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Enzymatic Sialylation of Synthetic Multivalent Scaffolds: From 3′-Sialyllactose Glycomacromolecules to Novel Neoglycosides

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Sialoglycans play a key role in many biological recognition processes and sialylated conjugates of various types have successfully been applied, e.g., as antivirals or in antitumor therapy. A key feature for high affinity binding of such conjugates is the multivalent presentation of sialoglycans which often possess synthetic challenges. Here, the combination is described of solid phase polymer synthesis and enzymatic sialylation yielding 3′-sialyllactose-presenting precision glycomacromolecules. CMP-Neu5Ac synthetase from *Neisseria meningitidis* (NmCSS) and sialyltransferase from *Pasteurella multocida* (PmST1) are combined in a one-pot reaction giving access to sequence-defined sialylated macromolecules. Surprisingly, when employing Tris(hydroxymethyl)aminomethane (Tris) as a buffer, formation of significant amounts of α-linked Tris-sialoside is observed as a side reaction. Further exploring and exploiting this unusual sialylation reaction, different neoglycosidic structures are synthesized showing that PmST1 can be used to derive both, sialylation on natural carbohydrates as well as on synthetic hydroxylated scaffolds.

1. Introduction

In particular, higher organisms make use of glycosylation – the covalent attachment of a carbohydrate motif onto another biomacromolecule such as a protein, lipid, RNA, or another glycan – to orchestrate a variety of biological processes, such as inflammatory response or viral and bacterial infections.[1–5] The biosynthetic introduction of carbohydrates occurs posttranslationally via glycosyl transfer reactions mediated by enzymes located both intra- and extracellularly.[6–10] An extraordinary role in the late-step cascade glycosylation plays *N*-acetylneuraminic acid (Neu5Ac), the most common member in the group of the sialic acids.[11–13] A well-known sialylated oligosaccharide is the sialyllactose glycan moiety[14–16] which descends of nucleotide-conjugated sialic acids being enzyme-specifically attached to lactosides[17] and belong to the family of human milk oligosaccharides (HMO). Neu5Ac is known as an important binding motif of glycan ligands involved, e.g., in recognition processes[18] and information transfer.[19] Sialylated glycans are often necessary for neuronal function[19,20] and are found on cellular tissue and major organs.[21,22] They are specifically recognized by a number of protein receptors such as selectins,[18] sialoadhesins (Siglecs),[23,24] and hemagglutinins.[23,24]

Based on the abundance and multitude of biological functions of sialylated compounds, great efforts have been devoted to the synthesis of natural sialylated glycans as well as synthetic sialylated conjugates such as neoglycosides or glycopolymers.[25–31] Sialylated polymers were introduced by Whitesides and coworkers[32,33] and characterized for their efficient binding to hemagglutinin and inhibition of the Influenza virus.[34] Today, various types of synthetic sialylated conjugates, e.g., based on dendrimers or nanoparticles have been realized and explored for their potential in biomedicine, e.g., as antivirals or anti-inflammatories.[35–38] A key feature of such glycan conjugates is their multivalency, where the presentation of multiple copies of sialic acid enables higher binding avidity.[39] One of the remaining challenges in developing sialylated glycoconjugates is realizing not only higher avidity but also selectivity towards a

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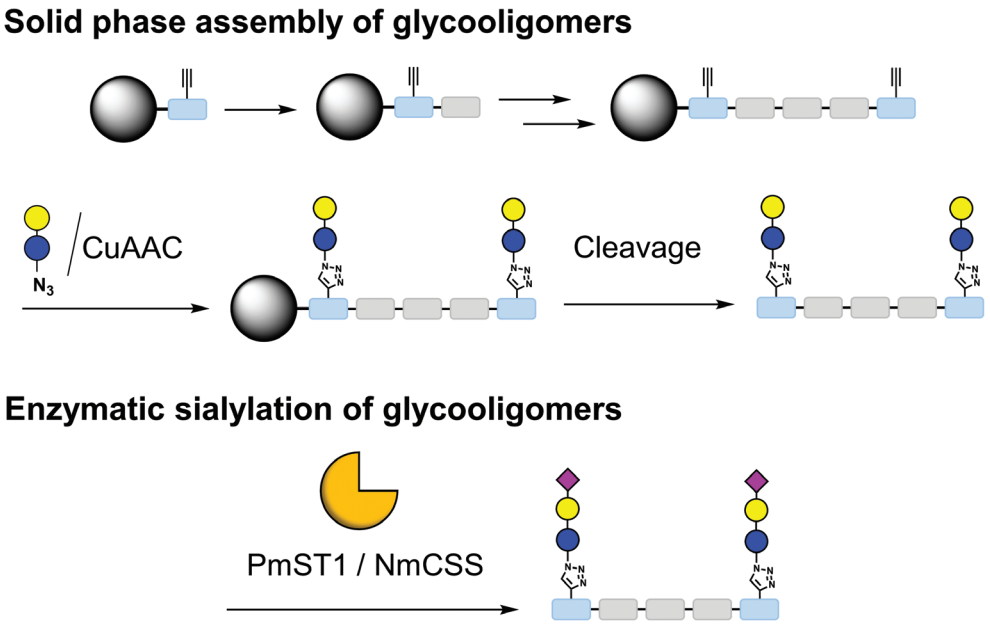

