Sialic Acid Engineering

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Glycan-Specific Metabolic Oligosaccharide Engineering of C7-Substituted Sialic Acids**

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Dedicated to Professor Werner Reutter on the occasion of his 75th birthday

Intact and integral glycosylation of membrane-associated as well as secreted glycoproteins has been shown to be essential for many aspects of the proper function of biological systems. Recombinantly expressed glycoproteins, such as antibodies, growth factors, hormones, vaccines, and contrast agents are key elements in medical applications.[1] The quality of these therapeutically administered glycoproteins can be efficiently improved by the incorporation of chemically functionalized monosaccharides into their glycan moieties, a process denoted as metabolic oligosaccharide engineering (MOE).[2] In addition to these pharmaceutical applications, MOE has greatly advanced diagnostics by localizing and visualizing glycans even in living animals.[2]

To date, a multitude of chemically modified monosaccharides have been designed for MOE applications. Owing to their terminal position at glycan structures of glycoproteins and relevance for cellular recognition, sialic acids and their metabolic precursor *N*-acetylmannosamine (ManNAc), are the most prominent targets for MOE.[3] Several ManNAc derivatives with N-acetyl side-chain modifications have been synthesized and metabolically incorporated by the sialic acid biosynthetic pathway into a corresponding sialic acid C5 analogue (Figure 1). This approach was beneficial to extending the understanding of the biological role of the N-acyl side chain of sialic acids, for example, in virus infection[4] or neuronal differentiation.[5] Alternatively, C9 modifications of sialosides have also been achieved by directly administering synthetic sialic acid analogues.[6] Additionally, selective cleavage of the glycol moiety led to a truncated sialic acid equipped glycans with an aldehyde for labeling reactions (Figure 1).[7] All of these modifications address sialylation of both, N- and O-glycosylation of glycoproteins, to almost the same extent.

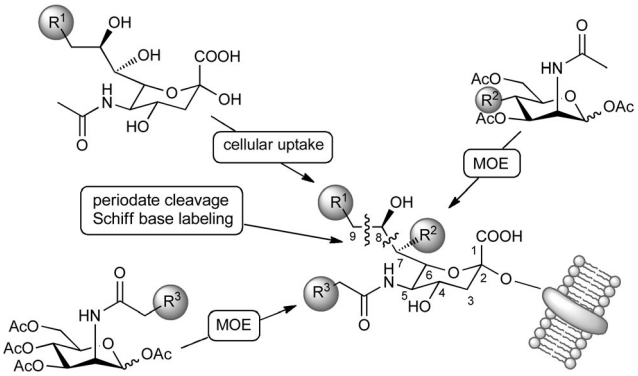

Figure 1. Methods for the structural modification of glycan-bound sialic acids by application of chemically modified ManNAc or direct periodate oxidation of glycan-bound sialic acids (left). Specific modification of the C7 position of sialic acids was achieved by C4-modified ManNAc in this study (right; note that to date these methods were carried individually, resulting in only one modification of a single sialic acid molecule).

Herein we investigate whether the biosynthetic machinery for sialic acids also tolerates other ManNAc derivatives as substrates, which are modified directly at the six-membered carbohydrate ring. The modification of the C4 position appeared most attractive, because it is not enzymatically modified during cellular glycoprotein production and would deliver previously unknown C7-modified sialic acid containing glycoproteins (Figure 1). To probe the biosynthetic promiscuity, we targeted a C4-modified ManNAc derivative, *N*-acetyl-4-azido-4-deoxymannosamine (4-azido-ManNAc, **1**), in our study to enable postglycosylational conjugation and visualization by bioorthogonal reactions.[8]

N-acetyl-(1,3,6-*O*-acetyl)-4-azido-4-deoxy-mannosamine (Ac_3-4-azido-ManNAc) was generated by an optimized literature method (Figure S1 in the Supporting Informa-

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Supporting information for this article (detailed information about the used materials, the chemical synthesis of compounds, and the biochemical methods) is available on the WWW under http://dx.doi.org/10.1002/anie.201108809.

