A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

CHEMBIOCHEM

SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY

Accepted Article

Title: Dienophile-Modified Mannosamine Derivatives for Metabolic Labeling of Sialic Acids: A Comparative Study

Authors: Jeremias E. G. A. Dold, Jessica Pfotzer, Anne-Katrin Späte, and Valentin Wittmann

To be cited as: *ChemBioChem* 10.1002/cbic.201700002

Link to VoR: http://dx.doi.org/10.1002/cbic.201700002

A Journal of

ChemPubSoc Europe

WILEY-VCH

www.chembiochem.org

10.1002/cbic.201700002

Dienophile-Modified Mannosamine Derivatives for Metabolic Labeling of Sialic Acids: A Comparative Study

Jeremias E. G. A. Dold, Jessica Pfotzer, Anne-Katrin Späte, and Valentin Wittmann*[a]

In memory of Professor Werner Reutter

[a] J. E. G. A. Dold, J. Pfotzer, Dr. A.-K. Späte, Prof. Dr. V. Wittmann
University of Konstanz
Department of Chemistry and Konstanz Research School Chemical Biology (KoRS-CB)
78457 Konstanz (Germany)
Fax: (+49) 7531-88-4573
E-mail: mail@valentin-wittmann.de

Abstract

Sialic acids play an important role in numerous cell adhesion processes and sialylation levels are known to be altered under certain pathogenic conditions such as cancer. Metabolic glycoengineering with mannosamine derivatives is a convenient way to introduce non-natural chemical reporter groups into sialylated glycoconjugates offering the opportunity to label sialic acids using bioorthogonal ligation chemistry. The labeling intensity not only depends on the rate of the ligation reaction but also on the extent to which the natural sialic acids are replaced by the modified ones, i.e. the incorporation efficiency. Here we present a comparative study of eight mannosamine derivatives featuring terminal alkenes as chemical reporter groups that can be labeled by an inverse-electron-demand Diels-Alder (DAinv) reaction. The derivatives differ in chain length as well as the type of linkage (comprising carbamates, amides, and a urea) that connects the terminal alkene to the sugar. As a general trend, increasing chain lengths result in higher DAinv reactivity and at the same time reduced incorporation efficiency. Carbamates are better accepted than amides with the same chain length; nevertheless do the latter result in more intense cell-surface staining visible in life-cell fluorescence microscopy. Finally, a urea derivative was shown to be accepted.

Introduction

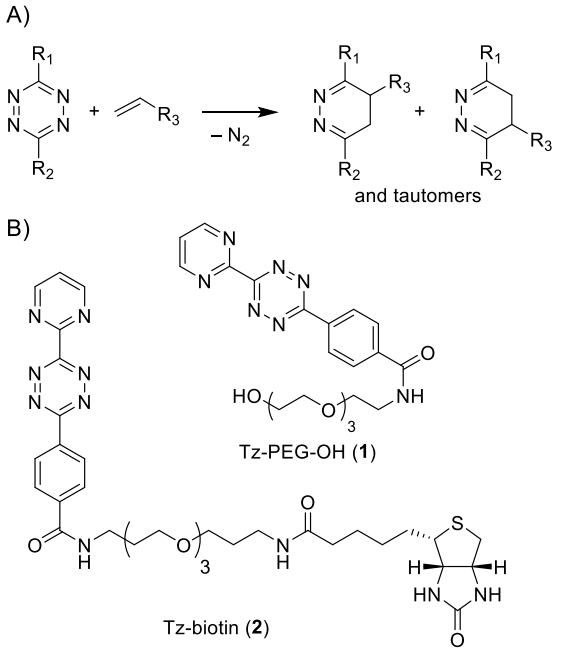
Carbohydrates are a highly important class of biomolecules and heavily involved in posttranslational protein modification. Of special interest are sialic acids that are positioned at the outer end of cell-surface glycan structures. They are involved in many recognition processes, for example during the inflammatory process or the adhesion of influenza viruses to host cells.[1] Labeling of sialic acids can be conveniently achieved by metabolic glycoengineering (MGE).[2] In this approach, mannosamine derivatives with an unnatural chemical reporter group are added to the culture medium of cells. After uptake the mannosamine derivatives are metabolized and incorporated into glycoproteins in the form of sialic acids.[3] The chemical reporter group can, subsequently, be reacted in a bioorthogonal ligation reaction[4] allowing the visualization of the sialic acid derivative.

