Enzymatic and chemical synthesis of sequence-defined macromolecules presenting oligosaccharides

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I assure in lieu of oath that the dissertation thesis was created by myself and without unauthorized external assistance in accordance to “principles to maintain good scientific practice at the Heinrich Heine University Düsseldorf”. This thesis has not been submitted to other faculties.

Düsseldorf, 15. July 2023 Patrick Benjamin Konietzny

The practical work was carried out from June 2018 until December 2021 within the institute of organic chemistry and macromolecular chemistry of Heinrich Heine University Düsseldorf. These studies included a research stay at the institute of chemistry and metabolomics of University of Lübeck within January and March 2019.

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Abstract

Glycans as well as glycoconjugates often appear as natural ligands in biological systems and are responsible for interactions with glycan-binding proteins. Here, complex carbohydrate motifs such as human milk oligosaccharides (HMO) are involved in multiple biochemical processes e.g. inflammatory response or cell-cell communication. Although HMOs can be used for synthetic glycan ligand design such as glycomimetics, they have been rarely used in the synthesis of multivalent and sequence-defined scaffolds among others due to the higher synthetic demands compared to their cropped terminal monosaccharide motifs.

Within these studies, it was focused on the synthesis of such glycomacromolecules and glycoconjugates via solid-phase peptide synthesis (SPPS) which allowed the access of monodisperse and sequence-defined scaffolds. In combination with chemoenzymatic and protection group-free glycosylation approaches differing from classical chemical carbohydrate synthesis, this method benefit from their tailor-made molecular design and the potential use as model ligands. Therefore, especially sialylated glycan motifs based on oligosaccharides as well as unusual ligand motifs were subjects of interest. As a result of this synthetic work, various glycosylated scaffolds were successfully isolated varying in glycan motif, glycan complexity, valency and macromolecular architecture.

First attempts of synthesizing HMO-functionalized macromolecules were carried out by introducing a 3‘-sialyllactose (3‘-SL) glycan motif in monodisperse and sequence-defined glycomacromolecules using a chemoenzymatic approach (see Scheme 1A). Therefore, lactose-functionalized macromolecule precursors were priorly synthesized using SPPS method with *N*-Fmoc-protected EDS and TDS building blocks formerly developed in the Hartmann group. The disaccharide motif was introduced using copper-catalyzed azide-alkyne cycloaddition (CuAAC) on resin-bound oligo(amidoamines) with two different acetylated, monoazidated β-lactose derivatives $LacN_3$ and $LacOPrN_3$ varying in the presence of *O*-glycosidic propyl linker. Eight different lactose-functionalized macromolecules were obtained being mono-, di- and trivalent and differing in ligand linker length and glycan spacing. These disaccharide motifs of these precursor macromolecules were subsequently elongated by sialic acid to give monodisperse products. This sialylation reaction was executed using a two-enzyme one-pot approach with *Neisseria meningitidis* CMP-sialic acid synthase (NmCSS) and *Pasteurella multocida* sialyl-transferase (PmST1) which was described by Chen group. Since PmST1 enzyme catalyzes both the formation of 3‘-sialyllactose motif as well as its desialylation reaction, reaction progress was analyzed using in-line NMR spectroscopy to adjust optimal reaction conditions and to circumvent undesired degradation reactions. The glycomacromolecule sialylation process window was extended by increasing CTP:Neu5Ac substrate ratio to 2:1 and by adding excess CTP shortly before work up procedure. Generally, the method benefit from easy synthesis and the broad applicability on multiple lactose-functionalized scaffolds independently from the glycan distance towards the macromolecule backbone.

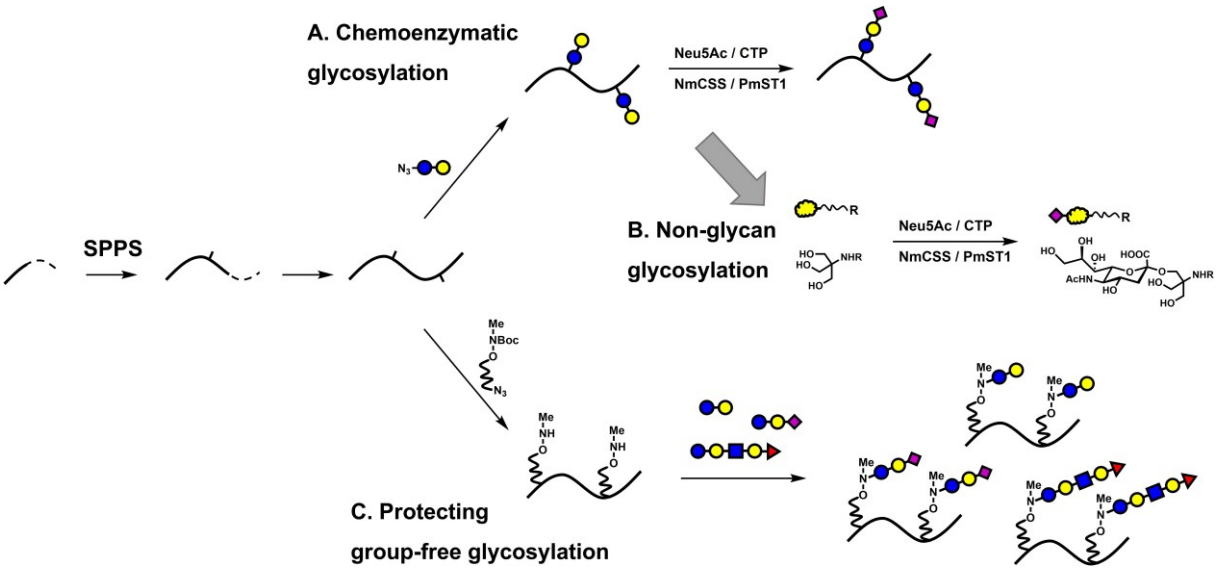
Besides glycomacromolecule synthesis, it was found that PmST1 also sialylates non-glycan substrates such as Tris(hydroxymethyl)aminomethane buffer. The novel Tris-sialoside conjugate was formed during irreversible, enzymatic CMP-Neu5Ac donor degradation processes and was successfully isolated. HMBC NMR analysis revealed the selective conjugation of α-sialoside to one of the symmetric Tris buffer hydroxyl groups. Surprisingly and in contrast to 3‘-SL derivatives, Tris-sialoside was stable

against PmST1 desialylation activity which might indicate higher neuraminidase stabilities of this neoglycoconjugate.

Based on these insights of PmST1 non-glycan sialylation activity, this newly described effect was further observed to exploit a synthetic purpose (see Scheme 1B). Therefore, structurally related non-glycan derivatives were tested on PmST1 sialylation such as buffers or derivatives carrying functional handles for potential CuAAC application. Prior to sialylation experiments Tris derivative substrates, it was inevitable to transfer in-line NMR spectroscopy analysis towards ESI-MS method. ESI-MS measurements confirmed the previous findings of Tris(hydroxymethyl)methyl motif sialylation by PmST1. First synthetic precursors were synthesized bearing this structural motif as well as azide or alkyne handles for CuAAC conjugation. Analytical challenges appeared due to the insufficient separation on RP-HPLC as well as poor UV absorption properties which led to lacking conversion quantification and reaction optimization. Hence, further Tris derivatives were developed carrying hydrophobic linkers for better C_{18} separation properties. A strongly deviated aromatic Tris derivative was isolated which allowed for quantification as found to show low conversions of up to 11 % using PmST1 P34H/M144L. The optimized reaction conditions from aromatic Tris derivative experiments were applied on other Tris derivatives on small preparative 0.11 mmol scale followed by first failed purification attempts via RP-HPLC. As an outlook for these studies, better purification strategies have to be considered for these neoglycoconjugates e.g. size exclusion chromatography or HILIC. Isolation and full characterization of these verified compounds are ongoing tasks as well as their conjugation onto macromolecules. Furthermore, this novel class of neoglycoconjugates need to be studied on neuraminidase stabilities as well their binding-properties towards sialic acid-binding proteins.

Another approach of synthesizing HMO-functionalized precision macromolecules was developed based on *N*-methyloxyamine glycosylation reaction previously described by Blixt group (see Scheme 1C). Functional linkers were derivatized by azide functionalization to enable CuAAC. In these works, *N*-methyloxyamine linker synthesis was diverged into two routes: the bifunctional *N*-Boc-protected azidated methyloxyamines and the synthesis of multivalent, symmetrical *N*-Boc-methyloxyamines. Whereas Boc-*N*-methyloxyamine linkers were used for synthesis of monodisperse and sequence-defined glycomacromolecule synthesis via SPPS or glycopolymer functionalization, symmetrical oxyamine molecules were converted into multivalent small molecule glycoconjugates via solvent-based chemistry. Prior to glycosylation reaction, the Boc-*N*-methyloxyamine residues required deprotection with trifluoroacetic acid (TFA). A special feature of the sequence-defined, SPPS-derived macro-molecules was the simultaneous Boc-deprotection with the cleavage from solid support yielding reactive macromolecules directly after isolation. The deprotected and preactivated *N*-methyloxyamine precursors were then glycosylated under aqueous, buffered conditions at 37°C using various non-functionalized carbohydrates such as glucose (Glc), *N*-acetylglucosamine (GlcNAc), lactose (Lac), 2‘-fucosyllactose (2‘-FL), 3‘-sialyllactose (3‘-SL), 6‘-sialyllactose (6‘-SL) and lacto-*N*-fucopentaose (LNFP1). The late-step introduction of carbohydrate motifs enable a high synthetic flexibility and was applied on three different oxyamine architectures to build up a glycoconjugate bibliography of a total of approximately 60 compounds. On the example of divalent, small molecule glycoconjugates it was shown that this approach can be exploited for heteromultivalent glycosylation reactions to combine unusual glycan pairs such as 3‘-SL/2‘-FL or 3‘-SL/LNFP1. The bioactivity of selected 3‘-SL- and 6‘-SL-

functionalized divalent macromolecules was shown on crystallization studies and binding studies with polyomavirus capsid proteins. Overall, this glycosylation approach benefit from easy glycosylation handling which allowed the introduction of complex glycan motifs without the need of advanced experience on carbohydrate synthesis.



Scheme 1: Project overview, **A:** Chemoenzymatic glycosylation of SPPS-derived glycomacromoecules, **B:** chemoenzymatic glycosylation of functionalized non-glycan substrates, **C:** protecting group-free glycosylation of SPPS-derived macromolecules via *N*-methyloxyamine linkers.

1. Introduction

1.1 The role of synthetic chemistry for targeting carbohydrate-binding proteins

In nature, organisms naturally use the ligand-receptor principle which is essential for many biological processes such as signal transduction or process regulation.[1–3] Steadily improved by evolution, the interplay between protein receptors and their ligand counterparts allows for communication between cells[4] and such receptor-ligand communication is an essential mechanism both for higher organisms[5] as well for simpler species such as unicellular organisms[6] or viruses.[7,8] Hence, understanding and controlling these processes is a popular subject of research and still contains many scientific challenges.[9,10]

Synthetic chemistry can support understanding these biochemical processes by enabling the synthesis and access of artificial bioactive ligands. Glycans form one class of such natural and synthetic bioactive ligands[11] which include non-conjugated carbohydrates[12] as well as their conjugates with e.g. lipids,[13] proteins,[14–16] peptides,[17,18] steroids[19,20] and biomolecules.[21] Studies on the application of glycans for therapy against diseases or malfunctioning regulation processes such as cancer or virus infections[22–25] are subject of many research fields in synthetic chemistry and related interdisciplinary biology.[26] Natural and synthetic glycans show characteristic and reversible binding to glycan recognizing proteins such as lectins,[27] selectins,[28], siglecs[29] and hemagglutinins.[30] Glycan binding is involved in various processes such as cell-cell communication,[31] host-cell interactions[32] or regulation of other processes e.g. tumor development,[33] immune regulation,[34] or growth factor signaling.[35] Although glycan-lectin interactions play an important role for these described processes, some mechanistical aspects are still not fully understood.[36,37] This also applies for unraveling of the overall role of glycan diversity in organisms and the decryption of the so-called glycocode.[38,39]

Since glycans possess high biological relevance and good bioavailability properties they are treated as potential candidates for selective therapies.[40] This leads to a synthetic focus on artificial glycan conjugates,[41] glycomacromolecules[42–44] and glycan-based drugs[45–47] as active pharmaceutical ingredients (API)[48] which allow for interfering with glycan binding processes. One example of such specific glycan recognition is the adhesion of bacteria[49] or virus surface proteins[50] onto the targeted host cell. Therefore, the pathogens navigate to the glycocalyx[51] which is an extracellular coating of many cells and is formed by membrane-conjugated glycans such as glyco(shingo)lipids, glycoproteins or proteoglycans (see Figure 1).[52] Here, pathogen-host adhesion is an early-stage step in the complete infection mechanism.[53] Pathogen lectins can bind to suitable host cell glycans which promote close contact between both spheres which allows subsequent internalization and infection of the host cell which is happening in e.g. Influenza A or Helicobacter pylori infection.[54–56] The described pathogen adhesion can be prevented or reversed by the addition of glycans similar to the ligands presented on the glycocalyx and allows for competitive inhibition or detachment of the bacteria or virus particle.[57]

The glycans presented on glycocalyx are diverse in their carbohydrate motifs and represent an organism- and cell-specific glycan pattern.[58] This glycan variation includes oligo- and polysaccharides

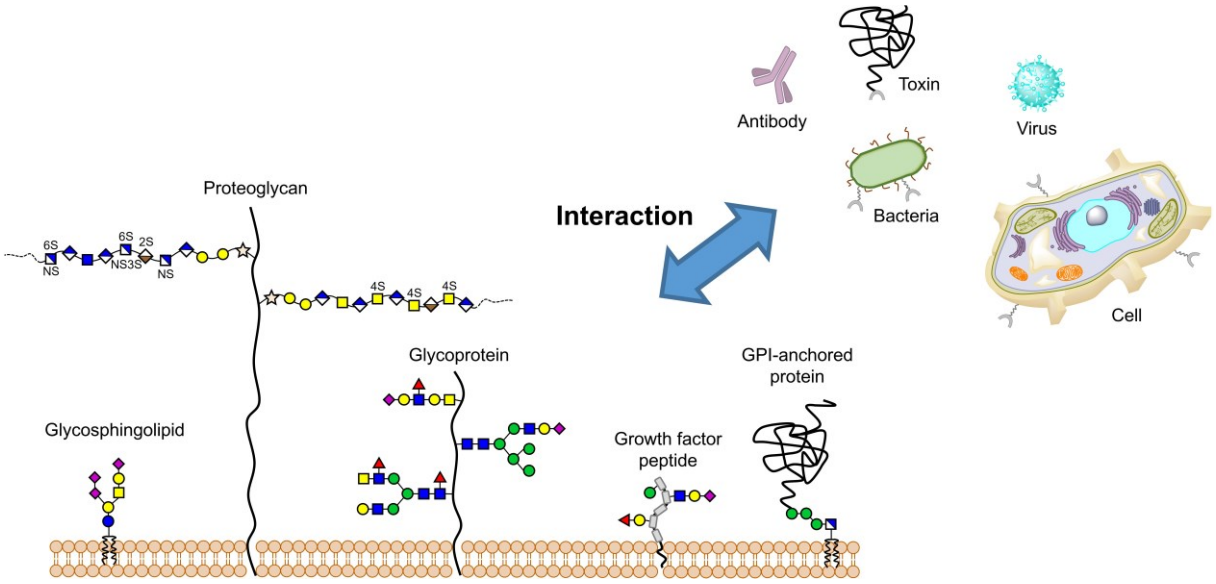


Figure 1: Schematic structure of glycocalyx consisting of bioderived, membrane-bound glycoconjugates such as glycoproteins, proteoglycans, growth factor peptides, glycosylphosphatidylinositol-anchored proteins or glycolsphingolipids. The biomolecules decorating the glycocalyx can show interactions with glycan-binding receptor proteins of antibodies, toxins, virus capsid proteins, bacteria or other cells (modified figure is shown with permission of Cold Spring Harbor Laboratory Press[59] and Springer Nature[25]).[52]

and allows the identification and thus differentiation between different tissues of an own organism and foreign particles by scanning the glycocalyx. As a result of glycan differentiation higher organisms can show immune responses when being exposed to unfamiliar antigens such as pathogens[60] or human blood group antigens (HBGA) from incompatible blood groups (see Figure 2).[61]

The natural glycan variety can be derived by intracellular glycosylation reactions of various monosaccharides towards oligo- or polysaccharides which can be varied[62,63] e.g. in the amount and assembly of monosaccharides, the linkage positions, anomerical configuration, branching and post glycosylation modifications. The specific oligosaccharide can either be copied, modified or synthetically mimicked

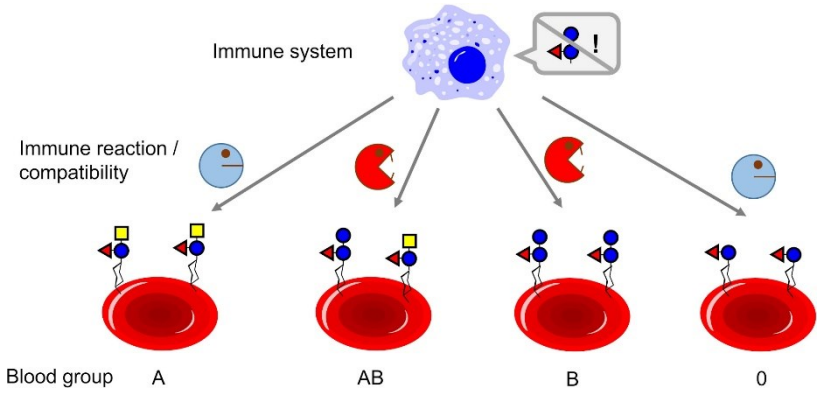


Figure 2: Immune response is regulated of by the ligand-receptor interaction between antibodies and erythrocytes. The HBGA covering the erythrocytes define the host blood group and is responsible for compatibility e.g. in terms of blood transfusions. It is exemplarily shown that blood group A hosts show immune response once B antigen is detected.[66]

using cropped glycan motifs like presenting mono- or disaccharides.[64,65] The access of synthetic glycans and their conjugates can be used to either prevent pathogens infection by the use as vaccines[67] or can help studying function of pathogen proteins by structural analysis[68] or quantitative selectivity studies.[69] Hence, studying glycan-protein interactions can provide information about their infection mechanisms and allow for the development of specific therapeutics.[70–73]

The binding properties of many specific glycan-protein pairs have been previously studied[74–76] and are commonly estimated by thermodynamic and kinetic parameters such as half maximal inhibitory concentration (IC_{50}) or equilibrium dissociation constant (K_d).[77–79] These values allows for precise quantification of a binding event and for comparison of different ligands via measurement techniques like frontal affinity chromatography (FAC), glycan microarray, surface plasmon resonance (SPR), isothermal titration calorimetry (ITC) or saturation-transfer difference nuclear magnetic resonance spectroscopy (STD-NMR).[80] The better binding properties between a single ligand motif towards a carbohydrate-recognition domain (CRD) of a targeted protein, the higher its specificity. This specificity between protein and glycan can be strongly adjusted with the choice oligosaccharide motif e.g. natural human milk oligosaccharide (HMO) or when using shorter mono- or disaccharide sequences as a glycan mimetic.[81–84] Even slight changes in monosaccharide linkage, spatial arrangement, post-glycosylation modifications (PGM) and the presence of non-glycan binding motifs can influence binding and hence selectivity which allows for synthetic ligand design. This effect can be explained with on studying binding properties of cropped A-tetrasaccharide motifs on Galectin-4N which resulted in higher Kd values the higher the deviation from the natural A-tetrasaccharide motif (see Figure 3).[85] Especially when targeting a single process within a complex system or a certain organ or tissue with e.g. *in vivo* bioassays, specifity is getting more important than in less complex *in vitro* protein studies.[86]

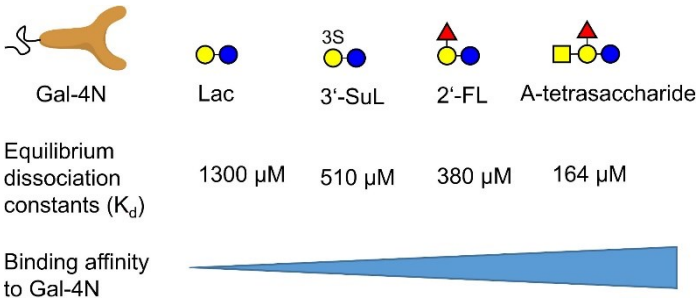


Figure 3: Binding affinities of lactose (Lac), 3'-sulfolactose (3'-SuL), 2'-fucosyllactose (2'-FL) and A-tetrasaccharide towards Galectin-4N showing differences in glycan complexity.[85]

Another very important method to increase or to control binding of glycans towards proteins is the use of spatial arrangement or multivalent glycan presentation on particles.[87] Compared to equivalent monovalent glycan ligands the binding affinity is increased due to thermodynamical reasons. This so-called multivalent effect often occurs in nature and is used by many organisms to enhance binding and has been precisely studied among others by the groups of Haag, Koksch and Kiessling.[88–91] The multivalent effect is composed of different mechanistical actions shown in Figure 4. Namely the chelate effect, the inclusion of aglycon subsite domains for binding events, receptor clustering on dynamical

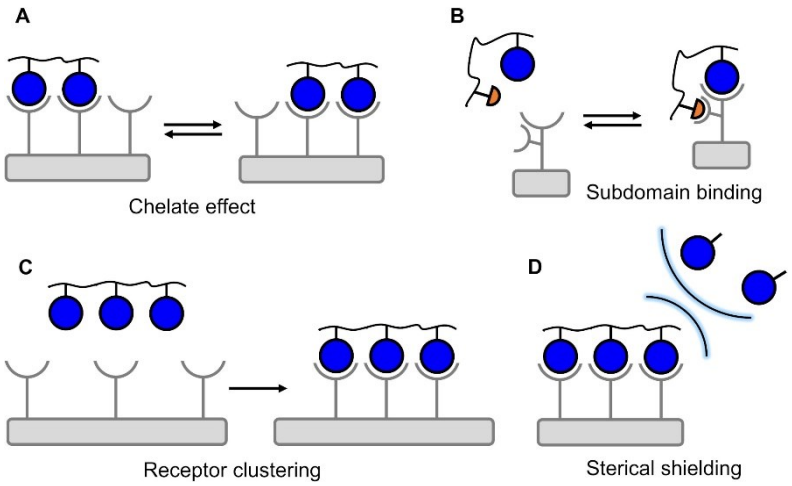


Figure 4: Multivalent effect and their stabilization mechanisms between multivalent ligands and receptors. **A:** Chelate effect, **B:** subdomain binding, **C:** receptor clustering, **D:** sterical shielding (modified figure is shown with the permission of Jon Wiley and Sons).[91]

surfaces and sterical shielding effects explain the overall tendency to prefer multivalent ligands towards monovalent ligands.[91] Macromolecule synthesis allow for such multivalent ligand assembly, studies of the structure-function relationship and thus for mimicking natural occurring glycan structures.[92,93] Although higher glycan valency often lead to better binding, this multivalent effect does not automatically lead to high selectivity towards a certain lectin or receptor e.g. when the glycan ligand motif shows weak binding behavior.[94,95] To address carbohydrate binding proteins selectively via synthetic macromolecules it is important to overcome the challenges of rational design and to consider both selectivity and overall binding affinity using the multivalent effect.

1.1.1 Rational design of synthetic glycomacromolecules

Derived from the challenges of targeting biologically relevant proteins, synthetic chemistry can be used to design applicable ligands, e.g. glycosylated macromolecules. Customized target structure requirements should be taken into account when designing synthetic glycan-bearing macromolecules and choosing between synthetic approaches. Thus, synthetic access of glycan conjugates allows for choosing between natural, complex glycan ligands or simpler, naturally derived glycan motifs.[96,97] Synthetic chemistry enables to expand the scope of biologically available glycans and hence increases the importance of trade-off decisions e.g. between high target specificity or a wider application range. Oligosaccharide motifs which show high selectivities in protein binding usually at the same time possess complex glycan structures[98,99] and thus are often more difficult to access than their cropped mono- or disaccharide components.[100–102] As a result the accessible amounts of carbohydrates vary e.g. depending on their structural complexity and thus the access of synthetic macromolecules or glycan conjugates is more difficult when using a more complex glycan motif.[103] Low carbohydrate amounts often occur either when complex oligosaccharides are isolated from biological sources[101,104] or when they are synthesized precisely via the assembly of monosaccharide building blocks, e.g. via solid phase synthesis of oligosaccharides.[105,106] Depending on the carbohydrate-binding protein, specific binding

appears with either complex oligosaccharides or smaller and more common mono- or di-saccharides.[107,108]

In general, the overall goal of synthetic ligand design is to increase binding affinity and specificity to be able to precisely address single proteins within organisms.[100,109,110] Therefore, the straightforward development of novel ligand architectures requires multiple optimization steps such as the ligand synthesis, spatial ligand assembly and the quantification of binding affinities by using bioassays to evaluate the synthetic ligands.[105,111]

Glycomacromolecules form a class of high molecular weight substances which can be isolated from natural sources and can be accessed synthetically.[112–114] These glycomacromolecules are defined as carbohydrate-based structures either conjugated to natural or synthetic macromolecules consisting of various assembled monomer repeating units[115,116] or directly formed by a monosaccharide backbone e.g. in polysaccharides.[117,118] The interest focus onto glycomacromolecules started in 1947 with studies of Horsfall and McCarty who found out that specific bacterial polysaccharides howed antiviral properties on behalf of *in vivo* studies with pneumonia virus infected mice.[119,120] Based on these findings further studies were performed with polysaccharides and specially designed non-glycan polymers for their virus inhibition behavior to develop selective therapeutics against virus infections and diseases.[121–123] Although first glycopolymers were already synthesized in 1930's by Reppe and Hecht,[124,125] the focus on synthetic glycosylated scaffolds gained further interest in late 1980's with the systematic polymerization of mono- or disaccharide-containing glycomonomers.[126–129] It was shown that synthetic

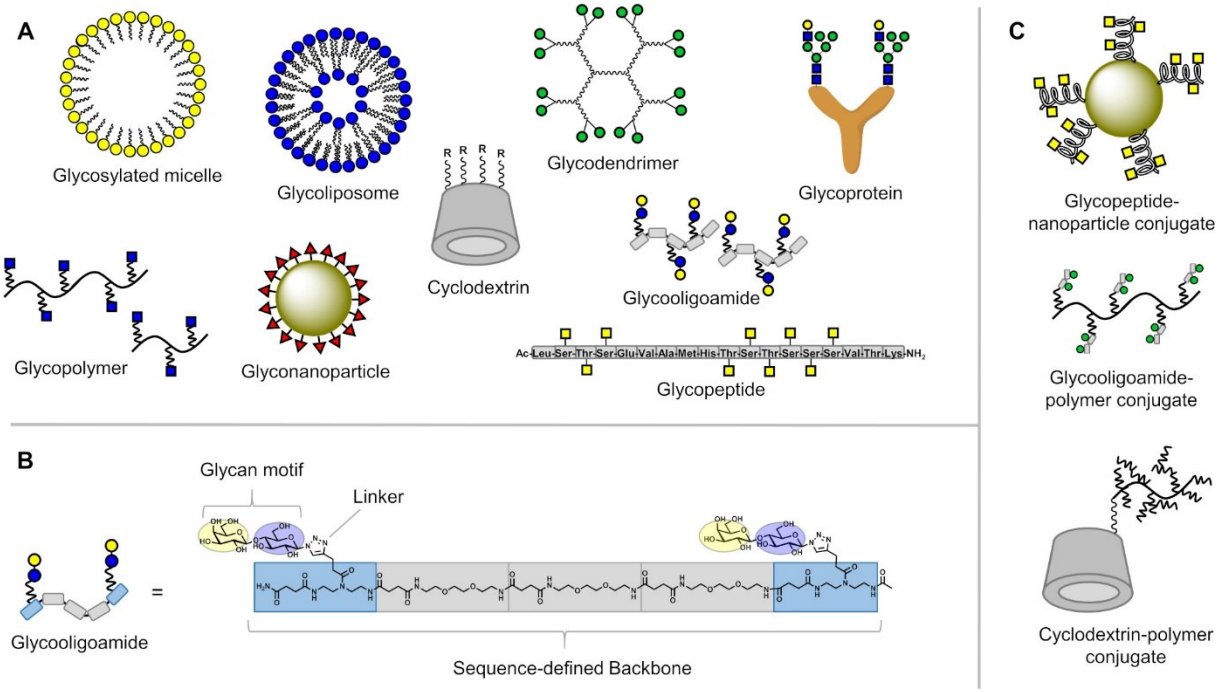


Figure 5: Overview of various glycomacromolecule architecture types. **A:** Different glycomacromolecule architectures varying in their spatial glycan presentation pattern and monomer assembly.[132] **B:** Glycooligoamides as an example of a monodisperse glycomacromolecule with a sequence-defined backbone.[133] The variation of monomer sequence leads e.g. to tunability of ligand distance. **C:** Examples of glycosylated hybrid structures accessible by combining synthetic methodologies.[134–136]

glycopolymers can effectively inhibit binding of e.g. Influenza virus hemagglutinins[130] or plant lectins.[128,131] This synthetic approach allows for tailor-made ligand design and structural variation of ligand assembly. Additional to natural glycan ligands, synthetic glycocmacromolecules opened up a new synthetic field and extended access to bioactive compounds.[93,137] Today the concept of glycopolymer or glycomacromolecule synthesis has been extended to various synthetic glycoconjugates such as glycodendrimers, cyclodextrins, glyconanoparticles, glycoliposomes, glycooligoamides and neoglycopeptides or neoglycoproteins (see Figure 5A).[132–136] These different glycomacromolecule classes strongly vary in their synthesis routes and molecular architectures and hence show characteristic properties.[138–140] These differences in e.g. topology, size, valency, dispersity and biocompatibility have to be considered when preparing a rational design for biological application or assay development. The presentation of glycan ligands of the whole glycomacromolecule is dependent e.g. on the topological macromolecule shapes.

Whilst glycosylated nanoparticles and liposomes appear in a strictly spherical shaped glycan surface layer, dendrimers can vary geometrically by either forming spheres[141] or branched-like dendrons[142,143] whereas cyclodextrins possess topological cavities allowing for small molecule encapsulation e.g. with pharmaceutical drugs or ingredients for food industry.[144] Glycopolymers and neoglycopeptides can be presented in a wide topological variety starting from linear to branched or star-shaped structures.[145,146] It was shown on multiple examples that glycan-bearing polymers and peptides[44,147] can be designed easily with high variability of the amount of ligand motifs and their spatial distances can be adjusted e.g. by using block copolymerization techniques[148,149] or amino acid-derived spacing building block assembly.[132,150,151] Although the synthesis of these described glycomacromolecule types allow for various scaffolds, their macromolecular ligand assembly can be further extended by combining architectural macromolecular assembly techniques to obtain hybrid conjugates with novel molecular properties.[152,153] Such glycomacromolecule hybrid conjugates can be obtained e.g. by conjugation of glycopeptides onto nanoparticles,[154,155] conjugation of glycodendrons onto proteins,[156] by forming a cyclodextrin-polymer conjugate[157] or by performing polymer-analogous reactions with sequence-defined glycooligomers grafted onto reactive ester polymers (see Figure 5C).[135] To be highlighted, the characteristic synthesis of glycosylated peptides or oligoamides allows customized assembly of monomeric building blocks, e.g. via stepwise coupling of functional building blocks.[158,159] This method can be used to achieve sequence-defined scaffolds with a discrete amount of monomer units per macromolecule as schematically shown in Figure 5B.[133]

The different glycomacromolecules presented in Figure 5 vary in their scaffold homogeneity which strongly depends on their architecture type. The homogeneity is indicated by the molecular weight distribution or dispersity which is a characteristic value describing the macromolecule. Many macromolecules appear as disperse compounds which may lead to practical limitations e.g. in analytical measurements, challenging structure-function relationship interpretation and potential regulatory difficulties as pharmaceutical drugs, e.g. on FDA approval procedures.[160–162] Glycomacromolecules with low dispersity avoid these limitations but are often connected to more effortful synthetic routes. Due to lack of precise control in monomer assembly it is challenging to access monodisperse particles with identical particle size or glycan valency.

Dispersities can be defined differently depending on the macromolecule architecture, e.g. large, spherical vesicles such as liposomes, micelles and nanoparticles are usually determined via their hydrodynamic radius[163–165] whilst linear and branched scaffolds can also be differentiated by their molecular weight distribution.[166,167] Furthermore, glycopolymers can show a wide dispersity range depending on the applied polymerization method, e.g. free radical polymerization lead to dispersity values Đ > 2.[168] Although anionic polymerization can rapidly reduce polymer dispersities close to Đ ≈ 1.0,[169] recent development of reversible-deactivation radical polymerization techniques, e.g. atom transfer radical polymerization (ATRP), reversible addition-fragmentation chain transfer (RAFT) or nitroxide-mediated radical polymerization (NMP), further expanded the synthetic toolbox to obtain low disperse polymers with typical values of 1.05-1.50.[170–172] In contrast to the examples being described above, glycodendrimers, glycooligoamides, glycoproteins or neoglycopeptides can be synthesized as monodisperse compounds.[173,174] Therefore, the straightforward use of efficient organic coupling reactions or conjugation of biosynthetically accessed precursor are used to access glycol-conjugates.[161,175] On the example of glycooligoamides, synthetic building blocks derived from amino acids can be used for polymerization via solid phase polymer synthesis (SPPS, further described in Section 1.3)[176,177] to give monodisperse and sequence-defined glycomacromolecules. The synthetic setup of SPPS allows for control of the monomer assembly and the amount of overall chain length as shown in Figure 5B.[97,133]

The utilization of low- or monodisperse macromolecule syntheses enhanced the synthetic access of structurally controlled or sequence-defined scaffolds and enabled a more precise analytical characterization (e.g. MALDI-MS,[178] native ESI-MS,[179] structural analysis via tandem MS experiments[180] or protein-ligand co-crystallization[181,182]). Synthetic methodologies had been regularly improved to further enhance method limitations. Since monodisperse glycomacromolecules such as glycopeptides or glycooligoamides show distinct chemical structures, they show characteristic advantages in terms of applicability and molecular design, e.g. the precise spatial adjustment of ligand arrangement.

For the synthesis and rational design of glycomacromolecules it is important to allow for right adjustment respective replacement of selected molecular motifs e.g. fluorescent labels,[183] His or biotin tags,[184,185] non-glycan motifs,[83,186,187] small molecule bioactive residues[188,189] or hydrophobic anchors.[190,191] Exemplarily, the toolbox-oriented and sequence-defined synthesis via SPPS shows better molecular control than conventional glycomonomer polymerization[192,193] and can be used for e.g. self-assemblying towards large uniform (nano-)particles.[194,195] These SPPS-derived macromolecules can be used for merging with other glycomacromolecule architectures using e.g. polymer-analogous coupling techniques.[125,196]

Independent from the architecture type of glycomacromolecules, different synthetic strategies can be applied to introduce the glycan motif side chains which was described in detail for brush polymers and mainly describes the order of monomer assembly and deprotection reactions.[197,198] The choice of synthetic polymerization strategy influences e.g. the side product formation or product functionalization and thus is highly important for the synthesis of low- or monodisperse glycomacromolecules. For side chain implementation different principles can be used, namely, the assembly of glycomonomers with

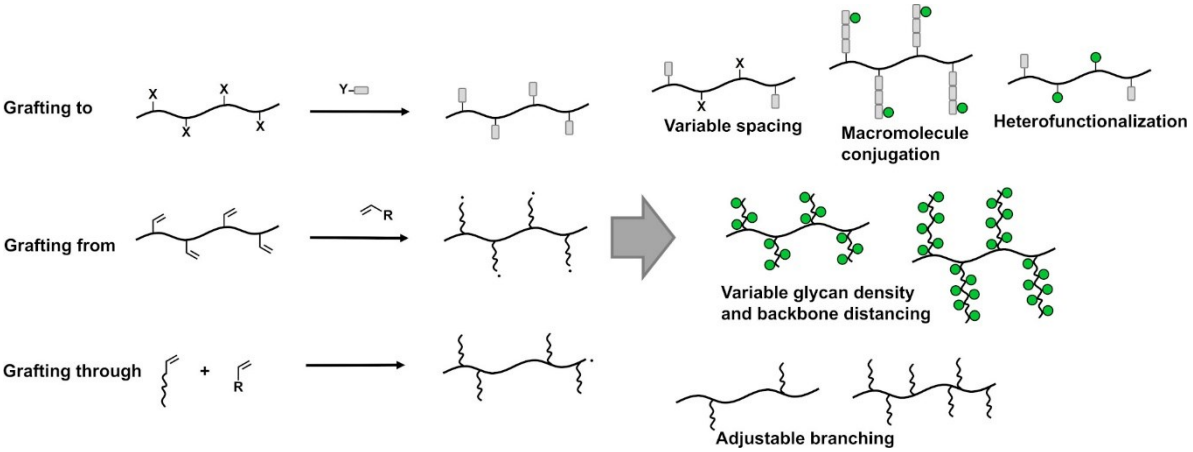


Figure 6: Graft polymerization of macromolecules and their relevance on for glycoconjugate synthesis.

introduction of functional side chains allowing for further side chain growth after completing the backbone sequence (grafting from), the polymer-analogous glycan attachment onto a previously formed macromolecule backbone (grafting to) and the assembly of already glycosylated macromonomers (grafting through) (see Figure 6).[199,200]

The polymer-analogous glycosylation or modification reactions via “grafting to” approach is a common method in polymer, peptide, protein or oligoamide glycosylation.[201–203] It allows e.g. a late-step introduction of the glycan motif without the prior synthesis of (macro-)glycomonomers and thus increase flexibility in synthesis planning. This divergent approach benefits from the possibility of flexibly exchanging glycan ligand motifs with other glycan motifs.[204–206] The distance between glycan ligand and backbone can be adjusted e.g. by both introducing a short, glycan-bound linker and by assembling various monomer building blocks with a “grafting from” approach (see Figure 7). Especially when synthesizing monodisperse glycomacromolecules, high conversions are important to enable feasible backbone sequence assembly as well as polymer-analogous glycan conjugations.[207]

Incomplete glycan incorporation can lead to a fragmentary distributed amount of glycans and full glycosylation is still an open challenge when obtaining large sequence-defined structures with high glycan valency.[160,208] Under real reaction conditions, conjugation reactions do not undergo complete conversions which show limitation for large, monodisperse macromolecules with high valency. Product and intermediate distribution were exemplarily calculated and shown in Figure 8 on behalf of mono- to tetravalent “grafting to” reactions at different conversion rates. At conversion rates of 80% the calculated

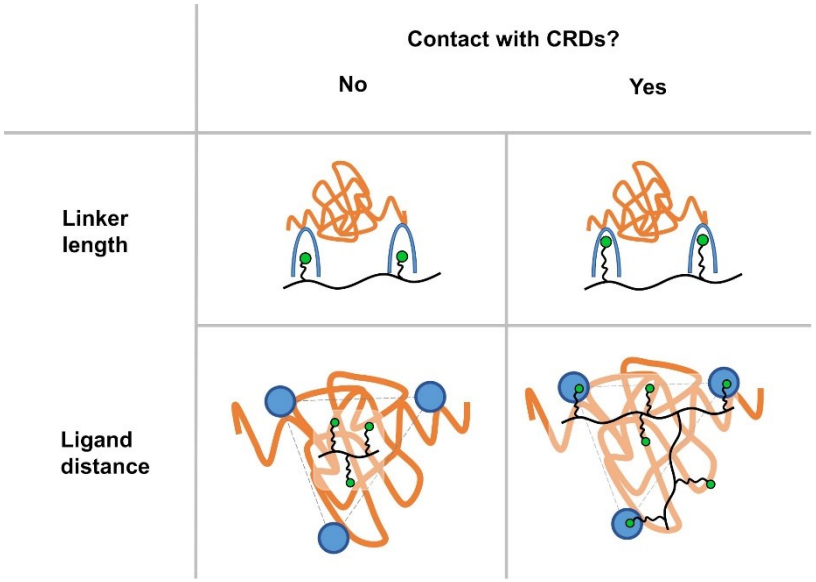


Figure 7: Effects of linker length and spacing for sufficient ligand-receptor interactions. Glycan ligands (green spots) and macromolecule backbone (black lines) describe the glycomacromolecule, whereas carbohydrate-recognition domain (blue spots and cavities) and protein scaffold (orange coil) describe the protein quaternary sequence acting as a glycan receptor.[209]

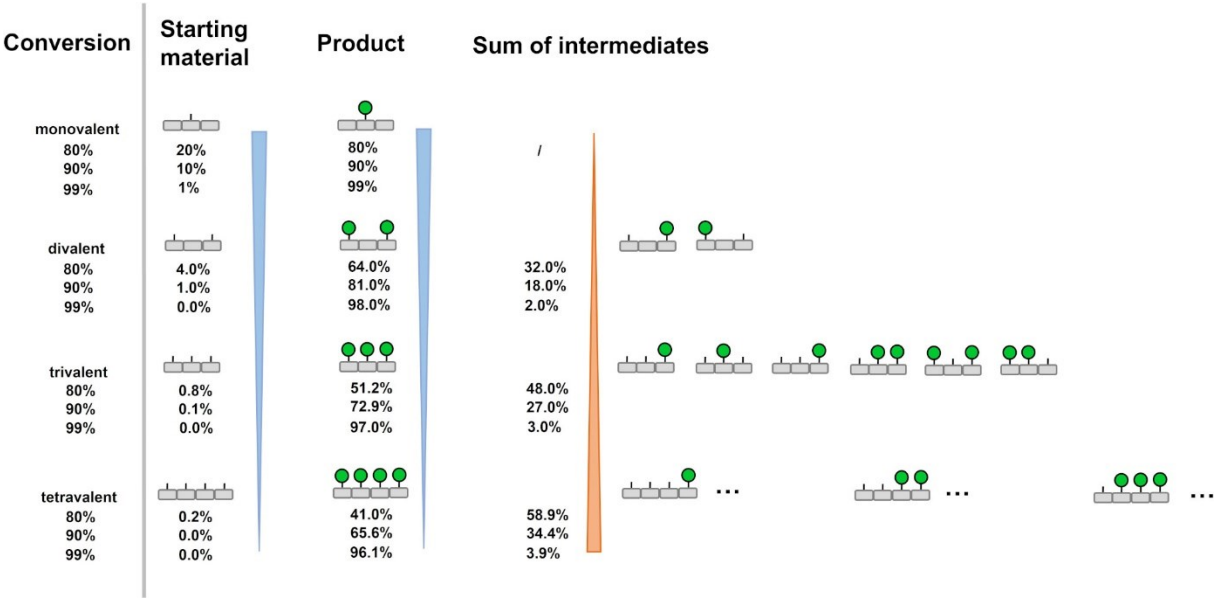


Figure 8: Product formation distribution via grafting to mechanism including the amount of starting material and intermediates. The effect of insufficient functionalization is exemplarily shown on behalf of mono-, di-, tri- and tetravalent functionalization. Values were calculated by the author assuming statistical glycan distribution and different conversion rates of 80%, 90% and 99%.

product yields decrease from 80% (monovalent) to 64% (divalent), 51% (trivalent) and 41% (tetravalent) whilst the sums of intermediates increase from 0% (monovalent) to 32% (divalent), 48% (trivalent) and 59% (tetravalent). Due to statistical distribution, the higher the target structure valency, the more purification effort has to be done with at the same time lower calculated product yields. This correlation states a general challenge for the multivalent conjugation in sequence-defined macromolecule synthesis.[210,211] In total, the quality of the polymer-analogous conjugation reaction defines the

feasability for fully converted glycomacromolecules. In the case of non-ideal conjugation reactions, a fundamental trade-off is required between low valency and mainly defined macromolecules or high valency and imperfectly glycosylated and therefore more disperse macromolecules.
Based on these described synthetic approaches to obtain glycomacromolecules, many examples were published which used rational design to find specifically binding scaffolds.[212–215] Different rational design approaches were studied such as supplying the structural analysis of protein topology[212,216,217] and the position of carbohydrate recognition (sub-)domains.[218,219] To obtain information about glycan specificity of proteins it is common to either determine thermodynamical and kinetical parameters or to perform structural analysis e.g. by protein co-crystallization with ligands being crystallized in the binding pocket.[81,212,220] With steady development of analytical methods low sample amounts are required which allows high variations of glycomacromolecule synthesis e.g. obtained by low-scale synthesis approaches.[221–224] Especially sequence-defined glycomacromolecules enable the tailor-made assembly of different monomeric building blocks and allow for the introduction of switchable groups via external triggers such as UV irradiation.[225] Small scales in glycomacromolecule synthesis combined with high control and good reproducibility of molecular properties allow for the preparation of molecule libraries e.g. by randomized single bead SPPS or the direct glycopolymer synthesis on solid support.[226,227] The use of biotin tags,[228,229] bioorthogonal conjugations[230] or the combination with fluorescent dyes[231,232] allow for various biological applications such as *in vivo* glycocalyx modifications[233–236] or the determination of proteins interactions towards resin-bound glycopolymers via fluorescent read-out.[237–240] Other techniques to obtain biological assays are e.g. surface plasmon resonance (SPR),[241] isothermal titration calorimetry (ITC),[242] protein crystallization studies,[243,244] saturation-transfer difference nuclear magnetic resonance spectroscopy (STD-NMR),[243,245,246] pull-down assays,[247,248] or native MS experiments.[245,249]

Generally, the decision between different glycomacromolecule architectures and their characteristics such as dispersity, valency, the structural variability or control have to be considered on behalf of the specific target protein to be studied for selective binding. In order to have a widely applicable system with feasible analytical characterization and possible tailor-made structural modifications, sequence-defined glycomacromolecules can be synthesized as being used by Hartmann group by means of glycooligoamide macromolecules.[250–252] To ensure glycomacromolecule monodispersity it is important to use efficient conjugation reactions both for backbone sequencing and for glycosylation reactions otherwise side products occur (analogue to Figure 7). Therefore, it is important to consider effective and broadly applicable glycosylation reactions.

