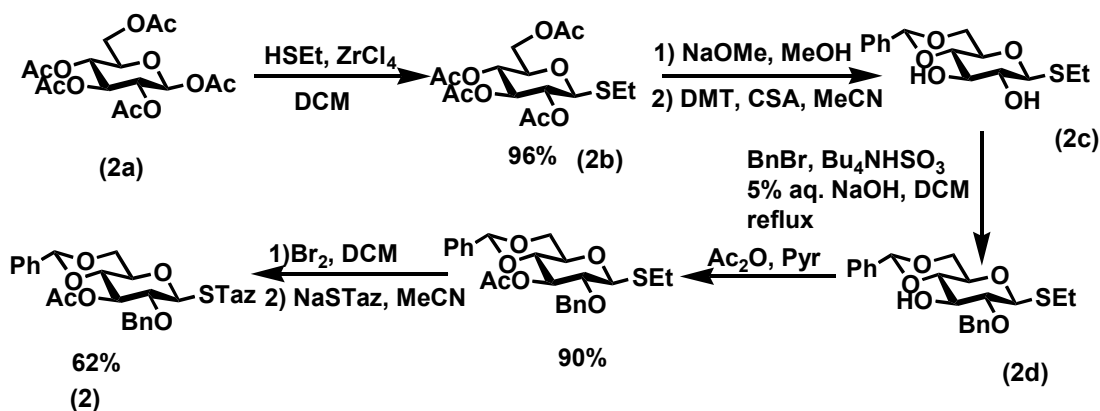
#### 3.5.1 Galactose Building Block

Peracetate of D- galactose (**1a**) was subjected to hydrolysis conditions using trifluoroacetic acid and water; hemiacetal formation followed by acetyl migration resulted in compound (**1b**) which was then subjected to benzylation using benzyl trichloroacetimidate in the presence of TfOH. The SBox moiety was introduced at the anomeric centre via the two step procedure involving bromination of the anomeric center followed by substitution with the potassium salt of 2- mercaptobenzoxazole.



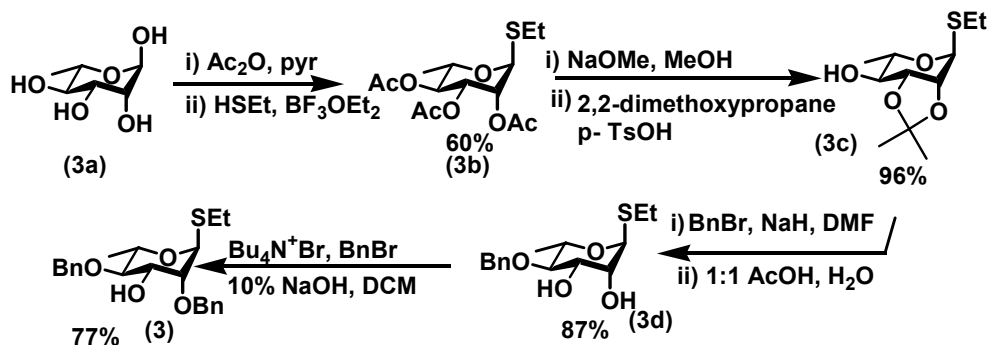
#### 3.5.2 Glucose Building Block

Peracetate of D-glucose (**2a**) was glycosylated with ethanethiol in presence of zirconium tetrachloride to obtain the  $\beta$ -anomer (**2b**) exclusively in 96% yield. Deacetylation followed by benzylidene introduction using dimethoxytoluene (DMT) in the presence of camphor sulfonic acid (CSA) yielded compound (**2c**). Benzylation under phase transfer conditions provided regioisomers which were separated by column chromatography to afford (**2d**). Final product (**2**) was obtained in 62% yield by acetylation of (**2d**) followed by STaz introduction at the anomeric position via two step bromination –S- glycosylation sequence. It should be noted that  $\beta$ -elimination was detected to take place as a side process during this two- step displacement at the anomeric center.