Various derivatives of *N*-acetylmannosamine (ManNAc) but also of other sugars with different reporter groups connected over different linkages have been used for MGE in the past.[2] Azides and alkynes, for example, can be visualized through the Staudinger ligation[5] and/or azide-alkyne cycloaddition[6] (also known as click reaction). More recently, alkenes have been introduced as chemical reporter groups for MGE.[7] They can undergo inverse-electron-demand Diels-Alder (DAinv) reactions[8] with 1,2,4,5-tetrazines (Scheme 1) that are fast, irreversible, and do not need toxic heavy metals as catalysts allowing applications even within living cells.[9] Terminal alkenes, besides their application in MGE, have proven to be useful in a number of studies with proteins[10] and nucleic acids.[11] Their advantages are that they are small, very stable, and easy to handle. However, their reactivity is strongly dependent on the electron density of the double bond. As expected for a $LUMO^{dien}$-$HOMO^{dienophile}$-controlled reaction, electron-rich alkenes react especially fast.[12]

The labeling efficiency of sialic acids on the surface of cells is not only dependent on the reaction rate of the bioorthogonal ligation reaction but also on the acceptance of the unnatural ManNAc derivative by the biosynthetic machinery of the cell. If only a small proportion of the natural sialic acids (the most common representative in human cells being *N*-acetylneuraminic acid, Neu5Ac[1a]) is replaced by the synthetic derivative, even a fast labeling reaction does not guarantee a high labeling intensity. Although the degree, by which Neu5Ac is replaced by the modified sialic acid, i.e. the incorporation efficiency, has such a high impact on the success of MGE, it has been studied only occasionally,[13] and further investigations would be of high interest. Here, we report a comparative study of the incorporation efficiencies of the series of mannosamine derivatives depicted in Figure 1. The compounds do not only differ in the length of the acyl side chain, that is terminated by an alkene serving as dienophile, but also in the type

10.1002/cbic.201700002

of linkage to the sugar. Beside the previously reported carbamate and amide linkage we also expand the repertoire of mannosamine derivatives by a urea derivative.



Scheme 1. A) DAinv reaction between a 1,2,4,5-tetrazine and a terminal alkene. B) Tetrazine derivatives **1** and **2** used in the current study.

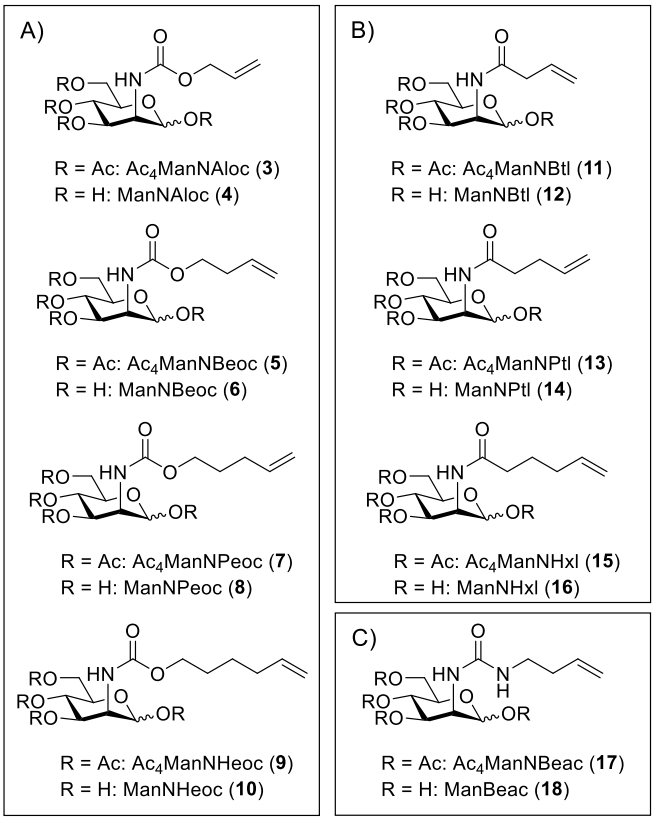
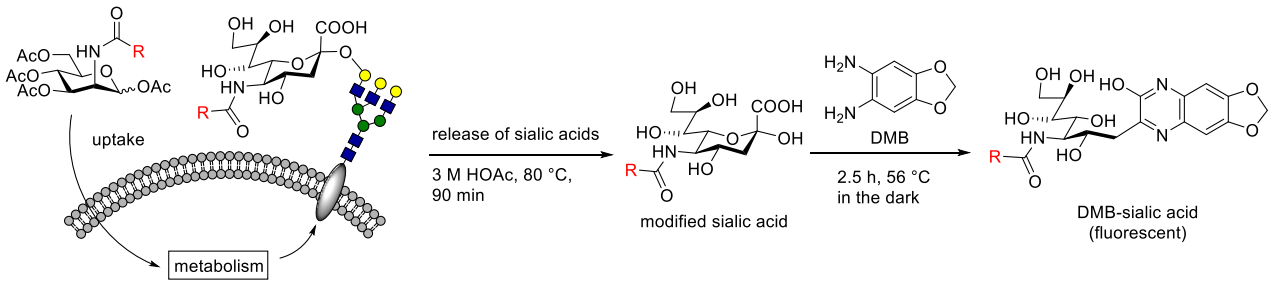


Figure 1. Mannosamine derivatives with terminal alkenes employed for MGE featuring (A) carbamate, (B) amide, and (C) urea linkages.