tion),[9] which included the peracetylation to ensure membrane permeability for the metabolic uptake. Subsequently, we explored whether this synthetic carbohydrate is a suitable substrate for MOE of cell surface glycoproteins of mammalian cells. We employed HEK293 cells lacking UDP-*N*-acetylglucosamine 2-epimerase/ManNAc kinase (GNE), the key enzyme of sialic acid biosynthesis, to ensure increased incorporation rates of ManNAc analogues compared to GNE expressing cells as demonstrated for N-acylated ManNAc derivatives.[10] Consequently, we incubated GNE-deficient as well as GNE-expressing cells with Ac_3-4-azido-ManNAc, along with peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz) and peracetylated ManNAc (Ac_4ManNAc, Figure 2a). Isolated membrane fractions were treated with alkynylated biotin and AlexaFluor 488 by copper-catalyzed cycloaddition (CuAAC) to label incorporated azido sugars.[11] After separation of the samples by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), azido-glycoproteins were detected by fluorescence and by western-blot analysis using a specific anti-biotin antibody (Figure 2b). In contrast to Ac_4ManNAc- or azido-sugar-treated GNE-expressing cells, concentration-dependent signals were detected in membrane fractions from Ac_3-4-azido-ManNAc-treated GNE-deficient cells, which were comparable to GNE-deficient cells treated with Ac_4ManNAz (Figure 2b). These results gave the first clear indication that Ac_3-4-azido-ManNAc is metabolized by cellular enzymes and is indeed incorporated into glycan structures of cell surface glycoproteins while maintaining the accessibility of its azido group for bioorthogonal conjugation with labeling tags.

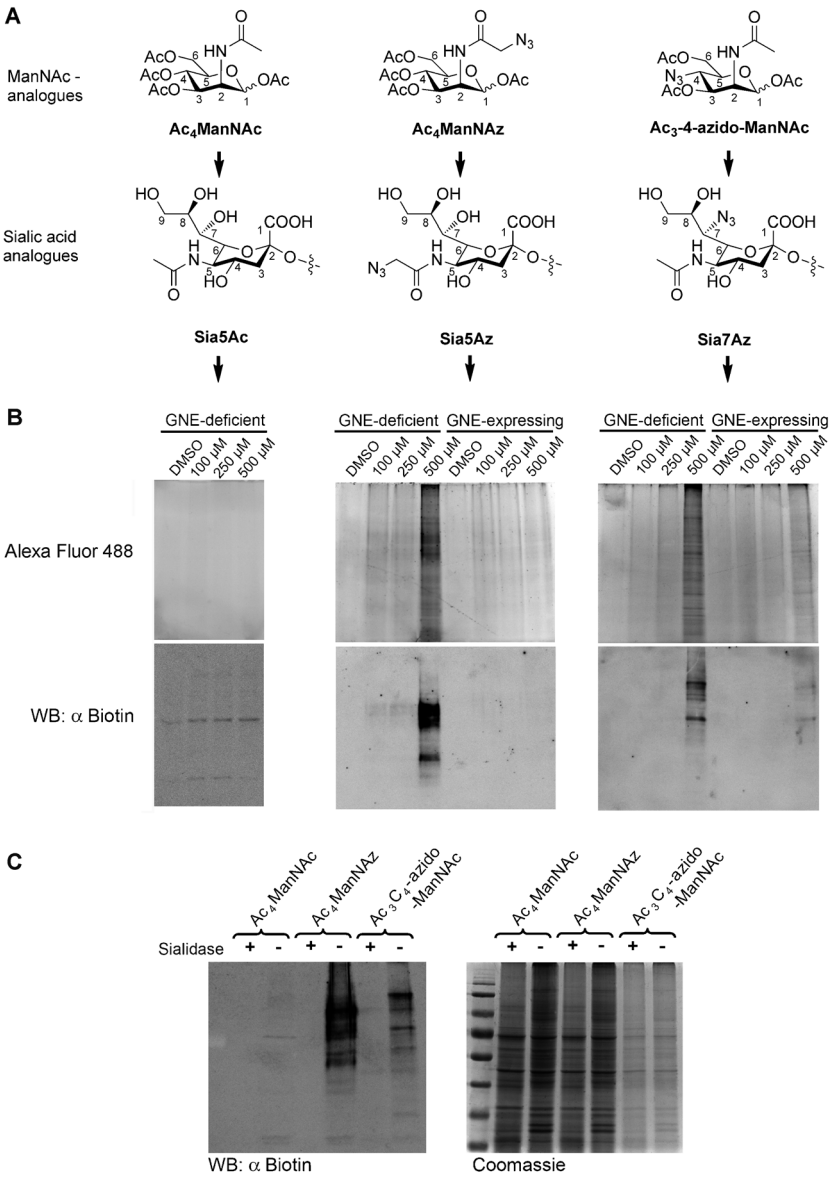

Figure 2. A) Biosynthetic incorporation of peracetylated (Ac_3,Ac_4-)ManNAc derivatives to the corresponding glycoconjugate-bound sialic acids. B) Incorporation of Ac_3-4-azido-ManNAc and Ac_4-ManNAz into cell surface glycoproteins of GNE-deficient HEK293 cells. Membrane fractions of sugar treated cells (3 days) were labeled with alkynylated AlexaFluor 488 or biotin by CuAAC enabling detection of incorporated azido sialic acids by fluorescence and western-blot analysis. C) Western blot analysis of glycoconjugate bound sialic acid derivatives by sialidase digestion of cells treated for three days with 500 µM solutions of ManNAc analogues. For further details see the Supporting Information.