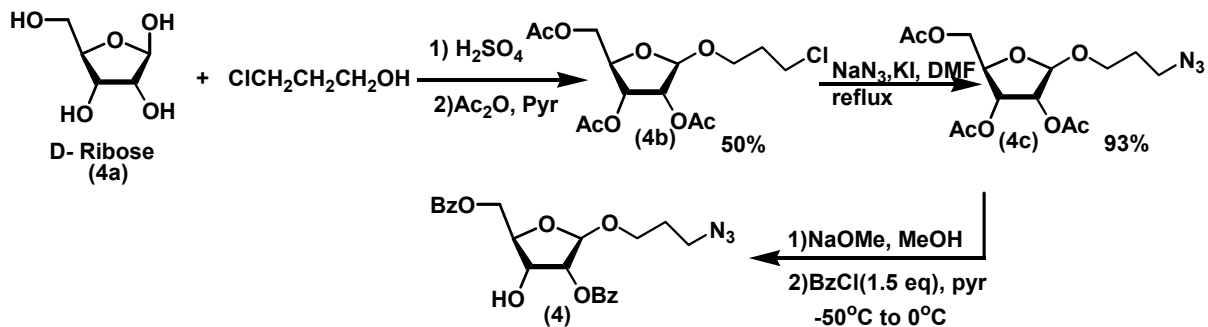
### 3.5.3 Rhamnose Building Block

L-Rhamnose (**3a**) was acetylated and glycosylated with ethanethiol in the presence of boron trifluoride etherate. The anomeric mixture obtained was purified by column chromatography to afford pure  $\beta$ - anomer (**3b**) in 60% yield. Deacetylation followed by isopropylidene introduction yielded compound (**3c**) in 96% yield. Benzylation at C-4 was carried out under conventional conditions (BnBr/ NaH) followed by isopropylidene cleavage afforded compound (**3d**) in 87% yield. Phase transfer conditions for selective benzylation at C-2 were then employed to obtain compound (**3**) in 77% yield.



### 3.5.4 Ribose Building Block

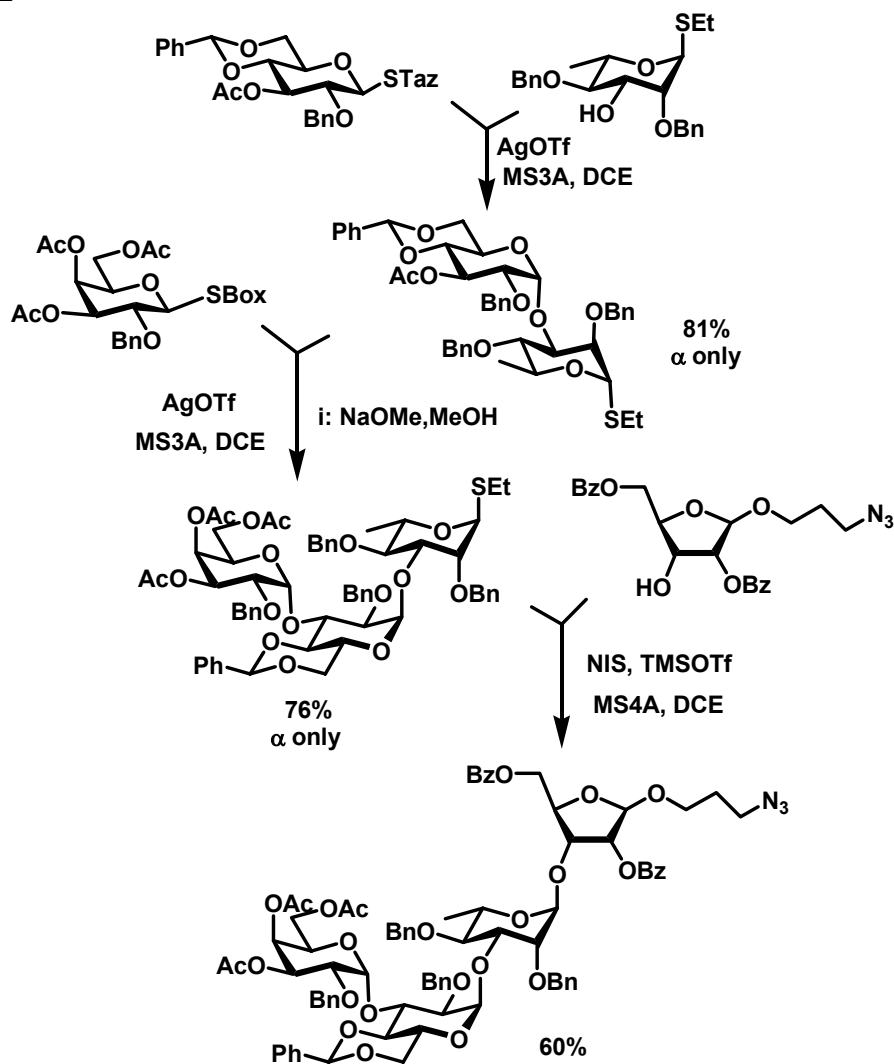
D-Ribose (**4a**) was glycosidated using 3-chloro-1-propanol in the presence of sulfuric acid. The product obtained was subsequently acetylated and the anomeric mixture obtained (1:1) was separated by column chromatography to get compound (**4b**) in 50% yield. Conversion of chloride to azide was carried out at reflux in DMF using sodium azide in the presence of KI. Deacetylation followed by selective di-benzylation, yielded compound (**4**) as a major regioisomer in 30% yield. The reaction was stopped before complete disappearance of starting material to avoid excess formation of other regioisomers.