Results and Discussion

DMB labeling

To determine the proportion of sialic acids that were modified through MGE (i.e., the incorporation efficiency), we added different mannosamine derivatives equipped with terminal alkenes to the culture medium of HEK 293T cells (Scheme 2). After two days the cells were treated with 3 M acetic acid at 80 °C for 90 min. Under these conditions, the sialic acids are cleaved off and can subsequently be labeled by treatment with 1,2-diamino-4,5-methylenedioxybenzene (DMB).[14] DMB selectively reacts with α-keto carboxylic acids such as Neu5Ac forming a quinoxaline fluorophore. This fluorogenic reaction thus allows to detect the only small proportion of α-keto acids within the complex mixture of the cell lysate by RP-HPLC using a fluorescence detector (λ_{ex} = 373 nm, λ_{em} = 448 nm).[13b] The incorporation efficiency *IE* was calculated from the integrals *I* of the RP-HPLC signals of DMB-labeled Neu5Ac (I_{Neu5Ac}) and the respective DMB-labeled modified sialic acid (I_{Neu5R}) according to the formula $IE = I_{Neu5R} (I_{Neu5R} + I_{Neu5Ac})^{-1}$.

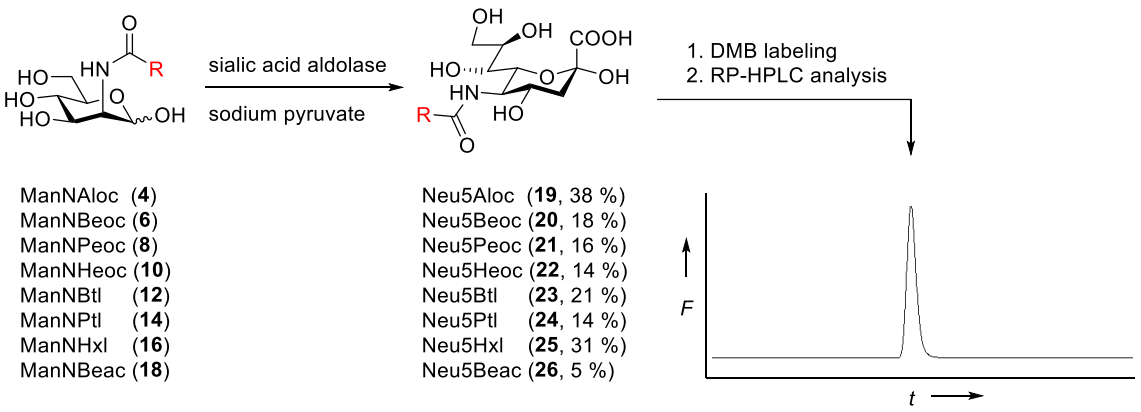


Scheme 2. DMB labeling of modified sialic acids obtained by MGE.

Synthesis of reference compounds

To be able to identify the DMB-labeled modified sialic acids by RP-HPLC, we first synthesized a series of reference compounds. Starting from the deacetylated mannosamine derivatives, an aldolase reaction[15] delivered the respective sialic acids which were subsequently derivatized with DMB (Scheme 3). We were pleased that the aldolase reaction could be performed successfully with all derivatives even the bulky ManNHeoc (**10**) and the urea-linked ManNBeac (**18**). The formulas and chromatograms of the DMB-labeled products are shown in the Supporting Information (Figures S2–S9).

10.1002/cbic.201700002



Scheme 3. Synthesis of reference compounds **19**–**26** followed by DMB-labeling and subsequent RP-HPLC analysis with fluorescence detection. *F* = fluorescence intensity, *t* = time

MGE with carbamate-modified mannosamines

Previously, we employed the series of carbamate-modified mannosamine derivatives depicted in Figure 1A (Ac_4ManNAloc (**3**), Ac_4ManNBeoc (**5**), Ac_4ManNPeoc (**7**), Ac_4ManNHeoc (**9**)) in MGE.[7g] We found that an increase of the length of the acyl side chain from 5 to 8 atoms is beneficial for the rate of the DAinv reaction as indicated by the increasing second-order rate constants k_2 given in Table 1 (left panel). Interestingly, Ac_4ManNBeoc (**5**) gave by far the best staining intensity on the cell surface within this series of sugars. We hypothesized that the length of the side chain has opposite effects on the DAinv reactivity and the incorporation efficiency.[7g] Although we expected Ac_4ManNAloc (**3**) to be incorporated most efficiently, its low DAinv reaction rate obviously prevented any staining. Ac_4ManNBeoc (**5**) seemed to have the best compromise between DAinv reactivity and incorporation efficiency leading to the most intense staining.[7g] Since the varying incorporation efficiencies were a hypothesis and we could not exclude that they are influenced by other factors beside the size of the side chain, we now determined the incorporation efficiencies of the whole series of these sugars by DMB labeling. As can be seen in Table 1 (left panel), there is a clear correlation between the incorporation efficiency and the length of the side chain. Ac_4ManNAloc (**3**) has by far the highest incorporation efficiency (50 %). With increasing chain length 15 % (Ac_4ManNBeoc, **5**), 3.7 % (Ac_4ManNPeoc, **7**), and 0.3 % (Ac_4ManNHeoc, **9**) of the cellular sialic acids are replaced by the non-natural derivatives.

