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Large-Scale Chemoenzymatic Synthesis of a Glycophorin A Tetrasaccharide Motif and Its Analogues

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A large-scale chemoenzymatic methodology has been developed to prepare a natural and substituted analogues of the Glycophorin A tetrasaccharide, in which the α-2,3-sialic acid is modified at either C5 or C9 either as F, OMe, CH_3, glycolyl or N_3. The strategy involved a chemical synthesis of a Gal-β-1,3-GalNAc disaccharide in which the galactose remains acetylated to first enable selective α-2,6-sialylation using a one-pot aldolase-NmCSS-Pd2,6-ST enzyme system. Finally, C5 or C9 modifed α-2,3 sialosides were placed at the galactoside moiety using C2 or C6 modified MaNAc derivatives catalyzed by aldolase NmCSS-Pd2,3-ST enzyme combination. The synthesised molecules can be useful in binding studies with a protein of interest or as an entry point to establish a library of analytical samples for mass-spectrometric studies.

1. Introduction

Gram-scale carbohydrate synthesis used to be a routine practice in the 20th century,[1] enabled by access to reliable synthetic protocols. In part, the advent of high-field NMR spectroscopy has stimulated small-scale synthesis, which has become more prevalent, often compromising the quality and reproducibility of results.[2] This is because small-scale protocols frequently fail to scale up, requiring extensive optimization and sometimes necessitating a rethinking of synthetic routes. We believe that large-scale carbohydrate synthesis should still be practiced to ensure reliable access to quantities sufficient for biological and structural research.

The work described herein demonstrates the value of the large-scale approach by synthesizing key components of the Glycophorin A glycan and its analogues in the context of an antimalaria drug discovery program.

Malaria, a mosquito-borne disease, poses a significant global health burden particularly in developing countries. In 2023, it was responsible for an estimated 219 million infections and 597,000 deaths with the majority of cases caused by *Plasmodium falciparum*.[3] The parasite's life cycle is complex, with the red blood cell (RBC) invasion stage being critical to the onset and progression of the disease.[4] Understanding the molecular mechanisms of RBC invasion could lead to the development of novel therapeutic strategies, as disrupting this process could halt the parasite's ability to multiply within human cells.

Central to the RBC invasion process is Glycophorin A, a protein terminally decorated with sialic acids and expressed on human erythrocytes.[5] Sialic acids are a family of nine-carbon sugars prominently displayed at the termini of glycoproteins and glycolipids on the cell surface.[6] Sialic acids, such as *N*-acetylneuraminic acid (Neu5Ac) play crucial roles in intercellular recognition and are involved in processes such as immune evasion and pathogen-host interactions.[7]

In the case of malaria, the *P. falciparum* parasite recognizes a sialylated tetrasaccharide (Figure 1) located on the RBC surface, enabling entry into host cells.[8,9] Specifically, erythrocyte binding antigen-175 (EBA-175), a parasite surface protein, binds to Neu5Ac residues on Glycophorin A facilitating RBC invasion and progeression to a disease.[10]

Given the significance of sialic acid-mediated interactions, modifications at the C9 position of sialic acids in glycoconjugates provide a valuable 'reporter' tool for probing the molecular details of parasite binding.[11,12] By altering the structure of sialic acids, insights into the specific interactions between the parasite and host cells could be obtained.[13] These modified sialic acids are expected to serve as key components for investigating the binding dynamics between sialylated glycans and EBA-175, offering a potential pathway for designing carbohydrate-based therapeutics.

This study focuses on the synthesis of C5/C9-modified sialic acids and their incorporation into glycoconjugates to explore their role in the interaction between sialylated glycoproteins and the *P. falciparum* protein EBA-175. Understanding these molecular interactions could pave the way for the development of novel

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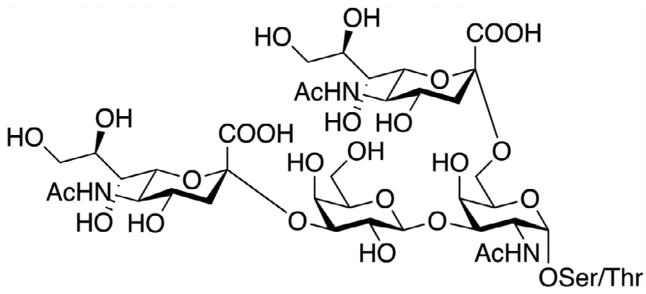


Figure 1. Structure of the major Glycophorin A *O*-glycan.

treatments to stop the parasite's ability to invade red blood cells.