1.2 Glycan integration in sequence-defined macromolecules

In order to synthesize glycomacromolecule scaffolds it is important to lay a special focus on glycosylation reactions. Principally, the covalent integration of selected glycan motifs is interchangeable with glycans of different complexities and protein selectivities. In advance of starting the macromolecule synthesis it is recommended to implement glycosylation strategy into the overall synthetic strategy respective rational design. Characteristic for carbohydrates is the high density of functional groups which requires

special handling compared to monomer or functional building block assembly e.g. regarding protecting groups[253,254] or stereoselectivity.[255]
Depending of the targeted synthetic glycomacromolecule architecture different structural variations can be integrated easily such as modifying the monomer assembly[256] or introducing functional handles or fluorescent dyes.[257,258] The tailored and precise adjustion of molecular properties can be conducted easily by using sequence-defined macromolecule synthesis e.g. with SPPS or dendrimer synthesis.[259] Since monodispersity and reliable ligand functionalization plays an important role for sequence-defined macromolecules, it is particularly important that glycan conjugation reaction proceed completely and the conjugated glycans remain stable under handling conditions.[260–263] Thus, the choice of glycosylation technique has to be orthogonal to the scaffold synthesis. Deglycosylation[264–266] or side reactions[267–269] should be prevented otherwise incompletely functionalized intermediates are formed lacking of spatial ligand arrangement control.[270,271] Whenever these undefined and undesired glycomacromolecule species occur, the successive compound analytics or biological assay interpretation of protein-ligand interactions is made difficult.[272] A very common potential cause of defect on glycomacromolecule synthesis is the final work up procedure which sometimes requires harsh conditions due to orthogonality reasons (e.g. concentrated trifluoroacetic acid in SPPS).[273–275] In contrast to monodisperse glycol-macromolecules, disperse macromolecules such as glycopolymers show a varying amount of ligand motifs conjugated to the macromolecule due to the scaffold dispersity, thus incomplete glycan functionalization is not fully preventable for the subsequent use as multivalent glycoconjugates.

Carbohydrates which are used for glycomacromolecule synthesis usually originate from biological sources, e.g. bacteria or plants.[276,277] The monosaccharides as well as their more complex oligo- and polysaccharides are synthesized via highly specialized biosynthetic pathways which allows for selective implementation of stereoinformation-containing carbohydrates.[278] The access to glycans is strongly dependent on the complexity and glycan size[279,280] which allows easy isolation either for very large, but disperse polysaccharides or for defined mono- or disaccharides. More complex oligosaccharides usually are present in organisms as compound mixtures or as glycoconjugates with peptides, proteins or lipids which require cleavage and challenging work up procedures prior to synthetic oligosaccharide use.[281–283] The availability of glycans from natural glycoconjugates is limited and is challenging due to limited glycan purity and sample amounts and is only limited to few examples.[281,282] e.g. for biological screening purpose[283] or for rare mono- or disaccharide motifs being previously post-glycosylation modified.[284,285] As a result from challenging availabilities of complex glycans, often mono- or disaccharides were used for the synthesis of sequence-defined glycomacromolecules which are commercially available in sufficient amounts and purities.[286,287] Meanwhile, the technologies on purification and synthesis of oligosaccharides using chemical,[288,289] chemoenzymatic[290,291] or biotechnological methods[292] were further improved. Especially progress from biotechnological industry helped gaining attention on commercial oligosaccharides such as human milk oligosaccharides (HMO).[102,293,294] These biotechnological syntheses use the bottom-up synthetic approach[295–297] which uses the bacterial assembly of small glycan fragments whereas top-down syntheses[277] are applied on the cleavage of natural glycomacromolecule structures such as polysaccharides or glycoproteins with subsequent purification of the glycan fractions.

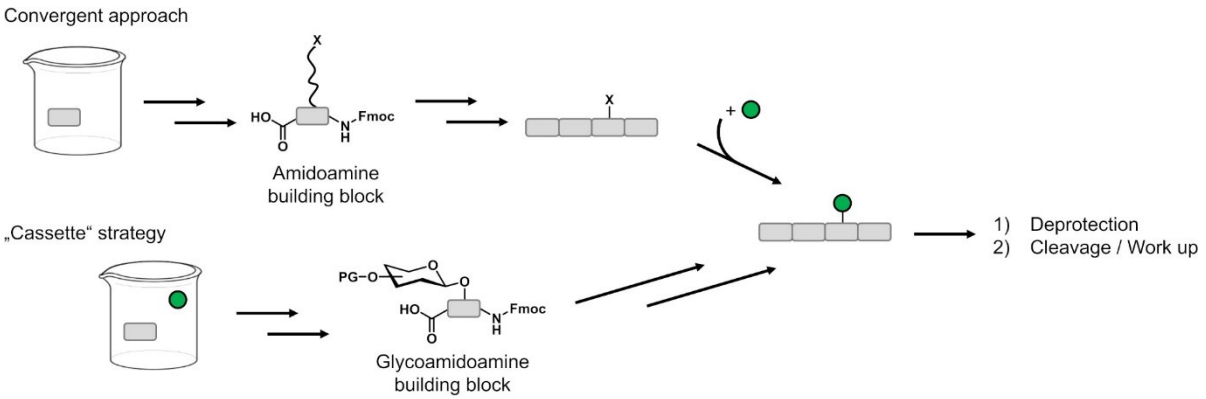


Figure 9: Comparison of glycan implementation strategies. Convergent approach and “cassette” strategy are based on building block assembly to obtain glycomacromolecules and differ from the moment of integrating the glycan motif.[298,299]

Although this allows for synthesis on preparative scale, mono- and disaccharides still show good advantages on glycomacromolecule synthesis in terms of availability, costs and synthetic handling. Because of the differences in availability often the less complex monosaccharides were used in glycomacromolecule glycosylation reactions. In many cases they show good binding affinities towards proteins when presented in multivalent fashion.[99,300] Hence, it is important to individually compromise between less complex monosaccharides and often more specific oligosaccharides in terms of carbohydrate availability, scalability, synthetic flexibility, specificity and synthetic effort. Glycosylation reactions are still challenging when oligosaccharides being conjugated onto glycomacromolecules especially in the context of sequence-defined and monodisperse scaffolds. When using conventional functionalization reactions, it has to be considered that each glycan respective oligosaccharide synthesis might require individual chemical modification and individual attention during synthesis.

In order to synthesize sequence-defined glycomacromolecules, two major synthetic approaches were established with specific advantages and disadvantages (see Figure 9). The convergent approach describes the linear or branched synthesis of macromolecules with the late-step introduction of functionalization, here specifically the glycan species.[299,301–303] Therefore, tailor-made building blocks were priorly synthesized which allow for subsequent, usually interchangeable glycan conjugation. A more specialized approach is the use of a “cassette” strategy which involves the prior synthesis of a glycoamidoamine building block with an already included glycan motif.[299,304,305] Both approaches can

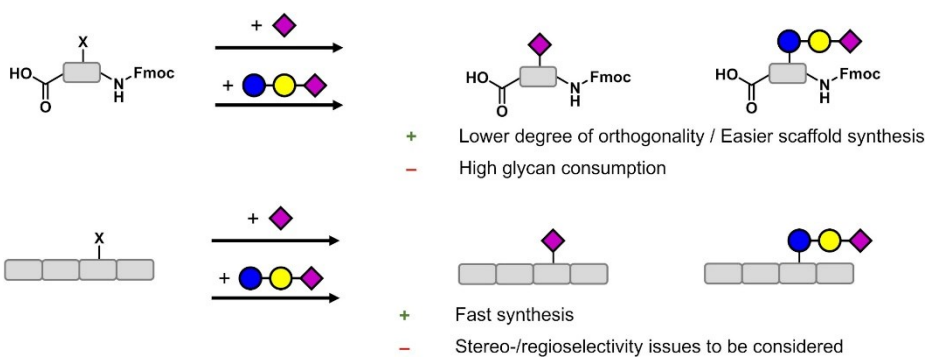


Figure 10: Advantages and disadvantages of both glycosylation strategies.

principally lead to similar glycomacromolecules but show method-specific properties. The convergent strategy allows for a flexible exchange of different carbohydrate motifs and it simultaneously requires a higher degree of orthogonality to differentiate between backbone assembly and the carbohydrate conjugation (see Figure 10). Whereas the “cassette” strategy developed by Danishefsky relocates attention towards the building block synthesis which allows for a more linear scaffold synthesis and thus is beneficial for challenging scaffold syntheses or automatized synthesis.[298,299] This “cassette” approach shows less flexibility in exchanging the glycan motif and is usually combined with a higher overall glycan consumption. Since glycan availability is a very common bottleneck for oligosaccharides or other rare glycan motifs, in these cases the choice of convergent glycomacromolecule synthesis is favorable (see Figure 10).[306]

In both approaches, the glycans usually require the use of protecting groups or functional handles for conjugation with the building block motif which are introduced by prior chemical functionalization.[307–309] This functionalization can be realized by introducing functional linkers at the reductive end as well as on *N*-acyl side chains or at other positions.

1.2.1 Chemical glycosylation

Glycosylation reactions combine a class of reactions in which covalent bonds between carbohydrates and another molecules are formed e.g. with functional linkers, macromolecules or another carbohydrates.[310] These glycosylation reactions can be differentiated in bottom-up construction of glycan motifs and the glycan attachment towards a natural or synthetic scaffold.[311,312] In general, these glycoconjugates or oligo-/polysaccharides originating from glycosylation reactions can be obtained using biological or organic chemical approaches. Progress in synthetic methods led to access to a high variety of both natural and non-natural glycoconjugates which allows for studying and mimicking biological processes.[313–315] Due to the high structural complexity of carbohydrates regarding stereochemical information or functional group density many carbohydrate reactions require the use of protection groups to maintain or adjust e.g. the linkage at the anomeric position or to regioselectively functionalize carbohydrates. Hence, when applying iterative glycosylation reactions often several synthetic steps are required to obtain the final glycosylated compound. The synthesis of complex oligosaccharide structures via chemical methods was successfully applied in literature on multiple examples and is often associated with low overall yields, high synthetic effort and harder purification procedures.[316]

The footing of chemical glycosylation reactions is the variety of monosaccharides which can be isolated from biological sources being used as feedstock.[277] The most common monosaccharides incorporated in mammalian glycans consist of 10 common members, namely D-glucose (Glc), D-galactose (Gal), D-mannose (Man), D-sialic acid (Sia), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc), L-fucose (Fuc), D-xylose (Xyl), D-glucuronic acid (GlcA) and L-iduronic acid (IdoA).[317] A further subclassification of these monosaccharides exist due to e.g. involvement of different biosynthetic carbohydrate derivatization[318–320] or post-glycosylation modifications.[321] Besides monosaccharide isolation, the industrial carbohydrate isolation in proper purity also includes disaccharides such as lactose,[322] trehalose,[323] sucrose[324] or maltose.[325] Simple oligosaccharides can be isolated and used

in food industry[325,326] but the isolation of more complex oligosaccharides from natural sources usually is not proceed due to inadequate compound purities.[327,328] The starting point of glycan construction is often performed either from mono- or disaccharides. To allow for selective synthesis protection groups are used to temporarily cap functional groups which should not participate during synthesis or to adjust stereodirecting effects.[329] Adhering stereochemistry is essential for the successful glycan synthesis as the involved stereoinformation is being used by carbohydrate-binding proteins such as lectins to identify specific monosaccharide or glycan motifs.[34] As a result from the selective adjustment of regio- and stereoselective functional groups in glycan chemistry, carbohydrate differentiation can take place although many monosaccharides show strong similarities to each other such as D-glucose and D-galactose. The functionalization of carbohydrates can occur on hydroxyl or amine groups to introduce side chains[307,330] or at the reducing end of the carbohydrate at C1.[331] Due to its chemical nature and the associated electronical effects of the hemiacetal group at C1 of reducing sugars, the so-called anomeric center is more prone to be attacked by nucleophiles which allows for facile functionalization of this position.[331] Both the pyranose and the furanose monosaccharide ring forms exist in a chemical equilibrium which contains the ring-opened form as well as the closed-ring forms which occur as α- and β-anomer. The formation of specific anomers can be influenced by external factors such as solvent, temperature, pH, etc.[332] The change between different configurations, this so-called muta-rotation,[333,334] allows the non-functionalized carbohydrate to interchange between both anomeric and open type and forms the thermodynamically favored anomer species. Once the anomeric position of carbohydrates is functionalized mutarotation does not occur spontaneously and thus enables the fixation of anomeric forms.[335] This fixation effect occurs after the formation of glycosidic linkages between monosaccharides and explains the anomeric stability of oligo- and polysaccharides. Based on the anomeric preference of the corresponding monosaccharide, they can be selectively functionalized to α-/β-anomeric form by controlling e.g. the choice of neighboring groups or the synthetic route.[336] Since α- and β-anomers of carbohydrates show differences in spatial ligand arrangement as well as torsion properties and thus might influence protein binding, the adequate anomeric selectivity during glycosylation is inevitable for a successful synthesis.[337,338] This need of high stereochemical control in glycan synthesis becomes more important when being used in monodisperse glycomacromolecules with fundamentally higher demands in structural control. For easy macromolecule glycosylation, glycans and the corresponding macromolecules can be prepared for “click reactions” e.g. with azide respective alkyne group which can be used for subsequent copper-catalyzed azide-alkyne cycloaddition (CuAAC) (see Figure 11A).[339,340] Another well-established conjugation method is the photo-induced thiol-ene coupling (TEC).[341,342]

Proven glycosylation reaction approaches based on the anomeric carbon position were intensively studied and lead to the development of several common used techniques such as the Fischer glycosylation[343–346] or the Koenigs-Knorr glycosylation (see Figure 11B).[347,348] Alternatively, reactive intermediates such as oxazoline[349] or trichloroacetimidate donors[350,351] can be used to preactivate glycan motifs for their subsequent transfer onto suitable acceptor motifs. These precursors favor the glycosylation reaction by using efficient leaving groups whereas they shift the chemical equilibrium

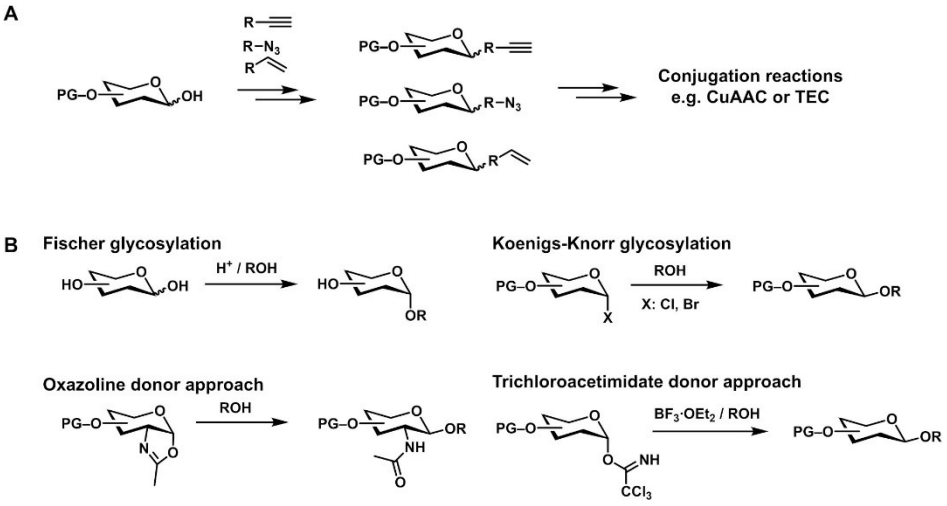


Figure 11: A: Integration of functional handles allowing for subsequent glycan conjugation reactions e.g. onto macromolecular scaffolds. **B:** Different types of glycosylation reactions.[349,352]

according to Le Chatelier. Exemplarily, the Fischer glycosylation is performed with unprotected carbohydrates and alkyl alcohols as reagents to give mono-alkylated carbohydrate derivatives being used e.g. as detergents.[344] The reaction can be completed by adding excess alcohol, by providing enough thermal energy and by removing water which is formed as a byproduct from substitution reaction. Although this reaction do not show good stereocontrol, this method allow for application on technical scale. The Koenigs-Knorr method uses a similar approach but requires priorly functionalized carbohydrate precursor to facilitate substitution reaction with alcohols.[347,353] Therefore, the carbohydrate has to be peracetylated which function as protecting groups and subsequently the anomeric center is halogenated e.g. by using hydrogen bromide. The introduced halogenide group is a good leaving group and the present acetyl protecting groups prevent side reactions such as undesired dimerization or oligomerization reactions. The acetyl groups further allow for sterical shielding and has the function of neighboring group effects, especially at C2 *O*-conjugated acetyls. Glycosylation reaction via Koenigs-Knorr mechanism lead to an anomerical inversion when adding nucleophiles such as alcohols.[354] Depending on the stereochemistry of the chosen monosaccharide type, this oxazoline-bearing key intermediate can be isolated or directly synthesized as being shown in Figure 11B. This principle of using preactivated carbohydrate precursors was further developed by introducing functional groups with better properties on anomeric control or substitution yields.[355] Further examples of such activated carbohydrate donor species are glycosyl carbonate,[356,357] glycosyl carbamate[358–360] or trichloroacetimidate derivatives[361–364] which also has been used in multiple reported glycosylation reactions. The corresponding acceptor molecule can vary so does the glycosylated product. When these reactions are performed specifically on another site-selectively unprotected carbohydrates with a single unprotected position, this conjugation leads to elongating the glycan species according to oligosaccharide synthesis. When using glycosylation on non-glycan acceptor motifs this method can be used for the introduction of functional handles e.g. fluorescent dyes,[365] hydrophobic linkers,[366] or polymerizable groups (e.g. methyl(meth)acrylates or acrylamides).[367]

These glycosylation reactions can be applied both in solution-based chemistry and on solid support as being applied on solid phase oligosaccharide synthesis.[316,368] Solid phase oligosaccharide synthesis can be performed with the help of automated synthesizers and heavily extends the synthetic access to both natural and non-natural oligosaccharides. Due to the high complexity of this approach including the tremendous product variety of solid-phase synthesis, this method circumvents time-consuming purification steps and is predominantly on synthesizing oligosaccharides on small scale.

Due to the steady development in the field of carbohydrate synthesis various glycosylation methods were developed to easily functionalize carbohydrates via their reducing end without the use of demanding protection group strategies (see Figure 12). The priorly described Fischer glycosylation is an example of such a protective group-free glycosylation but shows general limitation on the accessible product scope due to large required reagent quantities combined with common solubility issues.[369] Further examples are the reductive amination,[370,371] the use of glycosyl thiol functionalization,[372,373] the use of 2-chloro-1,3-dimethylimidazolium chloride-(DMC-)mediated reactions[372,374,375] or the oxyamine glycosylation.[376,377] Reductive amination uses the reaction of aldehyde groups of the ring-opened form with amines to form imines. These so-called Schiff base[378] can be reduced into the stable secondary amine form. Typical for this method is the broad applicability and the opening of the monosaccharide at the conjugation position.[379–381] Whereas reductive amination lead to changing the glycan topology whilst loosing stereochemical information at C1 position, the other glycosylation reactions summarized in Figure 12 maintain the initial hexose form.[370,372,377,382] The thiol glycoside conversion represents a selective 1,2-cis glycosylation reaction with alcohols where the thioglycan has to be priorly prepared by protection group-based synthesis.[373] DMC-based glycosylation reactions developed by Shoda group directly work out for non-functionalized carbohydrates and allow for the fast introduction of variable functionalities.[383–385] The further development of this DMC approach by Fairbanks and co-workers led to introducing azidated imidazolium reagents which were used for the direct synthesis of azidoglycans.[386,387] An alternative way of carbohydrate glycosylation is described with be oxyamine glycosylation. Depending on the hydroxyl-functionalization of the oxyamine precursor the glycans can either be conjugated via *O*- or *N*-glycosidic bond. *O*-glycosidic oxyamine functionalization require the use of basic additives whilst *N*-glycosylation can be applied on aqueous buffered environments at pH 4.5

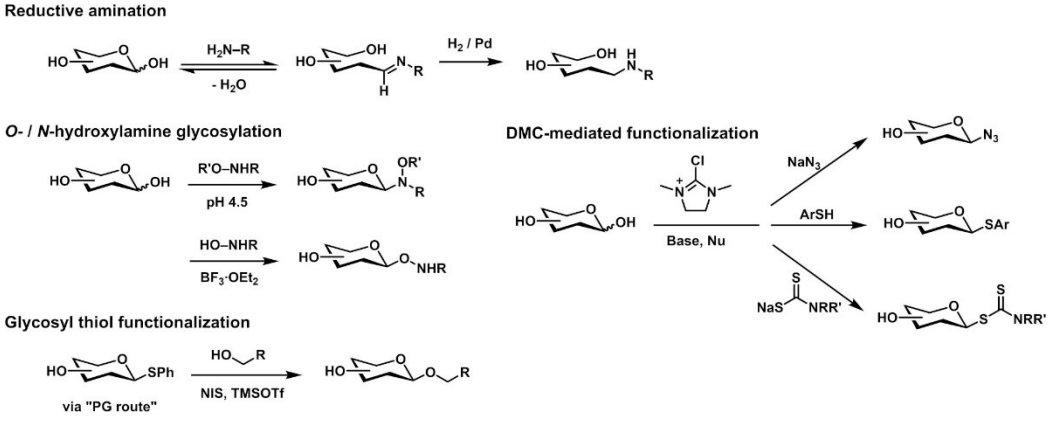


Figure 12: Examples of protection group-free glycosylation reactions.[370,372,377,382]

but are simultaneously described to be chemically sensitive against harsh conditions such as acidic pH < 4.[377,388–391] Originally used for the immobilization on glass chips working as glycan arrays for studying carbohydrate-lectin interactions,[377] the applicability of oxyamine glycosylation has already been shown on several published examples such as disperse glycomacromolecule synthesis[392,393] or the introduction of functional linkers containing amines,[377,394,395] azides,[390,396] thiols,[391,397] biotin linkers, fluorinated tags or UV-absorbing or fluorescent motifs.[390,392,393,398] Hence different oxyamine derivatives were synthesized and described in detail, namely *N*-methyloxyamines,[377,394,399] *N*-alkyl-*O*-methyl-oxyamines[390] and amino-oxy derivatives[399,400] which show slight differences in stability as well as stereoselectivity. Wittmann and co-workers showed on a comparison study that amino-oxy derivatives appears appears in three different glycoconjugate forms which are the α- and β-anomer and small amounts of the ring-opened form whereas *N*-methyloxyamines show better stereoselectivities on glucose-based carbohydrates.[399] The glycosylation selectivities strongly depend on the reducing end monosaccharide motif being glycosylated whereas glucosides showed high selectivity for β-anomeric conjugates, other monosaccharide motifs such as mannose or sialic acid did not show clear configuration preferences.[377,399,401] The glycosylation reactions with *N*-methyloxyamines under mild aqueous conditions is described as being incompatible with sulfated carbohydrates.[377]

Generally, these described chemical glycosylation reactions as well as their protection group-free approaches can be used for glycomacromolecule synthesis but might be associated to characteristic challenges. Some monosaccharide such as fucose or sialic acid do not show sufficient stereodirecting effects due to lack of neighboring group which often lead to anomerically impure conjugation reactions when using the chemical conjugation approach.[402–404] The resulting lack of stereocontrol can lead to anomeric mixtures, loss of yields and to challenging separations. This high synthetic demand requires additional effort when conjugating sialic acid motifs and still remains challenging even in specialized solid phase synthesis approaches or on multivalent scaffold conjugations.[207,405] Additional deglycosylation processes due to incomplete conversions or hydrolysis reactions might further complicate the isolation of complex glycoconjugates. To meet the final glycomacromolecule requirements regarding low dispersities, it should be considered to introduce sialic acid motifs lately or to apply sufficient protection group chemistry.[406] The timing of glycan introduction and thus indirectly the kind of glycosylation strategy is a matter of principle and needs special attention, e.g. when harsh conditions should be avoided on glycan motifs.[349,407,408] Whereas protecting group-free glycosylations principally allow for easy glycan introduction, synthetic requirements such as challenging stereoselectivity might be a reason for choosing alternatives towards chemical glycosylations. Therefore, solutions can be the use of readily glycosylated oligosaccharide motifs or alternative chemoenzymatic glycosylation approaches.

1.2.2 Chemoenzymatic glycosylation

Additional to chemical glycosylation reactions, the biochemical approach of chemoenzymatic glycosylation augment classical systems. Here, enzymes are being used as catalysts which benefit from extremely high regio- and stereoselectivity which usually outrange chemical methods.[409] These chemoenzymatic processes uses the similar principle of chemical glycosylation reactions by covalently

conjugating two carbohydrate motifs to each other using glycosyl transfer reactions or oxazoline donors and endoglycosidases.[410,411] Therefore the so-called glycosyltransferase enzyme identifies a biologically activated monosaccharide donor species which is selectively conjugated to an acceptor motif which is usually a mono-, di-, oligo- or polysaccharide.[412] Both parts, the donor as well as the acceptor are selectively recognized by the enzyme and are conjugated under mild reaction conditions.[413] The biological activation of the donor takes place using sugar nucleotide activation via synthase enzymes[414,415] which can proceed proximately to the transfer reaction as already occuring in natural glycosylation processes.[416] The enzymatic glycosylation reactions undergo strict anomerical control which can be exploited for the synthesis of complex glycan motifs or to introduce anomerically demanding monosaccharide motifs such as sialic acid or fucose.[417] Many examples have been reported in literature in which the combination of biological glycosylations with chemically derived precursors were described as advantageous for the synthesis of previously functionalized carbohydrates or glycoconjugates.[418,419]

The principle of enzymatic carbohydrate functionalization is derived from nature where it is already used by organisms for the synthesis of endogenous natural glycoconjugates being used e.g. for the implementation in the glycocalyx.[420–422] Due to the intracellular appearance of ubiquitous monosaccharide mixtures, organisms developed enzymatic systems which allowed for precise differentiation of both the donor and the acceptor species for the specific synthesis of glycans.[423] Therefore, the glycosylation process is formally separated into least two synthetic steps which includes the synthesis of the monosaccharide donor species and the transfer of this monosaccharide motif onto the acceptor.[424] The donor synthesis is catalyzed by enzymes of the group of donor synthases by the formation of sugar nucleotides. Sugar nucleotide synthesis uses organism-specific, complex pathways of either non-functionalized or phosphorylated monosaccharides depending whether the monosaccharide was synthesized *de novo* or was salvaged during metabolism pathway.[425] Here, the synthase enzymes stereoselectively forms sugar nucleotides from the anomerically mixed non-conjugated monosaccharides.[426,427] The subsequent glycosylation reaction via glycosyltransferase enzymes requires certain acceptor motifs which are mainly defined by the terminal chemical environment which is usually the terminal monosaccharide (see Figure 13).[428] To allow for the glycosylation of the whole variety of different monosaccharides, each organism respective cell requires various synthase and glycosyltransferase types which simultaneously coexist in the golgi apparatus.[62] In nature, the enzymes which are involved in the glycosylation process are located intracellularly either as soluble proteins or as lipid- or membrane-bound enzymes, e.g. presented in the golgi apparatus of the endoplasmatic reticulum to perform post-translational modifications.[422,429] For the synthesis of oligosaccharides and their glycoconjugates, glycosyltransfer processes occur in cascade reactions of donor syntheses and the subsequent monosaccharide attachments in the order given by the enzyme specificities and spatial glycosyltransferase localization.[423] Therefore, glycosyltransferase enzymes are naturally immobilized to the golgi apparatus being part of the secretory pathway to allow for streamlining

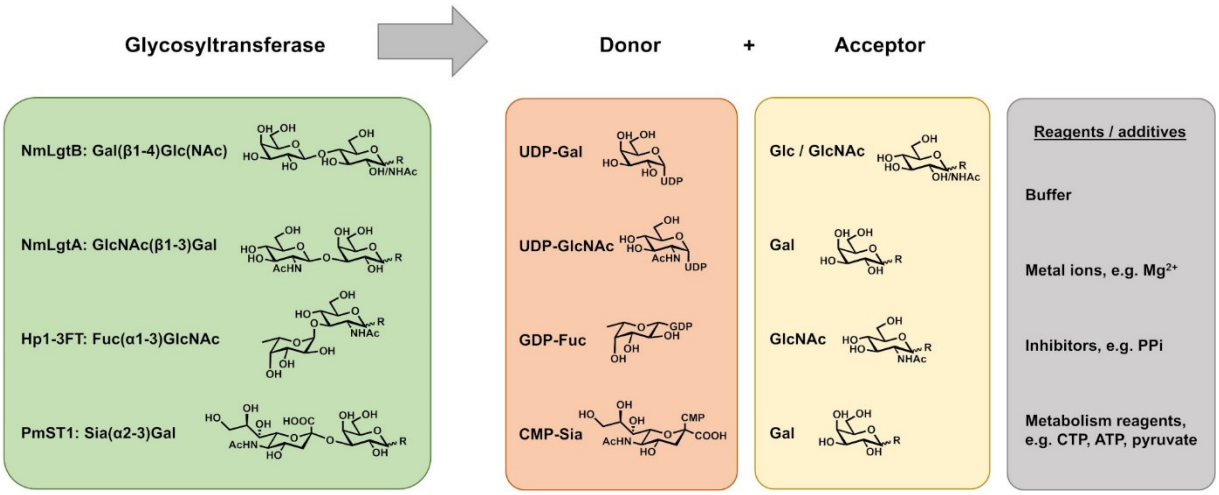


Figure 13: Principle of chemoenzymatic glycosylation reactions using glycosyltransferase enzymes.[428] The choice of glycosyltransferase defines the applied reaction conditions such as the corresponding donor and acceptor motifs as well the required reagents respective additives. Exemplarily shown are the enzymatic galactosylation, *N*-acetylglucosaminylation, fucosylation and sialylation reactions. The glycosyltransferases are abbreviated with NmLgtB: *Neisseria meningitidis* β1-4-galactosyltransferase,[430] NmLgtA: *Neisseria meningitidis* β1-3-*N*-acetyl-glucosaminyltransferase,[431] Hp1-3FT: *Helicobacter pylori* α1-3-fucosyltransferase,[432] PmST1: *Pasteurella multocida* sialyltransferase.[433], the nucleosides are abbreviated with: ATP: adenosine 5'-triphosphate, CMP: cytidine 5'-monophosphate, CTP: cytidine 5'-triphosphate, GDP: guanosine 5'-diphosphate, UDP: uridine 5'-di-phosphate, PPi: inorganic pyrophosphate.

the entire glycosylation process.[422] Due to the high demand of higher developed organisms such as mammalians, they have more complex glycan biosynthesis requirements and thus they developed enzymes with usually higher specificities and lower substrate tolerance compared to e.g. bacterial donor synthases or glycosyltransferases.[434,435]

The principle transfer of biosynthetic enzymatic glycosylation towards synthetic applications opens up new possibilities. Chemoenzymatic glycosylation reactions were applied on multiple examples of synthetic compounds to selectively elongate the glycan motif.[436–439] Since different approaches were examined to synthesize artificial compounds as an example enzymatic methods were combined with solid phase-immobilized precursors but glycosylation attempts did not lead to full conversions.[440–443] The selective *in vivo* incorporation of artificial monosaccharide precursors was presented by Bertozzi group and was used to decorate glycocalyx of mice and zebrafish cells which enabled fluorescent or metabolic cell labelling.[444,445] The protein expression from bacterial vectors allows for the isolation of glcosyltransferases or synthases which can be used for *in vitro* syntheses of oligosaccharides and glycoconjugates.[446,447] Chemoenzymatic glycosylation reactions can either be performed sequentially with separated donor synthesis and glycosylation or by combining both processes in a one-pot reaction. Although the one-pot synthesis approach profit from less effort in terms of work up and purification, the separated chemoenzymatic reactions benefit from a broader process window e.g. when applying donor synthesis and transfer reactions at different pH values or buffers.[428,448] The development of one-pot multienzyme synthesis approaches (OPME) has been studied by the groups of Wang and Chen and were proven on multiple examples.[428,430,431,433] For the sufficient handling of these described enzymes usually aqueous media are used with compatible reactions conditions such as buffer, temperature or

solvent stability. Furthermore, for the optimal activity of many enzymes the presence of specific cofactors such as metal ions is important as well the removal of specific inhibitor species.[449–451] The sequential use of OPME reactions can be used for iteratively synthesizing oligosaccharide sequences as exemplarily shown on Lewis x pentasaccharide synthesis (see Figure 14A)[428] or it can be used to supplement conventional chemical glycosylation reactions, e.g. to synthesize non-natural donor species respective monosaccharides.[452] This chemoenzymatic approach can be used with the help of automated glycan synthesizers as performed by Wong group[453] or in combination with chemical solid phase oligosaccharide synthesis.[454]

Enzymatic glycosylation reactions can be described on behalf of the specific example of sialylation reactions via the *Pasteurella multocida* sialyltransferase enzyme (PmST1). This enzyme recognizes terminally galactosylated glycans and elongates the carbohydrate motif by one sialic acid residue.[433,455,456] PmST1 requires a biologically activated CMP-sialic acid residue which in this case operates as the carbohydrate donor. The PmST1 catalyzes the transfer of this donor species onto the terminal galactoside motif which is exemplarily present on chemically functionalized lactose or Lewis x pentasaccharide.[428] The CMP-sialic acid donor can either be added to the reaction mixture as a substrate or it can be synthesized *in situ* by the use of synthases e.g. NmCSS (*Neisseria meningitidis* CMP-sialic acid synthase).[457,458] The Mg^{2+}-dependent NmCSS used here catalyzes the formation of β-anomeric donor by using CTP and sialic acid.[427,459] The CMP-sialic acid donor is then inverted during PmST1 glycosyl transfer to yield the characteristic regioselectively α-sialylated glycan with a linkage between anomeric sialic acid carbon (C2) and the C3 hydroxyl group of the acceptor galactoside. This specifically formed linkage is described as a α2,3-sialylation which includes information about the anomeric sialic acid configuration and the carbon linkage positions of donor and acceptor molecules. The multifunctional PmST1 WT was previously described to form α2,3-sialylated products but also show minor and thus negligible tendencies to form α2,6-linked byproducts.[433,455] A mentionable and more important side reaction of PmST1 WT is the desialylation of the sialylated products which is significantly slower than the product formation. Protein engineering[460] was applied on PmST1 which led to the creation of mutants with improvement of enzymatic properties such as decreased desialylation activity found on mutant M144D.[461,462] Although PmST1 M144D was reported to show no significant sialoglycan degradation, the sialyltransfer activity is at the same time strongly reduced which make the full conversion of glycosylation reaction more difficult. Other specially designed PmST1 mutants such as the P34H/M144L were developed to change the enzyme regiospecificity towards α2,6-sialylation.[463–465] Although these chemoenzymatic reactions were described in detail, reaction optimization is still important especially due to the desialylation activity of PmST1 WT. This effect usually requires close system monitoring since incomplete conversions can be reasoned either with an unfinished reaction or a degradation reaction after being fully sialylated.[462] Whereas incomplete reactions of monovalent substrates requires product purification procedures, the incomplete conversions of multivalent scaffolds can lead to a significant decrease of fully sialylated product (as described in Figure 8). Thus, the desialylation reaction as well as a slow reactivity can be challenging especially when accessing sequence-defined structures with precise control about the glycan valency or the distance between glycan ligand motifs.

When performing chemoenzymatic glycosylation reactions, it has to be considered that the enzymatic activities are strongly pH-dependent.[466] Thus, when performing a one-pot reaction of several enzymes it is likely that the involved enzymes do not meet their maximum activity conditions due to the given pH value of the aqueous medium. Furthermore, the interpretation of reaction kinetics may be challenging due to the dynamical change of substrate, product and intermediate concentrations. Therefore, in a two-enzyme one-pot sialylation reaction it is useful to allow for full conversion of the CMP-sialic acid intermediate by either, overdosing NmCSS and their substrates or to allow for synthase preconditioning with subsequent addition of the PmST1. By the use of various involved reagents for multienzyme reactions the concentration process window becomes more narrow due to the higher number of reagents resulting in less formulation options. Hence, the adjustment of stock solution concentrations has to be prepared precisely. The application of one-pot multienzyme synthesis using PmST1 was shown by Chen group to allow the simultaneous use of three enzymatic steps starting from *N*-acetylmannosamine (ManNAc) which yielded natural and artificial sialoglycans (see Figure 14B).[433] Whereas *N*-acetylneuraminic acid (Neu5Ac) is the most commonly used member of the sialic acid family other sialic acids can also be used for enzymatic glycan elongation reactions such as *N*-glycolylneuraminic acid (Neu5Gc), 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN) or synthetic *N*-acyl modified sialic acids.[402,433] These chemoenzymatic reactions can be used in combination with modular systems e.g. CuAAC[467] and benefit from a broad applicability on small preparative scales, the use of mild reaction conditions and the easy introduction of exotic sialic acid derivatives.

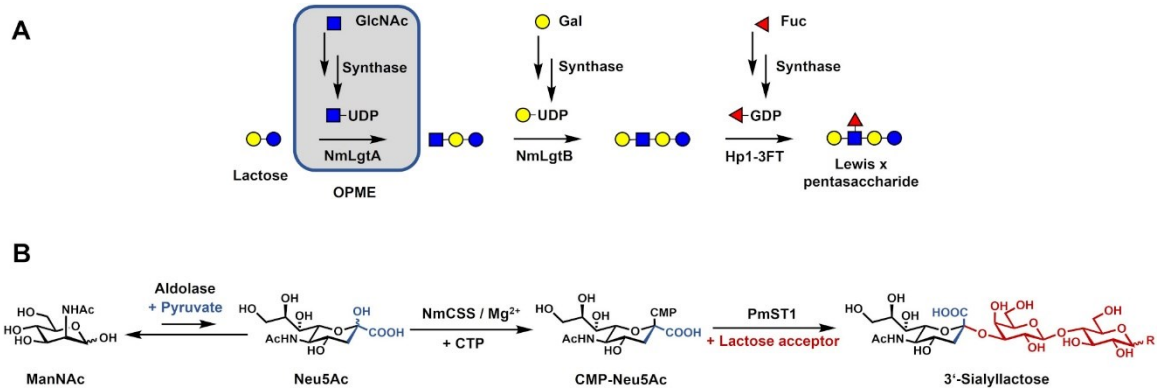


Figure 14: A: Chemoenzymatic cascade synthesis of Lewis x pentasaccharide using three sequential one-pot multienzyme syntheses (OPME). Enzyme abbreviations are listed in description of Figure 13.[428] **B:** Detailled one-pot multienzyme synthesis describing sialylation via PmST1.[433] Molecular parts introduced by aldolase (blue) and sialyltransfer reaction (red) are highlighted within the figure. Figure content was modified and printed with permission of Royal Society of Chemistry (Great Britain).[428]

1.3 Solid phase peptide synthesis

Solid phase peptide synthesis (SPPS) is a specially developed method to obtain macromolecules such as peptides or peptoids and was further developed to synthesize oligoamide scaffolds and their hybrid macromolecules.[468–470] This method allows for accessing various peptide-derived macromolecule architectures e.g. linear, branched or macrocircular constructs and is generally compatible with site-

selected synthetic post-translational and post glycosylation modification reactions such as glycosylation, sulfation, phosphorylation or methylation reactions.[471–473]

Prior to the development of SPPS, recent advances in peptide synthesis were made using organic chemical conjugation and classical solvent-based chemistry which led to the access to biologically active peptides. During this period small peptides such as the oxytocin nonapeptide was synthesized by the du Vigneaud group which was awarded with the Nobel prize in 1955.[474,475] Although at the time already existing methodology was applied to synthesize small peptides, the repetitive peptide conjugation reactions to complete the desired primary sequence was found to be extraordinary time-consuming due to the need of product purifying after each coupling step to remove excess starting material, coupling reagents or oligomerized monomers.[221,476] The high effort of product isolation using this procedure led to the motivation of Merrifield to discover the principle of SPPS in 1963 by isolating a sequence-defined model tetrapeptide Leu-Ala-Gly-Val using insoluble chloromethylated and divinylbenzene-crosslinked polystyrene resin as a solid support.[176] SPPS used the principle of conjugating amino acid building blocks onto the resin which allowed for the easy resin filtration after each coupling step. Whereas the conjugated amino acid remained on the resin, the residual starting material, reagents or non-defined, dissolved oligomers were rinsed off to allow for good molecular control of functional groups on the immobilized amino acid sequence.