Table 1. Incorporation efficiencies *IE* in MGE experiments and second-order rate constants k_2 of the investigated mannosamine derivatives. Left panel: carbamate derivatives, middle panel: amide derivatives, right panel: urea derivative.

Chain length[a]	Carbamate Ac_4ManNR	*IE* [%]	k_2 [$M^{-1}s^{-1}$][b]	Amide Ac_4ManNR	*IE* [%]	k_2 [$M^{-1}s^{-1}$][b]	Urea Ac_4ManNR	*IE* [%]	k_2 [$M^{-1}s^{-1}$][b]
4				R = Btl (**11**)	62 ± 1.7	0,0011 ± 0.0001[c]			
5	R = Aloc (**3**)	50 ± 1.2	0.0015 ± 0.0001[7g]	R = Ptl (**13**)	31 ± 5.6	0.021 ± 0.001[7b]			
6	R = Beoc (**5**)	15 ± 1.3	0.014 ± 0.003[7g]	R = Hxl (**15**)	8.3 ± 1.3	0.041 ± 0.0008[7b]	R = Beac (**17**)	6.6 ± 1.0	0.029 ± 0.001[c]
7	R = Peoc (**7**)	3.7 ± 1.0	0.038 ± 0.005[c]						
8	R = Heoc (**9**)	0.3 ± 0.2	0.074 ± 0.0013[7g]						

[a] Length of the acyl side chain including the carbonyl C atom. [b] Second-order rate constants were determined in all cases for reaction of the water-soluble deacetylated mannosamine derivatives with Tz-PEG-OH (**1**) in acetate buffer (pH 4.8). [c] For experimental details see Figure S1.

MGE with amide-modified mannosamines

Since the natural *N*-acetylmannosamine contains an amide moiety, also amide-linked alkenes are of high interest for MGE. Previous experiments with amide-linked mannosamine derivatives showed that they are accepted by the cells and can be visualized with the DAinv reaction.[7b] To find out whether amide-linked alkene-modified mannosamines show the same trend regarding the DAinv reactivity and incorporation efficiency, we investigated the series of compounds depicted in Figure 1B. To this end, Ac_4ManNBtl (**11**) was synthesized by the procedure published for the preparation of Ac_4ManNPtl (**13**) and Ac_4ManNHxl (**15**)[7b] (Scheme S1). The second-order rate constant k_2 for the reaction of ManNBtl (**12**) with Tz-PEG-OH (**1**) was determined to be 0.0011 ± 0.0001 $M^{-1}s^{-1}$ (Figure S1). Comparison with the published values of ManNPtl (**14**) and ManNHxl (**16**)[7b] revealed that also for the amide-linked alkenes the reaction rate increases with growing chain length (Table 1, middle panel).

Next, we investigated the incorporation efficiencies of the amide-linked sugars by DMB-labeling experiments. We observed that the alkene-modified mannosamines show the same trend as the series of the carbamate-modified sugars. The incorporation efficiency decreases with growing chain length from 62 % for Ac_4ManNBtl (**11**) to 31 % for Ac_4ManNPtl (**13**) and 8.3 % for Ac_4ManNHxl (**15**). This indicates that also the amide series shows an opposite effect of the chain length on the DAinv reactivity and on the incorporation efficiency.

To determine which derivative leads to the highest staining intensity on the cell surface, we examined metabolically labeled cells by confocal fluorescence microscopy. Cells were cultivated for 2 days with sugar and subsequently incubated with Tz-biotin (**2**) and then with streptavidin-

10.1002/cbic.201700002

AlexaFluor-555 and Hoechst 33342. The microscopy images are shown in Figure 2. Although Ac_4ManNBtl (**11**) has a very low DAinv reactivity after deacetylation, it leads to a decent staining probably due to its efficient metabolic incorporation. Ac_4ManNPtl (**13**) and Ac_4ManNHxl (**15**) lead to a comparable cell surface staining that is much more intense. Obviously, the higher incorporation efficiency of Ac_4ManNPtl (**13**) and the higher DAinv reactivity of Hxl derivative compensate each other, explaining the comparable staining intensity.