2. Results And Discussion

The scalable synthesis of Glycophorin A tetrasaccharide and its analogues relies on minimizing chromatography and utilizing inexpensive starting materials to ensure both efficiency and reproducibility. A key step in this process is the convenient preparation of the disaccharide core[14] **1**, which is a recognisable substrate for sialylation. Selective α-2,6 sialylation of the galactosamine residue is achieved by maintaining the terminal galactose acetylated.[15] Once sialylation is complete, the acetyl esters are quantitatively removed by using a base to give **2** allowing the remaining C5/C9 modified sialic acids to be enzymatically glycosylated[16] to yield the final tetrasaccharides.

Our synthetic route towards the core disaccharide, illustrated in Scheme 1, begins with the conversion of commercially available *N*-acetylglucosamine to a galactosamine-based acceptor via C4 configurational inversion.[17] First, per-*O*-acetylated glucosamine derivative **10** is treated with benzylamine,[18] followed by installation of a trichloroacetimidate,[19] resulting in donor **11** in 63% yield over two steps. Glycosylation of this donor with benzyl alcohol, catalyzed by trimethylsilyl trifluoromethanesulfonate (TMSOTf),[20] proceeds smoothly, yielding glycoside **12** in 86% yield after crystallization.

For activation, a higher temperature (60 °C) was necessary to ensure complete conversion of the oxazoline intermediate to the glycoside product **12**. Compound **12** was then deacetylated with NaOMe in methanol, and the resulting triol was treated with three equivalents of PivCl in pyridine, leading to a highly selective protection of the 4- and 6-positions to give **13** in 85% yield.[17] This regioselectivity is likely due to strong hydrogen bonding between the acetamido group and the C4 hydroxyl, making the latter a poor nucleophile.[21]

Configurational inversion of **13** was accomplished by treatment with triflic anhydride, followed by in situ addition of water, yielding the galactosamine derivative **14** in 74%. The pivalate esters were subsequently removed with NaOMe in methanol, and a 4,6-*O*-benzylidene acetal was installed, giving compound **15** in 76% yield over two steps. This intermediate was purified via crystallization, avoiding the need for chromatography.

Finally, acceptor **15** was coupled with acetobromogalactose using mercury cyanide (II) as a promoter,[22] delivering the disaccharide **16** in 78% yield. After the glycosidic bond formation, the benzylidene acetal was removed by treatment with aqueous acetic acid, affording the 4,6-diol **1** in 75%, which was used directly in subsequent enzymatic reactions.