Figure 1. Synthetic strategy towards 3′-sialyllactose-presenting glycomacromolecules applying solid phase assembly of the scaffold via stepwise addition of building blocks, chemical conjugation of the ligand and the subsequent enzymatic sialylation.

specific receptor. In nature, selectivity is often achieved by a unique spatial arrangement of a terminal sialic acid residue linked to a distinct glycan scaffold,[40] such as the stereoselective glycosidic linkage connecting Neu5Ac to a terminal galactose residue either via an α2,3- or an α2,6-glycosidic linkage which can modulate the binding specificity of certain receptors such as hemagglutinin of avian versus human-specific influenza virus A.[41–43] However, such stereoselectivity is still challenging to achieve by chemical synthesis. Other challenges in sialylation are posed by the steric demands during sialic acid conjugation,[44] and the need to eliminate byproducts (e.g., unsaturated Neu5Ac2en type products from elimination mechanism),[45] otherwise resulting in reduced yields.[46] An alternative strategy relies on the use of enzymes to perform sialylation reactions. Inspired by the posttranslational modification of proteins,[47–49] where sialic acid residues are attached enzymatically. This is the last step of the synthetic procedure,[20,26,50] thereby also preventing degradation of the rather pH-sensitive sialoside linkage and thus further improving yields.[51] In general, enzymatic methods often enable superior control of stereoselectivity, i.e., anomeric configuration and milder conditions, preventing unwanted side reactions such as formation of unsaturated sialic acid byproducts via elimination.[52,53] Such chemoenzymatic protocols have lately been successfully employed to synthesize complex branched sialylated oligosaccharides that were then used to study the role of sialylation in fertilization of human oocytes.[54]

In order to combine both, high control in multivalency as well as stereoselective sialylation, here we bring together our previously introduced solid phase polymer synthesis giving access to monodisperse, sequence-defined glyco(oligoamidoamines)[37,55,56] and the enzymatic sialylation on these multivalent scaffolds. Lactose-functionalized glycooligomers are readily available from our previous studies[57,58] and will be used for enzymatic sialylation of lactose moieties by well-established *Pasteurella multocida* sialyltransferase PmST1[53,59] in combination with a *Neisseria meningitidis* CMP-Neu5Ac-synthetase from NmCSS[60] in order to synthesize the required sialic acid donor CMP-Neu5Ac in situ (**Figure 1**). Product formation will be analyzed by in-line ^{1}H-NMR analysis giving direct access to reaction kinetics and yields enabling straightforward optimization of reaction conditions and comparison of wild type and mutant PmST1.

2. Results and Discussion

2.1. Developing Two-Step Enzymatic Sialylation and In-Line NMR Monitoring

For the synthesis of 3′-sialyllactose-presenting glycomacromolecules, we used a two-step enzymatic synthesis in a one-pot reaction based on published protocols, employing CMP-sialic acid synthetase from *Neisseria meningitidis*, NmCSS,[60] and sialyltransferase from *Pasteurella multocida*, PmST1, shown in **Figure 2**A. PmST1 was used as wildtype[61] (WT) and mutant[62] (M144D) enzyme as previously described by Chen and coworkers.[62,63] Compared to the wildtype enzyme, the M144D mutant has a significantly reduced Cytidine monophosphate *N*-acetylneuraminic acid (CMP-Neu5Ac) hydrolysis activity and, most importantly, a dramatically reduced sialidase activity,[64] which would potentially cause loss of product due to cleavage of the sialic acid. The α2,3-sialyltransferase activity is also decreased as reflected by k_{cat} K_M^{-1} values being about one order of magnitude lower.[62,64] However, suppression of the sialidase activity by far outweighs the slight disadvantage of longer reaction times needed for sialylation.