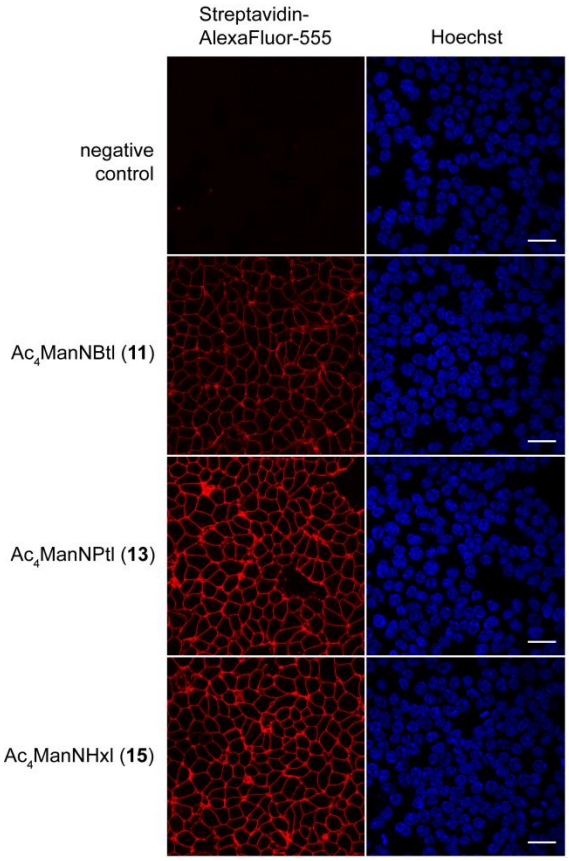


Figure 2. HEK 293T cells were grown with 100 µM Ac_4ManNBtl (**11**), Ac_4ManNPtl (**13**), Ac_4ManNHxl (**15**), or DMSO only (negative control) for 48 h. Cells were incubated with 1 mM Tz-biotin (**2**) for 6 h followed by incubation with streptavidin-AlexaFluor-555. Nuclei were stained with Hoechst 33342. Scale bar: 30 µm

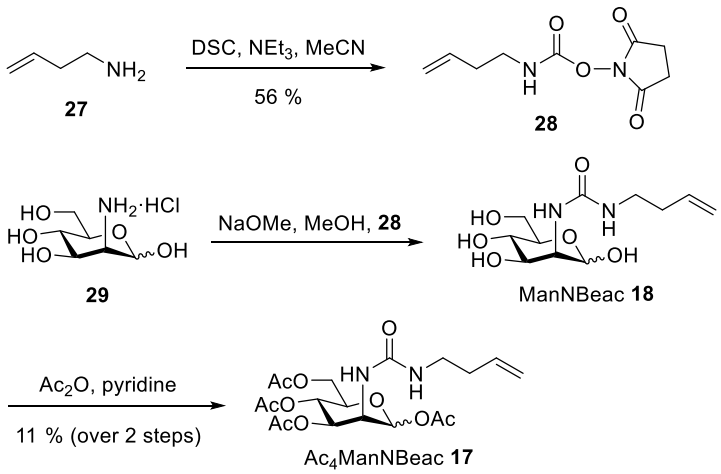
Comparison of amide- and carbamate-linked mannosamines

Having determined the reaction rates and incorporation efficiencies of the two series of sugars, a comparison of the influence of the type of linkage becomes possible. In Table 1 compounds with the same length of the acyl side chain are arranged in the same row. Comparison of Ac_4ManNAloc (**3**) with Ac_4ManNPtl (**13**), which both have a chain length of 5 atoms, and of Ac_4ManNBeoc (**5**) with Ac_4ManNHxl (**15**) with a chain length of 6 atoms reveals that the incorporation efficiencies of the carbamate-modified sugars are more than 1.6 times that of the amide-modified sugars. In contrast, the amide-linked derivatives with the same chain length feature higher DAinv reaction rates. For the Aloc derivative k_2 is 0.0015 $M^{-1}s^{-1}$ whereas for the

Ptl derivative k_2 is 0.021 $M^{-1}s^{-1}$ and for the Beoc derivative k_2 is 0.014 $M^{-1}s^{-1}$ whereas for the Hxl derivative k_2 is 0.041 $M^{-1}s^{-1}$. In summary, replacement of the CH_2 group in α-position of the carbonyl group by the heteroatom oxygen increases metabolic incorporation and reduces DAinv reactivity due to the high electronegativity of the oxygen atom. Thus, we wondered whether substitution of the oxygen with a less electronegative NH group could improve DAinv reactivity while retaining the higher incorporation efficiency. Accordingly, we synthesized the corresponding urea derivative Ac_4ManNBeac (**17**) with the same chain length as that of Ac_4ManNBeoc (**5**) and Ac_4ManNHxl (**15**).

MGE with a urea-modified mannosamine

The synthesis of Ac_4ManNBeac (**17**) is shown in Scheme 4. But-3-en-1-amine (**27**) was activated with *N,N'*-disuccinimidyl carbonate (DSC) and then reacted with mannosamine hydrochloride (**29**) followed by peracetylation.



Scheme 4. Synthesis of Ac_4ManNBeac (**17**).

So far, a mannosamine derivative carrying a urea moiety has not yet been tested for MGE. Therefore, we treated HEK 293T cells with Ac_4ManNBeac (**17**) for 2 days and determined the proportion of urea-modified sialic acids by DMB labeling. As shown in Table 1, right panel, the incorporation efficiency is 6.6 %. Interestingly, this number is much lower than that of Ac_4ManNBeoc (**3**, 15 %) and even than that of Ac_4ManNHxl (**15**, 8.3 %). The second-order rate constant for the DAinv reaction of ManNBeac (**18**) with Tz-PEG-OH (**1**) was determined to be k_2 = 0.029 $M^{-1}s^{-1}$. As expected, this represents an improvement over ManNBeoc (**6**, k_2 = 0.014 $M^{-1}s^{-1}$) but does not reach the performance of ManNHxl (**16**, k_2 = 0.041 $M^{-1}s^{-1}$).