### 3.5.5 Oligosaccharide assembly

Having obtained the above building blocks, glycosylation was carried out using the standard activation conditions. First glucose bearing the STaz leaving group at the anomeric position was selectively activated over 3-OH of the SET rhamnose acceptor using AgOTf as a promoter in the presence of 3Å molecular sieves and dichloroethane as solvent to obtain a disaccharide (Glu-Rha) in 81% yield with exclusively  $\alpha$  selectivity. (Scheme 2). Deacetylation was carried out to obtain disaccharide acceptor which was then coupled to galactose containing SBox moiety in the presence of AgOTf afford the trisaccharide (Gal-Glu-Rha) in 76% yield with  $\alpha$  selectivity. Final step involved the coupling with ribose containing spacer to obtain tetrasaccharide derivative in 60% yield.

Scheme 2



## Future Work

### 4.1 Synthesis of Structural Mimetics

Various structural mimetics of SPn6A containing ribofuranose with spacer will be considered. For example, disaccharide (Rha-Rib) and trisaccharide (Glu-Rha-Rib) will be prepared before further steps toward large-scale preparations have been undertaken. This will provide important initial steps toward our understanding of the structural features responsible for the biological activity and hydrolytic stability of the molecules of this class.

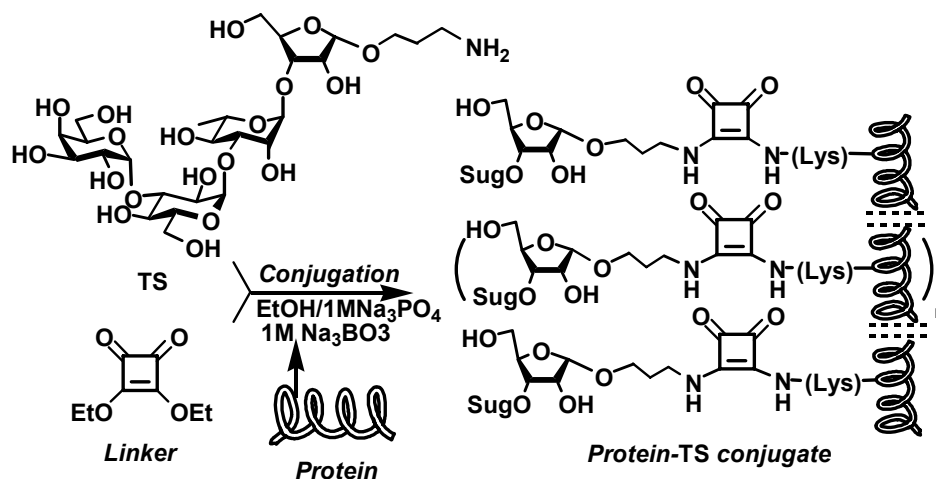
### 4.2 Global Deprotection and Purification

Deprotection of the oligosaccharides will be achieved by sequential deacylation (NaOMe in MeOH) and hydrogenolysis (Pd-C, H<sub>2</sub>) to obtain fully deprotected disaccharide (Rha-Rib), trisaccharide (Glu-Rha-Rib), or tetrasaccharide (TS). Final purification will be done by gel filtration chromatography using Sephadex G-15.