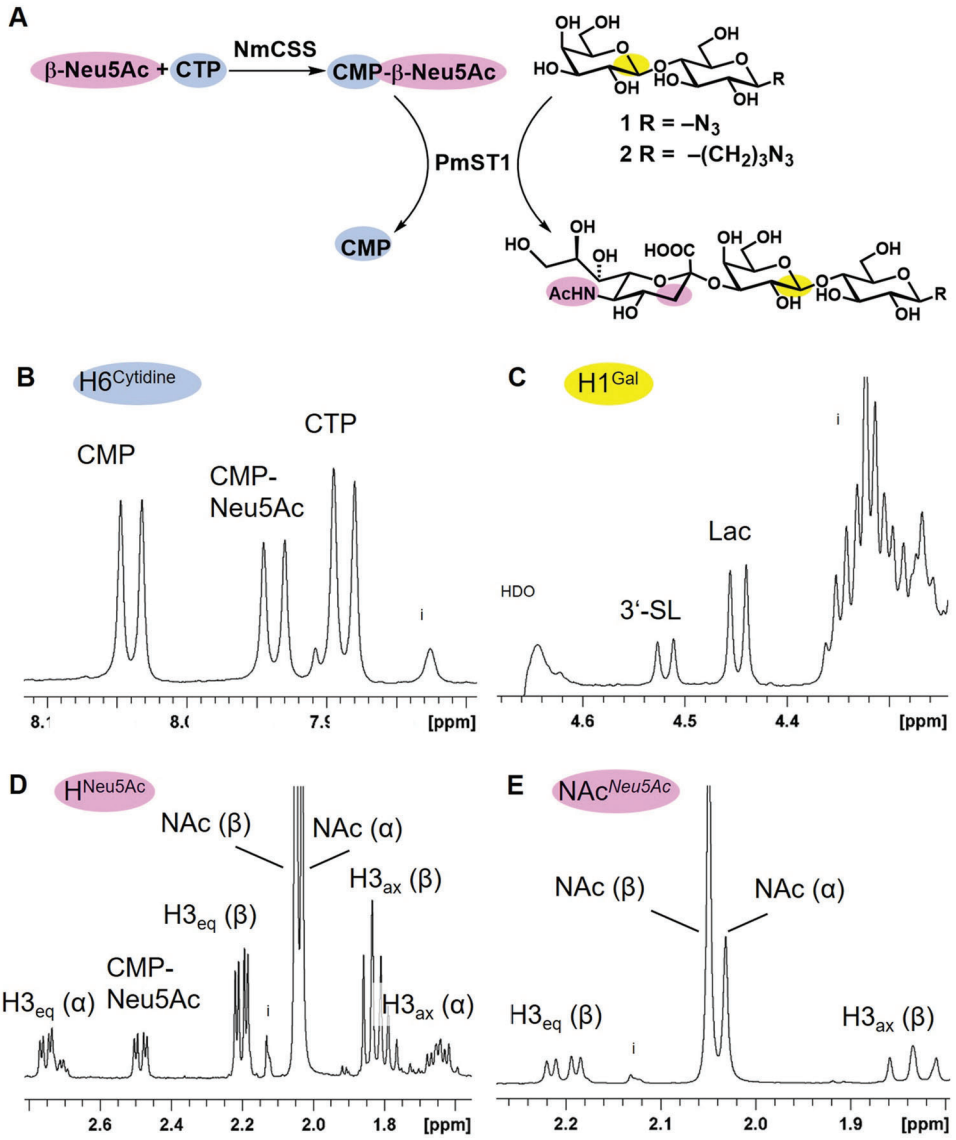

Figure 2. Enzymatic α2,3-sialylation of $LacN_3$ (1). A) Reaction scheme. B–E) Spectral regions of a proton ^1H NMR spectrum (500 MHz, 310 K) of a representative reaction mixture. B) $H6^{Cytidine}$ resonance signals of CTP (Cytidine triphosphate, δ = 7.89 ppm), CMP-β-Neu5Ac (δ = 7.94 ppm), and CMP (δ = 8.04 ppm) (blue). C) Resonance signals of $H1^{Gal}$ of lactose (δ = 4.45 ppm) and of 3′-sialyllactose (δ = 4.52 ppm) (yellow). D) Resonance signals of $H3_{eq}{}^{Neu5Ac}$ (δ = 2.75 respective 2.20 ppm), $H3_{ax}{}^{Neu5Ac}$ (δ = 1.94 respective 1.60 ppm), and of NAc^{Neu5Ac} (δ = 2.05 respective 2.03 ppm) of 3′-sialyllactose (NAc(α)), CMP-β-Neu5Ac (NAc(β)) and of free sialic acid (NAc(β)) (pink). E) Section enlargement of resonance signals of NAc^{Neu5Ac} (δ = 2.05 respective 2.03 ppm). The reaction mixture was composed of 11×10^{-3} M $LacN_3$ (1), 16.5×10^{-3} M CTP/Neu5Ac, 200×10^{-3} M Tris pH 8.8, 40×10^{-3} M $MgCl_2$, 400 µg mL^{-1} NmCSS, 50 µg mL^{-1} PmST1 WT, 200×10^{-6} M DSS, 166×10^{-6} M imidazole and D_2O.