During SPPS reactions it was extraordinary important that full conversion of the single reaction steps occurred such as the coupling onto chloromethyl resin groups or the distinct amino acid building block assembly steps. Incomplete coupling reactions leave behind reactive functional groups on solid phase particles which can participate in following coupling reactions and thus lead to deletion sequences.[477] Likewise for providing exact control in building block assembly or sequence pattern, it is important to avoid multiple amino acid conjugations on one single binding site leading in uncontrolled chain growth. Therefore, SPPS can be performed with *N*-protected amino acid building blocks to iteratively change between building block coupling and deprotection reaction procedure until the final peptide or polymer sequence is obtained (see Figure 15). The peptide sequence can undergo further reactions e.g. modification by *N*-acyl capping, glycosylation or conjugation of small molecular or macromolecular components or cleaved off the solid phase resin (see Figure 15). Beside the assembly of monomer sequence, SPPS allows for controlling the valency of functional groups respective ligands as well as their distance among each other which can have an effect e.g. on biological activity of the synthesized macromolecule.[478–480]

During the showcase of SPPS development, Merrifield simultaneously synthesized the same tetra-peptide by solvent-based synthesis and compared both synthetic strategies which highlighted the advantages of SPPS.[176] As a result of this comparison rational, SPPS-based oligopeptide synthesis was successfully applied on the oligopeptide structure with similar purities compared to conventional organic coupling techniques but in a shorter overall time and easier handling.[176] By exchanging the need of time-consuming purification procedures such as recrystallization or column chromatography with simple filtration technique, SPPS revolutionized and simplified the access of oligopeptides and was further improved to obtain better yields, higher purities and access to longer peptide chains.[481,482] The steady progress of SPPS methodology led to the development of automatized peptide synthesis,[221] supported distribution of commercial produced protected amino acids[177] and enhanced the scope of

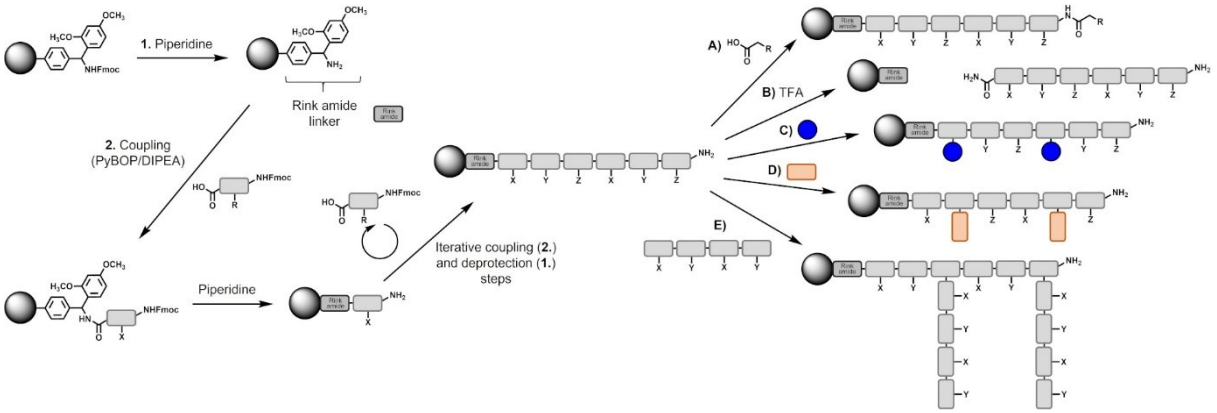


Figure 15: Basic principle of SPPS consisting of iterative deprotection (**1.**) with piperidine (25 vol% in DMF) and building block coupling (**2.**) with the help of coupling agents (e.g. PyBOP and DIPEA). The resin-bound and sequence-defined oligomer can be obtained by monomer assembly and can be used for various reactions e.g. **A:** *N*-terminal acyl capping with acetic acid or fatty acids (optionally with the help of coupling reagents or its activated anhydrides or acyl chlorides), **B:** acidic cleavage of rink amide linker using TFA/TIPS/DCM (95/2.5/2.5 vol%), **C:** glycosylation reactions e.g. via CuAAC, **D:** non-linear chain elongation with building blocks, linkers or alkyl residues, **E:** polymer-analogous conjugation of macromolecular building blocks.[250]

applicable protecting group strategies.[483] The further enhancement of solid phase method limitations allowed for synthetic access of difficult sequences,[484] peptides (e.g. Insulin[485,486] and Bradykinin[487,488]) and can also be used for large scale synthesis as exemplarily shown on the HIV-1 membrane fusion inhibitor enfuvirtide.[489] Starting from Merrifield's initially presented SPPS approach using carbobenzoxy-protected (Cbz) amino acid monomers for the peptide synthesis, SPPS methodology and protecting group strategies were further elaborated and differentiated.[490] Merrifield already observed limitations of his experimental strategy due to partial cleavage reactions during Cbz-deprotection which highlighted the need of precisely adjusted orthogonalities of the involved chemistries. Similar to solvent-based peptide synthesis, most SPPS spproaches inevitably required the use of protecting groups to regioselectively perform coupling reactions.[491–493] Due to the good feasibility of SPPS with *N*-protected building blocks, two major protecting group strategies were established by Carpino using *tert*-butyloxycarbonyl- (Boc) or fluorenylmethoxycarbonyl-protecting groups (Fmoc) for the linear assembly of the peptide backbone sequence.[494–496] Both strategies were used parallel with approach-specific characteristics and principally differ by the deprotection respective cleavage conditions. They are not fully compatible among each other and vary in reaction handling due to deprotection conditions respective stabilities. Whereas Boc deprotection occur under acidic conditions (e.g. with trifluoroacetic acid),[497,498] Fmoc-deprotection can be induced by using cyclic, secondary amines (e.g. with piperidine, see Figure 15).[177,499] Nowadays Fmoc-strategy was majorly implemented due to the broad and easy applicability, the commercial accessibility of the building blocks, the avoidance of highly corrosive deprotection reagents and the resulting simpler technical requirements on automated peptide synthesizers.[470,496]

Today numerous examples of commercially available building blocks exist derived from *N*-protected amino acids. Besides proteinogenic and non-proteinogenic α-amino acids, the amount of commercial building blocks for SPPS includes multiple artificial derivatives such as variations in side chain protection

or functionalization,[500,501] structural variation,[502] isotopic or fluorophoric labeling[503,504] or stereochemical modifications.[505] Another examples of functional building blocks are structurally deviated β- or γ-amino acids or further modified artificial oligoamide building blocks e.g. DNA-binding pyrrole-imidazole derivatives,[506–508] peptide nucleic acids[509] or peptoid building blocks.[468] The sequence-defined monomer assembly can be achieved by variable coupling strategies such as the submonomer approach with alternating coupling of compatible, symmetrically functionalized building blocks (AA and BB) or by a protecting group strategy with at least bifunctionalized but partially protected building blocks (AB-PG) as already described by Merrifield in 1963 (see Figure 16A).[510] To support coupling reaction between amines and carboxyl groups in SPPS, additional chemical effort needs to be pursued (see Figure 17). Therefore, reactivity and coupling efficiencies can be enhanced by e.g. using excess amounts of building blocks and by adding compatible activation reagents or, if necessary, by applying external energy sources such as heat or microwave radiation.[511–513] A common method for the activation of carboxyl groups use the of *in situ* transformation into active ester species which undergo easier amide coupling due to higher reactivity compared to the free acidic form.[514] Carbodiimides,[515] *N*-hydroxybenzotriazol derivatives,[516] uranium-type derivatives[517] and multiple other examples can be used as activation agents.[518] Frequently used reagents such as *N*,*N'*-diisopropylcarbodiimide (DIC),[519,520] *O*-(7-aza-benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HATU)[521,522] or benzotriazolyl-oxy-tris(pyrrolidino)-phoshonium hexafluorophosphate (PyBOP)[523] were optimized on their low epimerization properties which prevent racemization of COOH-neighbored stereocenters as typically present in most proteinogenic amino acids.[524]

Besides efficient building block coupling reactions, it is important to use a compatible solid support resin for SPPS which combine the occurrence of feasible amounts of functional groups, high stability against process conditions and broad solvent compatibility.[525–527] Hence, the assembling monomers can be coupled either directly onto the surface of porous, functionalized resin beads to form peptide chains immobilized on-resin or onto attached functional linkers.[528] These resin-bound linkers possess designed chemical properties in terms of cleavage conditions or stabilities or can be used for the terminal introduction of functional groups or labels onto the assembled macromolecule.[470,529] The solid phase resin beads can consist out of different materials such as polystyrene (PS),[176,530] polyethylene glycol

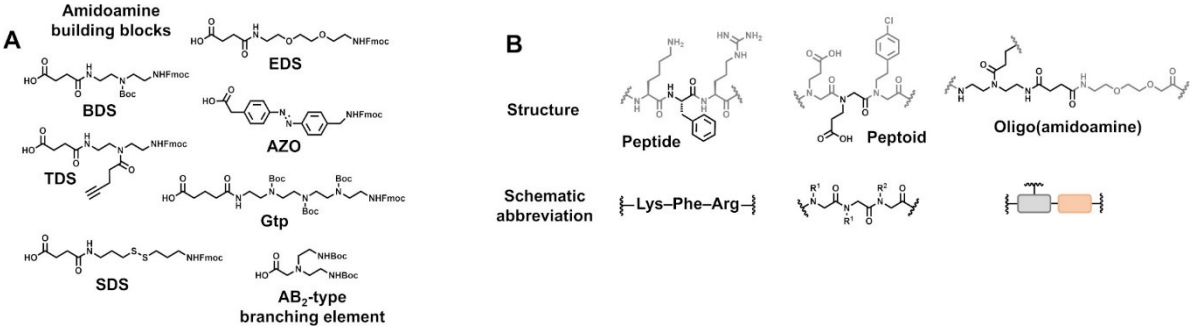


Figure 16: A: Examples of building blocks BDS,[537] TDS,[250] SDS,[538] EDS,[250] AZO,[538] Gtp,[539] and AB_2-type branching element.[540] **B:** Structural backbone comparison of exemplary SPPS-derived peptide,[513] peptoid[541] and oligo(amidoamine) scaffolds[542] as well as schematic structure abbreviations. The monomer repeating units were highlighted by color.

(PEG),[531,532] or modern hybrid material resins with optimized material properties such as poly(ethylene glycol)-polyacrylamide (PEGA),[533] Tentagel-based resins (TG),[534–536] or sucrose-based polymer support (SUBPOL).[543] Their role and function were described in detail and the choice of resin has to be taken empirically depending on the solvents, building block chemistry and activation reagents.[535,544,545] These porous materials usually strongly interact with surrounding solvents which leads in shrinking and swelling behavior once solvents are changed.[546] As a result of the dynamic change of surface properties, the location of functional coupling sites for solid phase synthesis can vary and thus might change accessibility e.g. through swelling and shrinking of the resin. In terms of precise reaction handling, it is preferable that each coupling site undergoes coupling respective deprotection reaction which allows for the steady building block assembly resulting in monodisperse sequence growth. Slight changes in surface presentation or potential cause of sterical hindrance of elongation sites can lead to inconsistently growing polymer chains which increases the amount of deletion sequences and the grade of dispersity. To prevent these undesired side reactions it is important to use the solid support in a well swelled condition and to use compatible solvents for reactions which are ideally within a narrow polarity window between high and very high polarity.[547,548] In many examples the loading of a polymer resin can indirectly influence the reaction handling e.g. incomplete deprotection reactions on high loadings[549] or uncontrolled conjugation of two spatially close growing peptide chains leading to unreactive adducts being inable for further sequence growth.[550,551]
For sufficient substance dispersity and purity as well as a generally feasible application of SPPS, these described aspects of choosing the right systems out of building blocks, activation reagents and solid phase resins are essential. Thus, the optimization of coupling conditions, good building block purities, the right choice of protecting group strategy as well as polymer resin stability can help to improve SPPS handling.

The product scope from SPPS can go from the synthesis of natural peptides, to site-directed functionalization with artificial motifs up to completely non-natural polymeric scaffolds such as oligo(amidoamines) or peptoids (see Figure 16B).[468,552] Prominent challenges of SPPS were e.g. the establishment of traceless cleavage from resin,[553,554] the formation of complex scaffolds such as macrocycles[555–557] or self-assembling vesicles.[558–561] Small proteins were synthesized by linear SPPS as shown on the example of ribonuclease A[562] or several peptide fragments can be selectively conjugated by native chemical ligation technique to yield proteins as shown on cytochrome C,[563] and chemotactic protein CP10.[521] The principle of solid phase synthesis of assembling small molecules towards macromolecular structures were successfully transferred to related solid phase synthesis methods for oligosaccharides,[316,368,564] oligonucleotides[565,566] and polymers.[567–569] The further deviation of this method can be used to design insoluble polymer-supported reagents[570,571] for better or to use polymer resins for small molecule organic reactions. This so-called solid phase organic chemistry (SPOC) can be used for the synthesis of e.g. drug-like heterocycles[572,573] or cyanine dyes.[574] The combination of conventional SPPS amide coupling can be enhanced with orthogonal assembly options with other conjugation reactions than amide formation. Depending on the used building blocks, e.g. substitution reactions,[541,575] rearrangement reactions or “click reactions” such as CuAAC, TEC or inverse Diels-Alder reactions[576–578] can be applied to synthesize the sequence-defined scaffolds (see

Figure 17).[513,541,542] This combination of different chemistries can be used to obtain hybrid macromolecules which merge different molecular properties e.g. by implementing glycan motifs[205,579,580] or fluorescent dyes.[561,581] This building block-oriented synthetic toolbox inspired by peptide-related macromolecules is a research subject of the Hartmann group which used SPPS-derived systems to synthesize glycomimetic scaffolds based on commercial and specially designed building blocks. These building blocks can be commercially available amino acids, polymeric macromolecules or tailor-made compounds as exemplarily shown on BDS (**B**oc-functionalized **d**iethylenetriamine conjugated with **s**uccinic acid),[537] TDS (**t**riple bond-functionalized **d**iethylenetriamine building block conjugated with **s**uccinic acid),[250] SDS (di**s**ulfide-containing **d**iamine building block conjugated with **s**uccinic acid),[538] EDS ((**e**thylene**d**ioxy)bis(ethylamine) building block conjugated with **s**uccinic acid),[250] AZO (**azo**benzene building block),[538] Gtp (**g**lutaryl **t**etraethylene**p**entamine)[539] and AB_2-type branching element (see Figure 16A).[540] The monodisperse glycomimetics function as model ligands for interactions with carbohydrate-binding proteins such as concanavalin A (Con A),[250,582,583] mammalian signaling lectins,[135,187,584] bacterial lectins[81,217,585] or virus capsid proteins.[218,220,586,587] With the help of SPPS method several different approaches of sequence-defined glycomacromolecule synthesis were presented such as the block copolymerization of defined oligomeric glycomimetic structures,[582] the assembly of heteromultivalently glycosylated scaffolds[133,561,588] or the implementation of non-glycan motifs into macromolecules.[186,187,581,589–591] These examples state the straightforward and flexible use of SPPS, especially for its use on the flexible synthesis of customized and glycosylated target structures.

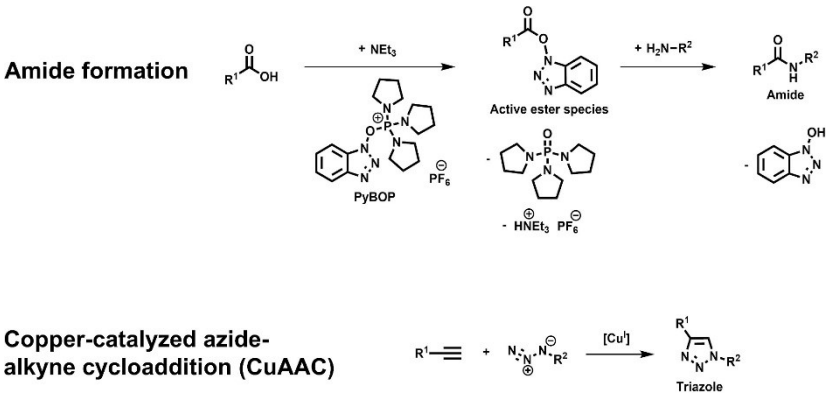


Figure 17: Amide coupling reaction[514] and CuAAC[339] as feasible conjugation reactions for SPPS. Figure content was modified and printed with permission of Elsevier.[514]

2. Motivation and outline

Glycosylated macromolecules and glycomimetics show interactions to carbohydrate-binding proteins and thus are treated as potential theraupeutics e.g. for applications against viral and bacterial diseases.[40] The synthesis of sequence-defined glycomacromolecules via solid phase polymer synthesis (SPPS) is an established strategy to obtain such artificial glycomimetics.[468–470] These synthetic scaffolds as well as the SPPS synthesis strategy possess characteristical advantages e.g. the tailor-made arrangement of molecular components, the precise spacing between molecular motifs and the control of total amount of ligands per molecule.[250,259,582]

Nevertheless, facing limitations and open challenges associated to SPPS become more important when synthesizing specific glycomacromolecules addressing glycan-binding proteins. On monodisperse glycomacromolecules, amongst others the conjugation method defines the maximum amount of feasible ligand valency. Additionally, the sensitivity for incomplete functionalization increases with increasing amount of ligands. This limitation is further amplified when using unusual or pH-sensitive ligand motifs, difficult sequences or new coupling strategies. Beside that it is recommended to maintain synthetic flexibility of the applied synthesic route to allow for easy modification or replacement of structural components or ligands e.g. in terms of reactivity or ligand specificity. A common practice on glycomacromolecule synthesis is the simplification of complex natural oligosaccharide ligand motifs by using their terminal mono- or disaccharide motif as usually done in the Hartmann group.[250,582] Especially when presenting these simplified ligands in a multivalent fashion, they often show sufficient overall binding affinities but can lack on protein binding specificity. Nevertheless, mono- and disaccharide motifs are still often the most feasible ligands due to their good availability and their established conjugation methods.[286,287] This limitation of targeting proteins specifically is accomplished e.g. when using simplified ligands for targeting sialic acid binding virus proteins.[56,73] Therefore, the synthesis of oligosaccharide-functionalized macromolecules requires more attention on the selection of ligands. Already established synthesis approaches described the prior functionalization of oligosaccharides and their subsequent attachment onto macromolecules which usually requires experienced handling on carbohydrate synthesis due to higher structural complexity compared to related mono- and disaccharides.

This thesis should be used to study and extend the yet available synthetic toolbox of SPPS. As a target, new methods should be employed to obtain sequence-defined glycomacromolecules and to conjugate more complex binding ligands than typically done e.g. HMOs or unusual neoglycoside motifs. Therefore, the previously established approaches of chemoenzymatic glycosylation reaction and carbohydrate conjugation via *N*-methyloxyamine chemistry should be exploited and adapted to be used with SPPS-derived macromolecules. Both approaches were investigated in past research work and were applied either on monovalent molecules[377,394,428,433] or on multivalent systems lacking on sequence definition.[392,393,440,441,443] Here, these approaches should be used to open up new synthetic pathways in terms of flexibility of carbohydrate ligand choice of glycomacromolecules. Based on the main focus of the research network „Virocarb“ especially oligosaccharide-binding virus proteins are subjects of research interest, which bind in particular to fucose or sialic acid motifs.

The first approach should be used to introduce sialic acid motifs onto suitable molecular acceptor motifs using a two-enzyme system with NmCSS/PmST1.[428,433,456] These enzymatic reactions with glycosyl

transferases should be studied for the regioselective introducing of terminal sialic acid motifs onto oligomeric backbones under mild conditions. In a first step synthetic lactose-functionalized macromolecules should be elongated to 3‘-sialyllactose-functionalized derivatives to obtain HMO-functionalized glycomacromolecules. Therefore, the NmCSS/PmST1 system should be tested for feasability for the synthesis of proposed glycomacromolecules. It should be examined if the synthetic macromolecule precursors fit into the catalytic domain of PmST1 and it should be observed if linker motifs between the oligomeric backbone and the ligand have an influence on macromolecule sialylation. Furthermore, this technique should be examined if it is applicable for the synthesis of monodisperse, multivalent macromolecules, e.g. if incomplete sialylation hinders feasible product synthesis. Finally, the general chemoenzymatic synthesis including its work up of the final compounds should be evaluated. Hence, a set of different synthetic glycomacromolecules should be used as precursors for the following enzymatic reaction with different valencies of one, two and three lactose ligands per molecule. Two different lactose derivatives with different linkers – a derivative azidated on the reductive end and another one lactoside with azidopropyl functionality – to the oligomeric backbone should be evaluated on behalf of this chemoenzymatic glycosylation. The NmCSS/PmST1 reaction should be performed on preparative scale and optimized to the given reaction requirements. The previously synthesized set of lactose-functionalized glycomacromolecules differing in spacing, valency and linker length should be sialylated using this chemoenzymatic method.

Derived from the findings of the chemoenzymatic approach, a second project emerged with the topic of NmCSS/PmST1 sialylation of unusual, non-glycan substrates based on Tris(hydroxymethyl)methyl structural motifs. This novel reaction should be adapted to synthetic purpose and it should be evaluated for ist potential for glycomacromolecule synthesis. First of all, a small selection of possible substrate derivatives should be synthesized and studied on behalf of PmST1 substrate tolerance. Hence, first enzymatic tests on synthetic substrates should give information about general feasability for non-glycan sialylation by PmST1. These chemically modified substances should be synthesized and were designed to carry functional handles to allow for subsequent conjugation via CuAAC which is compatible to TDS/EDS-based macromolecules as a commonly used tool in the Hartmann group.[250,582] To this point it is unclear if the further chemical modification of the molecular substrate motif has an influence on the acceptance by PmST1. For the isolation of the sialylated products and the separation from the likewise highly polar residual starting materials and reagents require a feasible work up procedure whereas HPLC methods are tested for purification. Ideally, the targeted sialylation products can be isolated, characterized and conjugated onto macromolecules. If possible, the obtained scaffolds should be tested for interactions with neuraminidases or sialic acid-binding proteins.

Although the chemoenzymatic glycosylation approach with PmST1 shows characteristic advantages, it exclusively accesses products with terminal sialic acid residues and does not allow for the flexible exchange of the carbohydrate ligand motif. Therefore, a different conjugation approach should be tested to extend ligand variety on sequence-defined macromolecules based on *N*-methyloxyamine chemistry. The *N*-methyloxyamine moiety was developed by Blixt group and has been used for the protection group-free conjugation of non-functionalized carbohydrates.[377] This method should be exploited on SPPS-based macromolecules for the individual introduction of simple carbohydrates as well as more complex human milk oligosaccharides (HMO). Furthermore, it should be evaluated whether this method

can be used for the synthesis of multivalently functionalized macromolecules and whenever conversion rates allow for the full glycosylation of the introduced reactive *N*-methyloxyamine linkers. Method applicability should be studied in detail especially in terms of glycomacromolecule handling and stability during synthesis. Hence, the overall potential shoud be evaluated regarding glycomacromolecule synthesis. From synthetic strategy perspective it should be tested to attach *N*-methyloxyamine moieties onto SPPS-derived macromolecules for the later in solution carbohydrate conjugation. The strategy of such reactive precursors can be used to circumvent potential instability issues of the carbohydrate-*N*-methyloxyamine bond. During the development of *N*-methyloxyamine linker adapted to TDS/EDS-based macromolecules it should be examined to derivatize these linker species and allow their transfer to other systems and scaffolds such as small molecules and polymers.

3. Results and discussion

3.1 Enzymatic sialylation of synthetic multivalent scaffolds: from 3'-sialyllactose glycomacromolecules to novel neoglycosides

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[1] Authors contributed equally

Own contributions:
Synthesis of functional building blocks and lactose-functionalized glycomacromolecules, collaborative development of glycomacromolecule sialylation protocols and their subsequent purifications, supervised protein expressions and activity measurements, recording of 1D NOESY NMR spectra for sialylation reaction monitoring, collaborative enzymatic experiment planning and interpretation, development of tool for quantitative sialylation conversion determination, data evaluation, HPLC purification of sialylated glycomacromolecules, detection and isolation of Tris-sialoside, collaborative experiments on evidence for Tris-sialoside, development of buffer sialylation hypothesis and selection of tested Tris-analogues, development of MS-based sialylation analysis method, analytical characterization of compounds using HPLC, ESI-MS, HR-ESI-MS, ^{1}H and ^{13}C NMR, collaborative writing of manuscript and supplement.

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3.2 Enzymatic sialylation of functionalized non-glycan acceptors using Pasteurella multocida sialyltransferase PmST1

Own contributions:
Collaborative concept development, development of MS-based analysis of enzymatic sialylation, further exploitation of enzymatic non-glycan sialylation, inspection of bis-tris-propane as commercially available sialylation substrate with divalent acceptor motif, design and synthesis of functionalized Tris derivatives, first studies on enzymatic sialylation artificial Tris derivatives, reaction optimization of sialylation reaction using hydrophobic Tris derivatives, first attempts on neosialoside isolation, non-glycan substrate tolerance screening for bacterial and human sialyltransferases, analytical characterization using HPLC, ESI-MS, HR-ESI-MS, ^{1}H and ^{13}C NMR, collaborative writing of manuscript first draft and supplement.

Abstract

The synthesis of glycoconjugates is of great interest for understanding the interactions with protein targets and the development of novel drugs. Recent proceedings in the enzymatic sialylation using PmST1 from *Pasteurella multocida* showed that besides galactose-bearing substrates also aglycon motifs can be sialylated as well which was exemplarily shown with the sialylation of Tris buffer. Arranged on the previous findings the method of non-glycan sialylation reaction was studied by using Tris-derived structures which were priorly functionalized to keep the possibility for later conjugation reactions to obtain e.g. macromolecules or fluorescent conjugates. Although the existence of this novel class of sialylated products from non-glycan precursors was shown on analytical scale, there are still open challenges of purifying the highly polar products and testing them on their binding behavior and stabilities towards neuraminidases.

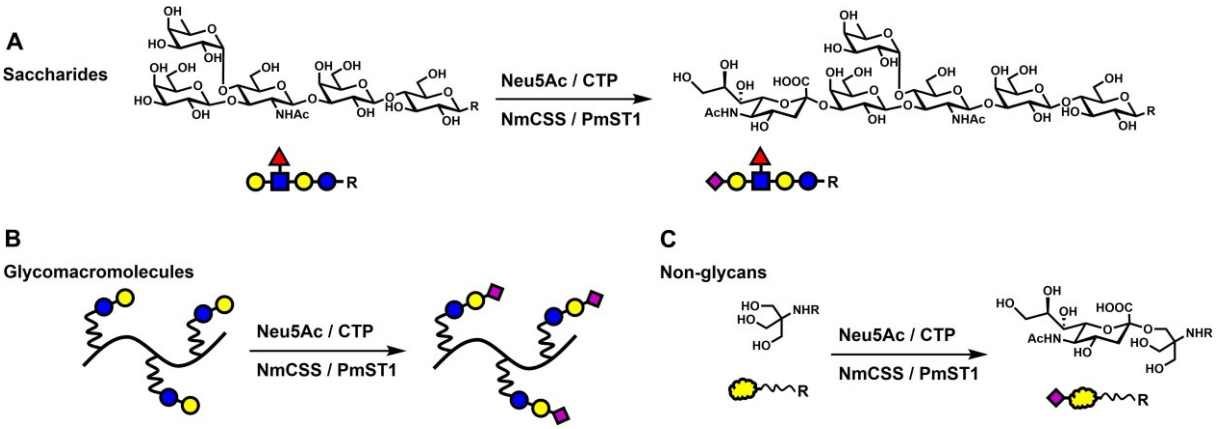
Introduction

In recent studies, we have shown the use of the enzymatic NmCSS/PmST1 system to introduce sialic acid residues on sequence-defined glycomacromolecules and to be applicable for this chemoenzymatic synthetic approach.[592] Although this chemoenzymatic approach using NmCSS/PmST1 was previously used for the sialylation of synthetic oligosaccharides (see Scheme 2B), we surprisingly found sialylation of the utilized Tris buffer as a non-glycan motif (see Scheme 2C). This formation of an artificial Tris-sialoside lead to severe degradation of the CMP-Neu5Ac donor species and thus hinders glycol-macromolecule formation. Although the desired glycomacromolecule sialylation were completed in accordance to published protocols by the group of Chen et al.,[461,462] we immediately intrigued by the opportunities this offers from synthetic perspective. Whereas enzymatic sialylations in previous publications were performed on glycan substrates (see Scheme 2A),[428,431,593] in this study we further explored the sialylation of non-glycan substrates. Therefore, we used compounds showing structural similarities to previously sialylated tris(hydroxymethyl)aminomethane. Hence, both readily available yet uncommon substrates were used for enzymatic sialylation as well as synthetically modified Tris derivatives which mimic the Tris motif and adding functional handles such as azide or alkyne moieties (see Scheme 2C).

Therefore, a two-enzyme one-pot system[427,433,456,592,594,595] was used consisting of the enzymes *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS)[457,458,596] and *Pasteurella multocida* sialyl-transferase (PmST1).[455,456,594] This system was described in detail by Chen and coworkers and this principle was often used for the sialylation of galactoside- or *N*-acetylgalactosamine-terminated carbohydrates such as functionalized lactose[456,594,597] or branched complex oligosaccharides.[454,598–600] In contrast to enzymatic sialylations, the Neu5Ac conjugation using organosynthetic methods do not show high stereospecificities due to missing neighboring effects on C3 leading to α/β-anomeric mixtures.[601–604] These unselective chemical sialylations lead to overall carbohydrate impurities due to the transferred Neu5Ac motif.[402,605,606] Whereas sialyltransferase enzymes such as the PmST1 catalyze the stereoselective reaction of alpha-sialosides, the challenges of conventional conjugation techniques could be circumvented.[461] PmST1 requires a suitable acceptor (galactoside, e.g. lactose) and a donor

substrate (CMP-sialic acid, e.g. CMP-Neu5Ac) which are conjugated to each other during inversion of the β-Neu5Ac donor motif.[427,461]

The required CMP-Neu5Ac species was formed by NmCSS from the substrates CTP and Neu5Ac under presence of Mg^{2+} ions.[459] The combined use of NmCSS and PmST1 offer a wide flexibility for synthetic purpose such as the introduction of non-natural sialic acid derivatives to label[432,597,607,608] or to modify synthetic carbohydrates.[183,609,610] Characteristically for the PmST1 WT is the tendency to act as a multifunctional enzyme.[402,611,612] Besides α2,3-sialylation activity, it was described to show significant desialylation tendencies which were adjusted by application of protein engineering.[462,463,613,614] Punctual mutations lead to several examples of PmST1 mutants being applicable for synthetic purpose such as M144D mutant[461,462] with reduced desialylation activity but also a lower α2,3-sialylation reaction rates or P34H/M144L mutant[463–465] with a changed regioselectivity from α2,3 to α2,6-sialylation.



Scheme 2: Chemoenzymatic sialylation using NmCSS/PmST1, **A:** sialylation of synthetic oligosaccharides,[610] **B:** sialylation of sequence-defined glycomacromolecules and Tris buffer,[592] **C:** method transfer towards sialylation of artificial Tris derivatives.

Results and discussion

Enzymatic sialylation of Good's buffers

In previous study[592] the context of chemical shifts in ^{1}H NMR spectra was discussed on the behalf of enzymatic buffer sialylation Tris, tricine and TES (see Figure 18). As a result it was found that Tris as well as tricine, TES an other commercial derivatives can be enzymatically sialylated and it is assumed that the sialylation of a single hydroxyl group can principally be transferred to similar substrates by using the same reaction pathway. To expand the analytical scope of PmST1 sialylation in a fast, qualitative and reproducible fashion, the method of mass spectrometry was used for further characterization of sialylated reagents instead of NMR technique. Therefore, the enzymatic reactions were performed in a slightly reduced setup since deuterated solvents, buffers and internal NMR standards become no longer necessary. For this MS-monitored reaction 200 mM buffer and 40 mM $MgCl_2$, pH 8.8, 16.5 mM Neu5Ac, 33.0 mM CTP, 1000 µg/ml NmCSS and 40 µg/ml PmST1 P34H/M144L were used and allowed for 20 h reaction at 37 °C in a total volume of 50 µl. Prior to PmST1 addition, the NmCSS/reagent mixture was allowed to react for 1 h at 37 °C. Direct ESI measurements confirm the previous findings of buffer sialylation from NMR data (see Figure 18). In MS data, masses of both non-sialylated starting material and sialylated product were found. Hence, it cannot be differentiated whether an incomplete conversion

occurred or gas phase fragmentation effects led to the degradation to their substrate forms. Nevertheless, applied MS analysis did not give significant information about α/β-anomeric configuration of Neu5Ac and strictly allows qualitative evidence. The formation of the sialylated product can be proven by the presence of their corresponding $[M+H]^+$ or $[M+Na]^+$ m/z signals, whereas M equal the sialylated products. Calculated and found m/z signals as well as their relative intensities can be found in Table 1. Regarding signal intensities, Tris sialylation showed the highest intensities which can indicate that it is a better acceptor than tricine or TES. Based on results from previous publication[592] and based on the strict formation of CMP-β-Neu5Ac by NmCSS,[427] it can be assumed that the formed sialosides show α-anomeric configuration. Using this MS technique, it was verified that PmST1 WT and its mutants M144D and P34H/P144L show sialylation activity on non-glycan acceptors Tris, tricine and TES (see Figure 19 B, C and D). When bicine is being used as a starting material it was shown to be not sialylated by PmST1 and no considerable CMP-Neu5Ac donor degradation behavior was observed. Positive control reactions were performed with azido-lactose ($LacN_3$) as a good acceptor for sialylation reaction. In general, we found to have a better sensitivity of MS experiments towards previous NMR experiments (see Table 1).

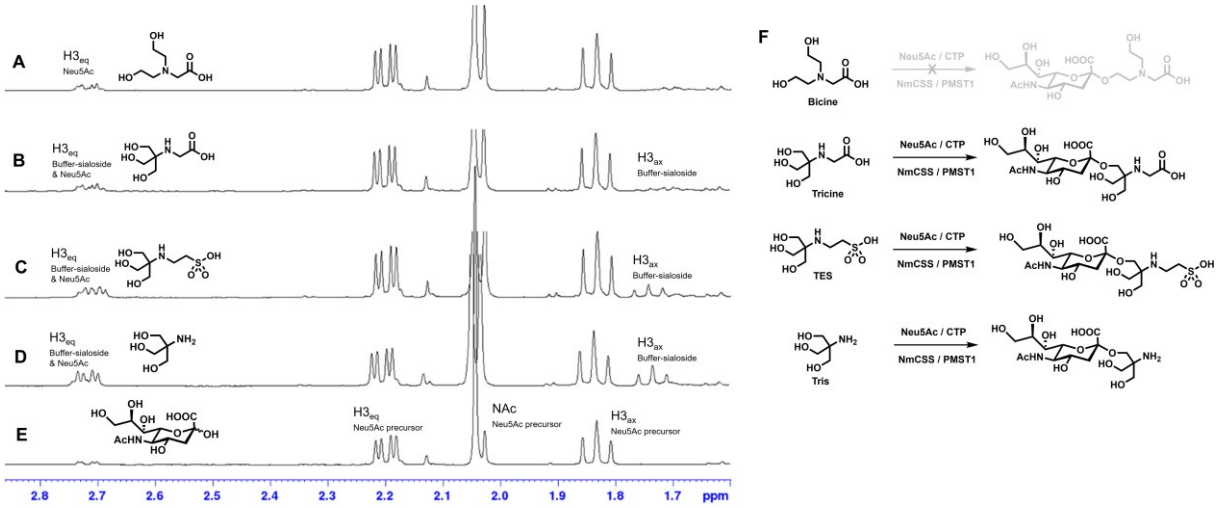


Figure 18: ^1H NMR spectra of enzymatic sialylation with Good's buffers using NmCSS/PmST1 WT (**A**-**D**), **A:** 100 mM bicine (negative control), **B:** 100 mM tricine, **C:** 100 mM TES, **D:** 100 mM Tris (positive control), **E:** 16.5 mM Neu5Ac (reference), **F:** enzymatic buffer sialylation using NmCSS/PmST1 method.[592]

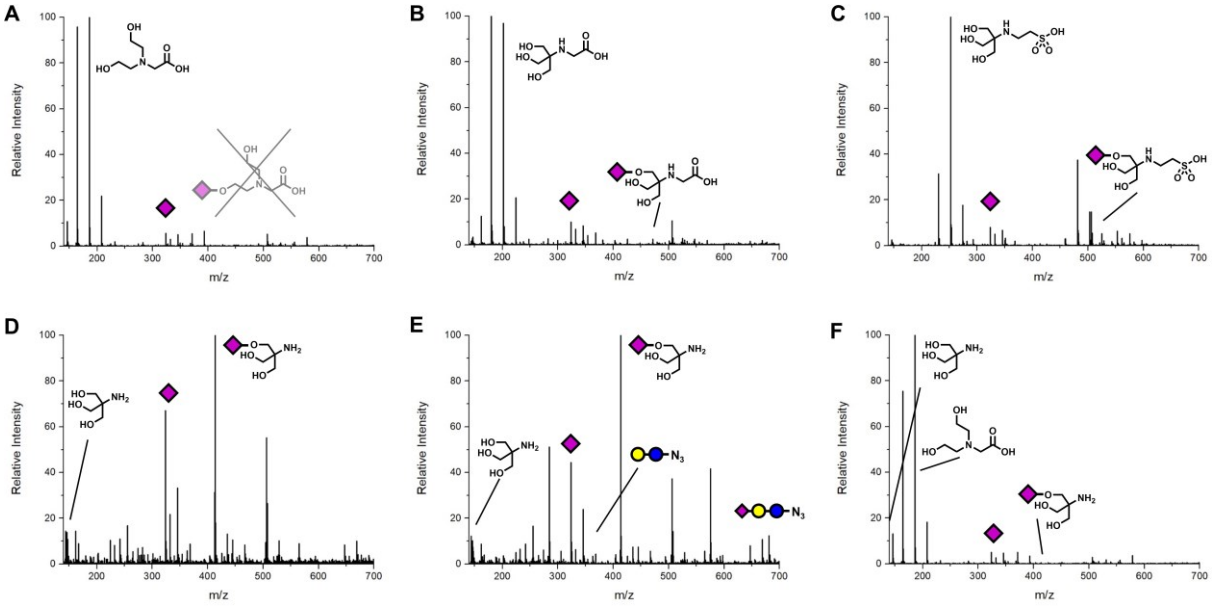


Figure 19: Mass spectra of enzymatic sialylation reactions using PmST1 P34H/M144L, **A:** 200 mM bicine (negative control), **B:** 200 mM tricine, **C:** 200 mM TES, **D:** 200 mM Tris, **E:** 11 mM $LacN_3$ in 200 mM Tris buffer (positive control), **F:** 11 mM Tris in 200 mM bicine buffer.

Table 1: Relative intensities of m/z signals of sialylated buffers using MS method after reaction with NmCSS/PmST1 P34H/M144L.

	m/z $[M+Sia+H]^+$			m/z $[M+Sia+Na]^+$		
	Found	Calculated	Relative intensities	Found	Calculated	Relative intensities
200 mM Bicine	455.2	455.2	0.45[a)]	477.3	477.2	0.13[a)]
200 mM Tricine	471.2	471.2	2.58[b)]	493.2	493.2	0.65[b)]
200 mM TES	521.2	521.2	1.15[c)]	543.2	543.2	1.82[c)]
200 mM Tris	413.2	413.2	100[d)]	435.2	435.2	12.72[d)]
11 mM $LacN_3$ in Tris	659.1	659.2	0.13[d)]	681.2	681.2	12.42[d)]
11 mM Tris in bicine	413.2	413.2	0.45[a)]	435.2	435.2	0.09[a)]

[a)] Normalized on m/z signals of bicine (146.2, 164.0 or 186.0 m/z), [b)] normalized on m/z signal of tricine (180.0 m/z), [c)] normalized on m/z signal of TES (252.0 m/z), [d)] normalized on m/z signal of sialylated Tris (413.2 m/z).

Transfer of reaction conditions using non-reactive bicine buffer for enzymatic sialylation

Hence, PmST1 WT did not use bicine as a substrate acceptor, it was considered in following experiments to use bicine buffer for adjusting pH 8.8 to guarantee enzyme stability and activity. The presence of non-reactive buffers is mandatory for the enzymatic sialylation reaction of glycan substrates which do not possess buffering properties itself, e.g. when sialylating lactosides.[456,594,597] This is particularly important when using sialyltransferases for the sialylation of non-glycan substrates.

Therefore, it was tried to perform MS experiments to show compatibility to changes of buffer concentrations from higher (200 mM) to lower concentrations (11 mM substrate, compared to 16.5 mM Neu5Ac and 33 mM CTP). In the last case non-reactive buffers such as bicine were used to allow for enzymatic activity whilst the *in situ* formed CMP-Neu5Ac donor was not degraded by bicine in the

presence of PmST1. The experiments showed that Tris, tricine and TES can be sialylated in presence of 200 mM bicine buffer (see Figure 19 F). The principle of substrate sialylation in the presence of an unreactive bicine buffer was tested on the divalent bis-tris-propane buffer which is a commercially available compound showing two tris(hydroxymethyl)methyl motifs. Although it can be assumed to divalently sialylate bis-tris-propane, it was only sialylated once due to insufficient conversions (see Figure 20).

Bicine shows strong signals on MS suppressing m/z signal intensities of the sialylated products (see Table 2). This signal imbalance between product and bicine buffer can be explained with different magnitudes of equivalents of substrate towards bicine buffer. The presented MS spectra as well as data shown in Table 2 highlight that the quantitative interpretation of this sialylation reaction is challenging when using MS method as performed here. Nevertheless, this principle can be used to qualitatively look for the presence of signals related to the sialylated products as further employed in this studies.

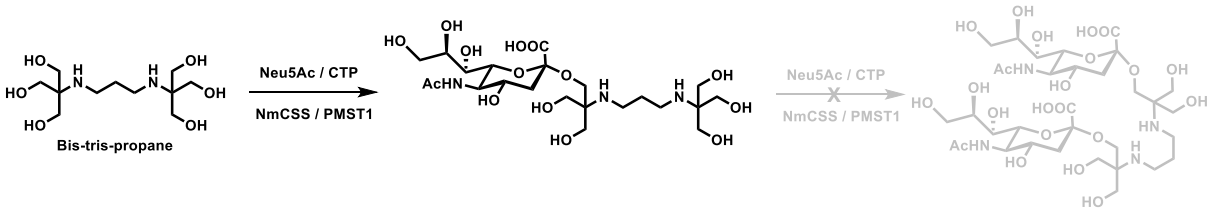


Figure 20: Enzymatic sialylation of bis-tris-propane using PmST1 P34H/M144L.