Membrane fractions of the GNE-deficient cells revealed, after sialidase digestion and incubation with an alkynylated biotin to target remaining azido sialic acids, no signals by western-blot analysis, which confirmed that Ac_3-4-azido-ManNAc was converted into the C7-modified 7-azido-7-deoxy-*N*-acetylneuraminic acid (Sia7Az) in glycans of cell surface proteins (Figure 2c). To determine the incorporation rate of Ac_3-4-azido-ManNAc we analyzed membrane fractions of sugar-treated cell lines by flourescence-based HPLC. For this purpose hydrolytically cleaved sialic acids were labeled with 1,2-diamino-4,5-methyleneoxybenzene (DMB) and identified in comparison to chemo-enzymatically synthesized Sia7Az (Figure 3).[9b,12] Sia7Az integrity was further verified by combined liquid chromatography and mass spectrometry (LC-MS; for details see the Supporting Information). We found that incorporated Sia7Az accounts for up to 50% of total sialic acids, which is in a similar range to the maximum incorporation rate as Sia5Az derived from Ac_4ManNAz (60% of total sialic acids). The high incorporation rate of modified sialic acids into glycoconjugates in this study is, at least in parts, due to the use of GNE-deficient cells, which promote efficient metabolization of ManNAc derivatives.[10] On the other hand, several studies have shown that ManNAz is also useful for the modification of sialic acids in "normal" GNE-expressing cells. The incorporation rate for 4-azido-ManNAc from this study therefore indicates, that the novel

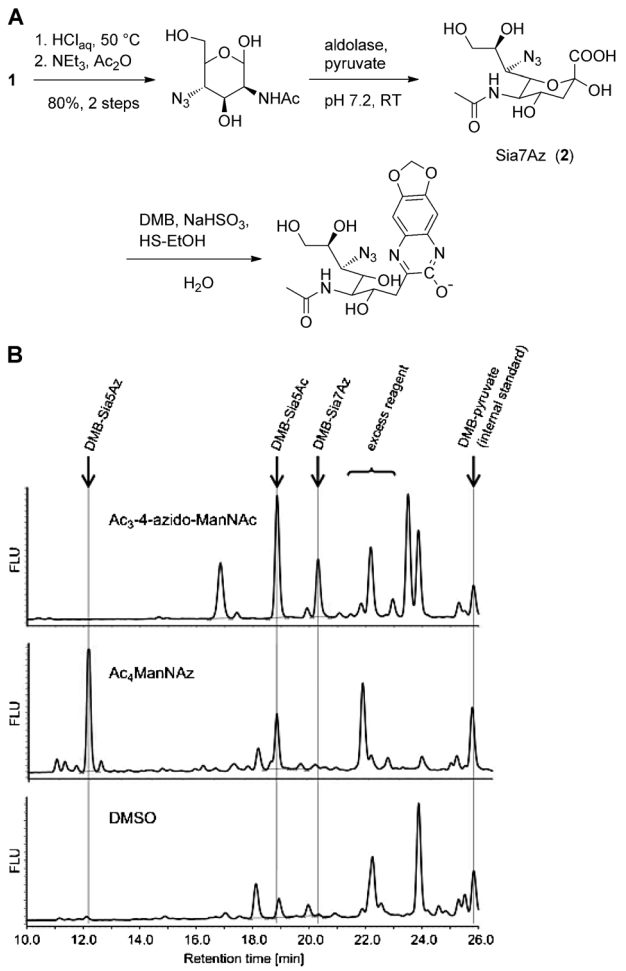

Figure 3. A) Chemoenzymatic synthesis of standards for HPLC assay. B) Quantitative analysis of Sia5Az and Sia7Az incorporation into glycans of GNE-deficient HEK293 cells. HEK293 cells were treated with a 500 μM solution of Ac$_3$-4-azido-ManNAc (top), Ac$_4$-ManNAz (middle), or corresponding amounts of DMSO (bottom) for 3 days. FLU = fluorescence light units. For further details see the Supporting Information.