In-line monitoring of enzymatic sialylation reactions by recording ^1H NMR spectra of reaction mixtures at fixed time intervals has been reported previously,[64] and was used here to optimize reaction conditions for enzymatic sialylation of lactose building block $LacN_3$ (1) (Figure 2A). Briefly, we used structural reporter group ^1H NMR signals[65] of protons $H3_{eq}{}^{Neu5Ac}$ (δ = 2.75 respective 2.20 ppm), $H3_{ax}{}^{Neu5Ac}$ (δ = 1.94 respective 1.60 ppm), NAc^{Neu5Ac} (δ = 2.05 respective 2.03 ppm) as well as $H1^{Gal}$ (δ = 4.45 respective 4.52 ppm) to follow the enzymatic glycosylation reaction (Figure 2C–E). Importantly, the chemical shifts of $H3_{eq}{}^{Neu5Ac}$ (δ = 2.75 respective 2.20 ppm) and $H3_{ax}{}^{Neu5Ac}$ (δ = 1.94 respective 1.60 ppm) report on the type of glycosidic linkage, i.e., 2,3 or 2,6.[66,67] Enzymatic turnover of CMP-Neu5Ac is reflected by the resonance signals of protons $H6^{Cytidine}$ (δ = 7.89, 7.94 respective 8.04 ppm, Figure 2B).

To follow product formation, we measured integrals I of protons $H1^{Gal}$ (δ = 4.45 respective 4.52 ppm) over time and calculated corresponding integral ratios $R_{3'SL}$ as given by Equation (1). Enzymatic turnover of CMP-Neu5Ac is reflected by the integrals I of protons $H6^{Cytidine}$ (δ = 7.89, 7.94 respective 8.04 ppm) and the corresponding integral ratios $R_{CMP\text{-}Neu5Ac}$ as given by Equation (2)

$$R_{3'SL} = I\left(H1^{Gal}\ \left(3'\mathbf{SL}\right)\right) \,/\, \left(I\left(H1^{Gal}\ (1)\right) + I\left(H1^{Gal}\ \left(3'\mathbf{SL}\right)\right)\right) \quad (1)$$

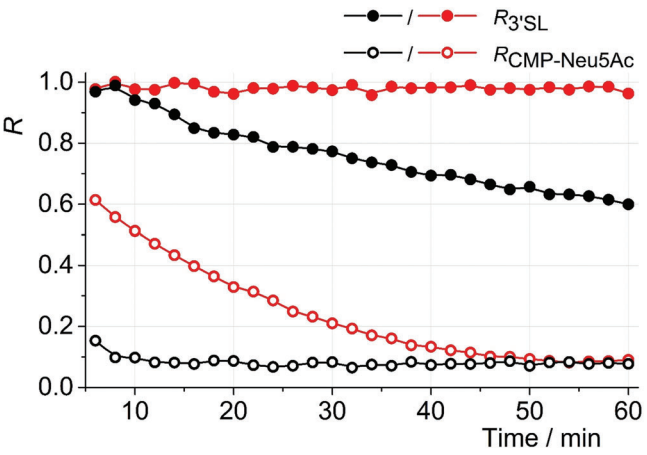

Figure 3. Enzymatic sialylation of $LacN_3$ (**1**) using PmST1 WT. Red circles: 40 μg mL^{-1} PmST1. Black circles: 280 μg mL^{-1} PmST1. Filled circles: $R = R_{3''SL}$ (cf. Equation (1)). Open circles: $R = R_{CMP\text{-}Neu5Ac}$ (cf. Equation (2)). The curve represented by the red filled circles reflects the improved stability of 3′′-SL. The curve represented by the black filled circles reflects product degradation in the absence of CMP-Neu5Ac (black open circles). All samples contained 11×10^{-3} M acceptor (**1**), 33×10^{-3} M CTP/Neu5Ac, 300 μg mL^{-1} NmCSS in 200×10^{-3} M Tris pH 8.8, 40×10^{-3} M $MgCl_2$ each.

$$R_{CMP-Neu5Ac} = I\left(H6^{Cytidine}\,(CMP - Neu5Ac)\right) / \left(I\left(H6^{Cytidine}\,(CMP - Neu5Ac)\right) + I\left(H6^{Cytidine}\,(CMP)\right) + I\left(H6^{Cytidine}\,(CTP)\right)\right) \quad (2)$$

Here we explore a modified strategy aiming at more flexible reaction handling employing PmST1 WT. Using lower concentrations of PmST1, adding CTP and sialic acid in excess over acceptor (**1**), we succeeded in effectively suppressing the α2,3-sialidase activity of PmST1 WT. In **Figure 3**, the integral ratios R as defined by Equations (1) and (2) are shown as a function of time at high and low concentrations of PmST1, reflecting the improvement in terms of product stability by using a lower concentration of PmST1 and applying CTP and sialic acid in excess. The first data point in **Figure 4** has been recorded approximately five minutes after initiating the enzymatic sialylation by adding PmST1. Since the enzymatic sialylation has come to completion within this time the curves in Figure 4 only reflect product (3′-SL) stability over time. Product stability is coupled to the presence of CMP-Neu5Ac: As long as CMP-Neu5Ac was present, no sialidase activity was observed.