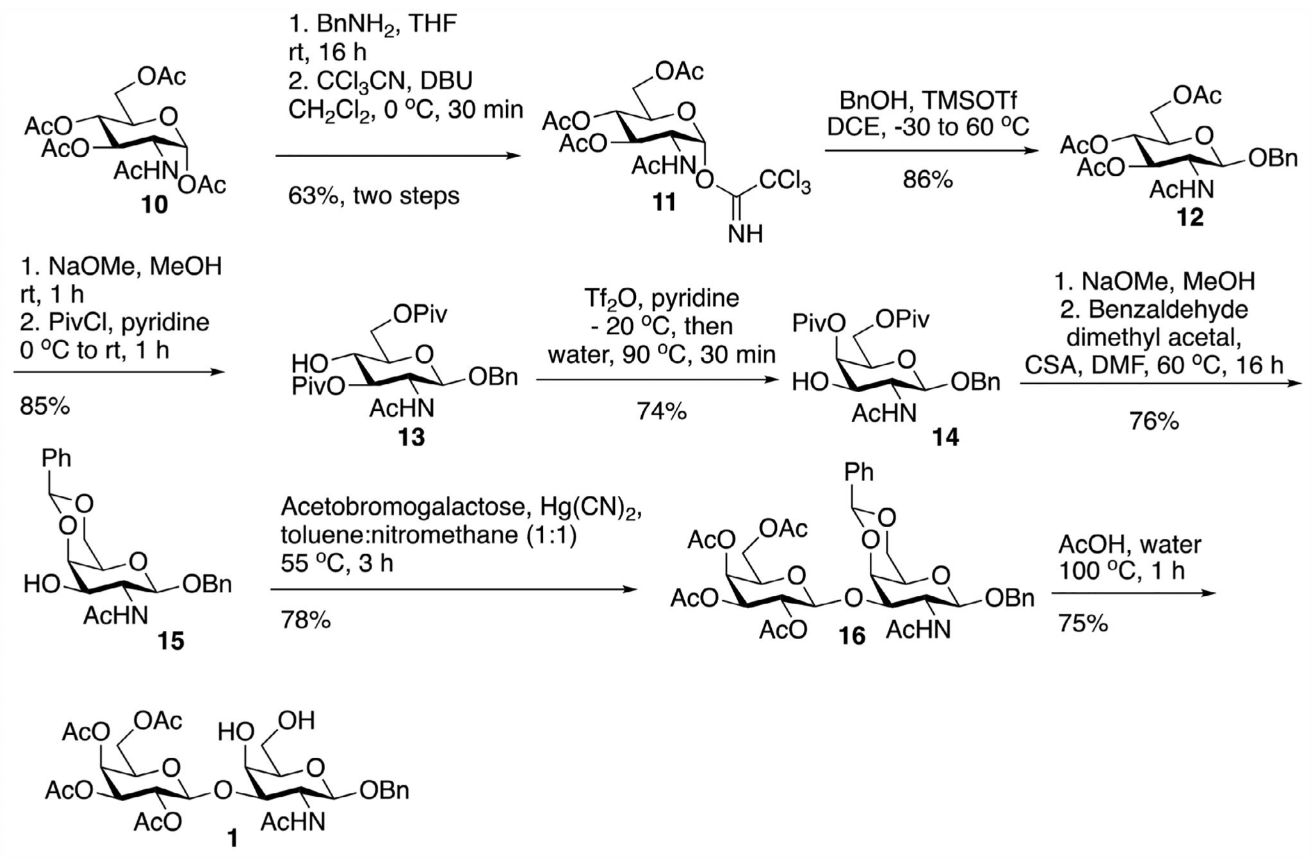
To incorporate C9-modified sialic acids we chose to use C6-modified derivatives of *N*-acetylmannosamine, which when exposed to a bacterial sialic acid aldolase, become C9-modifed sialosides.[12] The synthetic approach is outlined in Scheme 2. First, commercially available ManNAc was treated with benzyl alcohol in presence of acetyl chloride[23] to give the α-glycoside **18**, followed by selective installation of the *tert*-butyldiphenyl (TBDPS) group at C6 to afford **19** in 63% yield over two steps. The remaining hydroxyl groups were then benzylated to provide intermediate **20**, which without any further purification, was treated with a catalytic amount of AcCl in methanol[24] to furnish **21** in 55% yield over two steps. This species was then exposed to a variety of conditions[25] to introduce either an —OAc (**22**), —F (**23**), —CH_3 (**24**), or —OMe (**25**) groups in quantitative, 52%, 83%, and 86% yield respectively. Catalytic hydrogenolysis over palladium hydroxide (20%, w/w) afforded compounds **26**–**29** in quantitative yields ready for enzymatic transformaitons (Supporting Information).

With sufficient quantities of the disaccharide **1** in hand, the α-2,6-sialylation of the galactosamine moiety was undertaken. To this end, a multifunctional bacterial α-2,6-sialyltransferase (Pd2,6ST) derived from *P. damselae* was used.[12] This enzymatic reaction was conducted in buffer containing 5% DMSO to ensure the disaccharide acceptor **1** remains in solution. Thus, trisaccharide **2** (700 mg) was obtained in 60% yield after an additional base treatment, which is ready for final installation of C9-modifed sialic acids.