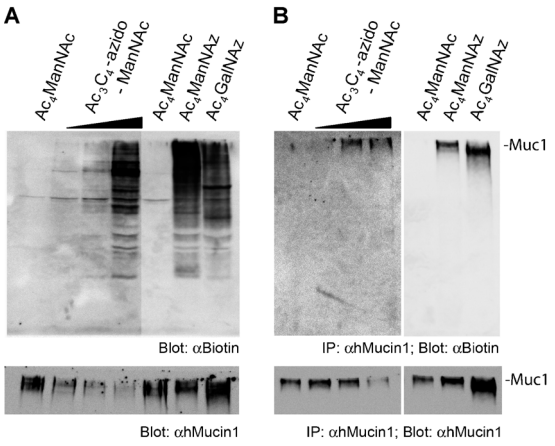

Figure 4. A) Concentration-dependent incorporation of Ac$_3$-4-azido-ManNAc into cell surface glycoproteins of Mucin-1-expressing MCF-7 cells. Cells were treated for 3 days either with increasing amounts of Ac$_3$-4-azido-ManNAc (100, 250, and 500 μM) or with Ac$_4$-ManNAc, Ac$_4$-ManNAz, and Ac$_4$-GalNAz (each 500 μM). Western blot analysis of biotin-labeled cell surface glycoproteins indicated azido-modified sialic acids or GalNAz in glycoproteins (top). Membrane associated Mucin-1 expression was detected by immunoblotting (bottom). B) Incorporation of Ac$_3$-4-azido-ManNAc into glycans of soluble O-glycosylated Mucin-1. Soluble Mucin-1 from cell culture media of azido sugar-treated MCF-7 cells (analogous to (A)) was immunoprecipitated, treated with alkynylated biotin by CuAAC and analyzed by western blotting. Ac$_4$-ManNAz and Ac$_4$-GalNAz treatment served as positive controls (top). Amount of precipitated Muc-1 was detected by immunoblotting (bottom).

compound we introduce herein is as promising as ManNAz as a general tool in glycoengineering.

After successful incorporation of Ac$_3$-4-azido-ManNAc into glycans of cell surface glycoproteins, we investigated the applicability of this sugar for MOE of recombinantly expressed glycoproteins. Ac$_3$-4-azido-ManNAc was first tested for glycan incorporation in the CEA-related cell adhesion molecule 1 (CEACAM1) and in lactotransferrin (LTF), which could be addressed successfully by Ac$_4$ManNAz.[10,13] Surprisingly, no incorporation of Ac$_3$-4-azido-ManNAc into the glycans of CEACAM1 or LTF could be found (Supporting Information, Figure S2). Since both proteins predominantly carry N-glycans,[14] we speculated that Ac$_3$-4-azido-ManNAc may be specifically incorporated into O-glycans.

O-glycan specificity of Ac$_3$-4-azido-ManNAc was probed by treatment of cells with PNGase F, an enzyme that specifically cleaves N-glycans. Cells treated with ManNAz, then by PNGase F, showed a strong but not complete decrease of the azido-specific signals (Figure S3, left), which indicates the predominant incorporation of the label into N-linked chains. Incubation of Ac$_3$-4-azido-ManNAc-treated cells with PNGase F had virtually no effect on the signal intensity of biotin labeled Sia7Az, as detected by western-blot analysis (Figure S3, middle). In agreement with this data, cell treatment with the N-glycosylation inhibitor tunicamycin indicated no decrease in the incorporation of Ac$_3$-4-azido-ManNAc (Figure S3, right).

To gain direct evidence for the specific incorporation of Ac$_3$-4-azido-ManNAc into O-glycans, we investigated Mucin-1, a heavily O-glycosylated protein highly expressed in MCF-7 cells.[15] MCF-7 cells did not only reveal incorporation of Ac$_3$-4-azido-ManNAc into glycans of cell surface proteins (Figure 4a) but also into glycans of secreted Mucin-1 (Figure 4b). For comparison, we also investigated the incorporation efficiency of Ac$_4$ManNAz into sialic acids, and the O-glycan-specific azido sugar Ac$_4$GalNAz.[16] Both were incorporated into cell surface glycans of MCF-7 cells as well as into glycans of soluble Mucin-1 efficiently and to a similar extent as Ac$_3$-4-azido-ManNAc (Figure 4a,b). The labeling of Mucin-1 by all three compounds used clearly indicates modification of O-glycans, whereby Ac$_3$-4-azido-ManNAc is the only molecule specifically targeting sialic acids in this kind of oligosaccharide.