2.2. Enzymatic Sialylation of Lactose-Functionalized Glycomacromolecules

It was previously described that PmST1 requires lactose or *N*-acetyllactosamine as acceptor entities for the successful, regioselective attachment of the sialic acid residue.[61] Therefore, lactose-functionalized glycomacromolecules were used as precursors for later chemoenzymatic reactions. Synthesis of these precursors followed previously presented protocols for the solid phase assembly of oligoamide scaffolds, including synthesis of azido-functionalized lactose derivatives employing CuAAC.[57,68,69] In short, oligoamide scaffolds were derived from tailor-made building blocks carrying a free carboxy group and a Fmoc-protected amine group. This allowed for the stepwise addition on solid support using standard Fmoc-peptide coupling protocols. Here, previously established functional building blocks were used for scaffold assembly. TDS building block (1-(Fluorenyl)-3,11-dioxo-7-(pent-4-ynoyl)-2-oxa-4,7,10-triazatetradecan-14-oic acid, triple bond diethylenetriamine succinylamide) was used to introduce an alkyne moiety in the side chain and EDS building block[64,66] (1-(Fluorenyl)-3,14-dioxo-2,7,10-trioxa-4,13-diazaheptadecan-17-oic acid, ethyleneglycol diamine succinylamide) was chosen to insert an ethylene glycol spacer into the oligoamide backbone. As for the β-lactose (Lac) derivatives, the azide functional group was either placed directly at the anomeric carbon atom ($LacN_3$, **1**) or was *O*-glycosidically attached via a propylene spacer ($LacOPrN_3$, **2**). After successful assembly of the desired scaffold on solid support and acetylation of terminal amine group, CuAAC was applied to attach the desired azido-lactose derivatives onto the scaffold as illustrated in Figure 1. Deprotection and cleavage from solid support resulted the final lactose-presenting glycomacromolecules. (see the Supporting Information for further details on synthesis and analysis). The synthesis and analysis of glycomacromolecules **3**, **4**, **6**, **7**, **8**, and **10** was presented previously.[51]

In total, eight lactose-presenting glycomacromolecules were used for later chemoenzymatic reactions (**3–10**). The first set was based on the use of $LacN_3$ (**1**) directly attaching the lactose through a triazole linker onto the scaffold. Mono-, di-, and trivalent glycomacromolecules were obtained by varying the number of TDS building blocks during scaffold assembly (**3**, **4**, and **6**). Furthermore, for the divalent structures, the spacing between the two side chains was varied by elongating the scaffold with additional EDS building blocks (**4** and **5**). Using the same scaffold structures but attaching $LacOPrN_3$ (**2**), the second set of glycomacromolecules was obtained now including an additional propylene spacer between scaffold and lactose (**7–10**) (see Figure 4). These structures will allow us to test whether glycan spacing has an effect on later sialylation reactions, e.g., in terms of yield and reaction rates. All oligomers were used for further enzymatic reactions as obtained after precipitation in diethyl ether, anion exchange and subsequent preparative reversed-phase HPLC (see the Supporting Information for spectral and analytical information).

Based on the optimized reaction conditions for wild type PmST1, we synthesized the α2,3-linked glycomacromolecules (**11–18**). NMR-analysis of the glycomacromolecules confirmed that exclusively α2,3-linked products were formed. Further refinement of the protocol involved adding CTP and Neu5Ac in three portions at fixed time intervals between 0.5 and 1 h, each portion containing 1.5 mol acceptor equivalents of CTP and Neu5Ac as well as 50×10^{-3} M Tris buffer (pH 8.7). Yields are given in **Table 1**. While complete conversion was observed during the reaction, final yields are decreased due to the multistep isolation of the products, including gel filtration and preparative HPLC. Potentially, also partial desialylation can take place again through the enzyme during the work-up. Future