Table 2: Relative intensities of m/z signals of sialylated buffers using MS method after reaction with NmCSS/PmST1 P34H/M144L.

	m/z $[M+Sia+H]^+$			m/z $[M+Sia+Na]^+$		
	Found	Calculated	Relative intensities[a)]	Found	Calculated	Relative intensities[a)]
11 mM Tris in bicine	413.2	413.2	0.45	435.0	435.2	0.13
11 mM TES in bicine	520.8	521.2	0.21	n.d.	543.2	n.d.
11 mM Tricine in bicine	471.2	471.2	0.21	493.0	493.2	0.19
11 mM $LacN_3$ in bicine	659.1	659.2	0.11	681.1	681.2	1.18
5.5 mM bis-tris-propane in bicine	574.3	574.3[b)]	0.27	596.4	596.3[b)]	0.03

[a)] Normalized on m/z signals of bicine (146.2, 164.0 or 186.0 m/z), [b)] monosialylated, n.d. – not determined.

Enzymatic sialylation of functionalized Tris derivatives

For following experiments, it was observed to use this principle for structurally similar compounds with functional handles such as azide or alkyne groups. Prior to testing on enzymatic sialylation behavior, some exemplary substrates were synthesized containing the characteristical tris(hydroxymethyl)methyl motif to get a small selection of potential sialylation substrates for PmST1. The synthetic design was chosen to enable easy conjugation using CuAAC click chemistry for e.g. glycomacromolecule or glycol-conjugate synthesis as similarly done in previous studies.[81,187,469] Hence, three options were presented to access substrates which functional handles.

On a first attempt, Tris was functionalized on its amine functionality according to reported protocols.[615,616] Therefore, Tris was converted into a chloroacetamide intermediate **1** by using ethyl chloroacetate in methanol and subsequent fractionated crystallization. The following azidation reaction using sodium azide in dimethylformamide (DMF) gave the azidoacetamide-functionalized Tris derivative **2** (see Figure 21A).[615,616] The second approach was using 2-(bromomethyl)-2-(hydroxymethyl)propane-1,3-diol as a commercially available precursor which was azidated to product **3** using sodium azide in DMF (see Figure 21A). Compared to the azidated product **2**, this bromide group is sterically hindered which required a higher reaction time and higher temperatures to bring the reaction to completion as reported by Mead et al.[617] Azido compound **3** possess an additional methylene group between tris(hydroxymethyl)methyl residue and the azide which results in different electronical effects compared to Tris-related structures. The third Tris derivative was obtained by the functionalization of Tris buffer with propargyl bromide in tetrahydrofuran (THF). The substitution with the amine led to a mixture of secondary and tertiary amine derivative **4** (see Figure 21A). Although the resulting mono- and difunctionalized alkynyl derivative **4** is an unselectively formed mixture of two compounds with an approximate ratio of 70 % monofunctionalized and 30 % dialkynylated Tris derivative, it was further used for first substrate screenings. First attempts to separate this mixture on C_{18} columns (RP-HPLC) or silica gel chromatography failed due to high polarities of the compounds.
All of the three attempts gave highly polar compounds which did not show sufficient interactions with the stationary phase of C_{18} columns and thus were not able to analyze efficiently via RP-HPLC. HLPC analysis of the artificial Tris derivatives lead to signals which appeared in respectively close to the injection peak using water/acetonitrile gradients. For the following enzymatic testing purpose, it is beneficial that the substances **2**-**4** show differences in electronical and sterical properties.

For testing the substrate tolerance width of PmST1 towards the synthesized Tris derivatives **2**-**4**, the substrates were tested separately in a similar manner to the described double-buffer experiments (see Figure 19). Therefore, 11.0 mM functionalized substrate **2**-**4**, 33.0 mM CTP, 16.5 mM Neu5Ac, 200 mM bicine, 40 mM $MgCl_2$ pH 8.8 and 1000 µg/ml NmCSS were mixed and allowed to react for 1 h at 37 °C and then 40 µg/ml PmST1 (WT, M144D or P34H/M144L) were added and reacted for 20 h at 37 °C. Reactions were performed on analytical scale in a minimal total volume of 30 µl by using HPLC vials with micro inlets. After 20 h, a sample was removed for mass spectrometrical analysis to obtain qualitative information if the mass of sialylated product occurred. The sialylation products of **2**-**4** were detected but no precise evidence to the conversion quantity was made due to the qualitative nature of the test method (see Table 3 and Figure 21B). Hence, a sufficient statement towards the reactivity differences of non-glycan substrates cannot be made when using this MS method.

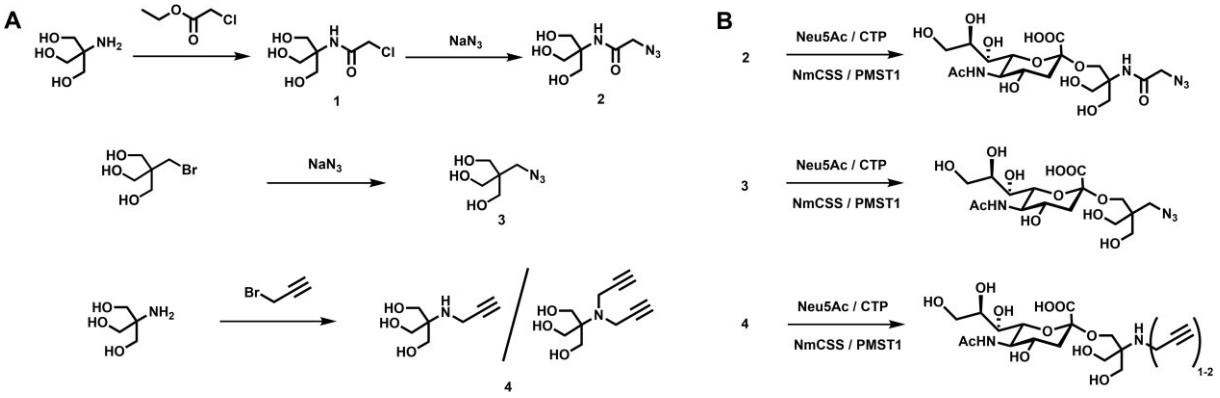


Figure 21: Synthesis of functionalized Tris derivatives, **A:** azidoacetamide-functionalized Tris **2**, 2-(azidomethyl)-2-(hydroxymethyl)propane-1,3-diol **3** and mono- and dialkynylated Tris **4**, **B:** enzymatic conversion of **2**-**4** using NmCSS/PmST1 method.

Table 3: Relative Intensities of m/z signals of sialylated synthetic Tris derivatives **2**-**4** using MS method after reaction with NmCSS/PmST1 P34H/M144L.

	m/z $[M+Sia+H]^+$			m/z $[M+Sia+Na]^+$		
	Found	Calculated	Relative intensities[a)]	Found	Calculated	Relative intensities[a)]
11 mM **2** in bicine	496.5	496.2	0.11	518.2	518.2	1.07
11 mM **3** in bicine	453.3	453.2	0.11	475.2	475.2	0.62
11 mM **4** in bicine						
monoalkynylated	451.0	451.2	0.79	473.0	473.2	0.14
dialkynylated	489.0	489.2	0.93	511.0	511.2	0.74

[a)] Normalized on m/z signals of bicine (146.2, 164.0 or 186.0 m/z).

Development of Tris derivatives with hydrophobic handle for quantitative analysis

To allow for reactivity comparison of the PmST1 mutants used in this study (WT, M144D and P34H/M144L) and to perform reaction optimization, quantitative data of enzymatic sialylation reaction had to be obtained. Since high polarity of substrates, products and byproducts hindered analytics respective compound separation, the synthetic Tris derivated were further structurally modified to gain more hydrophobic derivatives. Therefore, a next generation of chemically modified Tris derivative substrates was introduced by implementing hydrophobic motifs e.g. hydrocarbon chains or aromatic moieties via *N*-functionalization analogue to the synthesis of **1** and **2**. Due to increasing solubility differences of involved reagents (hydrophilic Tris vs. non-polar, long-chained ethyl esters), here acyl chlorides were used for amide formation.

First, the C_6 structural motif was obtained by *N*-amide coupling of 6-bromohexanoylchloride with Tris to access **5**. Due to the halogenide exchange justified by simultaneous presence of chloride and bromide functionalities in the starting material, the obtained product **5** appeared as a mixture of chlorinated and brominated species. Since the presence of this halogenide mixture did not disrupt with following azidation reactions, **5** was used to obtain Tris derivative **6** with an azidated C_6 linker (see Figure 22A). A similar synthesis strategy was applied for accessing aromatic derivative **7** by using 4-(chloromethyl)-benzoylchloride for amide formation and the following azide substitution gave product **8**. Both azidated,

less hydrophilic Tris derivatives **6** and **8** were found to show separation on analytical RP-HPLC using acetonitrile/water gradients with the addition of 0.1 % formic acid. Due to their different UV absorption properties at λ = 214 nm, the aromatic amide **8** showed better UV traceability than aliphatic amide **6**. Hence, aromatic Tris derivative **8** allowed for good substance purity quantification and was further used as a model compound for conversion quantification of enzymatic sialylation.

Prior to the determination of sialylation quantification, the Tris derivatives **6** and **8** were tested on substrate acceptance for PmST1 using the ESI-MS assays as previously performed on substrates **2-4**. The presence of the m/z signals of sialylated products gave evidence that sialylation took place when using further modified Tris derivatives **6** and **8**. Separation on analytical RP-HPLC showed that with both substrates sialylated product and non-sialylated starting material different retention times occur (see Figure 22B). In the case of the aliphatic derivative **6** both the sialylated and the non-sialylated species was separated on RP-HPLC but and their compounds were identified using MS detector but did not show sufficient UV absorption (see Table 4). Due to a poor signal-to-noise ratio in the UV chromatogram, no sufficient sialylation quantification was able. Whereas aromatic derivative **8** showed better UV absorption than **6**, sialylation conversions of approx. 5 % were found (see Table 4). Due to the good separation properties of **8** and its sialylated product, this compound was used for further reaction optimization and the comparison of different PmST1 mutants.

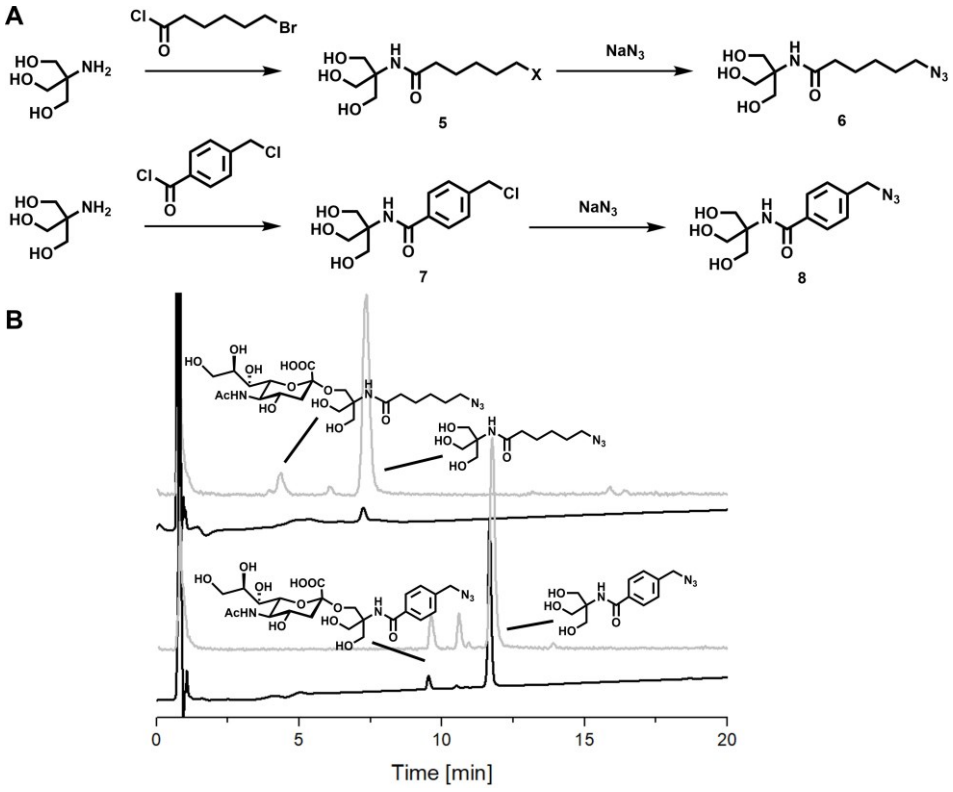


Figure 22: Synthesis of less polar Tris derivatives, **A:** azidohexanoylamide-functionalized Tris **6** and aromatic azido-functionalized Tris **8**, **B:** HPLC chromatograms showing sialylations of **6** and **8** by using NmCSS/PmST1 method.

Table 4: Relative Intensities of m/z signals of sialylated synthetic Tris derivatives **6** and **8** using MS method after reaction with NmCSS/PmST1 P34H/M144L.

	m/z $[M+Sia+H]^+$			m/z $[M+Sia+Na]^+$			
	Found	Calculated	Relative intensities[a)]	Found	Calculated	Relative intensities[a)]	Conversion [b)]
11 mM **6** in bicine	552.4	552.3	0.05	574.2	574.2	1.14	-
11 mM **8** in bicine	572.2	572.2	0.33	594.2	594.2	1.00	5.4 %

[a)] Normalized on m/z signals of bicine (146.2, 164.0 or 186.0 m/z), [b)] gradient of 5-50 % acetonitrile in water (+0.1 % formic acid) in 30 min.

To certain extend, this method was used to categorize the influence of reaction handling, e.g. with the choice of sialyltransferase mutant, substrate equivalents or enzyme concentrations. Therefore, the effect of reagent concentrations and the amount of buffer and enzymes was studied to find optimal reaction conditions as shown in the following experiments. The kinetic behavior of several PmST1 mutants[462,463] was already described in literature and based on kinetic data it was assumed that M144D mutant also shows a lower activity in this non-glycan sialylation than WT and P34H/M144L. Actually, these activity differences were confirmed on the sialylation of aromatic Tris derivative **8** as shown in Table 5 whereas P34H/M144L showed best sialylation conversions (7.6 %) under applied conditions. The other PmST1 WT (5.0 %) and M144D (1.2 %) showed significantly lower sialylation rates. Hence, the double mutant P34H/M144L was the preferred choice for further enzymatic sialylation reactions as used here in this study.

The influence of enzyme concentration on the sialylation reaction on aromatic Tris derivative **8** was investigated to use optimal conditions. The concentration of NmCSS was found to have no significant influence when being used in the concentration range of 100-1000 µg/ml within the reaction solution (see Table 6). A different behavior were observed when changing the PmSt1 P34H/M144L concentration. The more PmST1 was used for sialylation reaction the higher the conversions in a non-linear correlation (**40 µg/ml:** 6.9 %, **100 µg/ml:** 7.3 %, **300 µg/ml:** 7.8 %, average values of measurements with different NmCSS concentrations, see Table 6). Although high PmST1 enzyme concentrations lead to higher conversions, within multicomponent reactions high enzyme amounts respective volumes are often inconvenient due to the limited addition of stock solutions to the reaction solution. High stock concentrations might lead to substance solubility limitations or a decreased enzyme stability when using highly concentrated enzyme stock solutions.

Table 5: Sialylation conversions of aromatic Tris derivative **8** was used for activity comparison of PmST1 mutants.

	Bicine pH 8.8 $MgCl_2$	**8**	Neu5Ac	CTP	NmCSS	PmST1	Conversion[a)]
PmST1 WT	200 mM 40 mM	11 mM	16.5 mM	33 mM	100 µg/ml	40 µg/ml	5.0 %
PmST1 M144D	200 mM 40 mM	11 mM	16.5 mM	33 mM	100 µg/ml	40 µg/ml	1.2 %
PmST1 P34H/M144L	200 mM 40 mM	11 mM	16.5 mM	33 mM	100 µg/ml	40 µg/ml	7.6 %

[a)] Gradient of 5-95 % acetonitrile in water (+0.1 % formic acid) in 15 min then isocratic for 2 min.

Table 6: Enzymatic sialylation of aromatic Tris derivative **8** was used for studying the effect of enzyme concentrations.

	Bicine pH 8.8 $MgCl_2$	**8**	Neu5Ac	CTP	NmCSS	PmST1 P34H/M144L	Conversion[a)]
NmCSS **+** PmST1 **+**	200 mM 40 mM	11 mM	16.5 mM	33 mM	**100 µg/ml**	**40 µg/ml**	7.6 %
NmCSS **++** PmST1 **+**	200 mM 40 mM	11 mM	16.5 mM	33 mM	**200 µg/ml**	**40 µg/ml**	6.5 %
NmCSS **+++** PmST1 **+**	200 mM 40 mM	11 mM	16.5 mM	33 mM	**1000 µg/ml**	**40 µg/ml**	6.5 %
NmCSS **+** PmST1 **++**	200 mM 40 mM	11 mM	16.5 mM	33 mM	**100 µg/ml**	**100 µg/ml**	7.3 %
NmCSS **++** PmST1 **++**	200 mM 40 mM	11 mM	16.5 mM	33 mM	**200 µg/ml**	**100 µg/ml**	7.1 %
NmCSS **+++** PmST1 **++**	200 mM 40 mM	11 mM	16.5 mM	33 mM	**1000 µg/ml**	**100 µg/ml**	7.4 %
NmCSS **+** PmST1 **+++**	200 mM 40 mM	11 mM	16.5 mM	33 mM	**100 µg/ml**	**300 µg/ml**	7.8 %
NmCSS **++** PmST1 **+++**	200 mM 40 mM	11 mM	16.5 mM	33 mM	**200 µg/ml**	**300 µg/ml**	7.2 %
NmCSS **+++** PmST1 **+++**	200 mM 40 mM	11 mM	16.5 mM	33 mM	**1000 µg/ml**	**300 µg/ml**	8.5 %

[a)] Gradient of 5-95 % acetonitrile in water (+0.1 % formic acid) in 15 min then isocratic for 2 min.

Beside the enzyme selection and concentration, the impact of bicine buffer on enzymatic sialylation of **8** was observed. Different buffer and Mg^{2+} concentrations from 150 to 300 mM bicine respective 30 to 60 mM Mg^{2+} were tested (see Table 7). The experiments gave the result that it is beneficial to use at least 200 mM bicine buffer and 40 mM Mg^{2+} for sialylation reaction. Higher buffer or Mg^{2+} concentrations did not lead to significantly higher conversions. When using 250 mM bicine stock solution slight pH changes can lead to a decrease of sialylation conversion when using pH 8.7 instead of pH 8.8 or pH 9.1.

The information of previous optimization experiments which was obtained so far was used for the investigation of the effect of reagent amounts Neu5Ac and CTP. It was found that it is important to use an excess of CTP towards Neu5Ac led to better sialylation conversions of **8** (see Table 8). The ratio of 4.5 CTP and 1.5 eq. Neu5Ac towards Tris derivative **8** showed best results with conversions of 11.6 % sialylated species. The importance of using excess CTP can be illustrated by comparing reactions of 2.4 eq. CTP / 1.2 eq. Neu5Ac with 2.4 eq. CTP / 2.1 eq. Neu5Ac showing both conversions of about 6 %. In this case the amount of CTP strongly influences the conversion rate whilst the exact Neu5Ac concentration does not matter as long >1.0 eq. are used based on Tris substrate. Due to the importance of the used CTP amount, the effect of CTP post-dosing was evaluated. One hour after PmST1 addition, another 8.2 mM CTP were added to the reaction mixture and were compared to the non-supplemented reactions. Post-dosing of CTP can significantly increase conversions as shown in Table 8 but shows similar effect than using higher CTP equivalents right in the beginning.

Although PmST1 was added to the reaction mixture after NmCSS preconditioning of 1 h at 37 °C, experiments with initial PmST1 addition did not show significant differences (preconditioned: 7.9 %, initial: 8.1 %, see Table 8).

Table 7: Effect of buffer concentration and pH on the enzymatic sialylation of aromatic Tris derivative **8**.

Buffer	Bicine pH 8.8 $MgCl_2$	**8**	Neu5Ac	CTP	NmCSS	PmST1 P34H/M144L	Conversion[a)]
0.75x	**150 mM 30 mM**	11 mM	16.5 mM	33 mM	400 µg/ml	200 µg/ml	6.6 %
1.0x	**200 mM 40 mM**	11 mM	16.5 mM	33 mM	400 µg/ml	200 µg/ml	8.1 %
1.25x, pH 8.7[b)]	**250 mM 50 mM**	11 mM	16.5 mM	33 mM	400 µg/ml	200 µg/ml	7.1 %
1.25x, pH 8.8	**250 mM 50 mM**	11 mM	16.5 mM	33 mM	400 µg/ml	200 µg/ml	8.4 %
1.25x, pH 9.1[c)]	**250 mM 50 mM**	11 mM	16.5 mM	33 mM	400 µg/ml	200 µg/ml	8.3 %
1.5x	**300 mM 60 mM**	11 mM	16.5 mM	33 mM	400 µg/ml	200 µg/ml	8.3 %

[a)] Gradient of 5-95 % acetonitrile in water (+0.1 % formic acid) in 15 min then isocratic for 2 min, [b)] stock solution of 1 M bicine and 200 mM $MgCl_2$ pH 8.7 was used, [c)] stock solution of 1 M bicine and 200 mM $MgCl_2$ pH 9.1 was used.

Table 8: Effect of Neu5Ac and CTP buffer concentrations on the enzymatic sialylation of aromatic Tris derivative **8**.

Neu5Ac/CTP eq.	Bicine pH 8.8 $MgCl_2$	**8**	Neu5Ac	CTP	NmCSS	PmST1 P34H/M144L	Conversion[a)]
1.2 / 2.4 eq.	250 mM 50 mM	11 mM	**13.2 mM**	**26.4 mM**	400 µg/ml	200 µg/ml	5.9 %
1.8 / 2.7 eq.	250 mM 50 mM	11 mM	**19.8 mM**	**29.7 mM**	400 µg/ml	200 µg/ml	7.4 %
2.1 / 2.4 eq.	250 mM 50 mM	11 mM	**23.1 mM**	**26.4 mM**	400 µg/ml	200 µg/ml	6.0 %
1.5 / 3.0 eq.	250 mM 50 mM	11 mM	**16.5 mM**	**33 mM**	400 µg/ml	200 µg/ml	7.9 % (9.7 %[b)])
1.5 / 3.0 eq. initial	250 mM 50 mM	11 mM	**16.5 mM**	**33 mM**	400 µg/ml	200 µg/ml[c)]	8.1 % (9.6 %[b)])
1.5 / 3.0 eq. less **8**	250 mM 50 mM	**6.6 mM**	**16.5 mM**	**33 mM**	400 µg/ml	200 µg/ml	8.2 % (9.5 %[b)])
1.8 / 3.6 eq.	250 mM 50 mM	11 mM	**19.8 mM**	**39.6 mM**	400 µg/ml	200 µg/ml	9.8 % (10.8 %[b)])
1.5 / 4.5 eq.	250 mM 50 mM	11 mM	**16.5 mM**	**49.5 mM**	400 µg/ml	200 µg/ml	11.6 % (12.1 %[b)])
2.1 / 4.5 eq.	250 mM 50 mM	11 mM	**23.1 mM**	**49.5 mM**	400 µg/ml	200 µg/ml	11.1 % (12.1 %[b)])

[a)] Gradient of 5-95 % acetonitrile in water (+0.1 % formic acid) in 15 min then isocratic for 2 min, [b)] Conversion of Tris derivative **8** after additional dosage of 8.2 mM CTP 1h after PmST1 addition, [c)] initial addition of PmST1 P34H/M144L directly after NmCSS dosage.

First purification attempts of neoglycan products

After roughly optimizing sialylation reaction with PmST1 P34H/M144L on the example of aromatic Tris derivative **8**, the reaction conditions were transferred into a scale up of sialylation of other functionalized Tris derivatives which were not priorly quantified. Therefore, Tris derivatives **2**, **3**, **4**, **6** and **8** as well as the buffers Tris, TES and tricine were scaled up using total volumes up to 10 ml with the total amount of 0.11 mmol substrate (approx. 15-30 mg) respective 2.5 mmol reactive buffer (approx. 500 mg). Scaled reactions were performed with 250 mM buffer, 50 mM $MgCl_2$, 11.0 mM Tris derivative **2**, **3**, **4**, **6** or **8**, 16.5 mM Neu5Ac, 49.5 mM CTP, 100 µg/ml NmCSS and 40 µg/ml PmST1 P34H/M144L. The reaction was allowed to react for 1 h at 37 °C and after PmST1 was added subsequently the mixture reacted for another 20 h at 37 °C. The sialylation batches were lyophilized after 20 h reaction and the crude reaction mixtures were stored for subsequent purification. Analytical RP-HPLC did not show sufficient separation of the sialylated products of **2**-**4**, which requires alternative separation methods (see Supporting Information, Figure S1). Even though compounds **6** and **8** and their sialylated products showed separation on analytical RP-HPLC, their first purification attempts via preparative RP-HPLC failed (isocratic, 2 % acetonitril in water) probably due to the presence of concentrated polar components (e.g. reagents and buffers) or the choice of the wrong gradient or wrong mobile phase pH value.

In general highly polar compounds such as CTP and their byproducts, remaining Neu5Ac and buffers usually do not show interactions with reversed-phase stationary phase resulting in their low retention times appearing close to injection peak. The polar substrates and the more interesting sialylated products usually show peak overlapping with other hydrophilic reagents. Further challenges derive from the use of excess equivalents of buffers and reagents and the presence of aromatic cytidine derivatives (e.g. from CTP) which conceal the desired product peaks when using UV detectors.

The Tris-sialoside isolated in previous studies was purified by performing size exclusion chromatography prior to RP-HPLC which was found to be an efficient combination of methods.[592] Skipping size exclusion chromatography step and directly purifying with RP-HPLC did not work out for the isolation of sialylation products of **2**-**4**, Tris, tricine or TES. The use of size exclusion chromatography (SEC) required long columns with slow mobile phase flow which was technically not applicable within this present study. Generally, SEC is extraordinary challenging in this setup due to the narrow mass differences of the functionalized Tris derivatives towards their sialylation products, other reagents and byproducts. This effect is enhanced by the enlargement and thus approximation of the molecular weight of used substrates towards other components in contrast to the relatively small Tris molecule used in previous study.

To circumvent narrow mass differences and resulting separation issues of SEC, HILIC chromatography columns[618–620] was considered as a suitable solution for this challenging separation. First separation attempts with analytical HILIC columns showed sufficient separations combined with coupled mass detector but with poor detection with UV detectors due to poor signal-to-noise ratio (data not shown). Surprisingly, although the polar compounds did not show separations on reversed-phase columns, the HILIC method exploited structural differences of compounds such as the differentiation of zwitterionic structures (e.g. sialylated Tris or sialylated **4**) and mainly negatively charged compounds (e.g. sialylated TES or sialylated **2**). Thus, it will not be expected to find one general purification method fitting for every

sialylated Tris derivative purification. A quantification with HILIC columns using UV absorption has not been performed so far as well as the purification respective isolation of presented sialylation reactions are still open tasks for upcoming works.

Non-glycan sialylation activity of sialylatransferases from other organism sources

Since PmST1 WT and their bio-engineered mutants M144D and P34H/M144L show slightly different sialylation activity (see Table 5) and also show differences in CMP-Neu5Ac donor degradation behavior,[462,463] it is still unclear if this effect is only limited towards bacterial enzymes from *Pasteurella multocida*. So far the effect of Tris-derivative sialylation has not been described in detail which opens up the question if other organisms than *Pasteurella multocida* possess sialyltransferases which do show the same non-glycan sialylation effect. This question gains relevance since Tris buffer is characterized as biocompatible and was examined as an ingredient for mRNA vaccines,[621] bacterial disease vaccines[622–624] or anti-inflammatory drugs.[625,626]

Therefore, a set of different sialyltransferases was tested on their sialylation behavior, namely human originated ST6Gal1, ST3Gal1 and ST3Gal4[627–629] as well as bacterial PmST1 (as used previously), PmST3 (*Pasteurella multocida*)[630,631] and Pd2,3ST (*Photobacterium dagmatis*).[464,632] These enzymes were compared to each other and tested on the sialylation of selected substrates such as Tris buffer,

Table 9: Sialylation products of human and bacterial sialyltransferases of the substrates Tris, aromatic derivative **8** and $LacN_3$.

	m/z $[MSia+H]^+$			m/z $[MSia+Na]^+$			
	Found	Calculated	Relative intensities[a)]	Found	Calculated	Relative intensities[a)]	Conversion [b)]
Tris, ST6Gal1	n.d.	413.2	n.d.	n.d.	435.2	n.d.	-
Tris, ST3Gal1	413.4	413.2	0.01	n.d.	435.2	n.d.	-
Tris, ST3Gal4	413.2	413.2	0.08	n.d.	435.2	n.d.	-
Tris, PmST1 P34H/M144L	413.2	413.2	0.81	435.2	435.2	0.06	-
Tris, PmST3	413.4	413.2	0.04	435.4	435.2	0.09	-
Tris, Pd2,3ST	413.2	413.2	0.57	435.2	435.2	0.18	-
8, ST6Gal1	572.2	572.2	0.05	n.d.	594.2	n.d.	n.d.
8, ST3Gal1	572.0	572.2	0.09	594.0	594.2	0.05	n.d.
8, ST3Gal4	n.d.	572.2	n.d.	594.1	594.2	0.04	n.d.
8, PmST1 P34H/M144L	572.0	572.2	0.07	594.1	594.2	0.46	7.5 %
8, PmST3	572.0	572.2	0.01	594.4	594.2	0.02	n.d.
8, Pd2,3ST	572.3	572.2	0.03	594.1	594.2	0.29	3.6 %
$LacN_3$, ST6Gal1	659.1	659.2	0.87	681.1	681.2	0.90	-
$LacN_3$, ST3Gal1	659.2	659.2	0.73	681.2	681.2	0.01	-
$LacN_3$, ST3Gal4	659.2	659.2	0.45	681.2	681.2	2.04	-
$LacN_3$, PmST1 P34H/M144L	659.3	659.2	0.03	681.2	681.2	4.28	-
$LacN_3$, PmST3	659.0	659.2	0.73	681.3	681.2	1.18	-
$LacN_3$, Pd2,3ST	659.2	659.2	0.11	681.1	681.2	1.59	-

[a)] Normalized on m/z signals of bicine (146.2, 164.0 or 186.0 m/z), [b)] gradient of 5-95 % acetonitrile in water (+0.1 % formic acid) in 15 min then isocratic for 2 min. n.d. – not determined.

aromatic Tris derivative **8** and azidolactose $LacN_3$.[456,592,633] $LacN_3$ is similar to the natural acceptor motif and has been previously used for enzymatic sialylation reactions. In this setup the azidolactose $LacN_3$ was used as a positive control substrate to prove sialyltransferase activity under the applied conditions regarding e.g. pH value, NmCSS concentration and the chosen MS assay.

The sialylation screening showed that each sialyltransferase used in this setup, also showed sialylation of $LacN_3$ but showed differences in sialylation of Tris derivatives. Besides PmST1, the Pd2,3ST showed non-glycan sialylation of Tris buffer and aromatic derivative **8**. Both, the human sialyltransferases (ST6Gal1, ST3Gal1, ST3Gal4) as well as the PmST3 did not show sialylation of the Tris-derived non-glycan substrates (see Table 9). The obtained data indicates the different specificities of sialyl-transferases in terms of substrate tolerance and highlights the advantages of using bacterial enzymes for synthetic chemistry to obtain unusually sialylated structural motifs.

Conclusion

Based on previous results, PmST1 was found to show sialylation of non-glycan substrates which also can be used for converting synthetic substrates into neoglycosides. Starting from a buffer sialylation reaction which was first categorized as an undesired side reaction, this principle could be extended to derived structures with a polyol residue, here the tris(hydroxymethyl)methyl motif. To potentially use this method for the construction of neoglycoside conjugates via CuAAC, this proof-of-concept study was extended to specially designed small, azide- or alkynyl-functionalized molecules derived from Tris buffer. The synthesis of these compounds either used the principle of *N*-functionalization or halogenide substitution reactions. The functionalized precursor compounds **2**-**4** were tested in chemoenzymatic reactions with PmST1 WT, M144D and P34H/M144L and it was found that high polarities of involved reagents and products led to analytical challenges when using reversed-phase (C_{18}) HPLC columns. Hence, the more hydrophobic compounds **6** and **8** were functionalized with hydrophobic linkers to enable sufficient separations on RP-HPLC. It was found that PmST1 show sialylation of these synthetic derivatives **2**-**4**, **6** and **8** and it is assumed that these substrates were converted with the same hydroxyl group sialylation as previously found on Tris buffer.[592] On the behalf of aromatic derivative **8**, analytical experiments showed a separation of the substrates and their expected sialylation products and allowed for indication of conversions up to 12 %. The observed conversion rates of non-glycan sialylation are much lower than compared to the sialylation of lactose-functionalized conjugates or the Tris sialylation from previous study.[592] These low conversions of the synthetic Tris derivatives could be explained by the significant deviation of **2**-**4**, **6** and **8** from the natural sialyltransferase acceptors (usually lactose derivatives). During scaled-up reactions with bicine buffers it was noticed that bicine might not be the optimal buffer choice for this chemoenzymatic reaction. Bicine as well as tricine showed a slight delay in CMP-Neu5Ac donor formation (visible as inhibited precipitation of Mg^{2+} pyrophosphate complex). Based on few hints, it can be assumed that carboxylate groups show complex formation with Mg^{2+} ions which are required for NmCSS activity and further for subsequent sialylation. Thus, bicine and tricine can indirectly inhibit this sialylation reaction and can be principally substituted by other buffers in this pH range (e.g. HEPBS or glycinylamide) which should be exploited in following studies.

The aromatic derivative **8** allowed for sufficient quantification of PmST1 sialylation activity. First experiments showed that PmST1 P34H/M144L double mutant showed the highest sialylation

conversion followed by WT and then by M144D. It was observed that minimum buffer as well as reagent concentrations are required for sufficient reaction shown in Table 6-8. Based on experiments with sialyltransferases from different species origins, it can be assumed that only bacterial enzymes such as PmST1 or Pd2,3ST do accept this class of aglycon substrates and human sialyltransferases do not show this kind of activity with Tris derivatives. For following studies various structurally-related polyol motifs could be tested to be used as substrates such as sweeteners (e.g. xylitol or erythritol) or glycerol-derived compounds.

Although the formation of these newly discovered synthetic sialylated compounds was proven, first attempts of preparative purification with C_{18} columns did not succeed. Up to now, it is still necessary to establish purification protocols and to isolate and fully characterize these sialylated compounds which are subjects of following works. Therefore, HILIC chromatography is expected to be a suitable purification method to overcome high hydrophilicity of the sialylated products presented in this studies. Due to differences in the charge states of the sialylated products it is not expected to find a general purification method fitting for each of the substrates **2**-**4**, **6** and **8**. Once sufficient purification protocols are established, the sialylated structures can be conjugated onto macromolecular scaffolds and can be used for studying their biological properties e.g. lectin binding or stability against neuraminidases. Furthermore, in future studies the in solution azide-alkyne conjugation with hydrophobic azides or alkynes can be exploited for the precise conversion quantification of different Tris-derived substrates using C_{18} columns.

3.3 Synthesis of homo- and heteromultivalent fucosylated and sialylated oligosaccharide conjugates *via* preactivated *N*-methyloxyamine precision macromolecules and their binding to polyomavirus capsid proteins

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Own contributions:

Synthesis of functional builing blocks and *N*-methyloxyamine glycomacromolecules, method development of macromolecule glycosylation via *N*-methyloxyamines, design of multivalent *N*-methyloxyamine-functionalized small molecules, isolation of small molecule glycosides and monodisperse glycomacromolecules, method development of accessing asymmetrically glycosylated small molecules, purification of monodisperse glycosides, analytical characterization of monodisperse compounds using HPLC, ESI-MS, HR-ESI-MS, ^{1}H and ^{13}C NMR, assistance on method implementation on polymeric backbones, collaborative writing of manuscript and supplement.

The publication (Konietzny et al. **2022** *Biomacromolecules*) as well as related supporting information can be viewed and accessed via the following link:
https://pubs.acs.org/doi/10.1021/acs.biomac.2c01092?ref=pdf

4. Conclusion and perspectives

The presented appraoches were used to synthesize target structures and were further used to identify and explore further derived synthetic applications. Both, the chemoenzymatic approach as well as the chemical *N*-methyloxyamine glycosylation, were used to obtain macromolecular scaffolds with method-specific advantages and disadvantages.

The chemoenzymatic approach using NmCSS and PmST1 was successfully used for α2,3-sialylation reaction to obtain multivalent sequence-defined glycomacromolecules with a 3‘-sialyllactose ligand motif. The two-step enzymatic sialylation in a one-pot reaction was performed in solution on lactose-functionalized precursor macromolecules which were synthesized with TDS- and EDS-based solid phase synthesis, then lactose-functionalized via CuAAC and subsequently cleaved from solid support using TFA. The lactose-functionalized macromolecules were glycosylated with *in situ* formed CMP-Neu5Ac from CTP and Neu5Ac in assistance with NmCSS and PmST1. As a result 3‘-sialyllactose macromolecules were obtained which were worked up using the combined use of size exclusion chromatography and preparative HPLC. The synthetic strategy presented in this thesis took into account that the sialyl motif was introduced in a late-step reaction under mild conditions to circumvent potential desialylation reaction through hydrolysis. This chemoenzymatic approach was applied to obtain eight 3‘-sialyllactose-functionalized macromolecule structures differing in valency (from mono- to trivalent) and glycan linker motif (linkerless and propyl linker). The enzymatic sialylation showed yields of 24 to 55 % depending on the macromolecule valency whereas the synthesis of high valent structures showed lower yields than their monovalent analogues. The overall yields of 2 to 20 % include the SPPS of the lactose-functionalized precursor structures. Although the yields were lower than comparable 3‘-SL-macromolecules from previous studies using non-enzymatic synthesis,[220] the substance variety was higher in this study. The yields and the reaction handling did not show notable differences when using the two linker motifs used here. From NMR analytical perspective, the linkerless macromolecule derivatives showed advantages due to the clearly interpretable $H1^{Gal}$ signal around 4.52 ppm of the sialylated product species. Whereas the propyl linker derivatives showed overlaps in this chemical shift region and thus required additional analytical measurements e.g. HPLC.

In parallel with the macromolecule sialylation attempts, the reaction behavior of PmST1 wildtype (WT) and M144D mutant was examined allowing to obtain useful information for enzyme handling. Therefore, a few aspects are relevant for optimal reaction realization. As previously reported by Chen group,[455] PmST1 is a multifunctional enzyme and showed fast sialylation product formation but is simulataneously attended with a slow enzymatic sialyl group degradation. This detail is highly relevant especially for the conversion of multivalent molecules, whereas non-sialylated sites let the macromolecule formally count as a intermediate or side product on the behalf of sequence-defined macromolecules. As a result of statistically distribution of partially sialylated motifs within multivalent substrates, even in optimal work up procedures the observed enzymatic sialylation conversions exceeded the observed yields. Thus, a small process window between minimum and maximum reaction time as well as the right enzyme and reagent concentrations were required for this kind of enzymatic reaction. Therefore, NMR activity assays via Neu5Ac reporter groups were performed to obtain relevant quantitative information and to adjust the enzymatic reaction parameters. The signals of equatorial and axial $H3^{Neu5Ac}$ (2.75 to 1.60 ppm) and sialylated and non-sialylated $H1^{Gal}$ (4.52 to 4.55 ppm) allowed for the calculation of integrals ratios which

gave information about the sialylation conversion which extended the information content compared to just monitoring residual CMP-Neu5Ac concentration. Both PmST1 variants WT and M144D showed different sialylation behavior as expected.[462] PmST1 WT was used for these syntheses due to the easier completion of reaction. Although this wildtype enzyme showed a higher desialylation activity than M144D mutant, this effect was circumvented as described in the following discussion and thus was more feasible for the application on multivalent structures.

The undesired desialylation process took place during synthesis whenever the *in situ* formed CMP-Neu5Ac donor was completely consumed. This effect was overcome by either stopping the reaction in time, using excess CTP or by adding additional CTP shortly before quenching the reaction. The proportional amount of incompletely sialylated glycomacromolecules and thus the amount of potential side product formation increases with the valency. This effect can be explained with statistical reasons and limits the application on high valency 3‘-SL-functionalized glycomacromolecules. After the seperation of PmST1 enzyme and reaction work up no non-enzymatic sialic acid hydrolysis was observed.

Although this presented chemoenzymatic approach allowed for an easy sialyl introduction and thus the feasible assembly of small substance bibliographies compared to previous synthesis routes e.g. by Baier et al., this method was strictly limited to the introduction of terminal α2,3-sialyl residues when using PmST1 enzyme. This method benefits from easy application when using inline reaction control techniques such as NMR spectroscopy. The application of faster and technically less demanding methods is principally possible when indirectly tracking reaction via CTP or CMP-Neu5Ac concentration measurements and needs to be evaluated in the future. When changing the enzyme to other glycosyl transferases once again reaction optimization would be required due to different enzyme handling. As an outlook for these studies the obtained experience with chemoenzymatic glycosylation of glycomacromolecules can be extended and combined with other methods from the SPPS toolbox. Therefore, this method can e.g. be used for the heteromultivalent glycosylation with different carbohydrate ligands to address two different proteins with one macromolecule or the sialylation of glycosylated surfaces for adhering sialic acid binding proteins. The handling on SPPS-derived macromolecules was further extended by testing to transfer this method for its use on solid support which requires mild cleavage conditions to prevent hydrolytic desialylation.

During the closer inspection of the PmST1 reaction behavior it was found to also sialylate non-glycan substrates such as Tris buffer used here for stabilizing the reaction solution. The formed Tris-sialoside was isolated and was characterized as covalently bound to a hydroxy group of the Tris(hydroxymethyl)methyl residue. In contrast to the sialylation of lactose motifs as used in a model substrate $LacN_3$ or precursor glycomacromolecules, the Tris-sialoside surprisingly was not enzymatically desialylated by PmST1. This finding allows to hypothesize different neuraminidase stabilities of the novel Tris-sialoside compared to related 3‘-SL derivatives. For this reason this newly discovered enzymatic pathway can be potentially used for synthetic purpose to access novel neoglycosides. In a first experiment with PmST1 WT structurally similar Tris-based buffers such as tricine and TES were tested and indicated sialylation reactions catalyzed by PmST1.

In a continuing study this effect of non-glycan sialylation was further investigated with the overall goal of transfering this Tris-sialoside formation to a use on glycomacromolecules. This initially defined goal was not achieved within this thesis as further described. On this project the analysis of synthesized sialoside product formation needed to be changed from NMR analysis towards a mass spectroscopy (MS) based analysis.