Since azido sugars have been successfully applied to render cellular membranes[17] we tested the potential of Ac$_3$-4-azido-ManNAc as a tool for glycan labeling in living animals. Therefore, Ac$_3$-4-azido-ManNAc was injected into

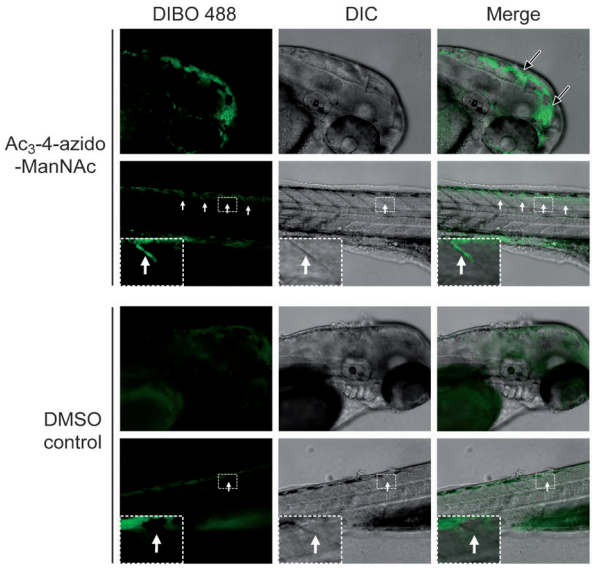

Figure 5. In vivo labeling of zebrafish glycans applying Ac_3-4-azido-ManNAc and AlexaFluor 488-DIBO. At 24 hpf, zebrafish larvae were intraventricularly injected with Ac_3-4-azido-ManNAc (top rows) or with DMSO (bottom rows). At 48 hpf, AlexaFluor 488-DIBO was injected as a chemical reporter for incorporated Ac_3-4-azido-ManNAc. At 72 hpf, fluorescence labeling was analyzed. Long arrows indicate midbrain and hindbrain regions, white arrows mark myosepts, magnified in insets. DIC = differential interference contrast.

the hindbrain vesicle of zebrafish larvae at 24 hpf (hours post fertilization). AlexaFluor 488-conjugated dibenzocyclooctyne (DIBO),[18] which reacts specifically with the azido group of modified sialic acids, was injected at 48 hpf, and live embryos were analyzed at 72 hpf. In embryos injected with Ac_3-4-azido-ManNAc, we detected a distinct labeling of the midbrain and the hindbrain, whereas these regions merely showed a slight background staining in DMSO-only controls (Figure 5). In addition we observed a faint staining of the dorsal myosepta, which was absent in the control embryos (Figure 5, white arrows and insets). The fluorescence staining of regions of the central nervous system and the myosepta presumably reflect high incorporation of Ac_3-4-azido-ManNAc into sialic acids of heavily O-glycosylated proteins such as dystroglycans, that display a very similar expression pattern in zebrafish larvae.[19] Further studies are required to corroborate our findings that indicate specific incorporation of Ac_3-4-azido-ManNAc into sialic acids of O-glycosylated proteins not only in mammalian cells but also in the developing zebrafish.

Taken together, we could demonstrate the successful biochemical and biological application of a synthetic C4-modified ManNAc analogue by showing its conversion into the corresponding C7-azido sialic acid in mammalian cell lines. Furthermore, we could address the biosynthetic accessibility of the C7 position of sialic acid for bioorthogonal functionalization for the first time. Attempts to chemically modify the C7 position of sialic acids incorporated in glycoproteins using periodate as a selective reagent had been made earlier. They always resulted, however, in a truncation of sialic acid molecules as a result of the removal of C8 and C9 upon periodate oxidation.[7] Our approach emphasizes the application of C4-modified ManNAc derivatives which are converted by cells, it thereby enables the functional characterization of C7-modified sialic acid with an otherwise maintained molecule composition. Interestingly, Sia7Az was not incorporated into N-glycans of recombinantly expressed proteins or of membrane proteins, but could be detected in the heavily O-glycosylated protein Mucin-1. This result makes the sugar a valuable tool for specific O-glycan analysis, which was not possible with the rather unspecific ManNAc derivatives used in previous MOE studies. In addition, Ac_3-4-azido-ManNAc was shown to be suitable for labeling of membrane associated glycoproteins of cultured mammalian cells and living animals, as demonstrated for zebrafish larvae. All in all, this study confirms the relevance of a new MOE tool in glycobiology research with distinct specifications differing from synthetically accessible azido sugars.

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