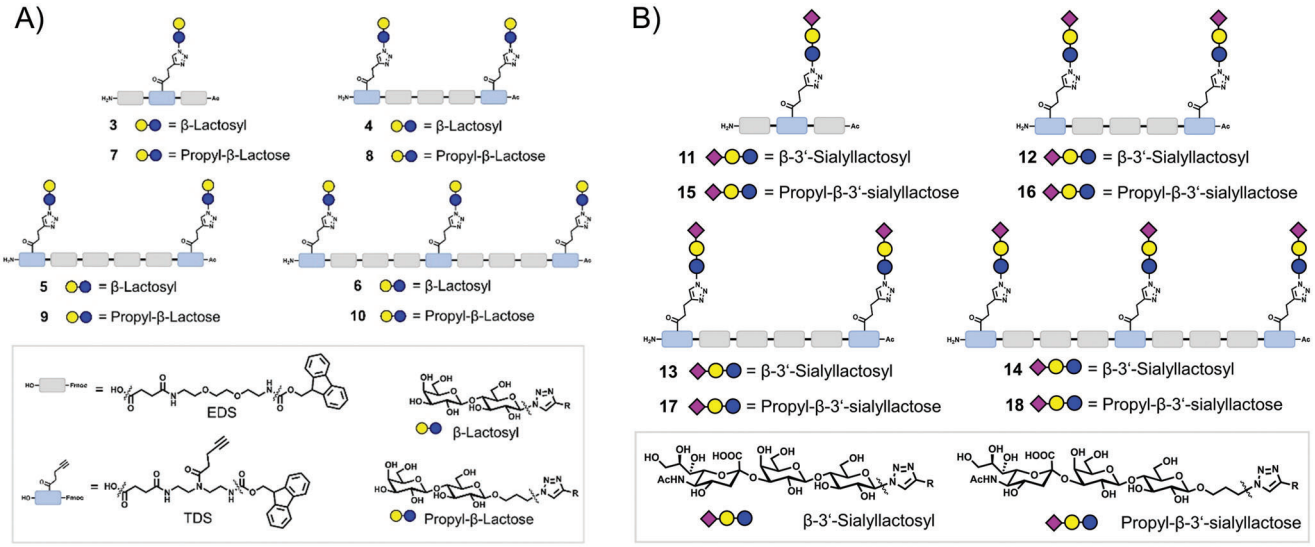

Figure 4. A) Lactose-presenting oligomers **3**, **4**, **6, 7, 8,** and **10**, synthesized via previously described SPPS,[57] that serve as precursors for chemoenzymatic reactions. B) 3′-Sialyllactose-presenting glycomacromolecules (**11**–**18**) after chemoenzymatic conversion.

Table 1. Yields for the enzymatic synthesis of glycomacromolecules (**3–10**) into sialylated products (**11–18**).

Precursor	Product	Yield[a)]	Relative purity[b)]	Molecular Weight [g mol^{-1}]
3	**11**	24%	>98%	1443.5
4	**12**	55%	97%	2597.6
5	**13**	31%	87%	2827.9
6	**14**	25%	98%	4212.3
7	**15**	37%	97%	1501.6
8	**16**	50%	>98%	2713.8
9	**17**	27%	97%	2944.0
10	**18**	38%	84%	4386.5

[a)] Yields after size exclusion chromatography and RP-HPLC; [b)] Relative purity was determined via UV absorption in HPLC.

work will optimize the purification steps, e.g., by using sialic acid recognizing lectins to isolate the product from the mixture.

2.3. PmST1 Catalyzes Sialylation of Nonglycan Acceptors

Very much to our surprise and to the best of our knowledge as not described before, we observed the formation of a sialic acid containing side product in large quantities. The chemical shifts of protons H3^{Neu5Ac} (δ = 2.75 respective 1.60 ppm) of this side product (see **Figure** S2 in the Supporting Information) are similar to values expected for α2,6-linked sialic acid residues known to be formed at more acidic pH,[61] which has been continuously controlled and adjusted during enzymatic synthesis. We identified this side product as the conjugate of the Tris hydroxy group and sialic acid as shown in **Figure 5**. This was confirmed by the mass of the purified compound as determined by ESI-MS matching the expected m/z value (see **Figure** S91 in the Supporting Information) and by an HMBC spectrum of a reaction mixture without acceptor (two protons at δ = 3.51 and δ = 3.85 ppm are connected to the anomeric carbon atom of sialic acid, C2^{Neu5Ac} (δ = 100.4 ppm), detection via long-range correlations, see **Figure** S4 in the Supporting Information).

Setting up the enzymatic reaction in the absence of any glycan acceptors but in the presence of Tris buffer leads exclusively to the Tris-sialoside (**19**), which was isolated and purified using size exclusion chromatography and subsequent standard RP-HPLC. This observation suggests an alternative pathway of CMP-Neu5Ac donor degradation by PmST1 and we hypothesized that other nonnatural, nonglycan substrates can be sialylated by the enzyme as well. First, we tested compounds similar to the Tris buffer molecule: bicine, tricine, and *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) (**Figure 5B**). Indeed, TES and Tris are acceptor substrates for PmST1 while for bicine no sialylation product is observed (see the Supporting Information), indicating the relevance of the Tris(hydroxy)-motif for sialylation reaction and offering an alternative system for the enzymatic sialylation that prevents side reactions with the buffer.