This MS-based method was found to be a suitable, sensitive and fast detection method which at the same time lacked on quantitative output. Enzymatic reactions were performed on analytical scale (50 µl, 6 µmol substrate) and diluted samples were measured either via direct ESI-MS or HPLC-coupled MS. Therefore, different sets of chemoenzymatic reactions with NmCSS/PmST1 were tested for the sialylation of structurally similar substrates. The sialylation of Tris, tricine and TES buffers by PmST1 was confirmed by identifying sialic acid adducts as previously indicated using NMR analysis.[592] The enzymatic sialylation was performed with different PmST1 mutants WT, M144D and P34H/M144L to compare their applicability for the non-glycan sialylation. Here, P34H/M144L showed highest conversion rates. Other sialyltransferases from various origin sources were tested in first experiments and showed mixed results on Tris sialylation. Human sialyltransferases ST6Gal1, ST3Gal1 and ST3Gal4 as well as PmST3 from *Pasteurella multocida* did not sialylate Tris and derivatives, whereas bacterial sialyl-transferase Pd2,3ST from *Photobacterium dagmatis* also indicated the formation of non-glycan sialylation. This result states that bacterial enzymes can be used for non-glycan sialylation processes. Whenever non-buffering substrates were used for enzymatic sialylation reaction it was necessary to use a non-reactive working buffer to adjust pH 8.8 to enable enzymatic activity e.g. bicine as used in this study. The bis(hydroxyethyl)amine motif was found to be not sialylated during PmST1 treatment.

For ongoing PmST1 experiments, a small selection of Tris derivatives was synthesized carrying small functional handles with azide or alkyne groups allowing for later conjugation via CuAAC. The synthetic Tris derivatives were designed to first sialylate and purify them prior to further conjugation reactions due to yet unknown sialylation yields. As most Tris derivatives used in these experiments were highly polar, this led to insufficient separation of reagents, (by-)products and substrates and thus indirectly did not allow for quantitative HPLC analysis. Due to this lack of quantitative data no significant differentiation of Tris derivatives in terms of substrate tolerance was made up to this point.

Based on these findings, two more hydrophobic Tris derivatives were designed and synthesized such as an azidated aromatic derivative or a Tris derivative with an azidated C_6 side chain. Both Tris derivatives were be successfully enzymatically sialylated in low conversions and likewise showed good separation on analytical RP columns. Whilst the C_6-functionalized Tris derivative only showed poor UV absorption behavior and thus sialylation conversion was not properly determined, the aromatic Tris derivative allowed for quantification via UV detector. The following reaction optimization was performed on PmST1 P34H/M144L using the aromatic Tris derivative as a substrate which was later used on the other Tris derivatives. First sialylation attempts according to attached protocols showed approx. 5 % conversion. The sialylation conversion were positively affected to a conversion of approx. 11 % by e.g. increasing bicine buffer concentration, adapting enzyme concentrations and adding excess 4.5 eq. CTP over 1.5 eq. Neu5Ac and 1.0 eq. aromatic Tris derivative **8**. Further increase of CTP and PmST1 concentration still led to very small conversion improvement, but they were evaluated as not practical

for synthetic use due to disproportionate high consumption of CTP and PmST1. Furthermore noteworthy is that the change of NmCSS concentration did not show significant effects on the sialylation yields. Whereas, a decrease of sialylation conversions can be observed when choosing PmST1 M144D mutant and when CTP:Neu5Ac ratio fall below 2:1 or when using pH < 8.7. In contrast to previous studies, the optimized sialylation conversions of the aromatic Tris derivative were smaller than the yields of the non-optimized, priorly isolated Tris-sialoside (28 % yield[592]) which allows to hypothesize that each Tris derivative is expected to show differences in PmST1 sialylation behavior.

The optimized reaction conditions were applied on small preparative scale sialylations (0.11 mol) using different Tris derivatives but they were not successfully worked up due to lack of product interaction with reversed-phase C_{18} columns. Strong signal overlap was observed using RP-HPLC. Further purification attempts respective product isolation, characterization and macromolecule conjugation are subjects of this project outlook which can be supported by the insights collected from the performed eperiments.
The extensive analysis and preparative purification of the sialylated Tris derivatives can be further tried using either HILIC or size exclusion chromatography columns. After isolation and characterization of the proposed sialylated neoglycosides, the feasability of the different Tris derivatives should be evaluated as well as their chemical and enzymatical stabilities. These stability tests can help developing a feasible overall synthetic strategy of sialylated neoglycoside-functionalized macromolecules and might contain information about biological activity. Justified by the observed neoglycoside stability against desialylation activity of PmST1, further testing with neuraminidases could be performed to examine functional differences between different sialylation modes, e.g. comparison with 3‘-SL, 6‘-SL and Neu5Ac-functionalized structures.
On behalf of the enzymatic sialylation reaction optimization it is further possible to apply molecular design onto Tris derivatives towards less polar compounds to allow synthesis of more feasible sialylation substrates, e.g. by the attachment onto monodisperse macromolecules, by introducing hydrophobic handles or by changing electronical effects working on the Tris(hydroxymethyl)methyl motif. These optimized Tris derivative substrates might lead to better sialylation conversions or easier purification procedures. At the same time, it should be evaluated if bicine as a non-reactive buffer can be substituted by other buffers to be applicable for enzymatic non-glycan sialylation. The present carboxylate moiety of bicine might show interactions with Mg^{2+} ions which are necessary for NmCSS activity and thus indirectly can influence CMP-Neu5Ac donor formation and the following sialylation reaction. Therefore, only a few buffers can be adjusted on pH 8.8 such as HEPBS or glycinylamide.
In the future, the scope of potential substrates for PmST1 enzymatic sialylation can be enhanced and further explored by sialylating e.g. larger macromolecule scaffolds functionalized with Tris(hydroxy-methyl)methyl motifs. Monodisperse Tris-functionalized oligomers can be tested on enzymatic sialylation in solution and then subsequently analyzed on RP-HPLC to gain quantitative information of Tris derivative sialylation which was not determined on most compounds within present studies. Another approach of in solution CuAAC of previously sialylated Tris derivatives can be developed to decrease molecule polarity and analyze product/substrate ratio via RP-HPLC analysis. These techiques can be principally used to compare among different Tris derivatives about their PmST1 substrate conversion and to allow evaluation for synthetic purpose. PmST1 sialylation behavior can also be tested on disperse

polymers whereas full conversion is not crucial for functional assays with sialic acid binding proteins. Another group of potential substrates might be small molecule polyol compounds such as erythritol, xylitol and their derivatives being known as artificial sweeteners in food industry.

Enzymatic glycosylation reactions bring along limitations of the introduced monosaccharide motif and its regioselective linkage, in the case of PmST1 the terminal α2,3-sialic acid motif. When synthesizing other carbohydrate ligand motifs via enzymatic pathway, different glycosyltransferases are usually being used with their own specific reaction handling and substrate requirements. For the integrated bottom-up synthesis of oligosaccharides several additional enzymes are required and thus a more biological project focus on protein expression and purification is necessary. In contrast to enzymatic glycosylation reactions whereas the product carbohydrate motif is defined by the use of glycosyltransferase, chemical glycosylations are defined by the carbohydrate availability, are less selective and but thus often more general in their applications. This enables a faster exchange of carbohydrate motif using chemical synthetic routes.

One method of synthetic glycosylation uses *N*-methyloxyamine functional group to attach protection group-free and non-functionalized carbohydrates as originally presented by Blixt group.[377] The past concept of glycosylating functionalized linker molecules was now further extended and modified by the development of azide-functionalized oxyamine linkers to enable compatibility with TDS-/EDS-based SPPS chemistry. The linker syntheses were sucessfully performed on symmetrical aliphatic and aromatic dibromo precursors which were functionalized with Boc-*N*-methyloxyamine motif, subsequently azidated in a second step and applied on SPPS. The symmetrical dibromo precursors were chosen to be functionalized with Boc-*N*-methyloxyamine inselectively to yield mono- and difunctionalized oxyamines. Whilst monofunctionalized Boc-*N*-methyloxyamines were further azidated to be used as functional linker molecules, the divalent Boc-*N*-methyloxyamines were used for multivalent small molecule glycosylation. This method was also exploited on tri- and tetrabromomethylated benzene precursors to develop a separate synthetic strategy for the facile synthesis of mutlivalent glycoconjugates. Boc-deprotection with trifluoroacetic acid lead to preactivated *N*-methyloxyamines which were glycosylated under NaOAc buffered conditions using 20 eq. carboyhdrate per *N*-methyloxy-amine group. The fulfillment of optimal reaction conditions was necessary, but still applicable on a high variety of scaffolds as shown in this study. Subsequent purification via preparative HPLC lead to the isolation of different β-anomeric glycoconjutages containing glucose, *N*-acetylglucosamine, lactose, 2‘-fucosyllactose, 3‘-sialyllactose, 6‘-sialyllactose and lacto-*N*-fucopentaose motif. When adding simultaneously two different carbohydrates it was shown that heteromultivalent glycans were formed by statistically conjugating the reactive oxyamine precursors. By using this method synthetically difficult glycoconjugate combinations e.g. with 2‘-FL and 3‘-SL motifs were isolated without prior carbohydrate functionalization. In general, this method of heteromultivalent glycosylation works easier when carbohydrates with different polarities were used such as fully hydroxylated vs. partially *O*- or *N*-acetylated carbohydrates or when small carbohydrates were used. It is noteworthy, that multivalent small molecule glycosylation was applied in a short synthesis sequence including a total of four steps and can be principally scaled-up due to precursor synthesis on gramm scale. The use of high

carbohydrate excess was compensated by skipping carbohydrate functionalization which is often challenging and is usually related with loss of yield.

The disadvantage of small molecule glycoconjugates is the lack of structural modularity, which were obtained by using different bromomethylated benzene derivatives. The construction of sequence-defined Boc-*N*-methyloxyamines was done by using the previously described azidated linkers in combination with SPPS. Therefore, *N*-methyloxyamine linkers were conjugated onto tailor-made oligomer backbones on solid support to arrange sequence-defined and multivalent structures. Similar to the small molecule derivatives, the *N*-methyloxyamine groups of the SPPS-derived macromolecules remained Boc-protected during synthesis. Macromolecule cleavage from solid support using TFA led to macromolecule isolation and simultaneous *N*-methyloxyamine deprotection. The macromolecule precipitation gave reactive molecules which undergo glycosylation with added carbohydrates once NaOAc-buffered conditions occur. Reactive macromolecule as well as small molecule handling was performed by using aqueous aliquotes which allowed for easy precursor storage and sample analysis. This SPPS-associated method was used to obtain mono-, di- and trivalent macromolecules with *N*-acetylglucosamine, lactose, 3‘-sialyllactose, 6‘-sialyllactose, 2‘-fucosyllactose and lacto-*N*-fucopentaose. The macromolecule glycosylations were performed in solution as its overall synthetic strategy was chosen for combining the sequence-definition by SPPS and the late-step glycosylation under mild conditions to prevent potential hydrolysis of the newly formed carbohydrate-oxyamine linkage.

During first experiments with monovalent SPPS-derived macromolecules and due to good separation properties on C_{18} columns, glycosylation byproducts were identified which derive from slow competition reaction of glycosylated *N*-methyloxyamines with acetate anions. Among other things, the synthesis of monovalent glycoconjugates was easier than divalent and trivalent structures since the probability of non-functionalized or degraded oxyamines increases with the overall oxyamine valency. When using this method on monodisperse molecules with higher valencies than three respective four *N*-methyloxyamine motifs, this synthetic method probably needs further optimization.

This valency limitation can be circumvented by transferring this method to polymeric backbone structures without sequence definition as already shown by Godula group.[392,393] Therefore, our azidated linkers were attached to poly(pentafluorophenyl acrylate) polymer with 50 repeating units using two different synthetic routes. Within the first route poly(pentafluorophenyl acrylate) was functionalized with propargylamine prior to *N*-methyloxyamine linker conjugation via CuAAC. Boc-deprotection and work up of the reactive polymers allowed for glycosylation reactions yielding glycopolymers functionalized with lactose, 2‘-fucosyllactose and 3‘-sialyllactose with a functionalization degree between 12 and 24%. The second route used monovalent SPPS-derived and amino-functionalized macromolecules as discussed before. The SPPS-macromolecules were also glycosylated with lactose, 2‘-fucosyllactose and 3‘-sialyllactose using *N*-methyloxyamine chemistry and were grafted to the poly(pentafluorophenyl acrylate) polymers giving functionalization degrees between 6 and 16%. Both polymer synthesis routes were applicable and showed their strength in higher valencies, higher ligand spacing and scaffold size. Although glycopolymers usually lack in definition compared to monodisperse systems and thus are harder to analyze, the work up after synthesis can be handled without technical demanding preparative HPLC.

In general, the use of *N*-methyloxyamine chemistry for glycosylation purpose is an interesting toolbox to skip prior synthetic carbohydrate functionalization by moving synthetic focus onto the simpler *N*-methyloxyamine linker synthesis. From the perspective of synthetic potential of this method, it should be highlighted that protection group-free glycosylation reactions as shown here can be used without having advanced knowledge in preparative carbohydrate chemistry. The method further benefits from the build up of structurally related substance bibliographies and the glycomacromolecule application in biological assays as shown in the experiments of SPPS-derived 3‘-sialyllactose- and 6‘-sialyllactose-functionalized macromolecules with JC and BK polyoma virus capsid. The direct comparison of different scaffold systems, the polymer glycosylation showed slightly better glycosylation yields of 18 to 87% compared to SPPS (6-50%) and small molecules (8-59%, homomultivalent glycosylation). The broad applicability of this glycosylation technique shows its potential on complex glycan introduction which enlarges general application range.

Both approaches, the chemoenzymatic as well the protection group-free glycosylation, provide additional synthetic access towards specialized glycomacromolecules and glycomimetics which can be used for the development of oligosaccharide-bearing therapeutic drugs. This can be realized by synthesizing molecules with glycan motifs which selectively address carbohydrate-binding proteins. Whereas the PmST1 chemoenzymatic method benefit from the strict introduction of 3‘-SL glycan motifs and the potential of introducing unusual Neu5Ac motifs with non-glycan structural motifs, the protection group-free approach is more suitable for the fast variation of the attached carbohydrate motif. The presented approaches of HMO-functionalized synthesis generally benefit from the circumvention of time-consuming oligosaccharide synthesis using organic chemical methods. At the same time, the industrial production and commercial access of oligosaccharides and HMOs from biotechnological sources has improved in the last century as shown in the commercialization of food grade HMOs.[102] Since these carbohydrate sources might play an emerging role in the future, these shown glycosylation approaches can be used for adaption onto other scaffolds which can be combined with SPPS-compatible (macro-)molecules.

5. Appendix

Supporting Information – Sialylation of synthetic tris(hydroxymethyl)aminomethane derivatives using α-2,3-sialyltransferase from Pasteurella multocida

Table of Content

Materials and methods

Analytical methods

Chemoenzymatic reactions were analyzed by using direct ESI method on an Agilent 1260 Infinity system. The automated injection via autosampler allowed for fast analysis of sequences of samples. More hydrophobic samples such as compound **6** and **8** as well as their sialylated species were additionally analyzed by RP-HPLC using analytical C_{18} column from Shiseido (Capcell Pak C18 type UG80, 20 x 250 mm) which was coupled to the electrospray ionization MS detector described before. The separation via HPLC was analyzed a coupled with UV absorption detector and allowed for the selective analysis of single compound fractions via ESI-MS.

The further characterization of compounds was proceed using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy (Bruker Avance III – 300 and Bruker Avance III – 600) and high resolution mass spectrometry (HRMS) (UHR-QTOF maXis 4G, Bruker Daltonics).

Semipreparative high-performance liquid chromatography method (Agilent 1260 Infinity) was tested to be used for separation of the sialylation reactions of **2**-**4**, **6**, **8** and **S5**-**S7**. Therefore, a C_{18} column was used from MZ Analysentechnik (MZ Aqua Perfect C18, 3 x 50 mm) in combination with gradients of mobile phase (A: MilliQ water, B: acetonitrile) without further additivation.

Enzyme expression

The applied enzymes kindly provided by Robert P. de Vries and coworkers from Utrecht University, including NmCSS WT (from *Neisseria meningitidis*), PmST1 WT (from *Pasteurella multocida*), PmST1 M144D (from *Pasteurella multocida*), PmST1 P34H/M144L (from *Pasteurella multocida*), PmST3 (from *Pasteurella multocida*), Pd2,3ST (from *Photobacterium dagmatis*), ST6Gal1 (from human origin), ST3Gal1 (from human origin), ST3Gal4 (from human origin).

Sialylation reaction protocols

A: Buffer sialylation: Whenever the non-glycan substrate possess buffering properties, 200 mM buffer (pH 8.8), 40 mM $MgCl_2$, 16.5 mM Neu5Ac, 33.0 mM CTP and 1000 µg/ml NmCSS were mixed and deionized water was added to give a total volume of 50 µl. The unready reaction mixture was allowed for donor synthesis for 1 h at 37 °C. Subsequently, 40 µg/ml PmST1 P34H/M144L were added and the reaction mixture was allowed to react for 20 h at 37 °C. The sample was analyzed by removing 3 µl reaction sample and subsequent dilution with 30 µl MilliQ water in micro inserts for HPLC vials. The samples were stirred well and remaining bubbles were removed by vial pivoting.

B: Non-glycan sialylation in bicine buffer: 200 mM bicine (pH 8.8), 40 mM $MgCl_2$, 11.0 mM Tris(hydroxymethyl)methyl derivative, 16.5 mM Neu5Ac, 33.0 mM CTP and 1000 µg/ml NmCSS were mixed and deionized water was added to give a total volume of 50 µl. Analogous to method **A**, the unready reaction mixture was allowed for donor synthesis for 1 h at 37 °C. Subsequently, 40 µg/ml PmST1 P34H/M144L were added and the reaction mixture was allowed to react for 20 h at 37 °C. The sample was analyzed by removing 3 µl reaction sample and subsequent dilution with 30 µl MilliQ water in micro inserts for HPLC vials. The samples were stirred well and remaining bubbles were removed by vial pivoting.

C: Sialylation using alternative sialyltransferase enzymes: 200 mM bicine (pH 8.8), 40 mM $MgCl_2$, 11.0 mM Tris(hydroxymethyl)methyl derivative, 16.5 mM Neu5Ac, 33.0 mM CTP and 1000 µg/ml NmCSS were mixed and deionized water was added to give a total volume of 50 µl. The unready reaction mixture was allowed for donor synthesis for 1 h at 37 °C. Differing from method **B**, 40 µg/ml sialyltransferase were added and the reaction mixture was allowed to react for 20 h at 37 °C. The sample was analyzed by removing 3 µl reaction sample and subsequent dilution with 30 µl MilliQ water in micro inserts for HPLC vials. The samples were stirred well and remaining bubbles were removed by vial pivoting.

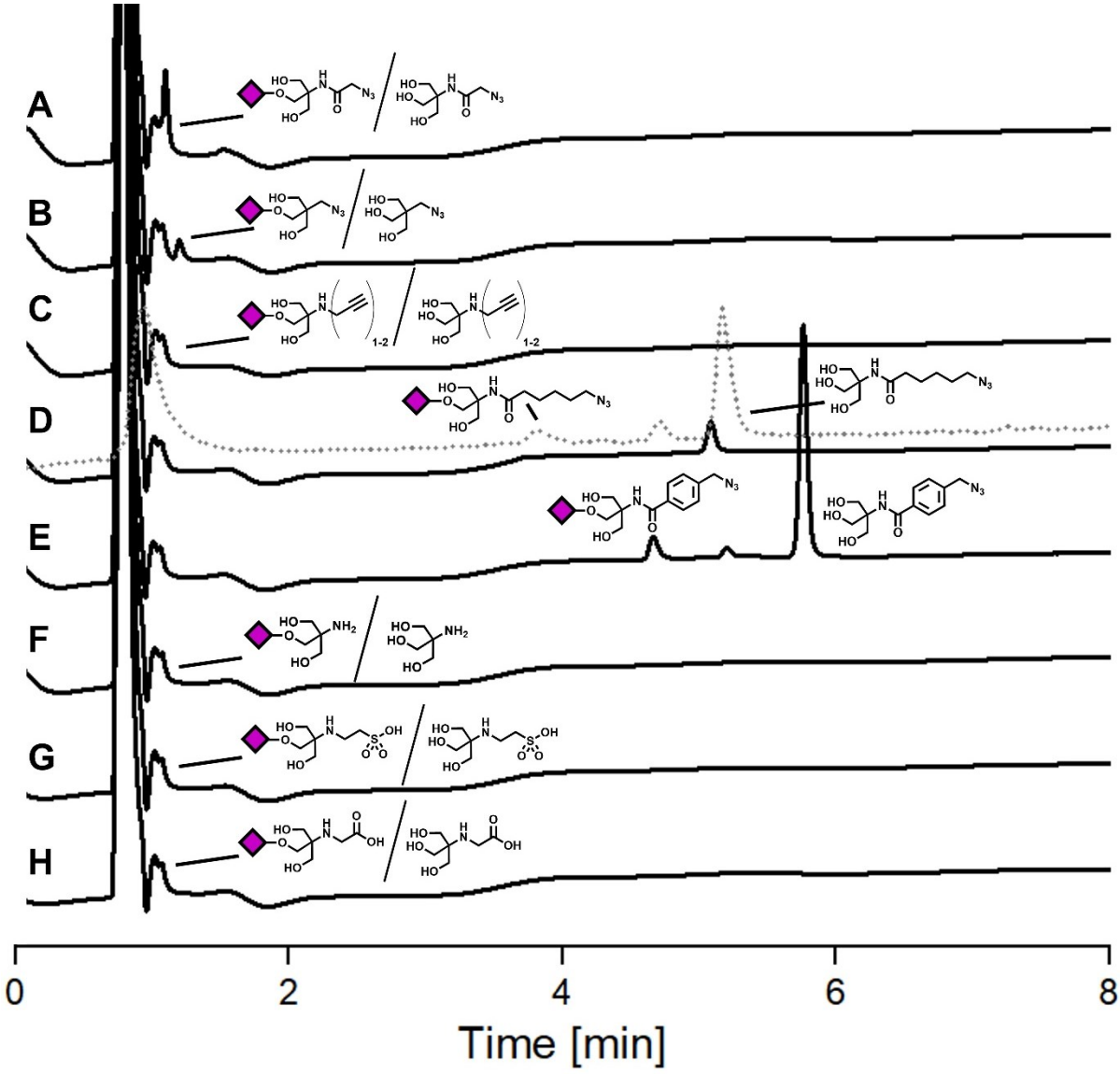
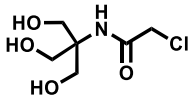


Figure S1: RP-HPLC chromatograms of enzymatic sialylation reactions using NmCSS and PmST1 P34H/M144L according to sialylation reaction protocol **B**. The following non-glycan substrates were used, **A:** compound **2**, **B:** compound **3**, **C:** compound mixture **4**, **D:** aliphatic compound (black: UV chromatogram, dashed: mass detector chromatogram) **6**, **E:** aromatic compound **8**, **F:** Tris, **G:** TES, **H:** tricine.

Syntheses and analytical data

Synthetic Tris(hydroxymethyl)methyl derivatives

N-[Tris(hydroxymethyl)methyl]-2-chloroacetamide, **1**



1

The product **1** was synthesized according to reported literature.[615] Therefore, ethyl chloroacetate (10.48 ml, 12.16 g, 99.22 mmol, 1.2 eq.) was dissolved in 8 ml methanol and was cooled with an ice bath. A suspension of 10.01 g Tris(hydroxymethyl)aminomethane (82.63 mmol, 1.0 eq.) in 32 ml methanol was slowly added to the cooled solution and were allowed to stir for two days at room temperature. The product was recrystallized from resulting solution by concentration to a total volume of approximately 5 ml and subsequent storage at -20 °C. A second recrystallization step lead to the product **1** which appeared as white crystals (3.73 g, 18.87 mmol, 23% yield).

^{1}H NMR (300 MHz, D_2O) *δ* 4.17 (s, 2H, –C*H*$_2$Cl), 3.81 (s, 6H, –C*H*$_2$OH).
^{13}C NMR (75 MHz, D_2O) *δ* 169.62 (*C*=O), 62.24 (–*C*(CH$_2$OH)$_3$), 60.11 (–C(*C*H$_2$OH)$_3$), 42.83 (–*C*H$_2$Cl).
RP-HPLC-MS: t_R = 1.09 min, relative purity not determined, linear gradient from 5 to 50 vol% acetonitrile in water (+ 0.1 vol% HCOOH) in 30 min.
MS (ESI) m/z calculated for M = $C_6H_{12}ClNO_4$: $[M+Na]^+$ calcd. 220.0, found 220.0.
HRMS (ESI, negative mode) m/z calculated for M = $C_6H_{12}ClNO_4$: $[M-H]^-$ calcd. 196.0382, found 196.0379.

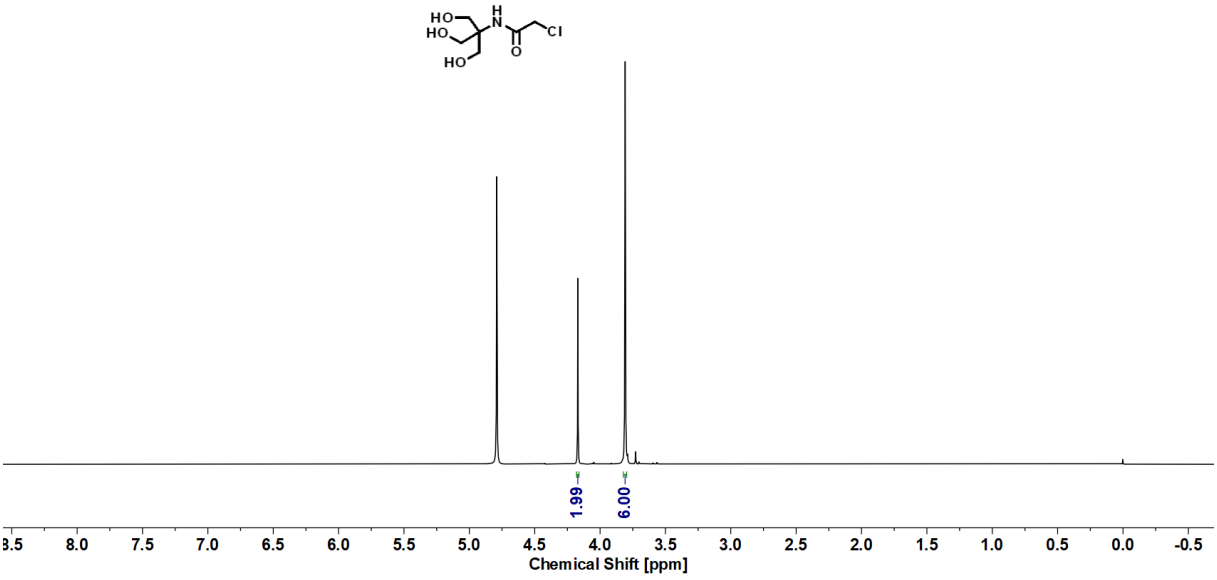


Figure S2: ^{1}H NMR spectrum of **1** (300 MHz, D_2O).

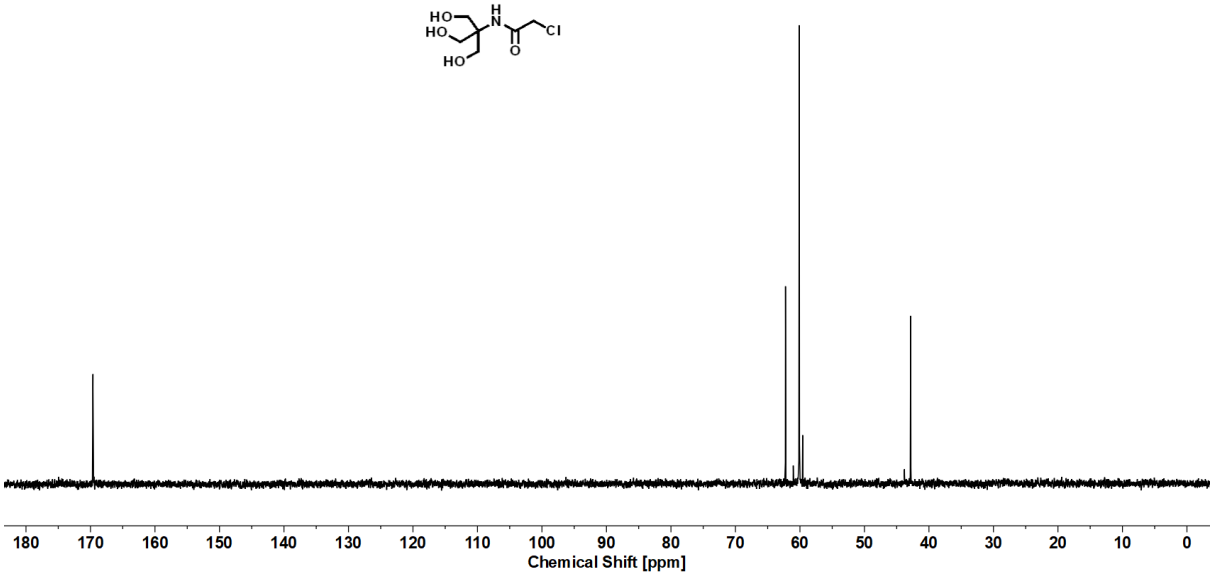


Figure S3: ^{13}C NMR spectrum of **1** (75 MHz, D_2O).

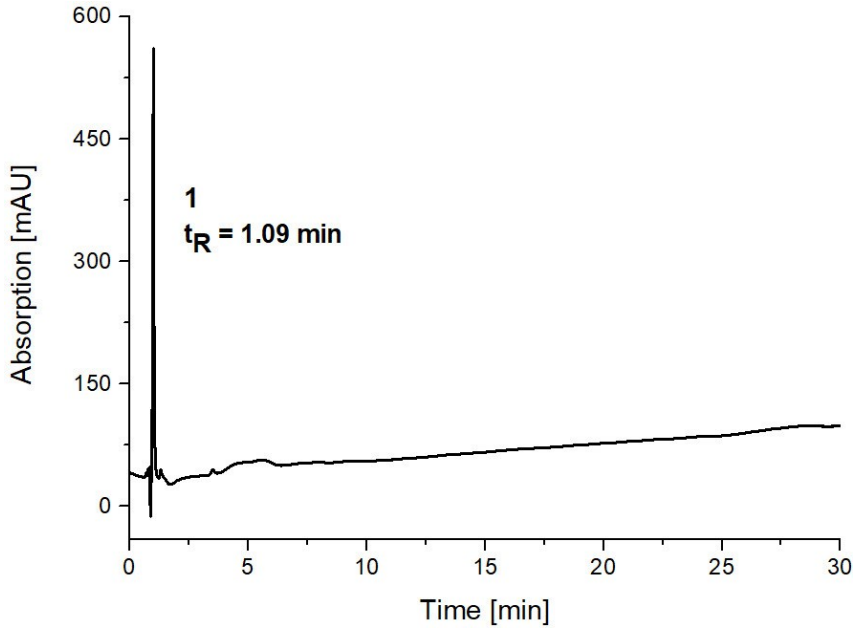


Figure S4: RP-HPLC chromatogram of **1**, linear gradient from 5 to 50 vol% acetonitrile in water (+ 0.1 vol% HCOOH) in 30 min.

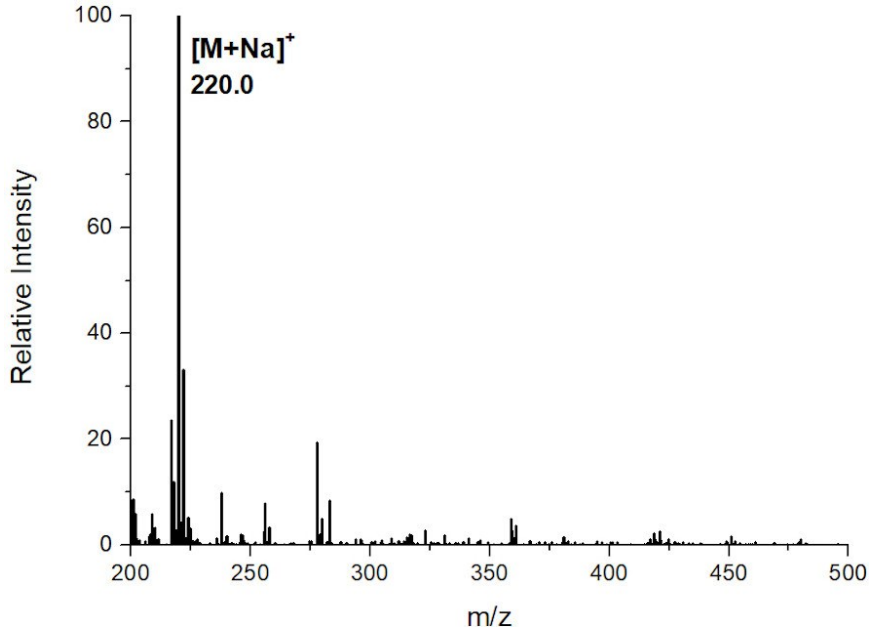


Figure S5: ESI-MS spectrum of **1** (positive mode).

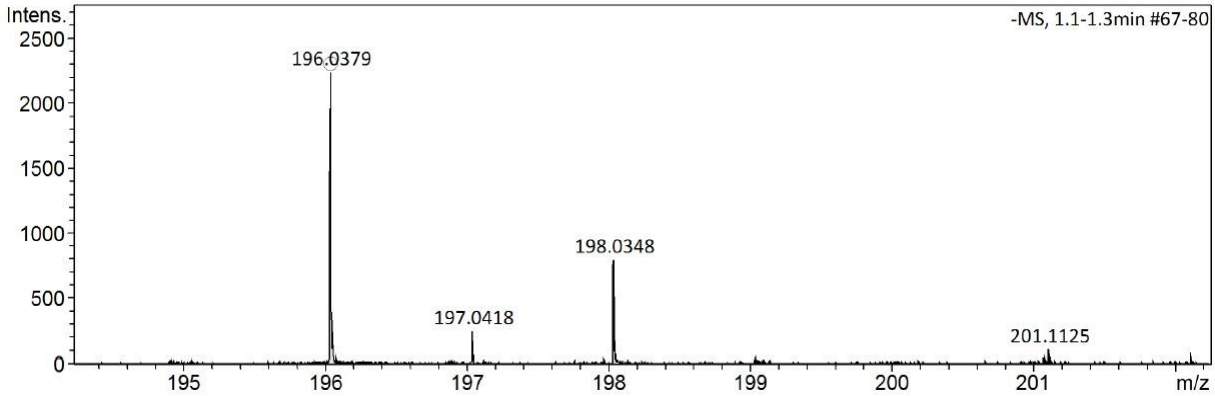
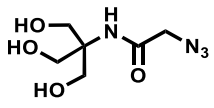


Figure S6: HR-ESI-MS NMR spectrum of **1** (negative mode).

N-[Tris(hydroxymethyl)methyl]-2-azidoacetamide, **2**



2

The product **2** was synthesized according to reported literature.[615] The chlorinated Tris derivative **1** (3.18 g, 16.09 mmol, 1.0 eq.) was dissolved in 8 ml DMF and 13.16 g sodium azide (23.50 mmol, 1.5 eq.) were added to the reaction mixture. The suspension was heated for two days (bath temperature: 60 °C). After reaction completion, the mixture was allowed to cool to room temperature and residual sodium azide were removed by precipitation and centrifugation with 40 ml acetonitrile. The acetonitrile solution was given to 40 ml diethyl ether and centrifuged. The etheric supernatant was isolated and concentrated in vacuo. The co-evaporation with water lead to the removal of remaining DMF traces. The product **2** was isolated as a white solid (0.86 g, 4.21 mmol, 26% yield).

^{1}H NMR (300 MHz, D_2O) δ 4.04 (s, 2H, –CH_2N_3), 3.80 (s, 6H, –CH_2OH).

^{13}C NMR (75 MHz, D_2O) δ 170.49 (*C*=O), 62.16 (–*C*($CH_2OH)_3$), 60.17 (–C(*C*$H_2OH)_3$), 52.00 (–*C*H_2N_3).

RP-HPLC-MS: t_R = 1.39 min, relative purity not determined, linear gradient from 5 to 50 vol% acetonitrile in water (+ 0.1 vol% HCOOH) in 30 min.

MS (ESI) m/z calculated for M = $C_6H_{12}N_4O_4$: $[M+H]^+$ calcd. 205.1, found 205.2, $[M+Na]^+$ calcd. 227.1, found 227.0.

HRMS (ESI) m/z calculated for M = $C_6H_{12}N_4O_4$: $[M+H]^+$ calcd. 205.0931, found 205.0931.

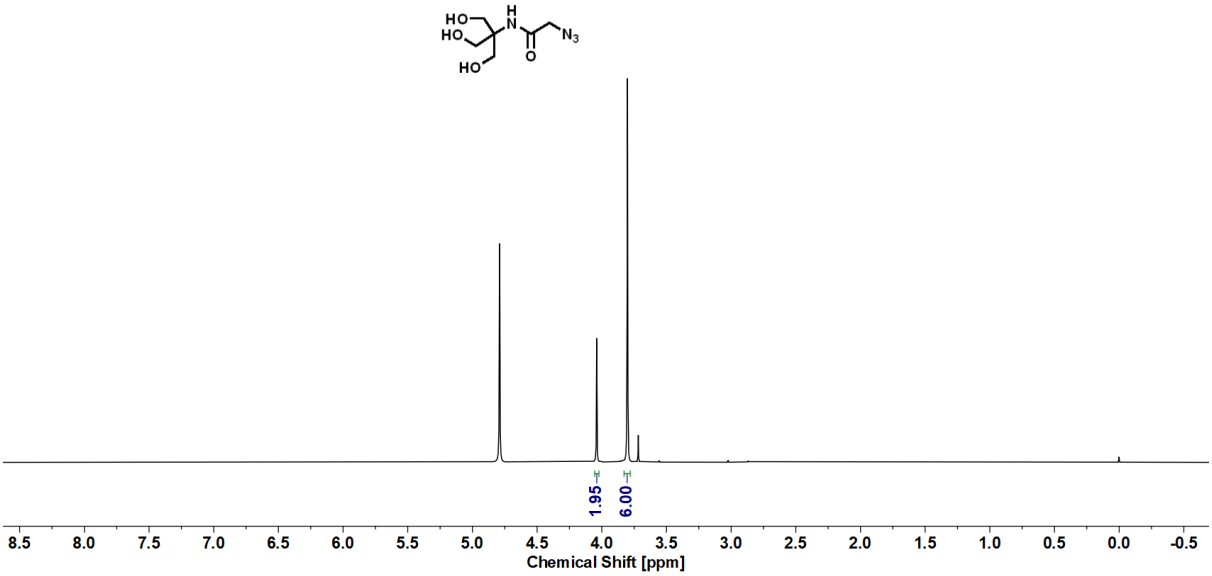


Figure S7: ^{1}H NMR spectrum of **2** (300 MHz, D_2O).

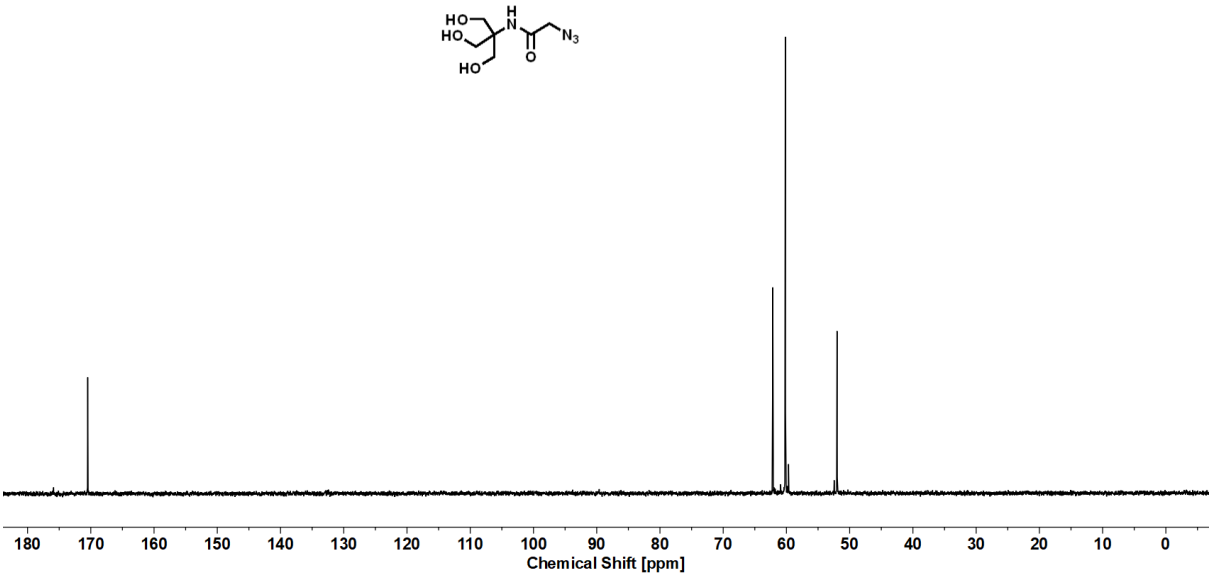


Figure S8: ^{13}C NMR spectrum of **2** (75 MHz, D_2O).

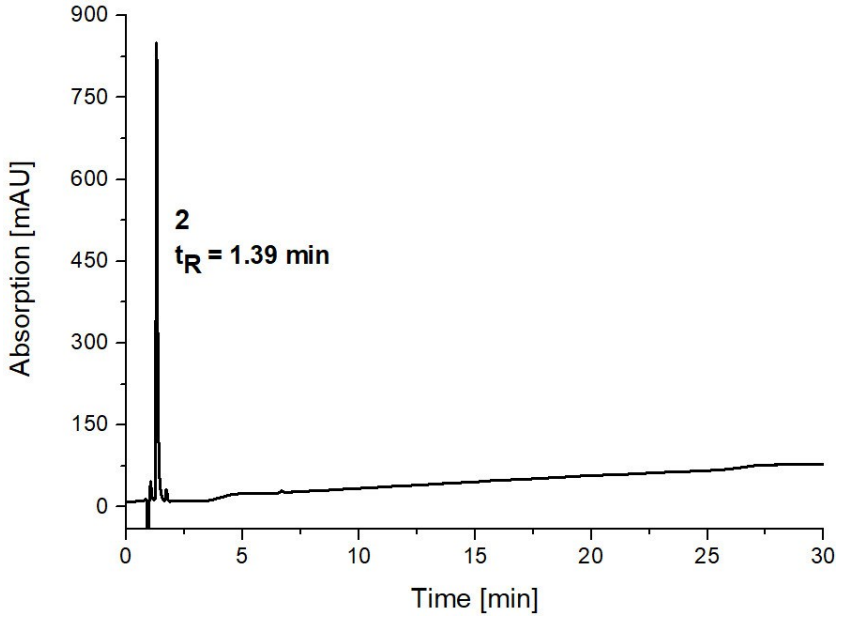


Figure S9: RP-HPLC chromatogram of **2**, linear gradient from 5 to 50 vol% acetonitrile in water (+ 0.1 vol% HCOOH) in 30 min.