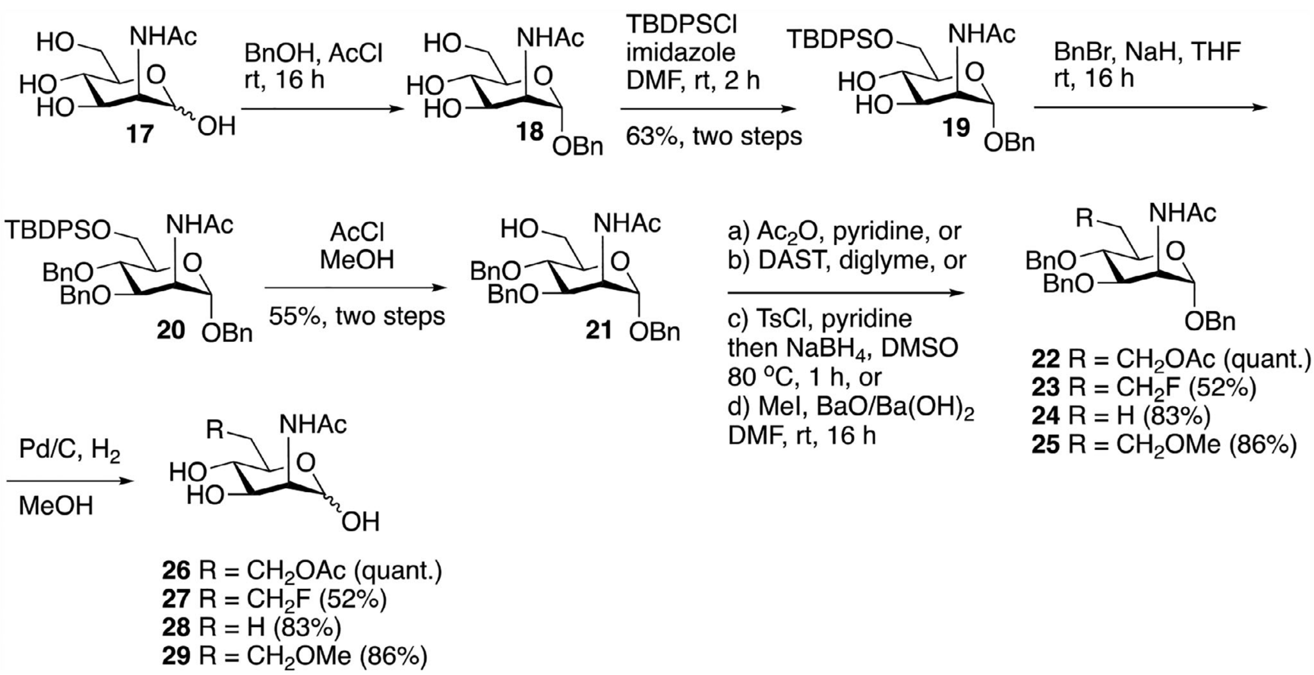
The final steps relied on using mannosamine derivatives **26** – **32**, which were in one pot exposed to *E. coli* aldolase in presence of pyruvate resulting in conversion to sialic acids (Scheme 3). NmCSS then converts the sialic acid to the corresponding C9-modified-CMP-Neu5Ac donor substrate, which are captured by PmST1 and transferred to the C3 position of the galactoside. This one-pot procedure[12] gave tetrasaccharide **3** in 62% yield, while higher yields were obtained for **4** (79%) and **7** (80%) suggesting that some C9 modifications enhance the efficiency of sialic acid transfer and are probably less resistant to hydrolysis by PmST1 (this step is reversible). Similarly, the Neu5Gc derivative **8** was obtained in 86% yield. On the other hand, no reaction was observed with the C9-OAc substrate **22**, likely because the acetate is a bulkier moiety requiring longer reaction times, at which point, at pH 8.5, it may undergo cleavage.