On one hand this unexpected sialylation is important to consider during reaction optimization of this and similar sialyltransferases. On the other hand, this offers new opportunities to create novel sialylated compounds. In order to gain first insights into the structural flexibility of this reaction, we tested a second set of nonnatural substrates, that contained aromatic moieties attached to the Tris(hydroxy)-motif. The aromatic moieties were mainly chosen for the ease in following enzymatic reaction via LC-MS but they also already test for further structural variation deviating from the original Tris motif. The commercially available compound 2-((6-(3,5-dimethyl-1*H*-pyrazol-1-yl)-1,2,4,5-tetrazin-3-yl)amino)-2-(hydroxymethyl)propane-1,3-diol (**20**) contains such a heterocyclic substituent while the in house synthesized *N*-(1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl)benzamide (**22**) contains a benzyl residue (**Figure 6**). Since Tris and similar buffer molecules cannot be used for such unconventional sialylation

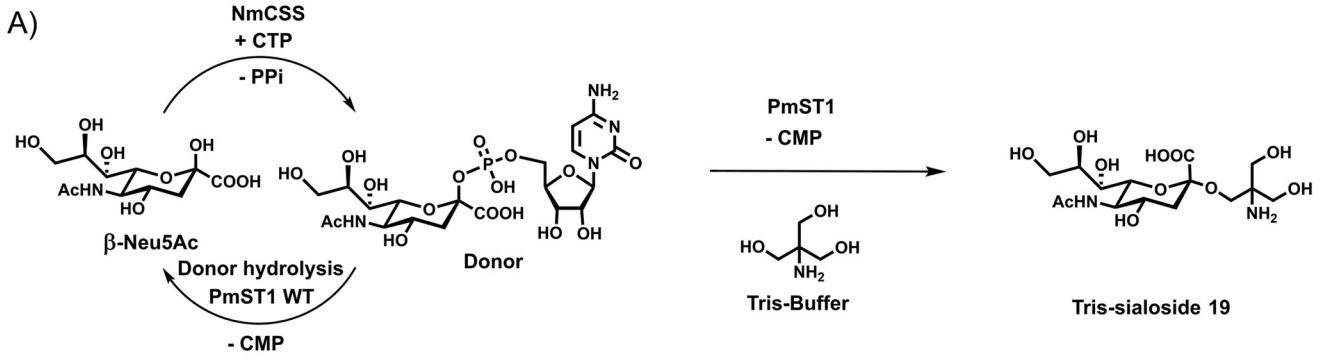

Figure 5. A) Reaction scheme of enzymatic sialylation with PmST1 showing Tris buffer as acceptor resulting in Tris-sialoside (**19**). B) Overview of other molecules with structural similarity to Tris buffer (marked with *) that were tested as acceptors for enzymatic sialylation with PmST1.