Next we employed the three sugars Ac_4ManNBeoc (**5**), Ac_4ManNHxl (**15**) and Ac_4ManNBeac (**17**) with the same acyl chain length of 6 atoms in MGE followed by confocal fluorescence microscopy. As can be seen in Figure 3, Ac_4ManNHxl (**15**) which combines the highest reaction

rate with a medium incorporation efficiency results in the brightest staining. Ac_4ManNBeoc (**5**) featuring the slowest reaction rate still shows a distinct staining ascribed to its superior incorporation efficiency. Surprisingly, Ac_4ManNBeac (**17**) shows hardly any staining, although its incorporation efficiency is almost as high as that of Ac_4ManNHxl (**15**). Possibly, its reaction rate is not high enough to overcome the low incorporation efficiency.

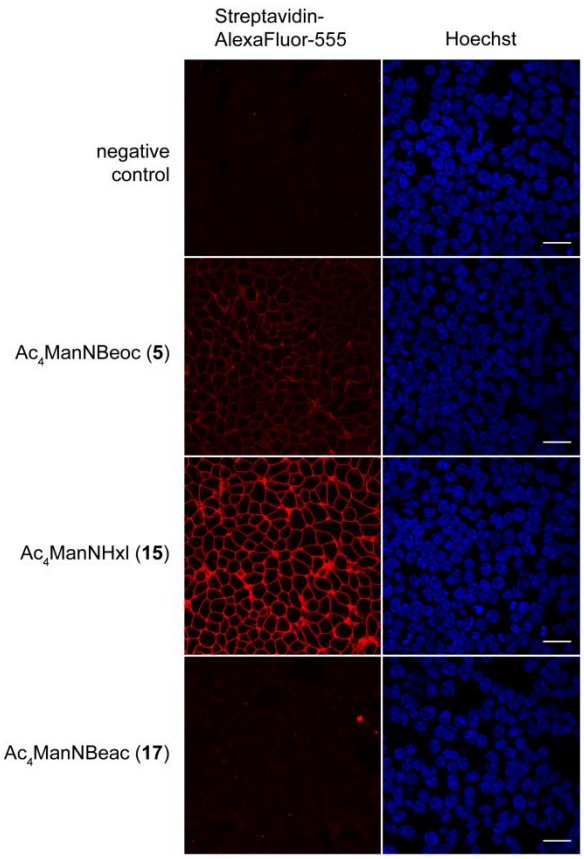


Figure 3. HEK 293T cells were grown with 250 µM Ac_4ManNBeoc (**5**), Ac_4ManNHxl (**15**), Ac_4ManNBeac (**17**) or DMSO only for 48 h. Cells were incubated with 1 mM Tz-biotin (**2**) for 6 h, followed by incubation with streptavidin-AlexaFluor-555. Nuclei were stained with Hoechst 33342. Scale bar: 30 µm

Conclusion

MGE with mannosamine derivatives is a simple means to introduce non-natural functional groups into sialylated glycoconjugates. This offers the opportunity to label cellular sialic acids using biorthogonal ligation chemistry. The approach bears great potential for the diagnosis of cell-surface sialic acid levels which are known to be altered under certain pathogenic conditions, such as cancer. Prerequisite for quantification, however, is knowledge on the extent to which the natural sialic acids are replaced by the modified ones and the reactivity of the introduced functional group in the subsequent labeling reaction. We have studied a whole series of

mannosamine derivatives that are equipped with terminal alkenes that can undergo a DAinv reaction. We compared different lengths of the side chains of the mannosamine derivatives and three different types of linkages for attachment of the side chain: carbamates, amides, and a urea. Both in the series of amides and carbamates the incorporation efficiency increases with decreasing chain length. The butenoyl moiety in Ac_4ManNBtl (**11**) represents the smallest modification within all compounds tested and leads to the highest incorporation efficiency of 62 %. When comparing carbamates and amides with the same chain length, carbamates are generally better accepted than amides. The length of the side chain has an opposite effect on the rate constants of the DAinv reactions of the terminal alkenes. Higher reactivity is observed in all cases with longer side chains. In each series a certain chain length leads to an optimal balance between incorporation efficiency and reactivity visible by a maximal labeling intensity of glycoconjugates on the surface of engineered cells. In the carbamate series Ac_4ManNBeoc (**5**) has been shown to give the best staining.[7g] In the amide series Ac_4ManNPtl (**13**) and Ac_4ManNHxl (**15**) give the best staining. The shorter butenoyl group leads to a less intense staining, and the same can be expected for longer chains than the hexenoyl group. Finally, we evaluated the suitability of a new type of mannosamine derivative featuring a urea linkage for MGE. Ac_4ManNBeac (**17**) has a reasonable incorporation efficiency of 6.6 %. However, its overall labeling intensity on the surface of engineered cells was rather low. Our comparative study thus provides an overview of scope and limitations of terminal-alkene-modified mannosamine derivatives which is of high interest for the development of future applications.