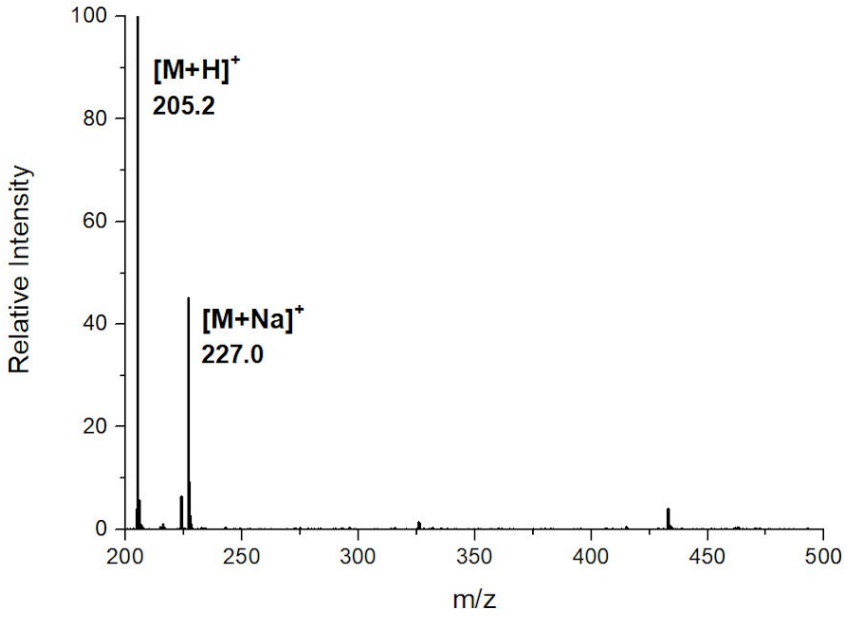


Figure S10: ESI-MS spectrum of **2** (positive mode).

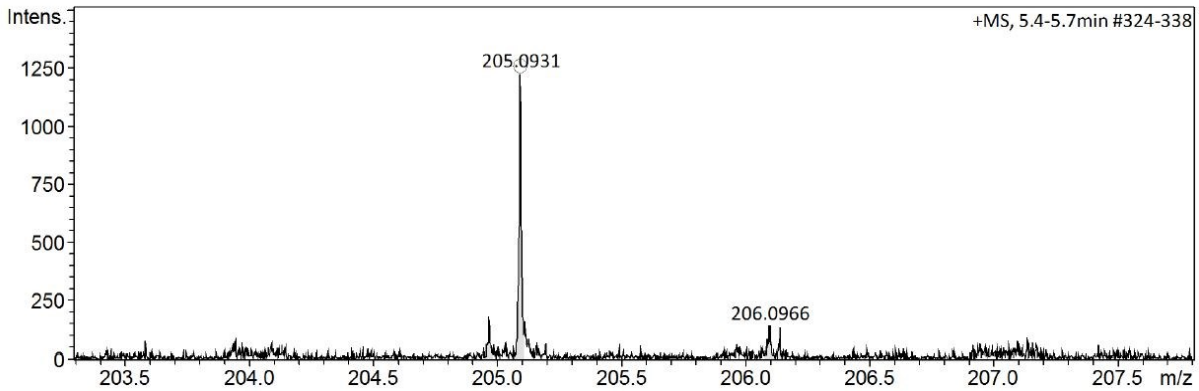
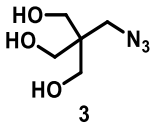


Figure S11: HR-ESI-MS NMR spectrum of **2** (positive mode).

2-(Azidomethyl)-2-(hydroxymethyl)propane-1,3-diol, **3**



3

2-(Bromomethyl)-2-(hydroxymethyl)propane-1,3-diol **S1** (1.27 g, 6.38 mmol, 1.0 eq.) was given to a 100 ml round bottom flask and were dissolved in 8 ml DMF according to previously published protocols.[617] 0.64 g sodium azide (9.84 mmol, 1.5 eq.) were added and the suspension was heated for 4 days (bath temperature : 90 °C). Then the reaction mixture was allowed to cool and 10 ml water were added. The mixture was slowly concentrated in vacuo to a volume of 2-3 ml and were purified using silica column chromatography. Therefore, a gradient of 10–20 vol% methanol in DCM was applied. Concentration lead to hygroscopic, viscous product **3** (0.76 g, 4.72 mmol, 74% yield, >95 % relative purity determined via NMR).

^{1}H NMR (300 MHz, D_2O) δ 3.56 (s, 6H, –CH_2OH), 3.43 (s, 2H, –CH_2N_3).
^{13}C NMR (75 MHz, D_2O) δ 60.84 (–CH$_2$OH), 50.89 (–CH$_2$N$_3$), 45.26 (C(CH$_2$R)$_4$).
MS (ESI) m/z calculated for M = $C_5H_{11}N_3O_3$: $[M+Na]^+$ calcd. 184.1, found 184.0, $[M-N_3+CN+H]^+$ calcd. 146.1, found 146.2.

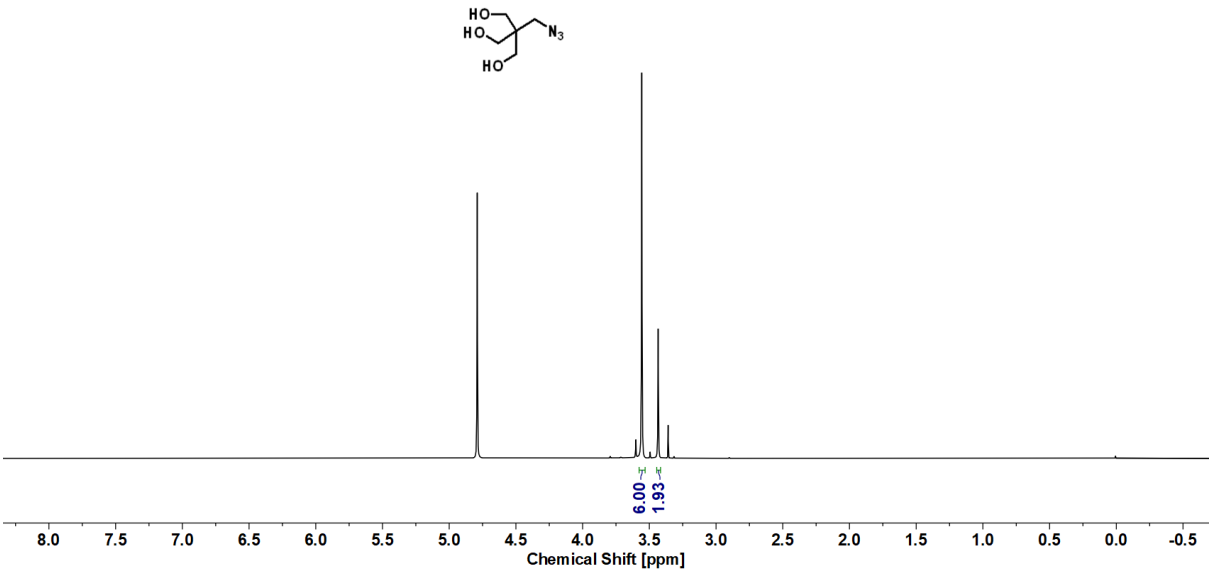


Figure S12: ^{1}H NMR spectrum of **3** (300 MHz, D_2O).

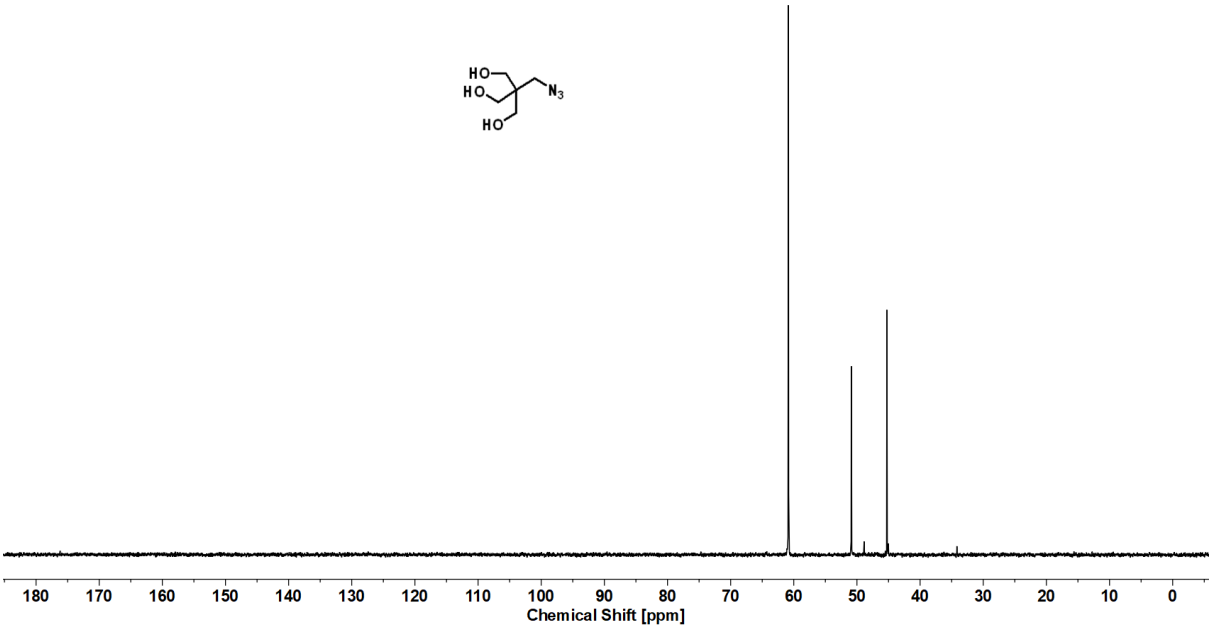


Figure S13: ^{13}C NMR spectrum of **3** (75 MHz, D_2O).

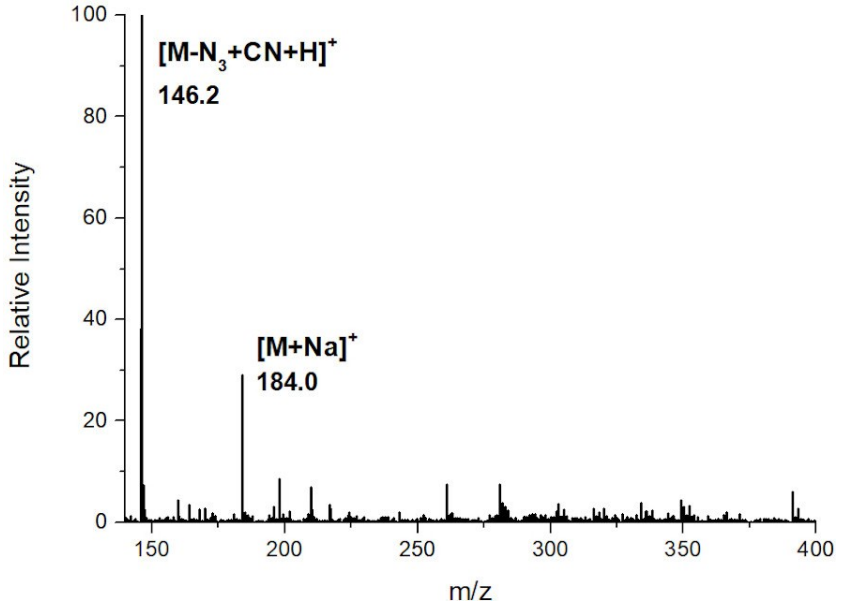
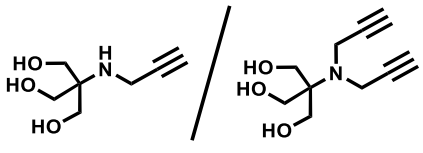


Figure S14: ESI-MS spectrum of **3** (positive mode).

N-[Tris(hydroxymethyl)methyl]-3-aminopropyne, mixture with *N*-[Tris(hydroxymethyl)methyl]-3,3-aminodipropyne, **4**



4

0.51 g Tris(hydroxymethyl)aminomethane (4.21 mmol, 1.0 eq.) were given to a round bottom flask and were suspended in 25 ml THF. 2 ml propargyl bromide solution (80% in toluene, 18.58 mmol, 4.4 eq.)

were added and the reaction mixture was heated under reflux conditions for 23 h (bath temperature: 80 °C). The mixture was concentrated in vacuo, were dissolved in THF/H_2O mixture (1:1) and were concentrated for three times. Lyophilization from an aqueous solution lead to a yellow solid. The crude product formed a mixture of mono- and dialkynylated Tris **4** and were used without further purification due to poor separation properties (0.81 g, $Ratio_{mono:di}$ = 70:30 (NMR), approx. 4.2 mmol, quant.).

^{1}H NMR (300 MHz, D_2O) δ 4.00 (s, 0.7H, –C≡C*H*$_{mono}$), 3.86 – 3.83 (m, 1.9H, –C*H*$_2$C≡CH$_{di}$ + impurity), 3.81 (s, 2.8H, –C*H*$_2$OH$_{di}$), 3.79 (s, 2.2H, –C*H*$_2$C≡CH$_{mono}$), 3.75 (s, 6.0H, –C*H*$_2$OH$_{mono}$), 2.69 (t, *J* = 2.2 Hz, 0.5H, –C≡C*H*$_{di}$).

^{13}C NMR (75 MHz, D_2O) δ 80.97 (–CH$_2$*C*≡CH$_{di}$), 73.86 (–CH$_2$*C*≡CH$_{mono}$), 65.22 (–CH$_2$C≡*C*H$_{di}$), 64.46 (–CH$_2$C≡*C*H$_{mono}$), 61.42 (–*C*(CH$_2$OH)$_{3,mono}$), 60.78 (–*C*(CH$_2$OH)$_{3,di}$), 59.34 (–C(*C*H$_2$OH)$_{3,mono}$), 58.60 (–C(*C*H$_2$OH)$_{3,di}$), 36.57 (–*C*H$_2$C≡CH$_{mono}$), 31.62 (–*C*H$_2$C≡CH$_{di}$).

RP-HPLC-MS: t_R = 0.74 min and 0.94 min, mixture of mono- and dialkynylated Tris, relative purity not determined, linear gradient from 5 to 95 vol% acetonitrile in water (+ 0.1 vol% HCOOH) in 15 min then 2 min isocratic at 95 vol% acetonitrile in water.

MS (ESI) m/z calculated for $M_{monoalkyne}$ = $C_7H_{13}NO_3$ and $M_{dialkyne}$ = $C_{10}H_{15}NO_3$: $[M_{monoalkyne}\text{-CHCCH+H}]^+$ calcd. 122.1, found 122.2, $[M_{monoalkyne}\text{-O}]^+$ calcd. 142.1, found 142.0, $[M_{monoalkyne}\text{+H}]^+$ calcd. 160.1, found 160.2, $[2M_{monoalkyne}\text{+H}]^+$ calcd. 319.2, found 319.0, $[M_{dialkyne}\text{-CHCCH+H}]^+$ calcd. 160.1, found 160.0, $[M_{dialkyne}\text{+H}]^+$ calcd. 198.1, found 198.0.

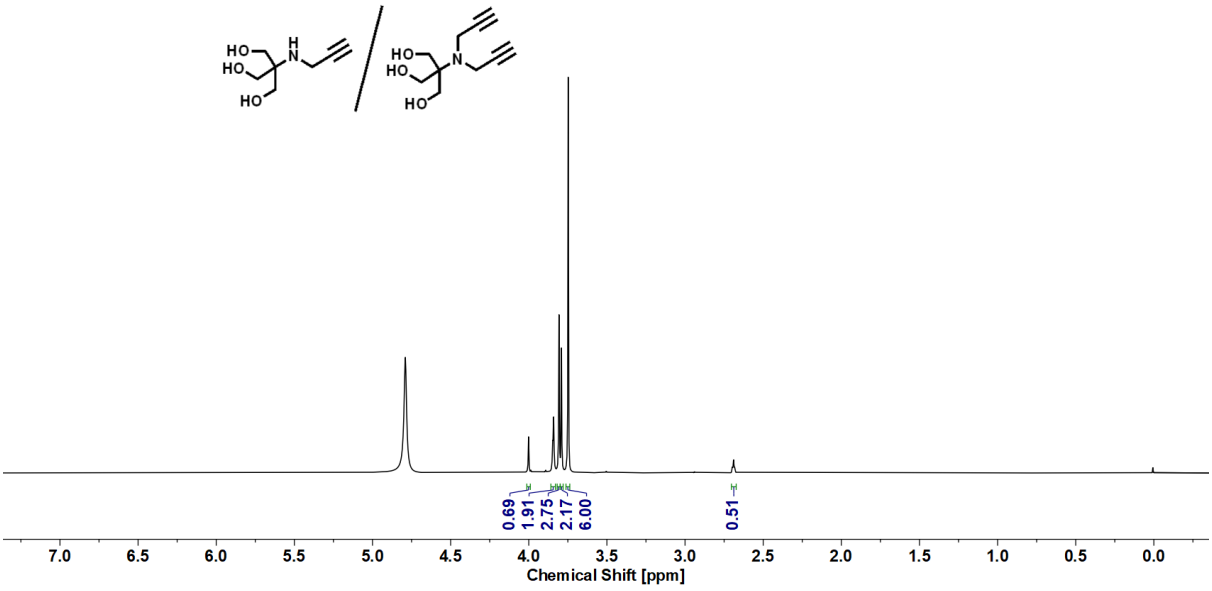


Figure S15: ^{1}H NMR spectrum of **4** (300 MHz, D_2O).

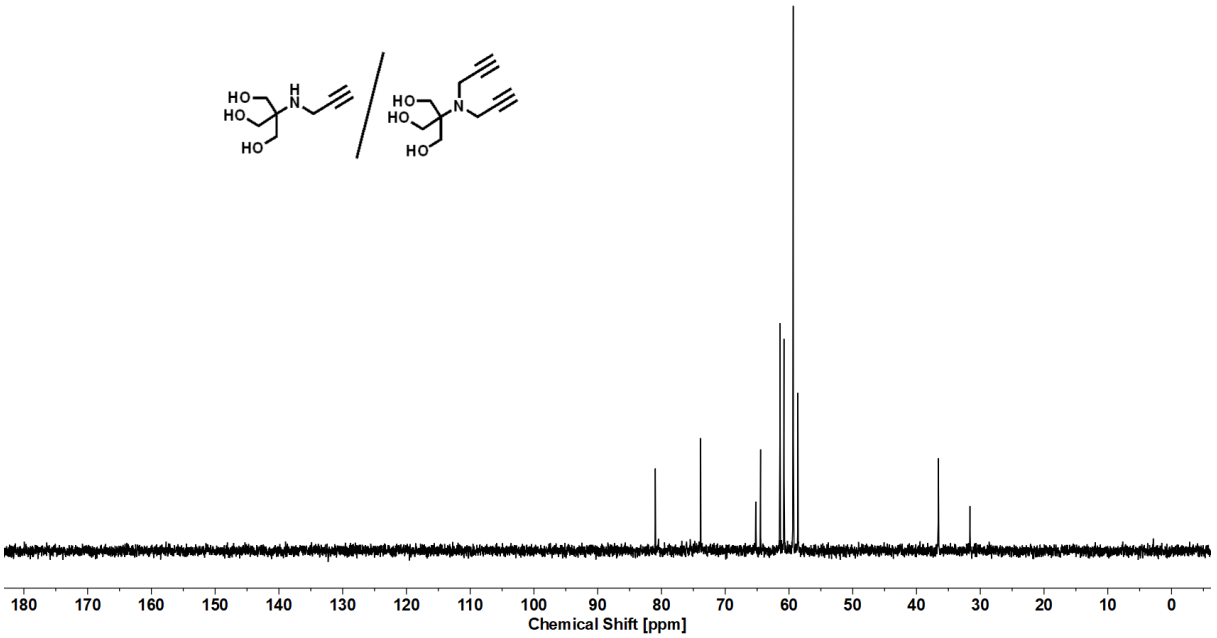


Figure S16: ^{13}C NMR spectrum of **4** (75 MHz, D_2O).

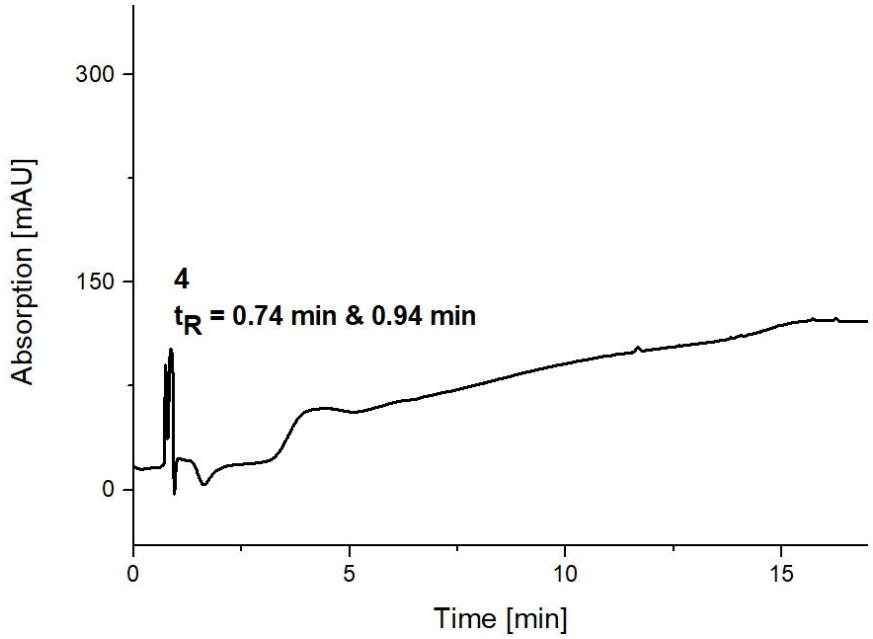


Figure S17: RP-HPLC chromatogram of **4**, linear gradient from 5 to 95 vol% acetonitrile in water (+ 0.1 vol% HCOOH) in 15 min then 2 min isocratic at 95 vol% acetonitrile in water.

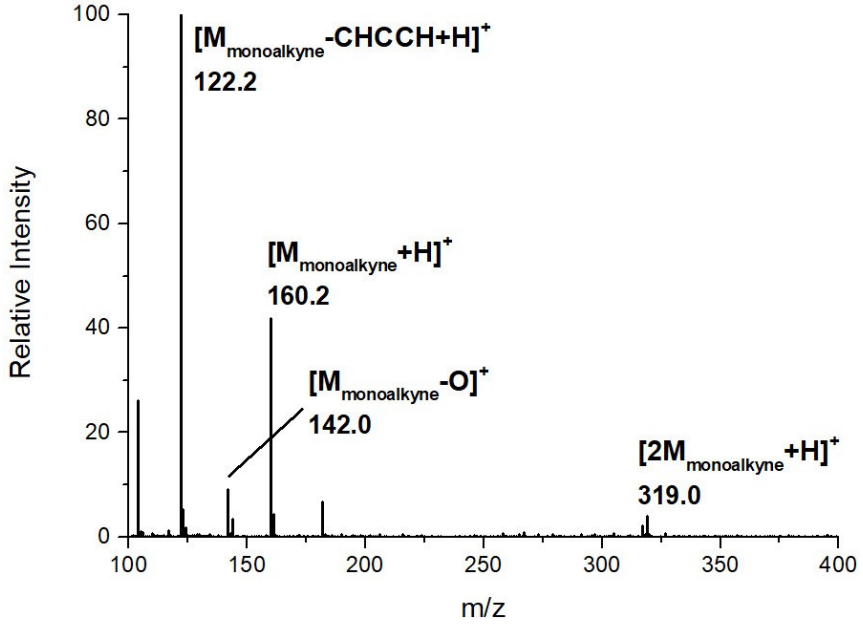


Figure S18: ESI-MS spectrum of monoalkynylated **4** (positive mode).

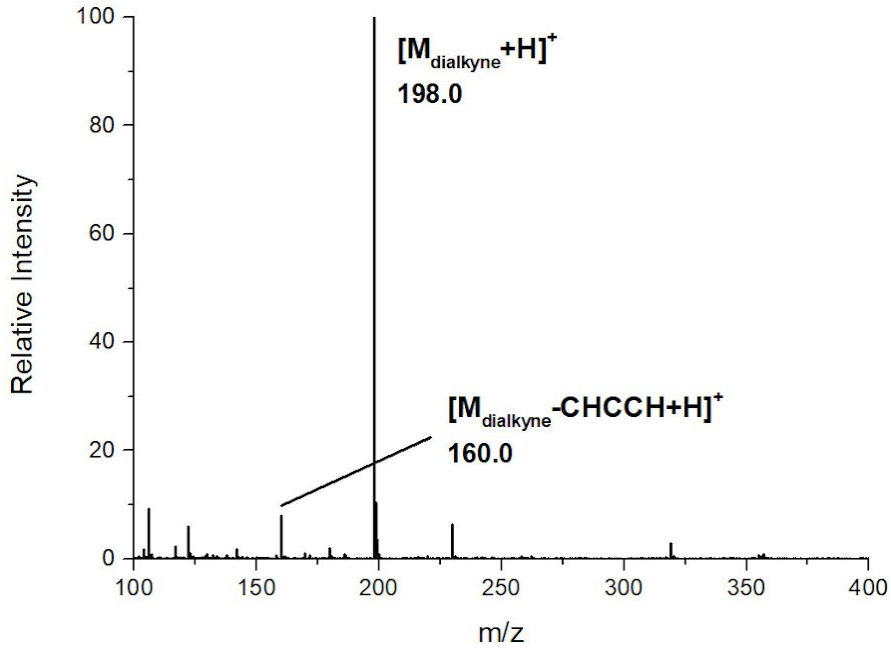
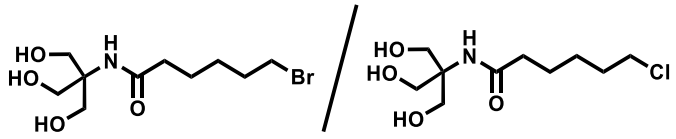


Figure S19: ESI-MS spectrum of dialkynylated **4** (positive mode).

6-Halo-*N*-(1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl)hexanamide, mixture of bromide and chloride derivative, **5**



5

2.31 g Tris buffer (19.07 mmol, 1.0 eq.) was suspended in 100 ml DMF and 5.1 ml triethylamine (3.72 g, 36.76 mmol, 1.9 eq.) was added. The mixture was cooled with an acetone/dry ice bath and 3 ml

6-bromohexanoylchloride (4.50 g, 21.08 mmol, 1.1 eq.) were added dropwise. The acetone bath was removed and the reaction was allowed to stir for 14 h. The solvent was removed in vacuo and the oily crude product was purified via silica chromatography using a gradient of 2-7% MeOH in DCM. The product **5** appears as a yellowish oil which is a mixture of brominated and chlorinated derivatives (1.41 g, 5.34 mmol, $Ratio_{Cl:Br}$ = 73:27 (HPLC), R_f = 0.41 at 10% MeOH in DCM, 28% yield).

^{1}H NMR (600 MHz, CD_3OD) δ 3.72 (s, 6.0H, –C*H*$_2$OH), 3.57 (t, *J* = 6.6 Hz, 1.5H, –CH$_2$C*H*$_2$Cl), 3.45 (t, *J* = 6.7 Hz, 0.5H, –CH$_2$C*H*$_2$Br), 3.22 (q, *J* = 7.3 Hz, 2.5H, EtOH impurity), 2.27 (t, *J* = 7.5 Hz, 2H, –CH$_2$CH$_2$C*H*$_2$C=O), 1.90 – 1.84 (m, 0.5H, –C*H*$_2$CH$_2$Br), 1.82 – 1.76 (m, 1.5H, –C*H*$_2$CH$_2$Cl), 1.67 – 1.60 (m, 2.0H, –CH$_2$C*H*$_2$CH$_2$C=O), 1.52 – 1.46 (m, 2.0H, –C*H*$_2$CH$_2$CH$_2$C=O), 1.32 (t, *J* = 7.3 Hz, 4.0H, EtOH impurity).

RP-HPLC-MS: t_R = 5.34 min and 5.84 min, mixture of halogenides (73% chloride, 27% bromide), linear gradient from 5 to 95 vol% acetonitrile in water (+ 0.1 vol% HCOOH) in 15 min then 2 min isocratic at 95 vol% acetonitrile in water.

MS (ESI) m/z calculated for M_{Br} = $C_{10}H_{20}BrNO_4$ and M_{Cl} = $C_{10}H_{20}ClNO_4$: $[M_{Cl}\text{-}O]^+$ calcd. 236.1, found 236.0, $[M_{Cl}+H]^+$ calcd. 254.1, found 254.0, $[M_{Cl}+Na]^+$ calcd. 276.1, found 276.0, $[M_{Br}\text{-}O]^+$ calcd. 280.1, found 280.0, $[M_{Br}+H]^+$ calcd. 298.1, found 298.0, $[M_{Br}+Na]^+$ calcd. 320.1, found 320.0.

HRMS (ESI) m/z calculated for M_{Br} = $C_{10}H_{20}BrNO_4$ and M_{Cl} = $C_{10}H_{20}ClNO_4$: $[M_{Br}+H]^+$ calcd. 298.0648, found 298.0650, $[M_{Cl}+H]^+$ calcd. 254.1154, found 254.1153, $[M_{Cl}+Na]^+$ calcd. 276.0973, found 276.0973.

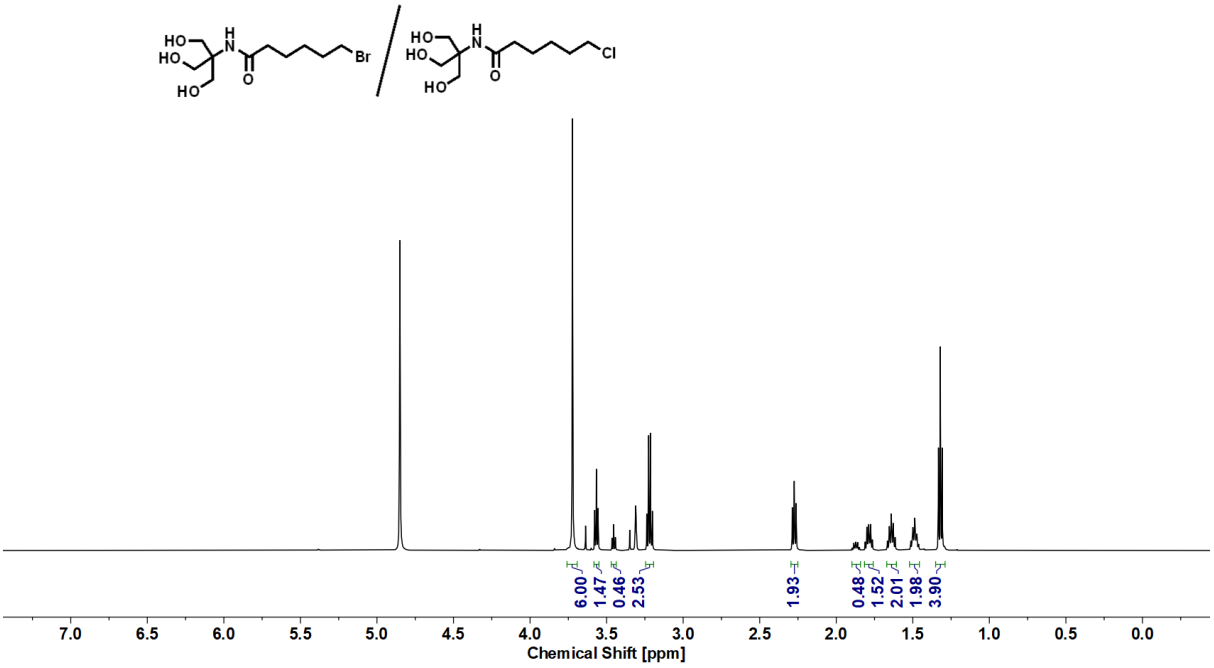


Figure S20: ^{1}H NMR spectrum of **5** (600 MHz, CD_3OD).

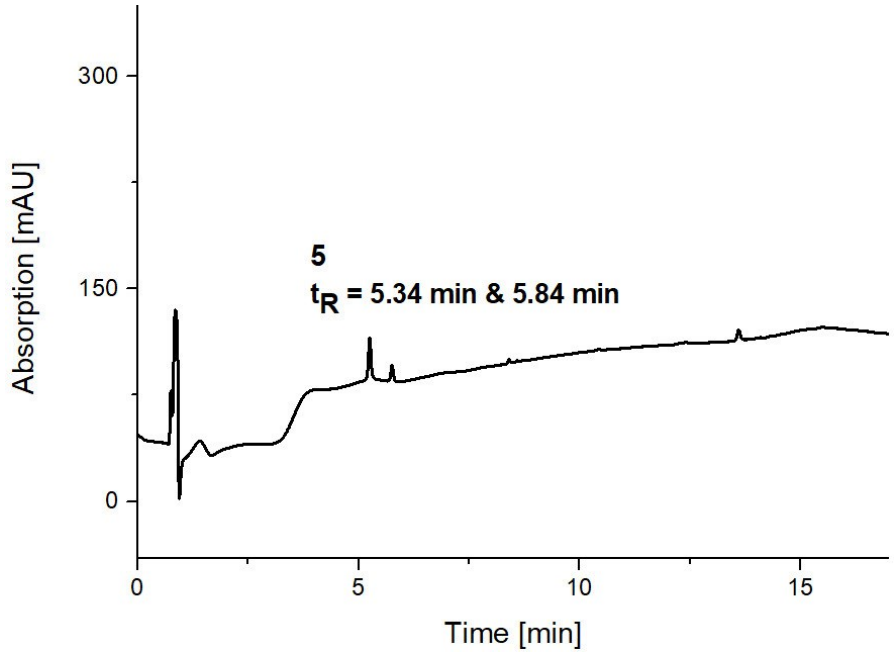


Figure S21: RP-HPLC chromatogram of **5**, linear gradient from 5 to 95 vol% acetonitrile in water (+ 0.1 vol% HCOOH) in 15 min then 2 min isocratic at 95 vol% acetonitrile in water.

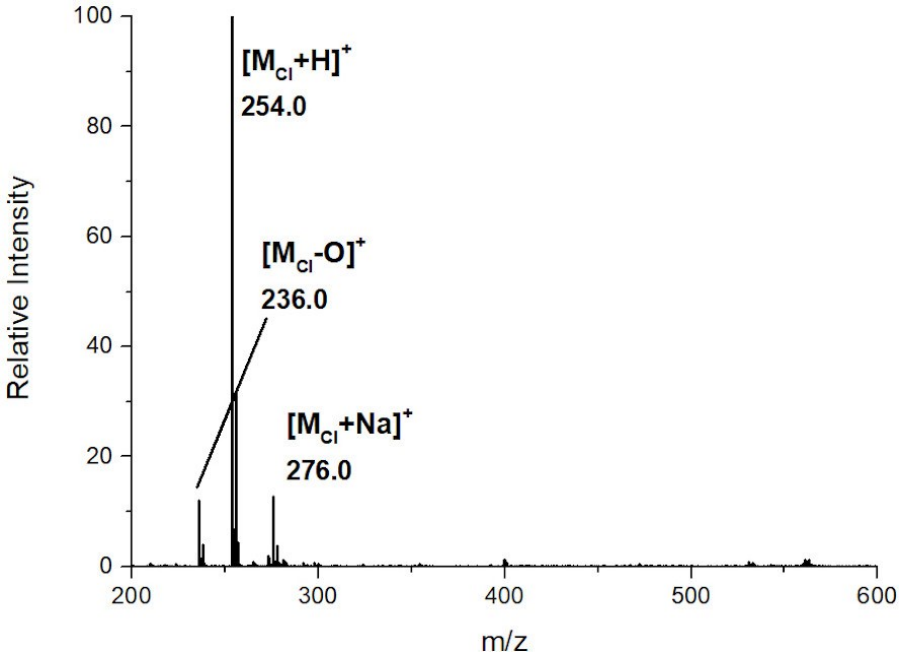


Figure S22: ESI-MS spectrum of chlorinated **5** (positive mode).

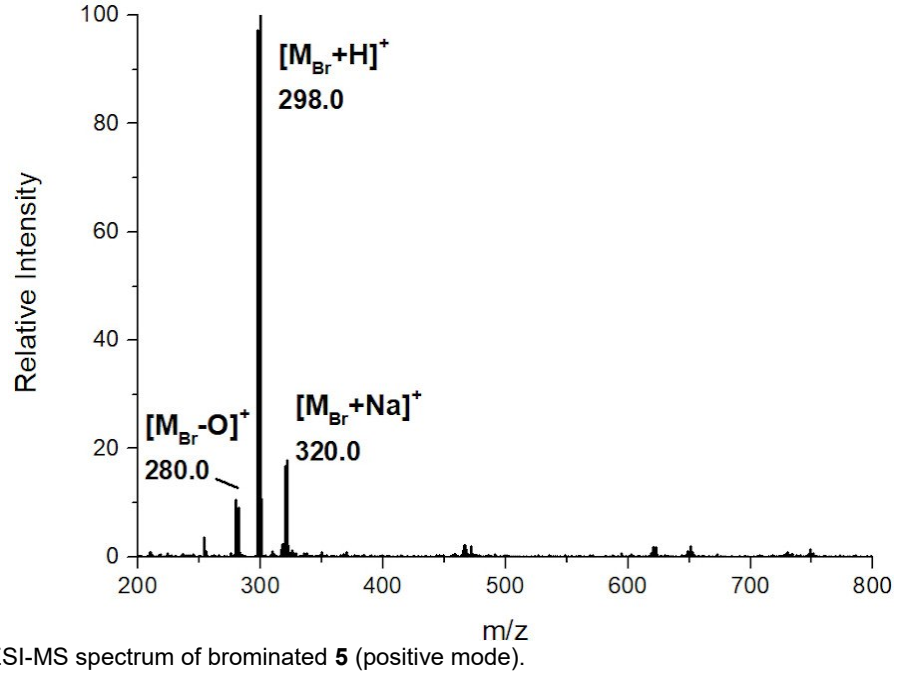


Figure S23: ESI-MS spectrum of brominated **5** (positive mode).

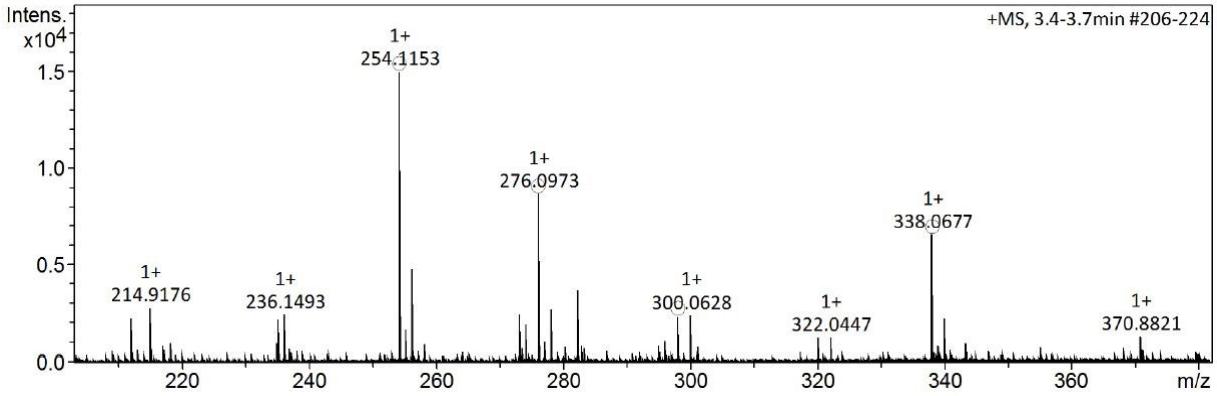
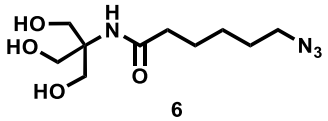


Figure S24: HR-ESI-MS NMR spectrum of **5** (positive mode).

6-Azido-*N*-(1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl)hexanamide, **6**



6

The halogenated Tris derivative mixture **5** (1.41 g, 5.34 mmol, 1.0 eq.) was dissolved in 10 ml DMF and 1.79 g sodium azide (27.53 mmol, 5.1 eq.) were added to the reaction mixture. The suspension was heated for 27 h (bath temperature: 80 °C). After reaction completion, the mixture was allowed to cool to room temperature and directly loaded to a silica column. The crude product was purified using a 5-7% MeOH in DCM gradient. The water soluble product **6** appears was lyophilized and appears as a slightly yellow solid (0.99 g, 3.79 mmol, R_f = 0.26 at 5% MeOH in DCM, 71% yield) which requires heat (bath temperature: 60 °C) for dissolving in water.

^{1}H NMR (600 MHz, CD_3OD) δ 8.00 (s, DMF impurity), 3.74 (s, 6H, –CH_2OH), 3.35 – 3.29 (m, 2.5H, –$CH_2CH_2N_3$ overlap with CD_3OD signal), 3.02 (s, DMF impurity), 2.88 (d, J = 0.7 Hz, DMF impurity), 2.30 (t, J = 7.5 Hz, 2H, –$CH_2CH_2CH_2$C=O), 1.70 – 1.60 (m, 4H, –$CH_2CH_2CH_2$C=O & –C$H_2CH_2N_3$), 1.48 – 1.41 (m, 2H, –C$H_2CH_2CH_2$C=O).

^{13}C NMR (151 MHz, CD_3OD) δ 177.10 (C=O), 63.55 (–C($CH_2OH)_3$), 62.63 (–C($CH_2OH)_3$), 52.28 (–$CH_2CH_2N_3$), 37.27 (–$CH_2CH_2CH_2$C=O), 29.61 (–$CH_2CH_2N_3$), 27.28 (–$CH_2CH_2CH_2$C=O), 26.41 (–$CH_2CH_2CH_2$C=O).

RP-HPLC-MS: t_R = 6.75 min, >98% relative purity, linear gradient from 5 to 50 vol% acetonitrile in water (+ 0.1 vol% HCOOH) in 30 min.

MS (ESI) m/z calculated for M = $C_{10}H_{20}N_4O_4$: $[M\text{-}O]^+$ calcd. 243.2, found 243.2, $[M+H]^+$ calcd. 261.2, found 261.1, $[M+Na]^+$ calcd. 283.1, found 283.1.

HRMS (ESI) m/z calculated for M = $C_{10}H_{20}N_4O_4$: $[M+H]^+$ calcd. 261.1557, found 261.1558.