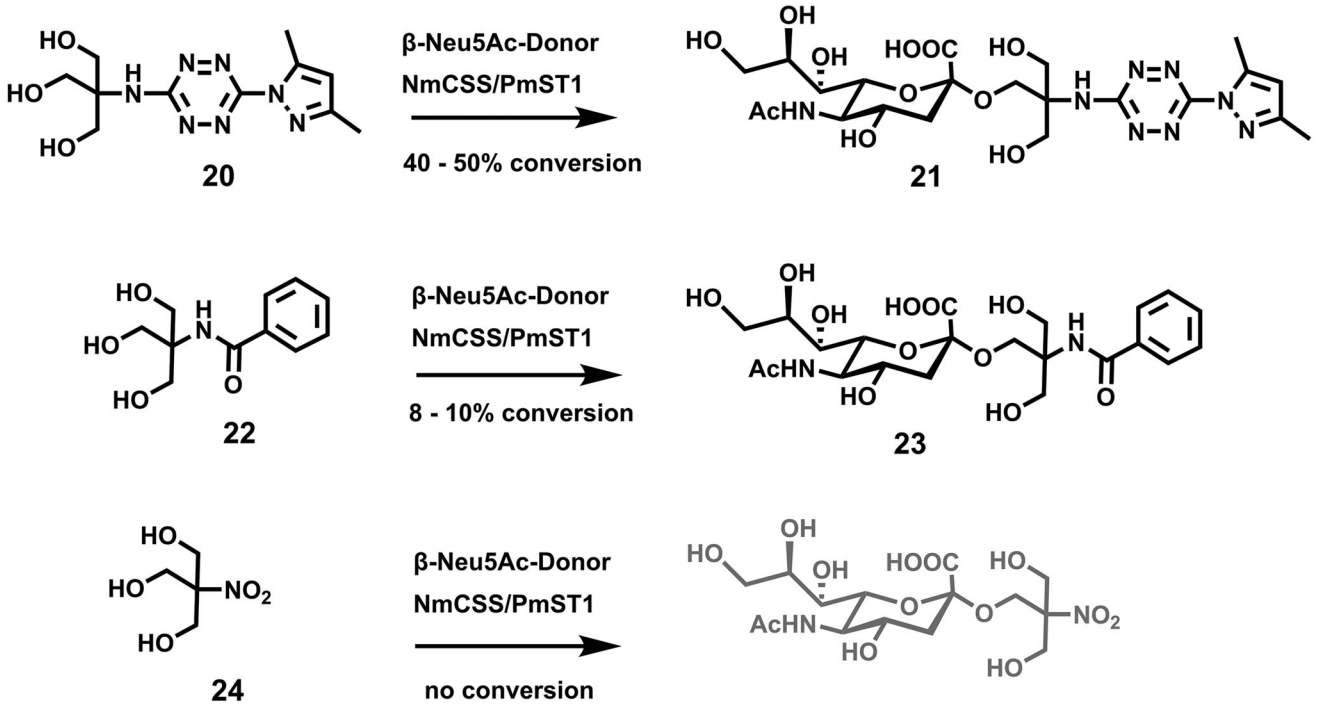

Figure 6. Selection of Tris-derivatives that were tested for their potential to serve as nonnatural substrates for enzymatic sialylation.

reactions, we also tested the enzymatic sialylation of (**20**) and (**22**) in different, alternate buffers to be used as a solvent system (**Table 2**).

For both scaffolds, sialylation was observed. Different yields were achieved depending on the particular buffer that was used for the enzymatic reaction, with glycinamide giving the overall best yields (Table 2). We observed higher yields for the formation of the sialylated aglycon (**21**) in comparison to the product (**23**), see (Figure 6/Table 2). The difference between the two aglycon substrates lies in the electron-donating (compound **20**) or electron-withdrawing (compound **22**) substituent on the nitrogen of the Tris motif (Figure 6). We hypothesize that electron-donating groups are supporting the sialylation reaction while electron-withdrawing groups have a limiting influence. This is supported by the reaction using compound (**24**) with a nitro-substituent attached to the tris(hydroxy)-motif (Figure 6) with an even more pronounced electron-donating effect showing no enzymatic sialylation under the same reaction conditions.

Table 2. Relative proportion of enzymatically sialylated product formed in different buffers.

Buffer	Proportion of (**21**) formed in [%]	Proportion of (**23**) formed in [%]
HEPBS	41.2 ± 2.2%	10.3± 0.3%
Glycinamide	38.5 ± 2.3%	11.6 ± 0.5%
Bicine	52.8 ± 2.8%	8.6 ± 0.3%
CHES	0.3 ± 0.2%	–

The conversion of the enzymatic sialylation was followed with analytical liquid chromatography coupled to MS-spectrometry (LC-MS). The percentage of sialylated product (21 and 23) formed in different buffers was quantified by integration of the particular peaks in the UV-spectra. The values represent the average of the three individual reactions and the standard error of the mean.

3. Conclusion

We successfully implemented α2,3-sialylation of glycooligomers using the CMP-Neu5Ac synthetase NmCSS and the sialyltransferase PmST1 in a one-pot reaction system. Lactose-presenting sequence-defined oligomers accessible via solid phase synthesis were subsequently enzymatically modified providing the sialylated glycomacromolecules **11–18** demonstrating that this route is applicable to macromolecules of different valency, with different spacing of glycan ligands and for different linkers connecting the glycan motif and scaffold.

To our surprise, our experiments showed that both wild type as well as mutant PmST1 also sialylate nonglycan structures such as the Tris buffer which was present in the reaction mixture. On the one hand, this critically affects the choice of buffer in the sialylation reaction when working with these enzymes. On the other hand, we made use of this finding and further explored the chemoenzymatic sialylation of nonglycan substrates. We show that the tris(hydroxy) motif can be further modified, e.g., by using commercially available derivatives with aromatic moieties at the *N*-position, with electron-donating groups being preferred and giving higher overall yield of the sialylated aglycon product. This now paves the way for synthetic schemes giving access to nonnatural sialylated compounds – aglycons and glycoconjugates – using enzymatic sialylation also on nonglycan substrates. Ongoing studies now also combine these two approaches creating sialylated glycomacromolecules from nonlactose functionalized scaffolds.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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P.B.K. and H.P. contributed equally to this work. L.H. is corresponding author for synthetic chemistry and enzymatic reactions leading to the aglycon scaffolds, T.P. for enzymatic chemistry and in-line NMR analysis. The authors thank the German Research Foundation for support through the research group "VIROCARB" (FOR 2327, P6/P7). The authors also thank Robert Creutznacher and Thorsten Biet for assistance with NMR experiments.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

chemoenzymatic synthesis, glycomacromolecules, neoglycoside, sialic acid, solid phase polymer synthesis

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