Experimental Section

General methods: All chemicals were purchased from *Sigma-Aldrich*, *Apollo*, and *Carbosynth* and used without further purification. AlexaFluor-555-labeled streptavidin and Hoechst 33342 were purchased from *Invitrogen*. Sialic acid aldolase was purchased from *Carbosynth* (MS110801004). Reactions were monitored by TLC on silica gel 60 F254 (*Merck*) with detection under UV light (λ = 254 nm). Additionally, acidic ethanolic *p*-anisaldehyde solution or basic $KMnO_4$ solution followed by gentle heating were used for visualization. Ac_4ManNAloc (**3**),[7g] ManNAloc (**4**),[7g] Ac_4ManNBeoc (**5**),[7g] ManNBeoc (**6**),[7g] Ac_4ManNPeoc (**7**),[7b] ManNPeoc (**8**),[7b] Ac_4ManNHeoc (**9**),[7g] ManNHeoc (**10**),[7g] Ac_4ManNPtl (**13**),[7b] ManNPtl (**14**),[7b] Ac_4ManNHxl (**15**),[7b] and ManNHxl (**16**)[7b] were synthesized according to published procedures. Preparative flash column chromatography (FC) was performed with an MPLC-Reveleris X2 system from *Grace*. NMR spectra were recorded at room temperature with Avance III 400 and Avance III 600 instruments from *Bruker*. Chemical shifts are reported relative to solvent signals ($CDCl_3$: δ_H = 7.26 ppm, δ_C = 77.16 ppm; CD_3OD: δ_H = 4.87 ppm, δ_C = 49.00 ppm; D_2O: δ_H = 4.73

ppm). Signals were assigned by first-order analysis; when feasible, assignments were supported by two-dimensional $^{1}H,^{1}H$ and $^{1}H,^{13}C$ correlation spectroscopy (COSY, HSQC, and HMBC). Numbering of structures is given in the supporting information. High resolution mass spectra (HRMS) were obtained with a micrOTOF II instrument from *Bruker Daltonics*. Semi-preparative high performance liquid chromatography (HPLC) was conducted on a LC-20A prominence system (high-pressure pumps LC-20AT, auto sampler SIL-20A, column oven CTO-20AC, diode array detector SPD-M20A, ELSD-LT II detector, controller CBM-20A, software LC-solution) from *Shimadzu* under the following conditions. Column: Eurosphere 100 C18 from *Knauer* (16 x 250 nm), flow: 9 mL min^{-1}; mobile phase: gradient of acetonitrile with 0.1 % formic acid (solvent B) in water with 0.1 % formic acid (solvent A). Analytical RP-HPLC-MS was performed on a LCMS2020 prominence system (pumps LC-20AD, column oven CTO-20AC, UV/Vis detector SPD-20A, RF-20A prominescence fluorescence detector (λ_{ex} = 372 nm, λ_{em} = 456 nm), controller CBM-20A, ESI detector, software LC-solution) from *Shimadzu* under the following conditions. Column: EC125/4 Nucleodur C18 from *Macherey-Nagel*, flow: 0.4 mL min^{-1}; mobile phase: gradient of acetonitrile with 0.1 % formic acid (solvent B) in water with 0.1 % formic acid (solvent A). UV/Vis absorption was measured with a Cary 50 instrument from Varian and software Cary WinUV scanning kinetics. Confocal fluorescence microscopy was performed with a Zeiss LSM 880 instrument equipped with a 40x 1.4 NA Plan-Apochromat oil immersion objective and a GaAsP-detector array for spectral imaging. Analysis of the obtained data was performed using image J software version 1.45.

Sialic acid aldolase reaction (general procedure 1, GP1): In a polypropylene vial the deacetylated mannosamine derivative is dissolved in phosphate buffer (100 mM, pH 7.18) to give a concentration of approx. 0.1 M. Sodium pyruvate (15 equiv) and sialic acid aldolase are added and the mixture is gently shaken at room temperature until completion of the reaction. The mixture is lyophilized and the sialic acid derivative is purified by RP-HPLC.

2,5-Dioxopyrrolidin-1-yl but-3-en-1-ylcarbamate (28): But-3-en-1-amine (**27**, 0.45 g, 6 mmol) was dissolved in dry MeCN (15 mL) under nitrogen atmosphere. *N*,*N*´-Disuccinimidyl carbonate (2.7 g, 10.5 mmol) and NEt_3 (2.13 g, 21 mmol) were added. The mixture was stirred overnight at room temperature. The solvent was removed and the product was purified by FC (silica, ethyl acetate 20%-80% in petroleum ether for 30 min). Product **28** was obtained as a white solid (0.714 g, 56%). TLC: R_f = 0.59 (petroleum ether/ethyl acetate 1:1); ^{1}H NMR (400 MHz, $CDCl_3$), δ = 6.00 (t, *J* = 5.8 Hz, 1H; NH), 5.72 (ddt, *J* = 17.1, 10.2, 6.8 Hz, 1H; H-3´), 5.15 – 4.97 (m, 2H; 2H-4´), 3.25 (q, *J* = 6.7 Hz, 2H; 2H-1´), 2.76 (s, 4H; 2H-3, 2H-4), 2.27 (m, 2H; 2H-2´) ppm; ^{13}C NMR (101 MHz, $CDCl_3$), δ = 170.4 (C2, C5), 151.5 (NHC=O), 134.3 (C3´), 117.65 (C4´), 41.1 (C1´), 33.5 (C2´), 25.5 (C3, C4) ppm.