### 4.3 Glycoconjugate Synthesis

The spacer will enable the conjugation of the TS to a commercially available protein, CRM197 of diphtheria toxin, a well-defined non-toxic protein that has been used as a carrier in other types of SPn vaccines.<sup>33</sup> Amongst conjugation techniques available,<sup>34</sup> the plan is to apply Tietze's squarate linker approach<sup>35</sup> as shown in **Scheme 3**.

**Scheme 3**



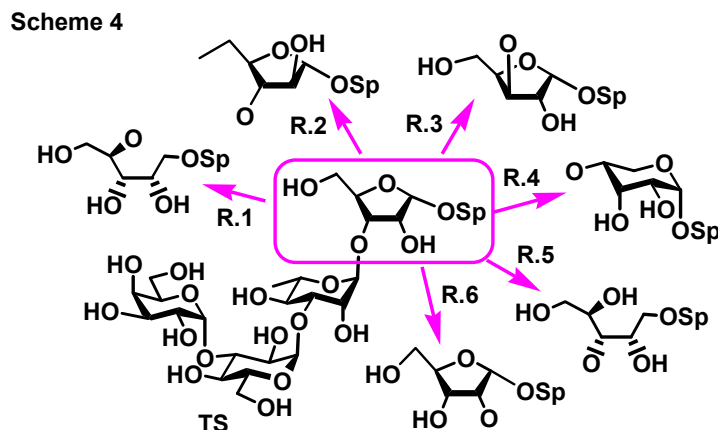
#### 4.4 Biological determinations.

We will then determine whether the synthetic TS-CRM197 conjugate is recognized by immunoglobulin antibodies (IgG) raised against SPn6A (and 6B). One of the conventional techniques to evaluate the efficacy of pneumococcal vaccine candidate is on an ELISA (enzyme-linked immunosorbent assay) plate, which is an accurate method for measuring the concentration of human antibodies directed against pneumococcal saccharides.<sup>14</sup> This testing will be done by our collaborator, Professor Dr. Moon Nahm, at the University of Alabama at Birmingham Medical School whose laboratory serves as a WHO reference laboratory for pneumococcal serology.

#### 4.5 Structure-Hydrolytic Stability Studies.

In order to understand the lower hydrolytic stability of SPn6A vs. SPn6B, we will engage the synthetic material in comparative studies. These hydrolyses will be monitored by HPLC, while the final mixtures of products will be analyzed by mass-spectral techniques, such as MALDI or electron spray. For these studies, we plan to obtain a library of structurally diverse tetrasaccharides, in which ribose unit (**R**) is substituted with other pentofuranoses, for instance D-arabinose (**R.2**) D-xylose (**R.3**), or positional isomers or (1→2)- and (1→4)-linked D-ribose (**R.4** and **R.6**) (**Scheme 4**).

The prepared Gal-Glc-Rha trisaccharide can then be used in direct one-step coupling with suitably protected spacer-containing building blocks **R.1-R.6**. This approach would allow to quickly obtain a small library of compounds with main effort focused on the preparation of relatively simple pentose building blocks **R.1-R.6**. Subsequently, the modified synthetic saccharides will be deprotected, conjugated, and tested.



## **Expectations**

- It is expected that we would obtain a number of fully synthetic conjugates within a reasonable period of time that will provide important initial steps toward our understanding of the structural features responsible for the biological activity of the molecules of this class.
- The synthesis and evaluation of the tetrasaccharide conjugates proposed will be sufficient to draw preliminary conclusions and plan further steps toward subsequent investigations.
- By identifying the most reactive conjugates we will make the first important step toward the development of excellent vaccine candidates against SPn serotype 6A (or both 6A and 6B).

## **Acknowledgement**

- ❖ Dr. Alexei V. Demchenko, Professor UMSL (Department of Chemistry and Biochemistry)
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