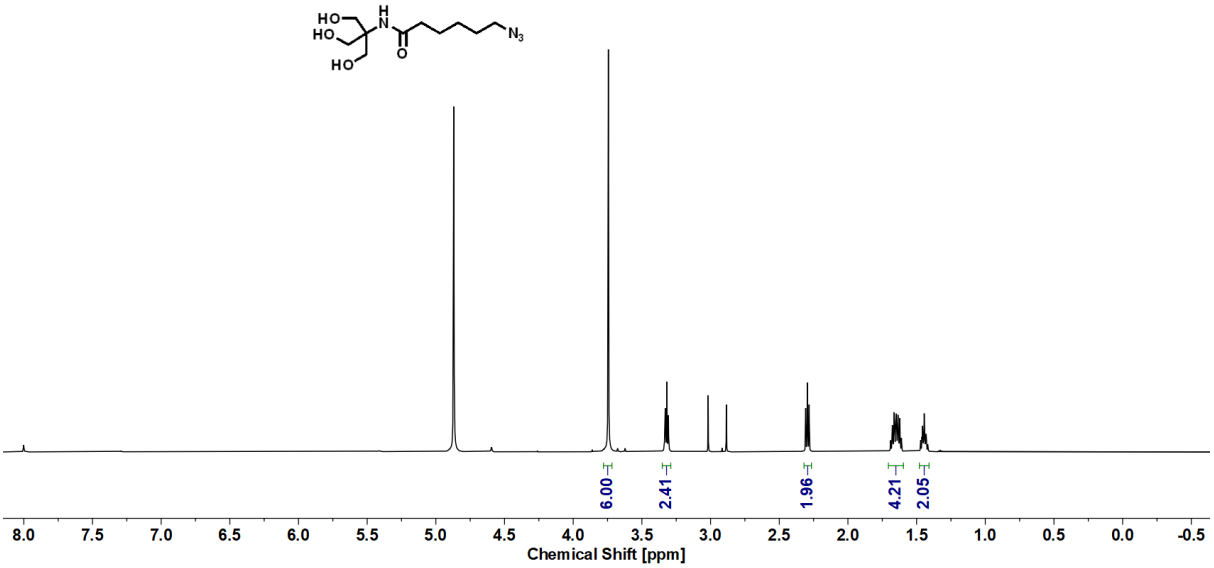


Figure S25: ^{1}H NMR spectrum of **6** (600 MHz, CD_3OD).

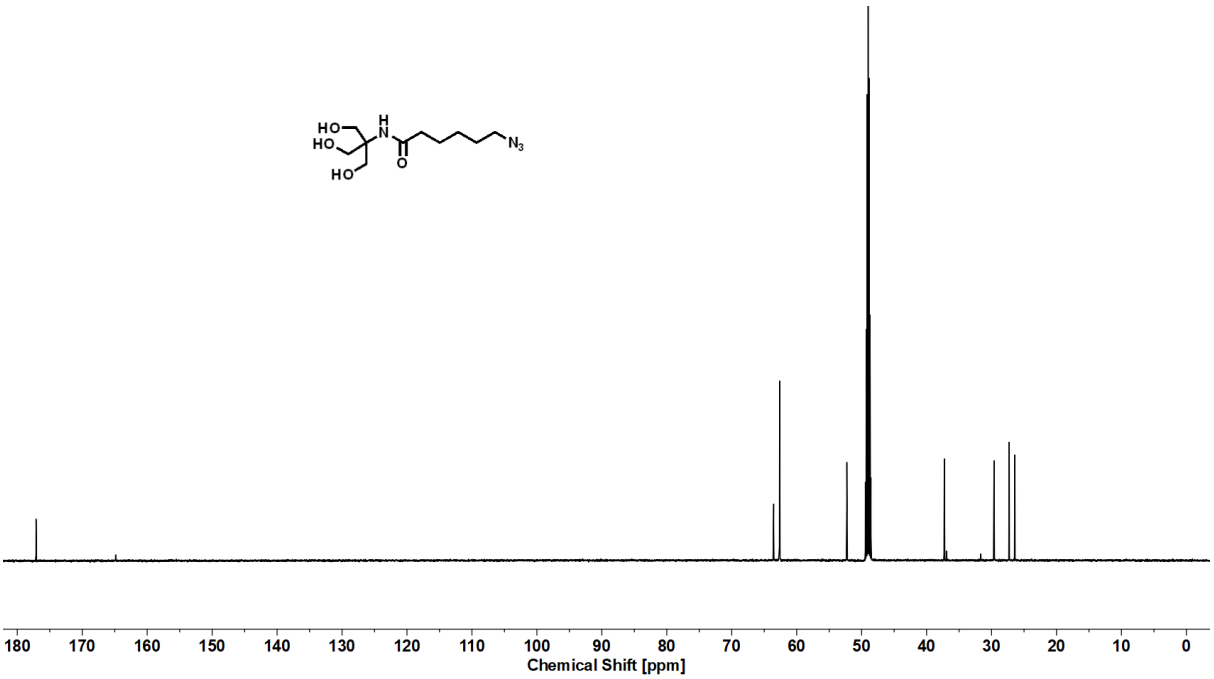


Figure S26: ^{13}C NMR spectrum of **6** (151 MHz, CD_3OD).

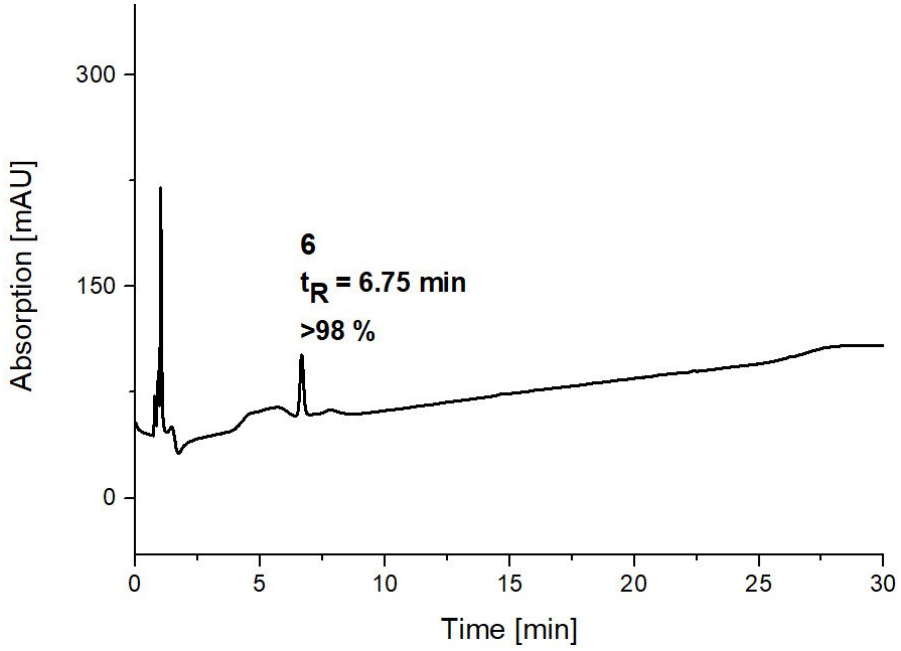


Figure S27: RP-HPLC chromatogram of **6**, linear gradient from 5 to 50 vol% acetonitrile in water (+ 0.1 vol% HCOOH) in 30 min.

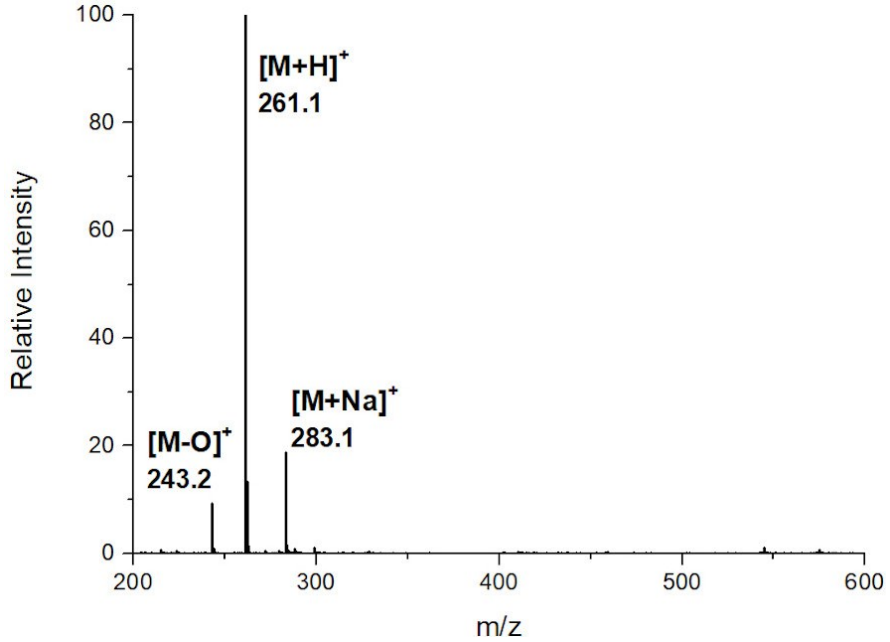


Figure S28: ESI-MS spectrum of **6** (positive mode).

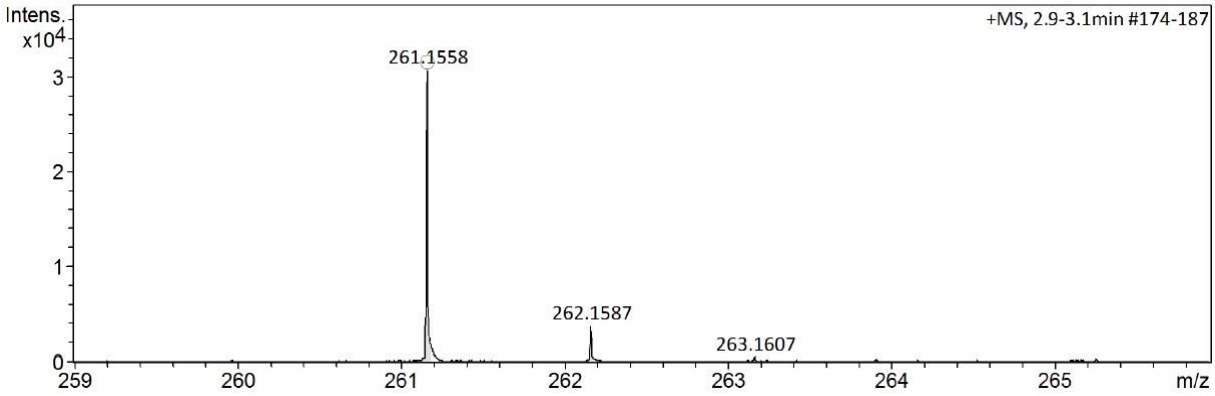
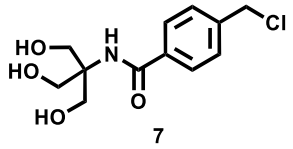


Figure S29: HR-ESI-MS NMR spectrum of **6** (positive mode).

4-(Chloromethyl)-*N*-(1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl)benzamide, **7**



7

4.21 g Tris buffer (34.75 mmol, 1.0 eq.) was given into a two-necked round bottom flask and then suspended in 40 ml DMF. Then 9.2 ml triethylamine (6.72 g, 66.4 mmol, 1.9 eq.) was added and the suspension turned into a solution. The solution was flushed with nitrogen for 30 min and cooled with an acetone/dry ice bath. A solution of 6.57 g 4-(chloromethyl)benzoylchloride (34.8 mmol, 1.0 eq.) in 15 ml DMF was added via a dropping funnel, the acetone bath was removed and the reaction mixture was allowed to stir for 20 h. The solution was transferred into a round bottom flask with the help of methanol and the solvent was removed in vacuo. The crude product was purified via silica chromatography using a gradient of 4-10% MeOH in DCM. The product **7** appeared as a colorless oil (0.55 g, 2.01 mmol, R_f = 0.28 at 5% MeOH in DCM, 6% yield).

^{1}H NMR (600 MHz, CD_3OD) δ 7.82 – 7.78 (m, 2H, C*H*$_{arom}$), 7.52 – 7.48 (m, 2H, C*H*$_{arom}$), 4.68 (s, 2H, –C*H*$_2$Cl), 3.86 (s, 6H, –C*H*$_2$OH).
^{13}C NMR (151 MHz, CD_3OD) δ 170.46 (*C*=O), 142.91 (*C*$_{arom}$), 136.01 (*C*$_{arom}$), 129.74 (*C*H$_{arom}$), 128.74 (*C*H$_{arom}$), 63.88 (–*C*(CH$_2$OH)$_3$), 62.50 (–C(*C*H$_2$OH)$_3$), 46.02 (–*C*H$_2$Cl).
RP-HPLC-MS: t_R = 6.08 min, 74% relative purity, linear gradient from 5 to 95 vol% acetonitrile in water (+ 0.1 vol% HCOOH) in 15 min then 2 min isocratic at 95 vol% acetonitrile in water.
MS (ESI) m/z calculated for M = $C_{12}H_{16}ClNO_4$: $[M\text{-}O]^+$ calcd. 256.1, found 256.0, $[M+H]^+$ calcd. 274.1, found 274.0, $[M+Na]^+$ calcd. 296.1, found 296.0.
HRMS (ESI) m/z calculated for M = $C_{12}H_{16}ClNO_4$: $[M+H]^+$ calcd. 274.0841, found 274.0845.

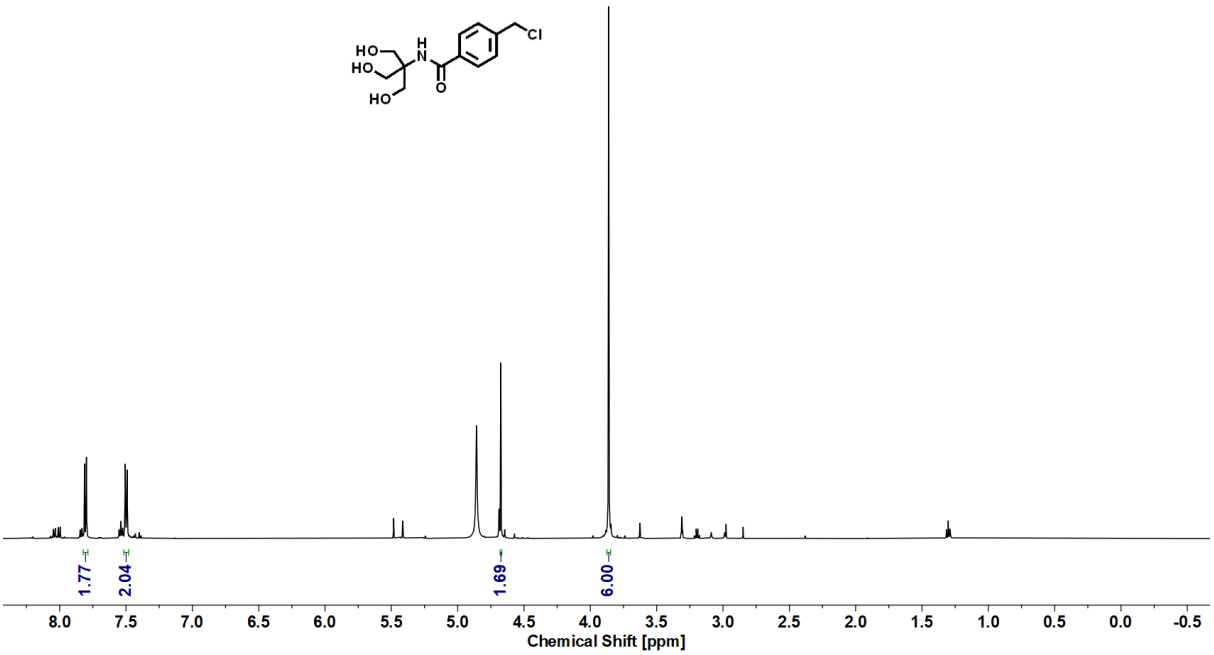


Figure S30: ^{1}H NMR spectrum of **7** (600 MHz, CD_3OD).

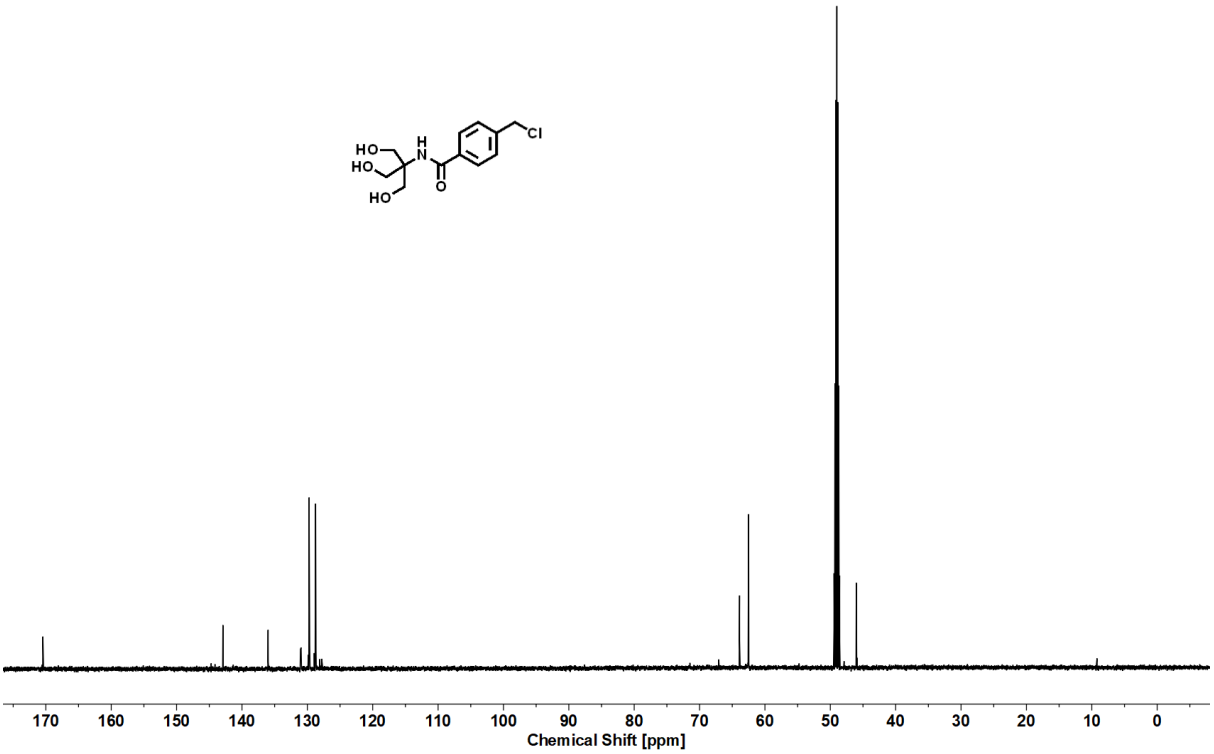


Figure S31: ^{13}C NMR spectrum of **7** (151 MHz, CD_3OD).

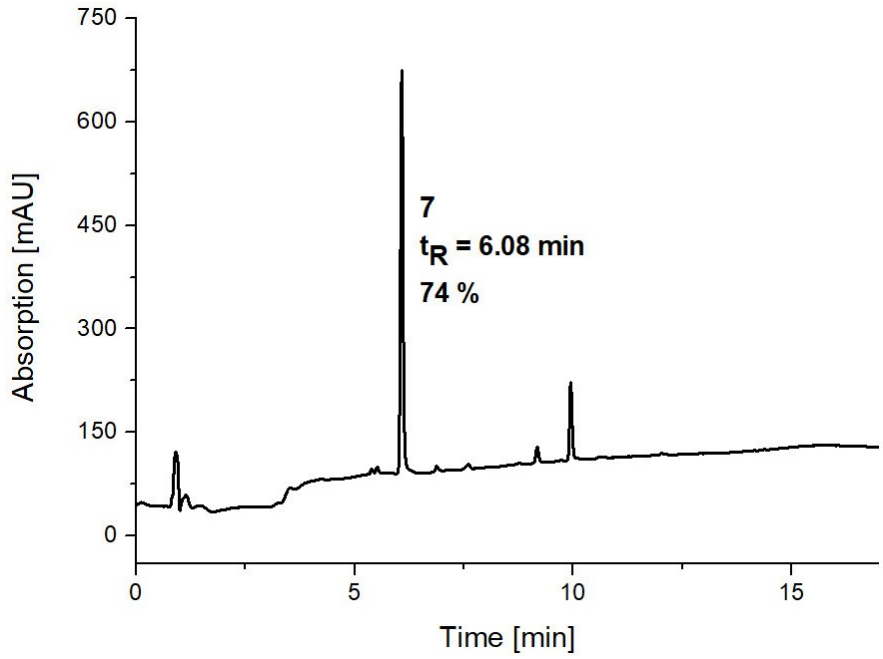


Figure S32: RP-HPLC chromatogram of **7**, linear gradient from 5 to 95 vol% acetonitrile in water (+ 0.1 vol% HCOOH) in 15 min then 2 min isocratic at 95 vol% acetonitrile in water.

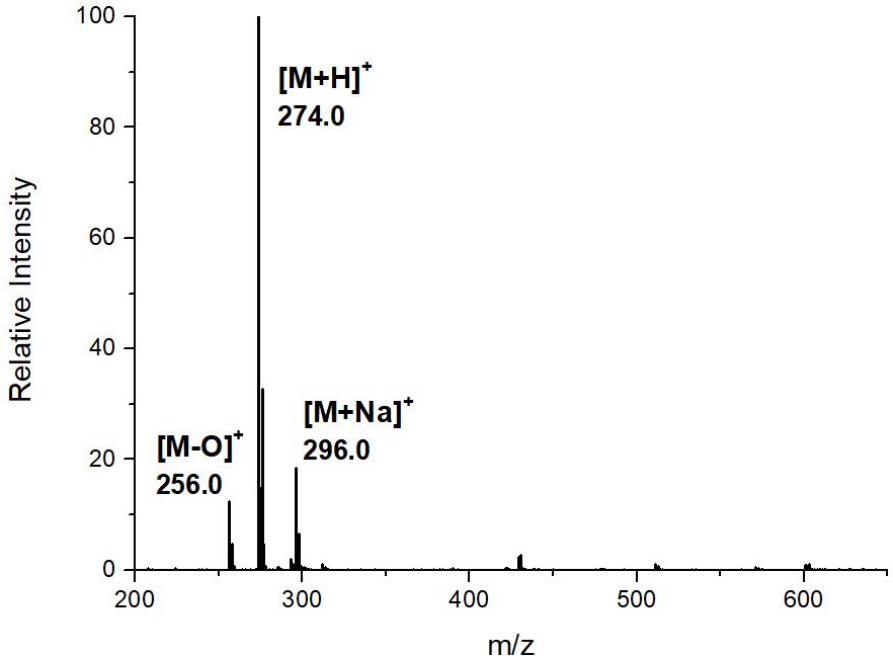


Figure S33: ESI-MS spectrum of **7** (positive mode).

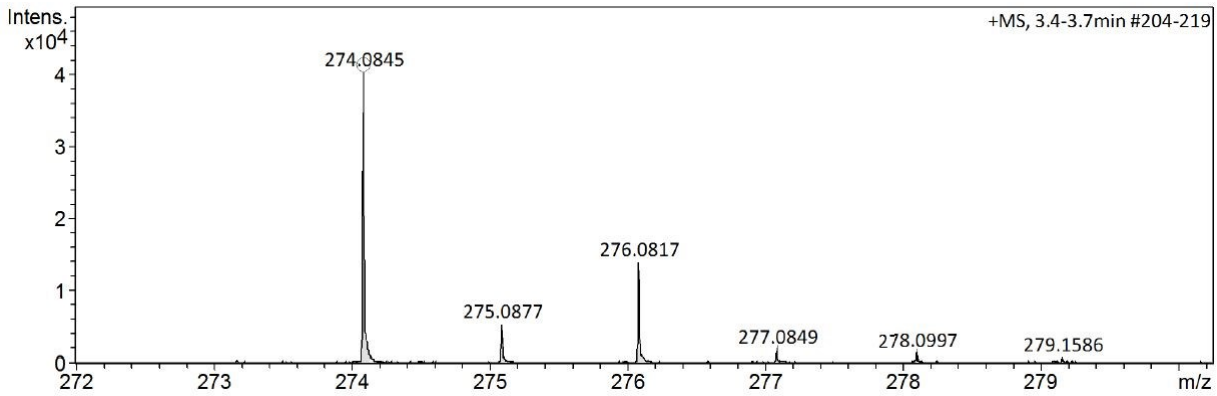
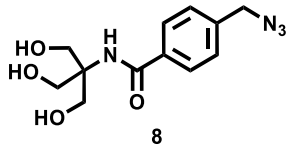


Figure S34: HR-ESI-MS NMR spectrum of **7** (positive mode).

4-(Azidomethyl)-*N*-(1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl)benzamide, **8**



8

The chlorinated Tris derivative **7** (1.38 g, 5.04 mmol, 1.0 eq.) was dissolved in 10 ml DMF and 1.64 g sodium azide (25.23 mmol, 5.0 eq.) were added to the reaction mixture. The suspension was heated for 21 h (bath temperature: 80 °C). After reaction completion, the mixture was allowed to cool to room temperature and the solvent amount was reduced to a total volume of ca. 5 ml (bath temperature: 50 °C). The residual sodium azide was precipitated with 30 ml acetonitrile and was removed by centrifugation. The acetonitrile/DMF solution was concentrated in vacuo and the crude product was purified via flash chromatography using a gradient of 3-7 % MeOH in DCM. The purified product **8** appeared as a colorless oil (0.65 g, 2.32 mmol, 46% yield) which requires heat (bath temperature: 60 °C) for dissolving in water.

^{1}H NMR (300 MHz, CD_3OD) δ 7.88 – 7.79 (m, 2H, CH_{arom}), 7.48 – 7.40 (m, 2H, CH_{arom}), 4.43 (s, 2H, –CH_2N_3), 3.86 (s, 6H, –CH_2OH).

13C NMR

^{13}C NMR (75 MHz, CD_3OD) δ 170.54 (*C*=O), 140.92 (*C*$_{arom}$), 135.93 (*C*$_{arom}$), 129.30 (*C*H$_{arom}$), 128.82 (*C*H$_{arom}$), 63.87 (–*C*(CH$_2$OH)$_3$), 62.54 (–C(*C*H$_2$OH)$_3$), 54.94 (–*C*H$_2$N$_3$).

RP-HPLC-MS: t_R = 9.48 min, 82% relative purity, linear gradient from 5 to 50 vol% acetonitrile in water (+ 0.1 vol% HCOOH) in 30 min.

MS (ESI) m/z calculated for M = $C_{12}H_{16}N_4O_4$: $[M\text{-}O]^+$ calcd. 263.1, found 263.1, $[M+H]^+$ calcd. 281.1, found 281.0, $[M+Na]^+$ calcd. 303.1, found 303.0.

HRMS (ESI) m/z calculated for M = $C_{12}H_{16}N_4O_4$: $[M+H]^+$ calcd. 281.1244, found 281.1248.

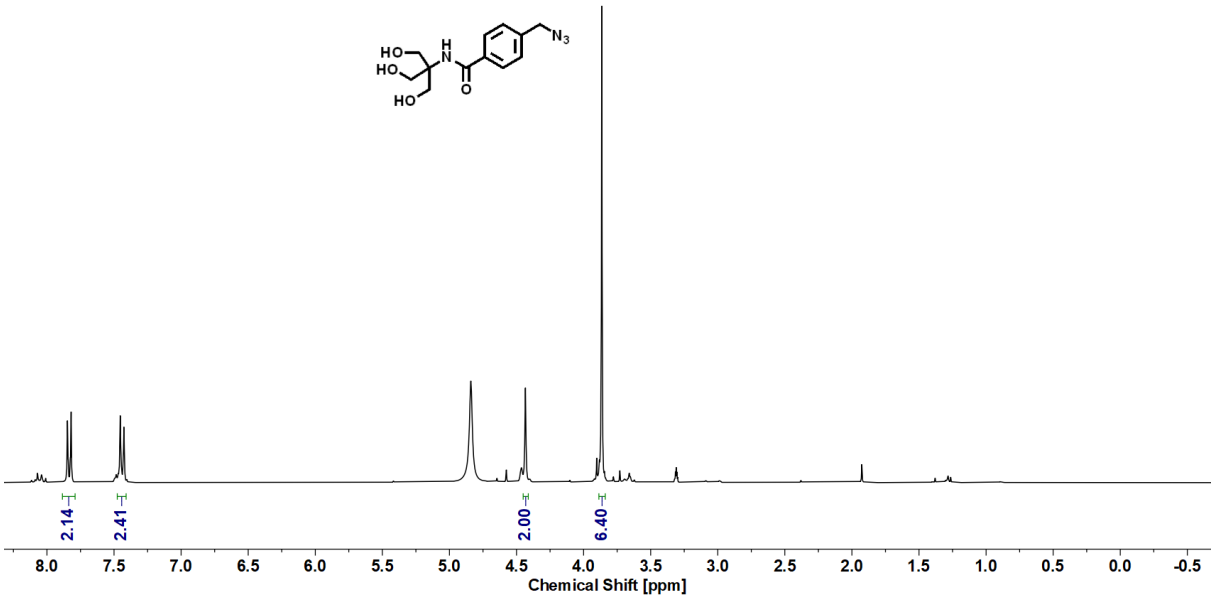


Figure S35: ^{1}H NMR spectrum of **8** (300 MHz, CD_3OD).

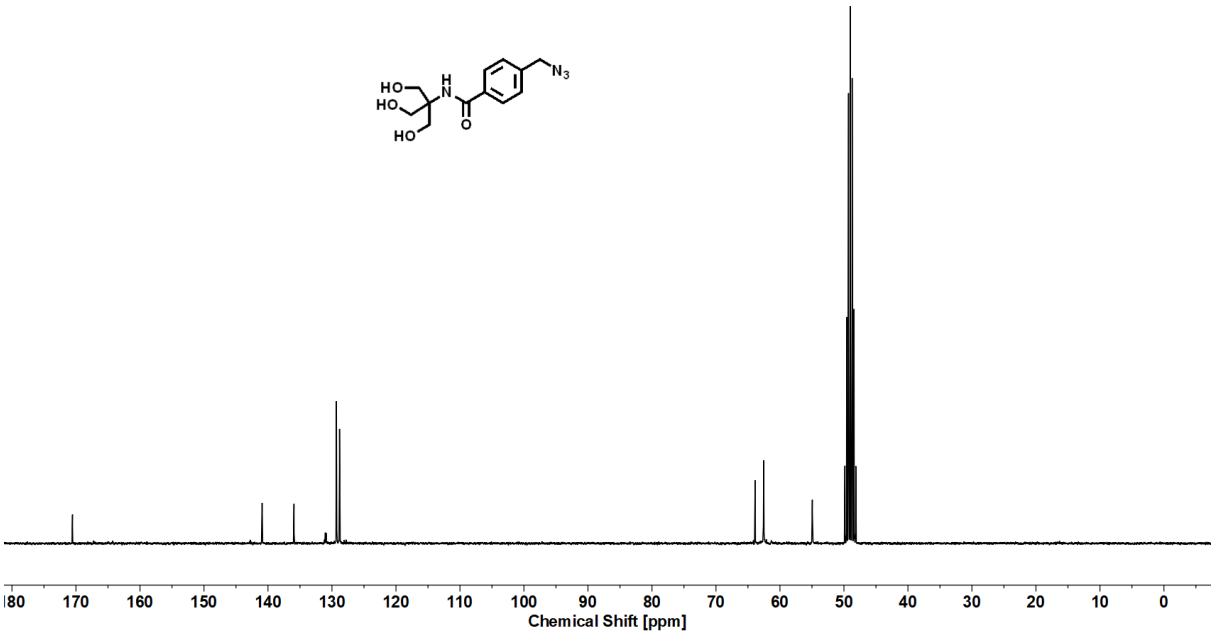


Figure S36: ^{13}C NMR spectrum of **8** (75 MHz, CD_3OD).

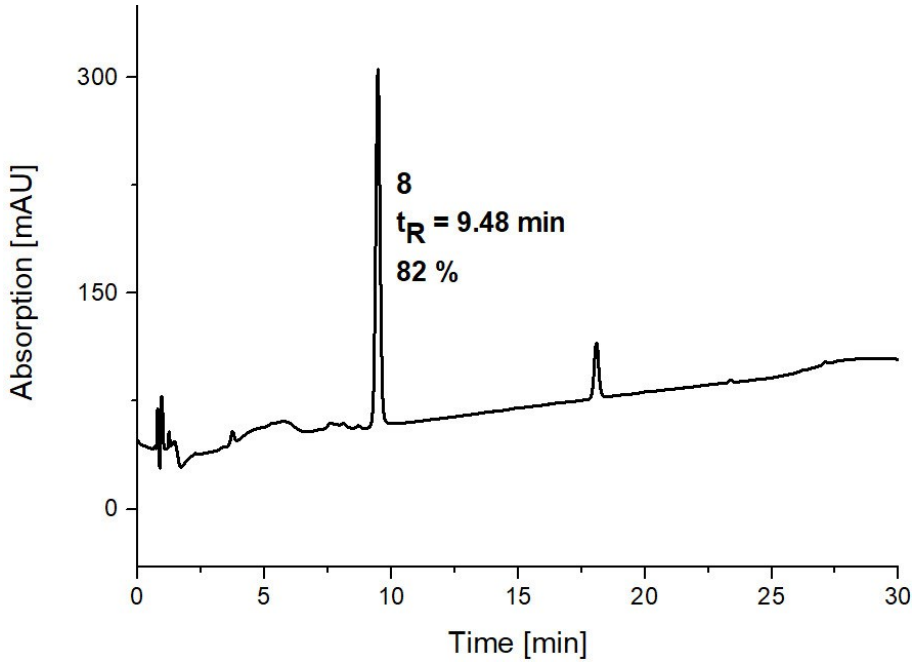


Figure S37: RP-HPLC chromatogram of **8**, linear gradient from 5 to 50 vol% acetonitrile in water (+ 0.1 vol% HCOOH) in 30 min.

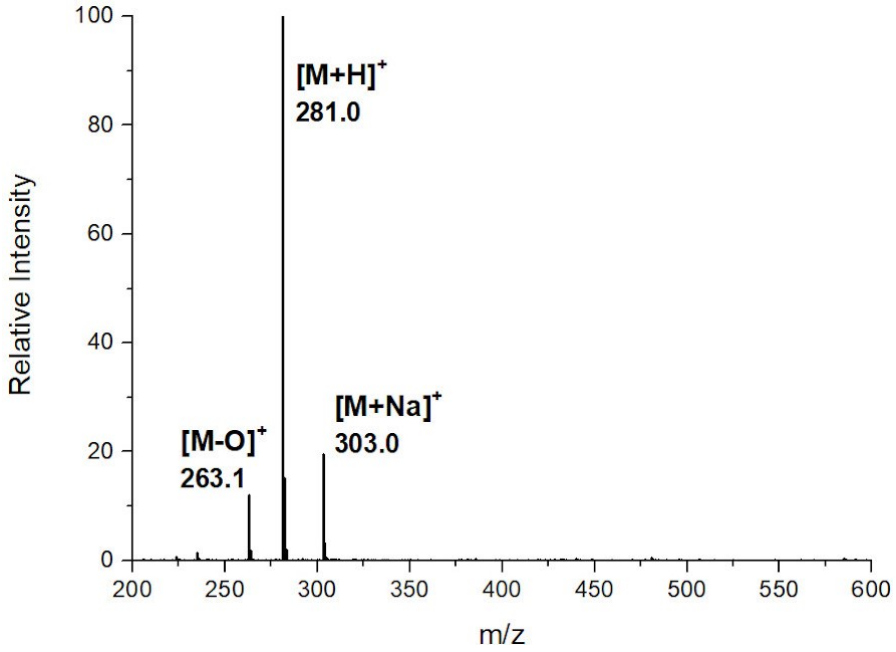


Figure S38: ESI-MS spectrum of **8** (positive mode).

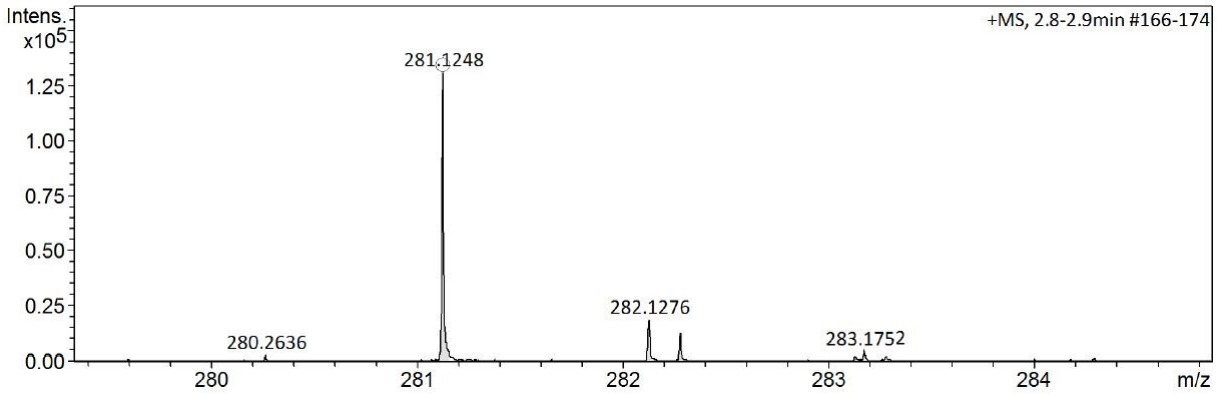
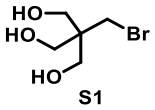


Figure S39: HR-ESI-MS NMR spectrum of **8** (positive mode).

Commercial substrates and reagents

2-(Bromomethyl)-2-(hydroxymethyl)propane-1,3-diol, **S1**



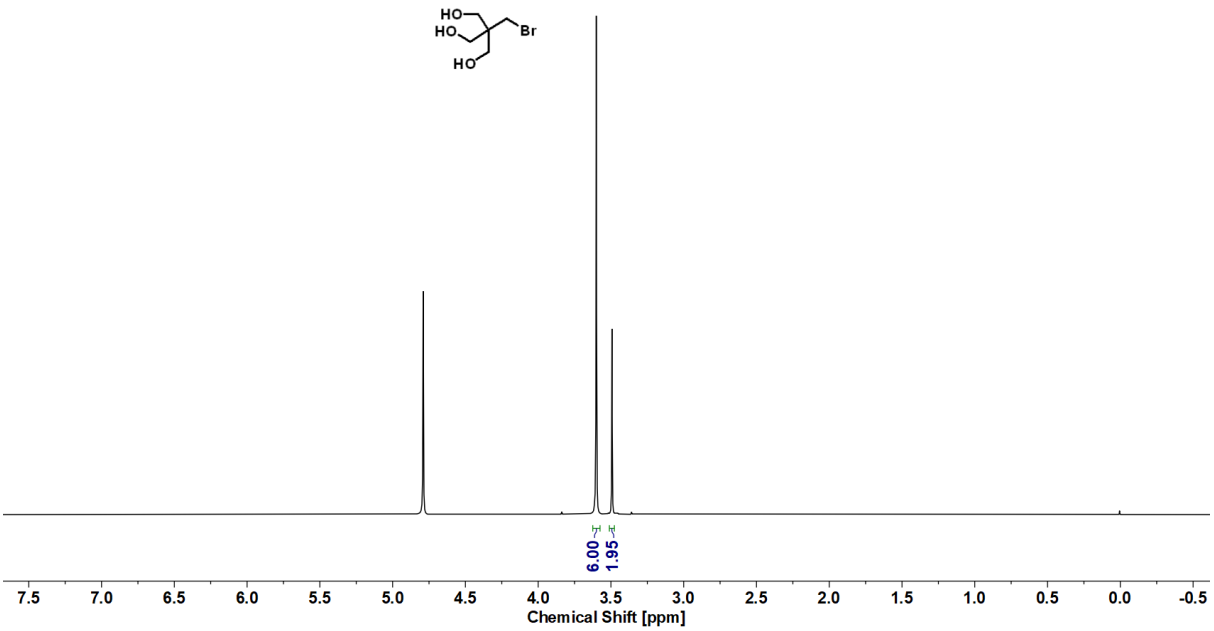
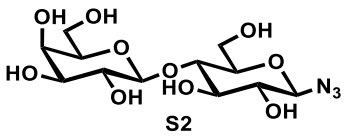


Figure S40: ^{1}H NMR spectrum of **S1** (300 MHz, D_2O).

β-D-Galactopyranosyl-(1→4)-β-D-glucopyranosylazide, $LacN_3$, **S2**



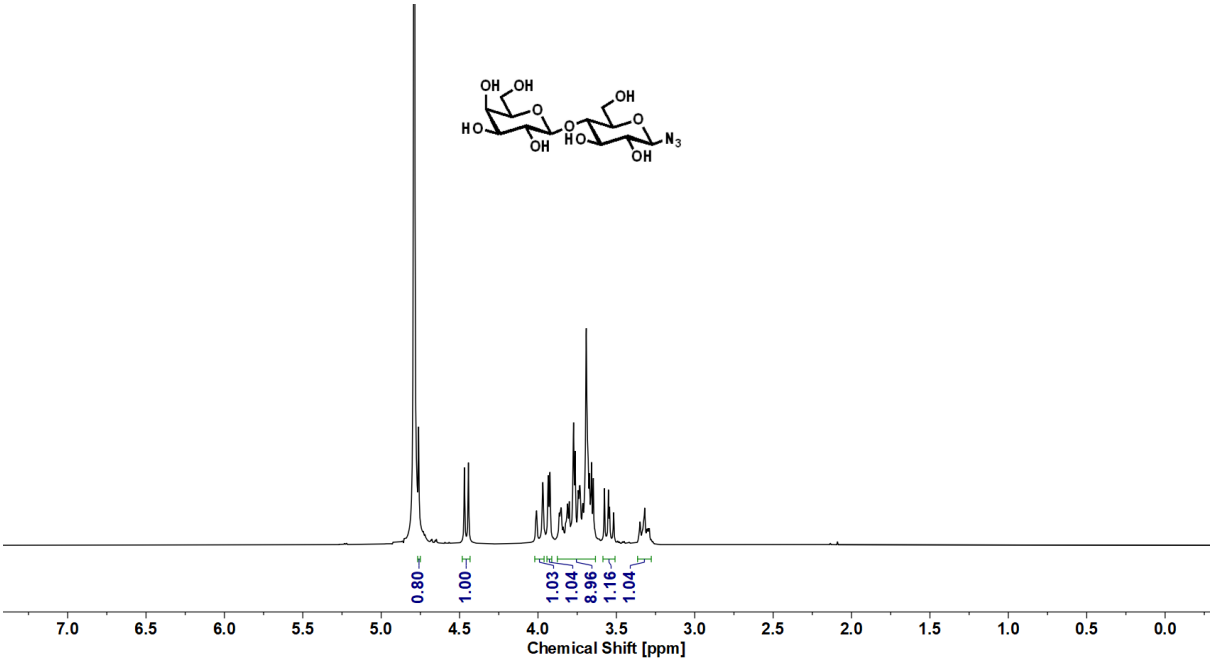


Figure S41: ^{1}H NMR spectrum of **S2** (300 MHz, D_2O).