3. Conclusions

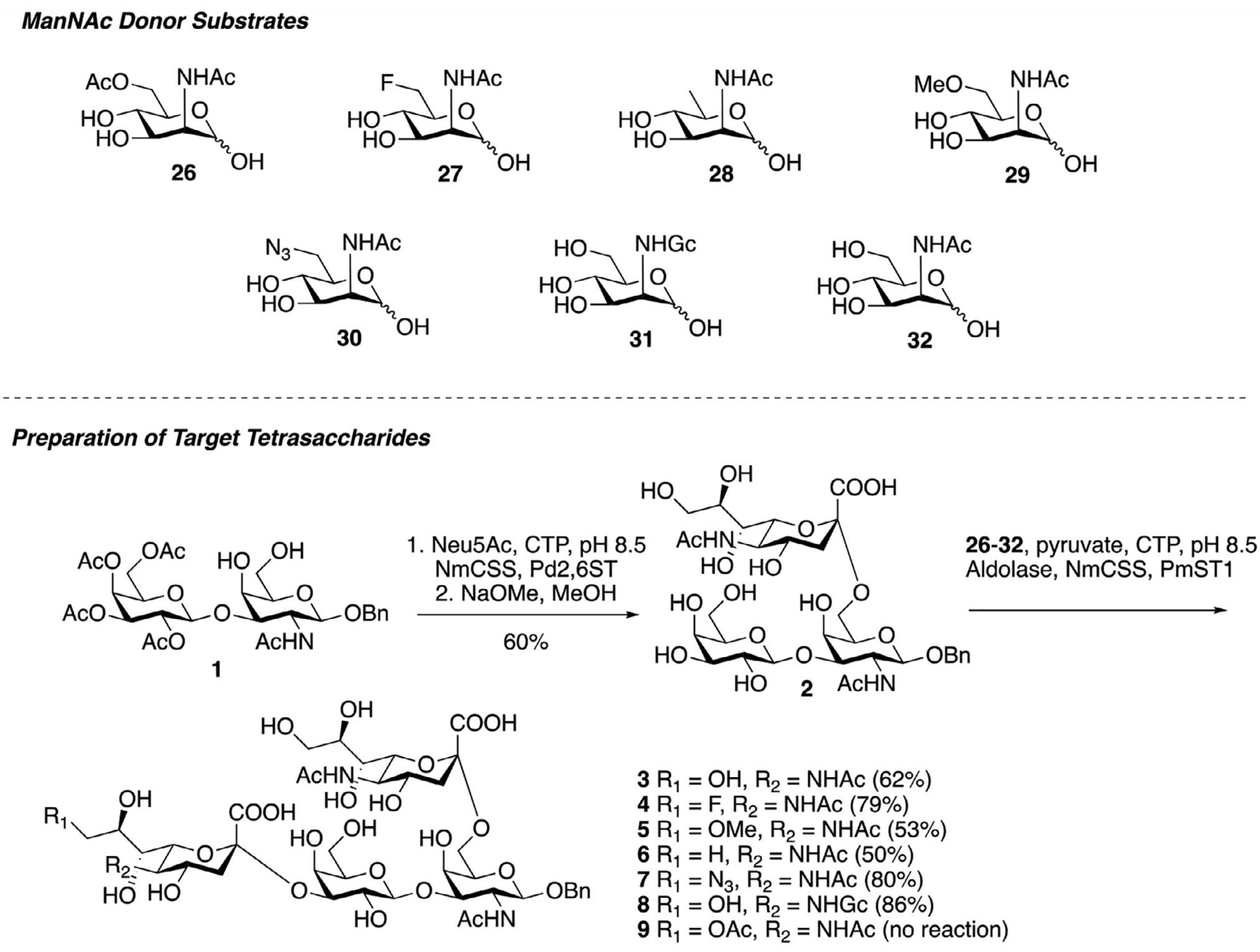
In conclusion, the scalable synthesis of Glycophorin A motif tetrasaccharides and their analogues has been optimized by employing cost-effective starting materials and minimizing chromatography. The key trisaccharide core was prepared from the



Scheme 1. Synthesis of acceptor **1**.



Scheme 2. Synthesis of C6-modified ManNAc derivatives.



Scheme 3. Chemoenzymatic assembly of tetrasaccharides.

chemically-synthesized disaccharide **1** employing a highly selective sialylation of the nonacetylated GalNAc moiety followed by enzymatic incorporation of C9-modified sialic acids on the galactose residue. This streamlined approach, involving both selective α-2,6- and α-2,3-sialylation, results in high yields of tetrasaccharides **3** – **8** with certain C9 modifications enhancing the transfer efficiency (**4** and **7**). These results provide a robust and reproducible method for large-scale glycan synthesis, which is important, for example when defined glycans or glycan fragments are required for mass spectrometric or protein binding experiments.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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