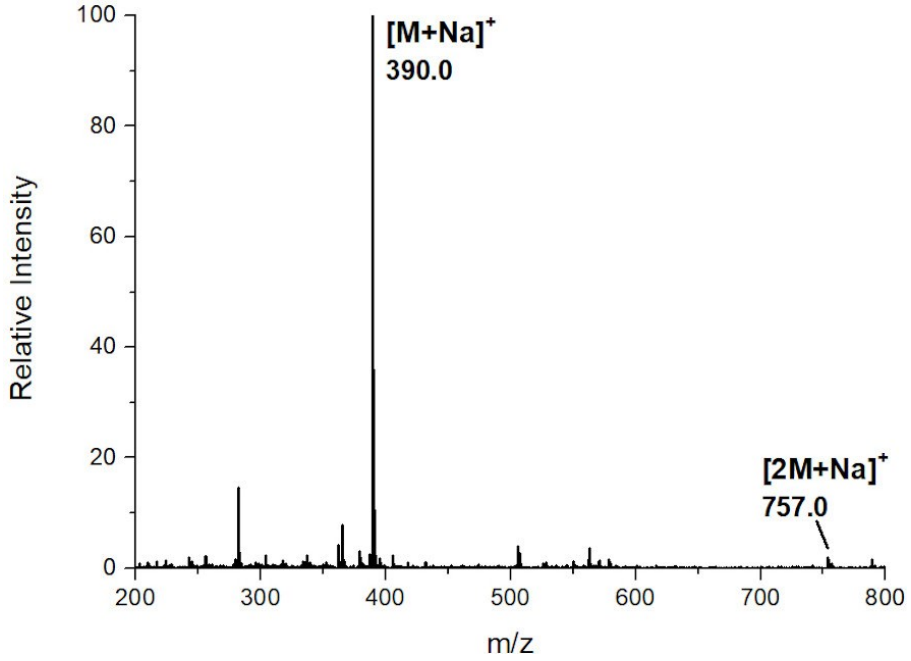


Figure S42: ESI-MS spectrum of **S2** (positive mode, M = $C_{12}H_{21}N_3O_{10}$).

Cytidine-5‘-triphosphate, disodium salt, CTP, **S3**

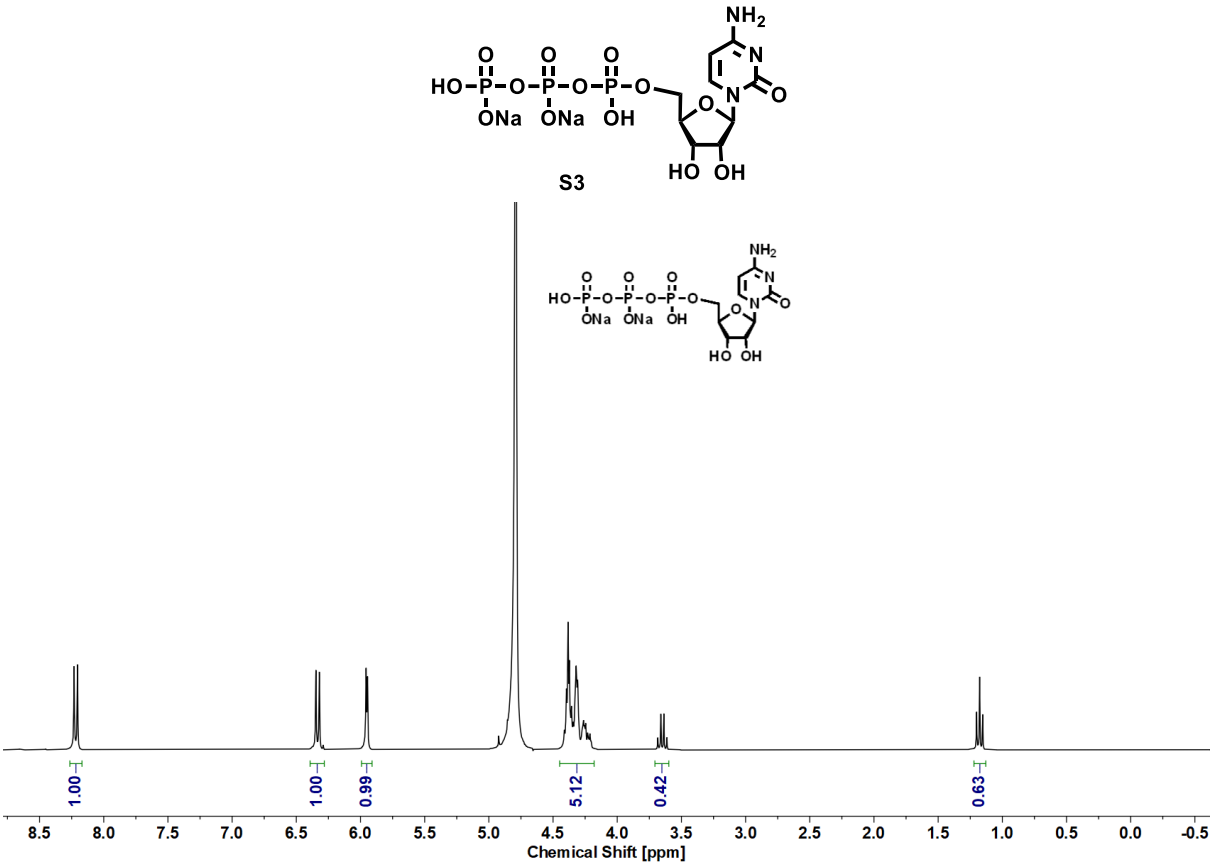


Figure S43: ^{1}H NMR spectrum of **S3** (300 MHz, D_2O).

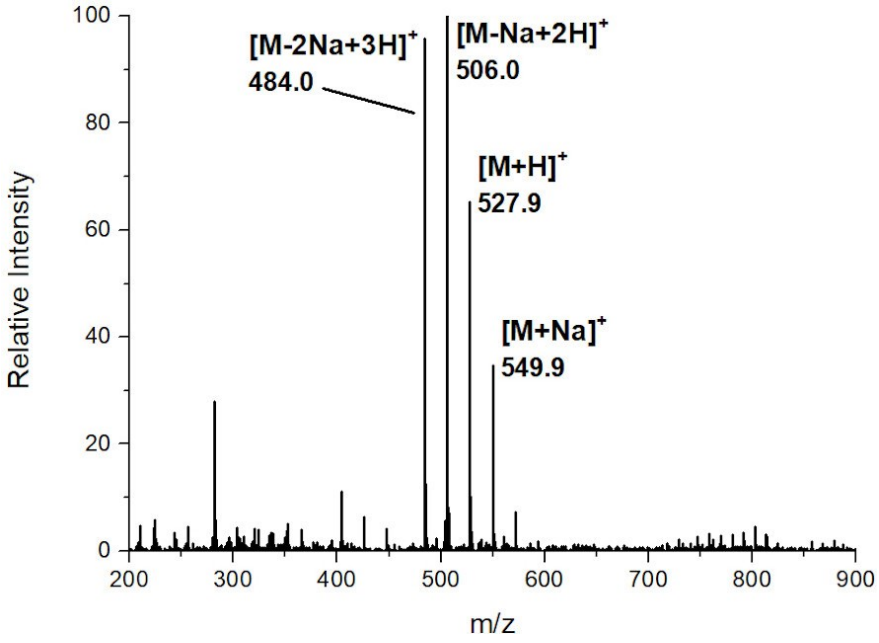


Figure S44: ESI-MS spectrum of **S3** (positive mode, M = $C_9H_{14}N_3Na_2O_{14}P_3$).

N-Acetylneuraminic acid, Neu5Ac, **S4**

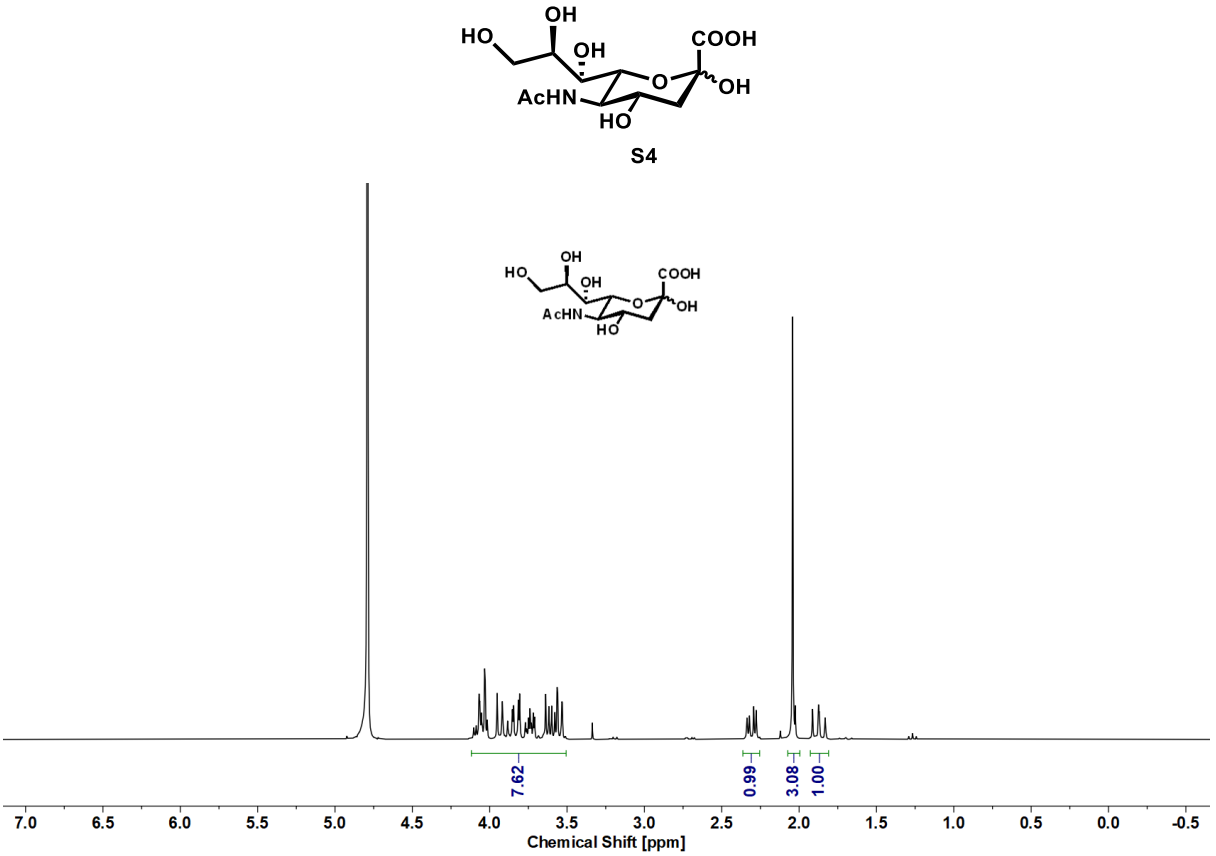


Figure S45: ^{1}H NMR spectrum of **S4** (300 MHz, D_2O).

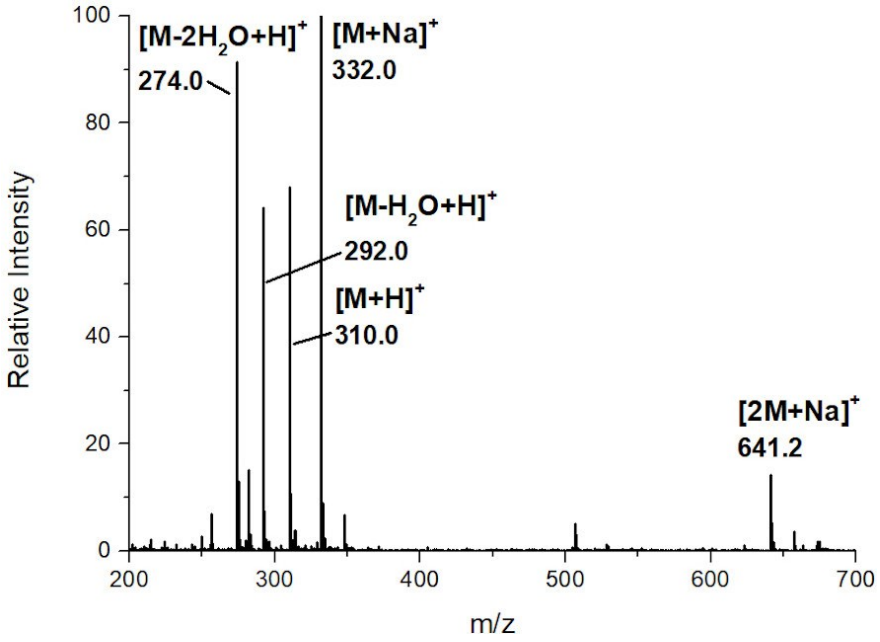


Figure S46: ESI-MS spectrum of **S4** (positive mode, M = $C_{11}H_{19}NO_9$).

Tris(hydroxymethyl)aminomethane, Tris, **S5**

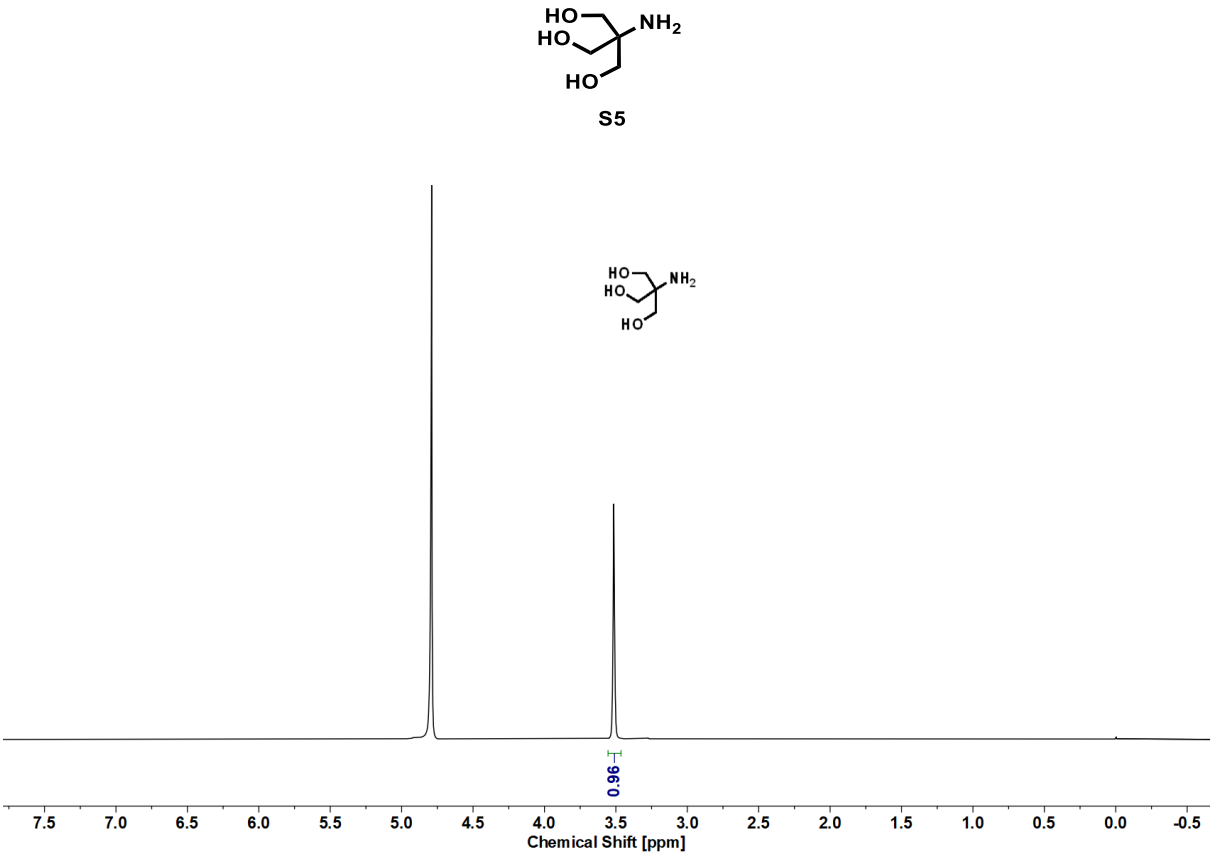


Figure S47: ^{1}H NMR spectrum of **S5** (300 MHz, D_2O).

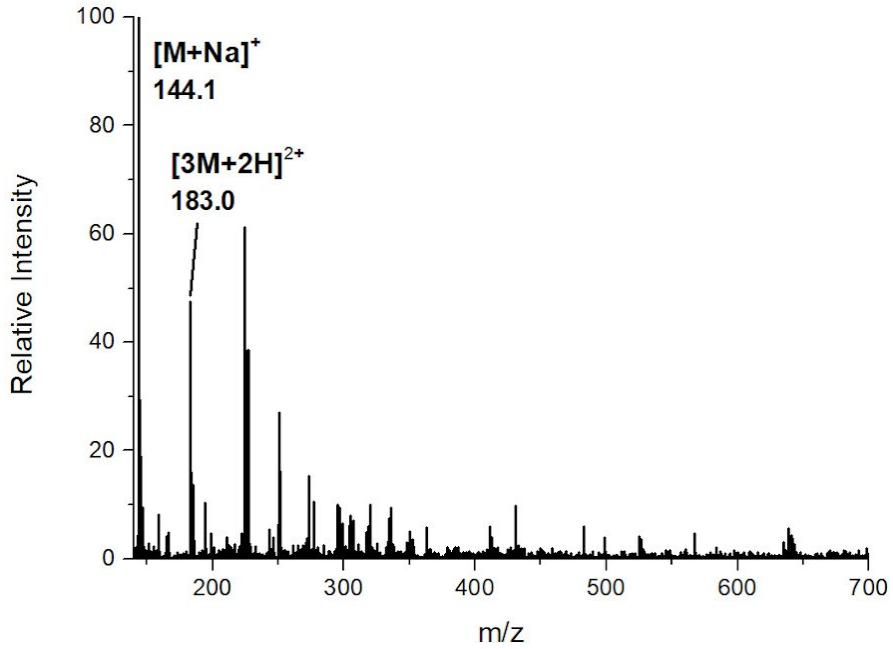


Figure S48: ESI-MS spectrum of **S5** (positive mode, M = $C_4H_{11}NO_3$).

N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid, TES, **S6**

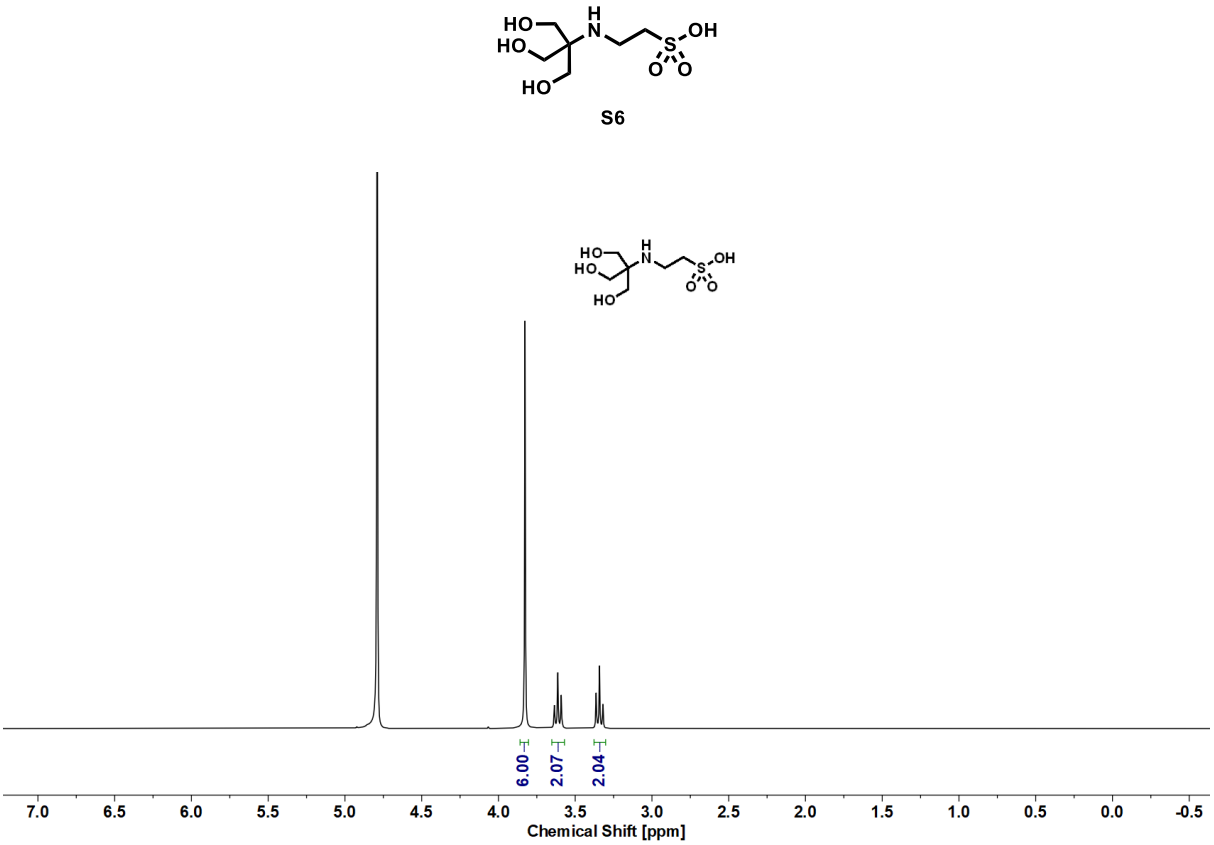


Figure S49: ^{1}H NMR spectrum of **S6** (300 MHz, D_2O).

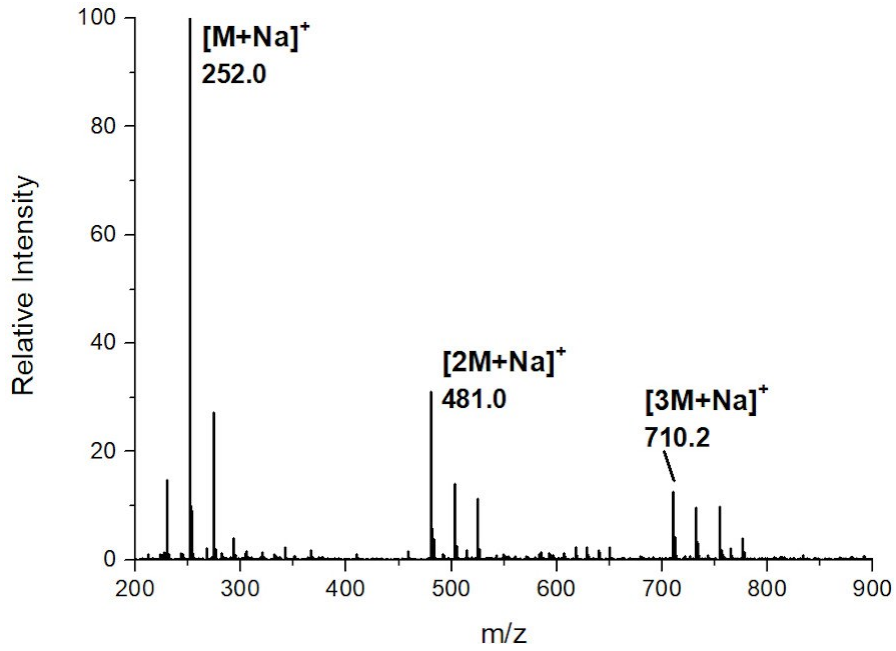


Figure S50: ESI-MS spectrum of **S6** (positive mode, M = $C_6H_{15}NO_6S$).

N-[2-hydroxy-1,1-bis-(hydroxymethyl)-ethyl]-glycine, Tricine, **S7**

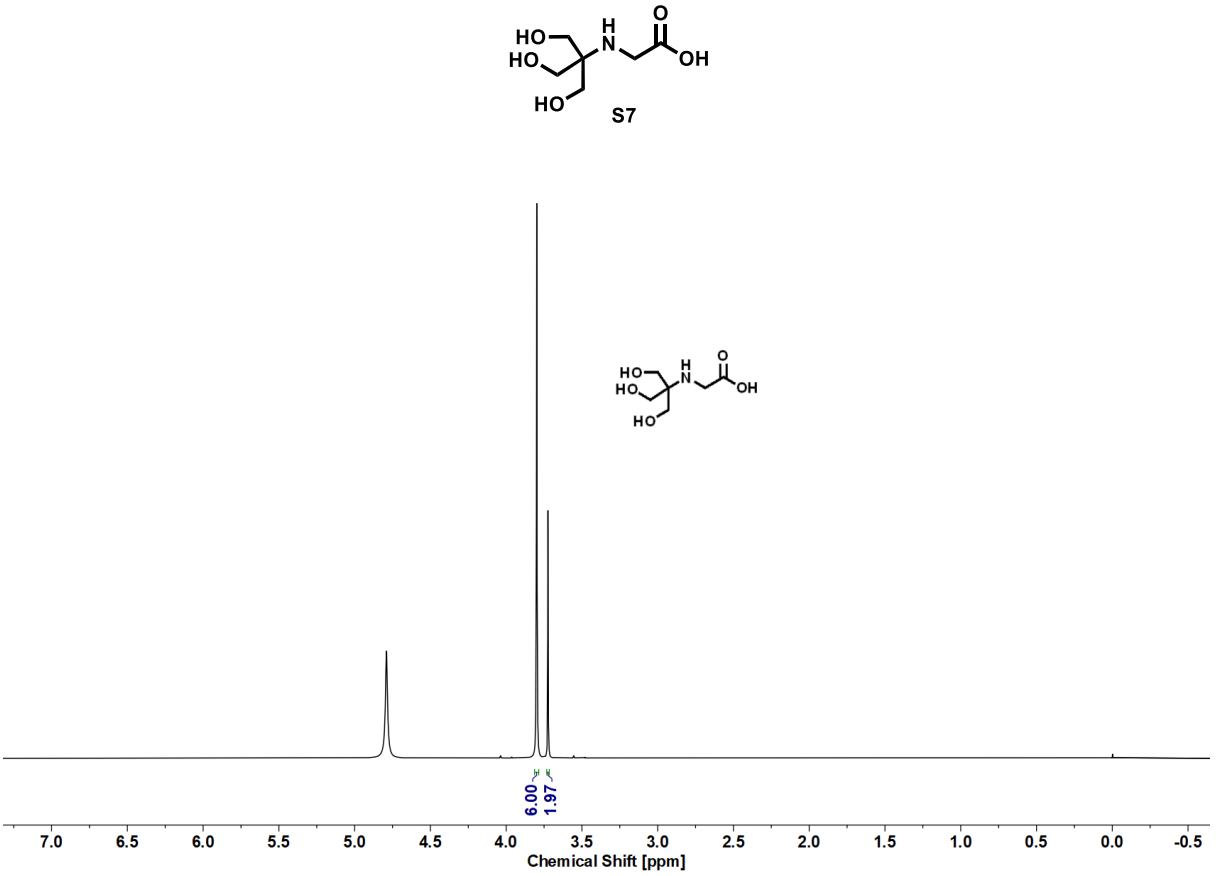


Figure S51: ^{1}H NMR spectrum of **S7** (300 MHz, D_2O).

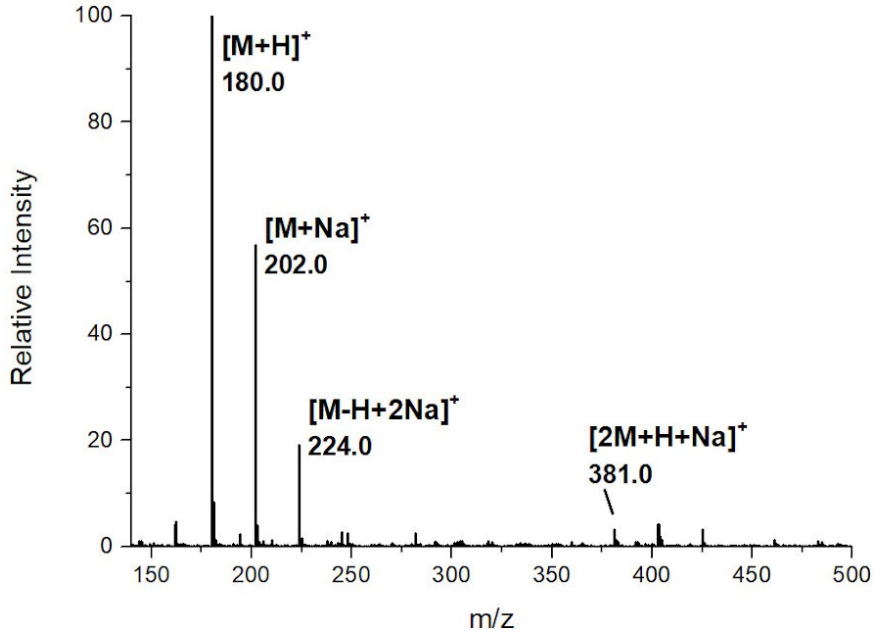


Figure S52: ESI-MS spectrum of **S7** (positive mode, M = $C_6H_{13}NO_5$).

N,*N*-Bis-(2-hydroxyethyl)-glycine, Bicine, **S8**

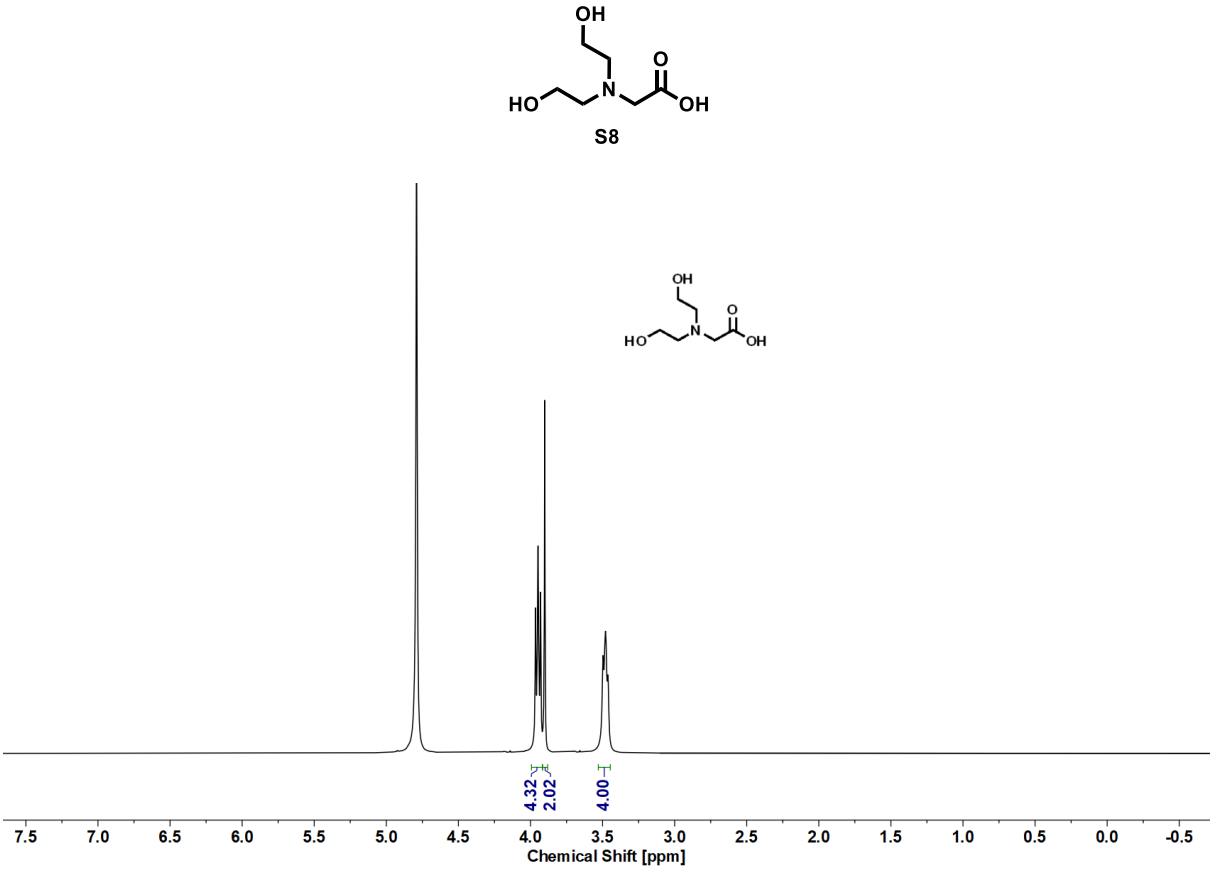


Figure S53: ^{1}H NMR spectrum of **S8** (300 MHz, D_2O).

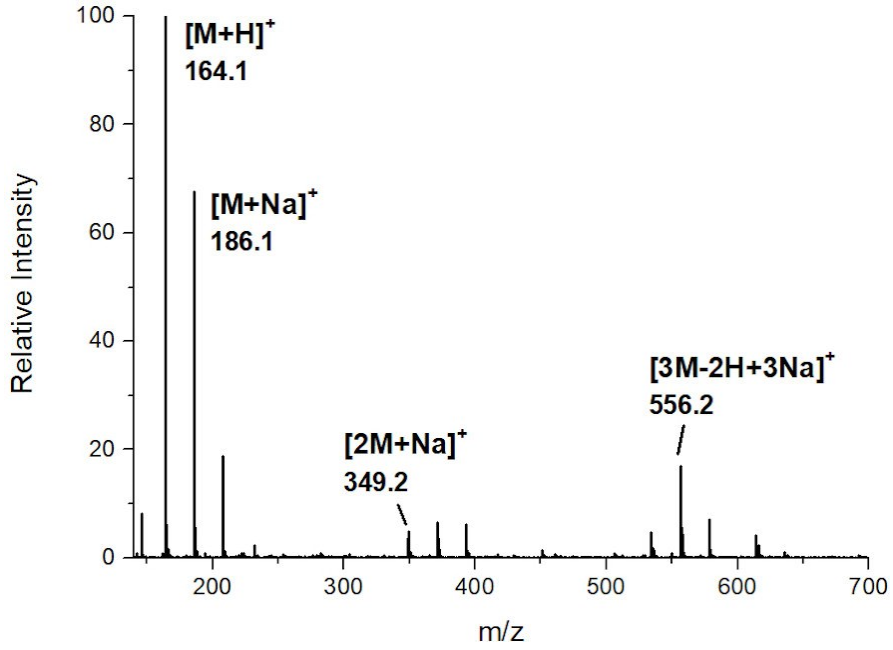
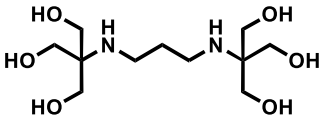


Figure S54: ESI-MS spectrum of **S8** (positive mode, M = $C_6H_{13}NO_4$).

1,3-Bis[tris(hydroxymethyl)methylamino]propane, BIS TRIS propane, **S9**



S9

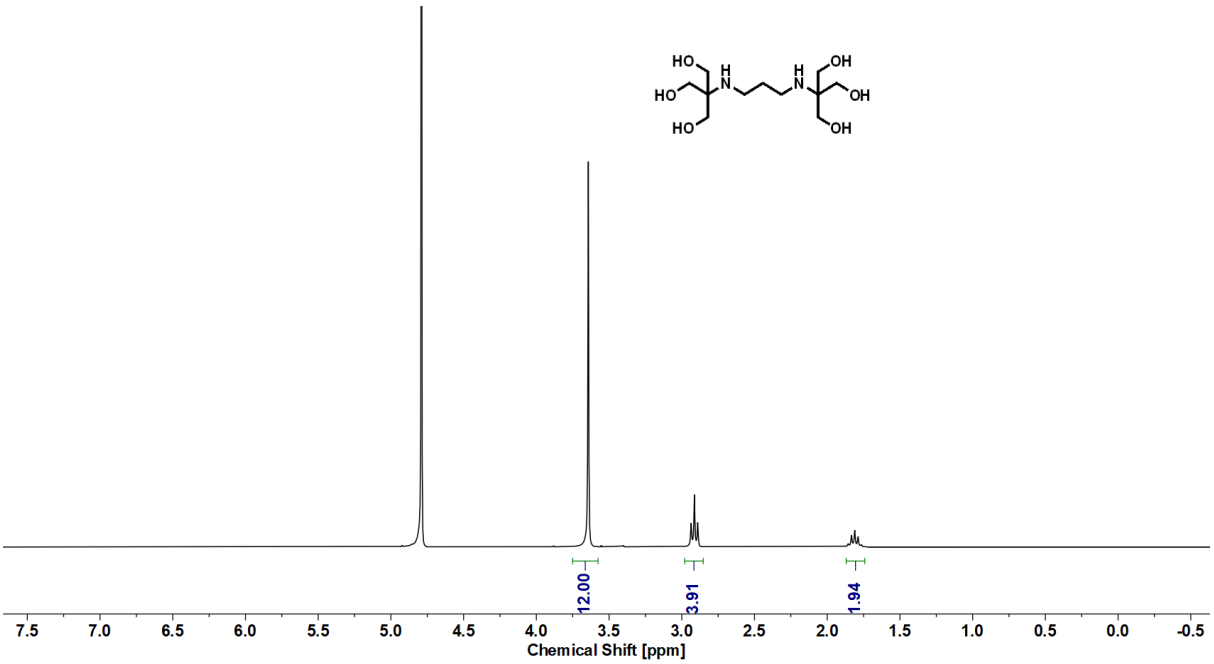


Figure S55: ^{1}H NMR spectrum of **S9** (300 MHz, D_2O).

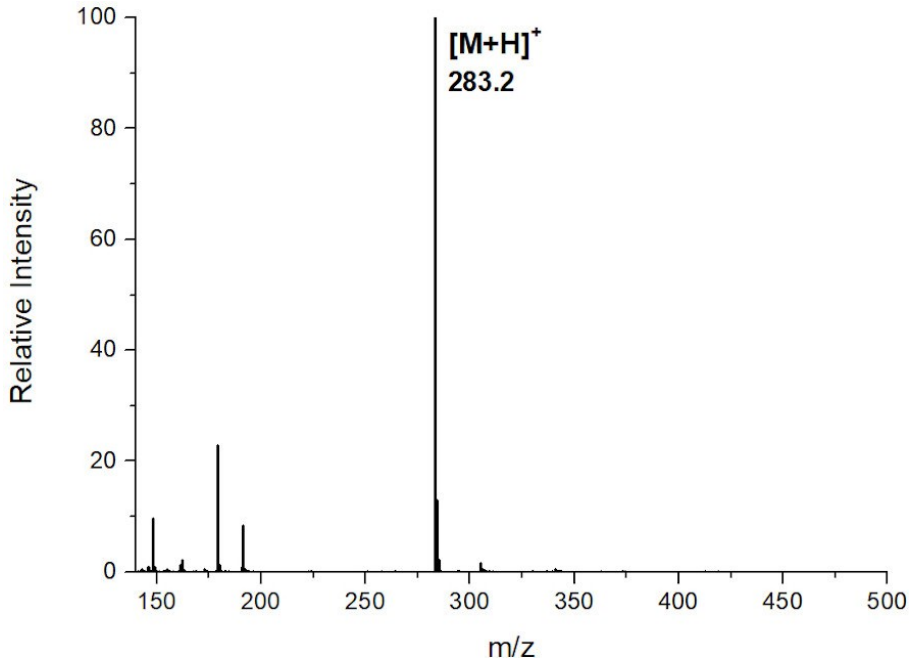


Figure S56: ESI-MS spectrum of **S9** (positive mode, M = $C_{11}H_{26}N_2O_6$).

6. Own publications

Publications included in this thesis:

P. B. Konietzny, H. Peters, M. L. Hofer, U. I. M. Gerling-Driessen, R. P. de Vries, T. Peters, L. Hartmann, *Macromol. Biosci.* **2022**, *22*, 2200358.

Own contributions: Synthesis of functional building blocks and lactose-functionalized glycomacromolecules, collaborative development of glycomacromolecule sialylation protocols and their subsequent purifications, supervised protein expressions and activity measurements, recording of 1D NOESY NMR spectra for sialylation reaction monitoring, collaborative enzymatic experiment planning and interpretation, development of tool for quantitative sialylation conversion determination, data evaluation, HPLC purification of sialylated glycomacromolecules, detection and isolation of Tris-sialoside, collaborative experiments on evidence for Tris-sialoside, development of buffer sialylation hypothesis and selection of tested Tris-analogues, development of MS-based sialylation analysis method, analytical characterization of compounds using HPLC, ESI-MS, HR-ESI-MS, ^{1}H and ^{13}C NMR, collaborative writing of manuscript and supplement.

P. B. Konietzny, J. Freytag, M. I. Feldhof, J. C. Müller, D. Ohl, T. Stehle, L. Hartmann, *Biomacromolecules* **2022**, *23*, 12, 5273–5284.

Own contributions: Synthesis of functional builing blocks and *N*-methyloxyamine glycomacromolecules, method development of macromolecule glycosylation via *N*-methyloxyamines, design of multivalent *N*-methyloxyamine-functionalized small molecules, isolation of small molecule glycosides and monodisperse glycomacromolecules, method development of accessing asymmetrically glycosylated small molecules, purification of monodisperse glycosides, analytical characterization of monodisperse compounds using HPLC, ESI-MS, HR-ESI-MS, ^{1}H and ^{13}C NMR, assistance on method implementation on polymeric backbones, collaborative writing of manuscript and supplement.

Other publications not included in this thesis:

P. B. Konietzny, H. Peters, M. Baier, T. Freichel, K. S. Bücher, N. L. Snyder, T. Peters, L. Hartmann, *Access to sialylated precision glycoacromolecules via solid phase synthesis: Enzymatic elongation as a tool of refinement*, Abstract on *1st International Symposium on Glycovirology* **2018**, Schöntal.
Own contributions: Abstract writing, preliminary results from Konietzny et al. **2022** *Macromol. Biosci.*

K. S. Bücher, P. B. Konietzny, N. L. Snyder, L. Hartmann, *Chem. Eur. J.* **2019**, *25*, 3301–3309.
Own contributions: Synthesis of functionalized lactose ligand and corresponding characterization via ESI-MS, ^{1}H and ^{13}C NMR, editorial adjustions on manuscript and supplement.

T. Freichel, D. Laaf, M. Hoffmann, P. B. Konietzny, V. Heine, R. Wawrzinek, C. Rademacher, N. L. Snyder, L. Elling, L. Hartmann, *RSC Adv.* **2019**, *9*, 23484–23497.
Own contributions: Synthesis of functionalized lactose ligands, synthesis and purification of high-spacing glycomacromolecules, analytical characterization of synthesized compounds using HPLC, ESI-MS, HR-ESI-MS, ^{1}H and ^{13}C NMR.

P. B. Konietzny, D. S. Ohl, M. A. Schmitter, H. Peters, J. Hofmann, T. Peters, L. Hartmann, *Multivalent Conjugation of Oligosaccharides onto Reactive Precision Macromolecules*, Abstract on *Virocarb PhD Symposium* **2021**, Münster.
Own contributions: Abstract writing, preliminary results from Konietzny et al. **2022** *Biomacromolecules*.

M. G. Lete, M. Hoffmann, N. Schomann, A. Martínez-Castillo, F. Peccati, P. B. Konietzny, S. Delgado, N. L. Snyder, G. Jiménez-Oses, N. G. A. Abrescia, A. Ardá, L. Hartmann, J. Jiménez-Barbero, *ACS Omega* **2023**, *8*, 19, 16883–16895.
Own contributions: Synthesis of functionalized lactose ligand and corresponding characterization via ESI-MS, ^{1}H and ^{13}C NMR.

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