2-(3-(But-3-en-1yl)ureido)-2-desoxy-D-mannopyranose (ManNBeac, 18): Mannosamine hydrochloride (**29**, 471 mg, 2.2 mmol) was suspended in dry MeOH (10 mL), neutralized with 0.5 M NaOMe solution (3.37 mL), and stirred for 1 h at room temperature under nitrogen. A solution of **28** (500 mg, 2.73 mmol) in dry MeOH (8 mL) was added and the reaction mixture was stirred overnight at room temperature. The solvent was evaporated and the product was purified by FC (silica, MeOH 0%-10% in ethyl acetate for 30 min, to yield ManNBeac (**18**) as a yellow solid (180.6 mg, 29%). This material was used for kinetic measurements. Full characterization was carried out after acetylation as described in the following procedure.

1,3,4,6-Tetra-*O*-acetyl-2-(3-(but-3-en-1yl)ureido)-2-deoxy-D-mannopyranose (Ac_4ManNBeac, 17): Mannosamine hydrochloride (**29**, 549 mg, 3 mmol) was suspended in dry MeOH (18 mL), neutralized with 0.5 M NaOMe solution (3.6 mL), and stirred for 1 h at room temperature under nitrogen. A solution of **28** (635.3 mg, 2.99 mmol) in dry MeOH (6 mL) was added and the reaction mixture was stirred overnight at room temperature. After complete removal of the solvent the residual syrup was dissolved in dry pyridine (18 mL), treated with acetic anhydride (3.8 mL), and stirred overnight at room temperature. The solvent was evaporated, and the remainder was dissolved in CH_2Cl_2. After washing with $KHSO_4$ (10 %), sat. $NaHCO_3$, and brine, the organic layer was dried over $MgSO_4$ and concentrated. The product was purified by FC (silica, ethyl acetate 20%-50% in petroleum ether in 40 min) to yield Ac_4ManNBeac as a white solid (143.5 mg, 11%) as an anomeric mixture (α/β 9:1). ^{1}H NMR (400 MHz, $CDCl_3$), α-anomer: δ = 6.03 (d, *J* = 1.8 Hz, 1H; H-1), 5.77 (ddt, *J* = 17.0, 10.1, 6.7 Hz, 1H; H-3'), 5.30 (dd, *J* = 10.2, 4.4 Hz, 1H; H-3), 5.24 – 5.05 (m, 3H; 2H-4', H-4), 5.00 (d, *J* = 8.8 Hz, 1H; NH), 4.51 – 4.42 (m, 1H; H-2), 4.35 – 4.19 (m, 1H; H6a), 4.10 – 3.95 (m, 2H; H-6b, H-5), 3.26 (t, *J* = 6.7 Hz, 2H; 2H-1'), 2.28 (q, *J* = 6.9 Hz, 2H; 2H-2'), 2.17 (s, 3H; OAc), 2.08 (s, 3H; OAc), 2.05 (s, 3H; OAc), 2.02 (s, 3H; OAc) ppm; ^{13}C NMR (101 MHz, $CDCl_3$), α-anomer: δ = 170.8 (C=O), 170.2 (C=O), 169.9 (C=O), 168.6 (C=O), 157.5 (NC=O), 135.3 (C3´), 117.6 (C4´), 92.6 (C1), 70.3 (C-5), 69.3 (C4), 65.8 (C3), 62.4 (C6), 50.4 (C2), 39.9 (C1´), 34.2 (C2´), 21.0 (OAc), 20.9 (OAc), 20.9 (OAc), 20.8 (OAc) ppm; HRMS: *m*/*z* calcd for $C_{19}H_{28}N_2O_4$: 445.1817 $[M+H]^+$; found: 445.1810.

2,5-Dioxopyrrolidin-1-yl but-3-enolate (30): But-3-enoic acid (534 mg, 6.21 mmol) and *N*-hydroxysuccinimide (1.0 g, 8.69 mmol) were dissolved in dry THF (18 mL) under nitrogen atmosphere. Dicyclohexyl carbodiimide (1.54 g, 7.45 mmol) was suspended in dry THF (18 mL) and added to the reaction mixture. The mixture was stirred over night at room temperature and controlled by TLC. The solvent was removed and the product was purified by FC (silica, petroleum ether/ ethyl acetate 2:1) to yield **30** as a white solid (867 mg, 76 %). TLC: R_f = 0.53 (petroleum ether/ethyl acetate 1:1); ^{1}H NMR (400 MHz, CDCl3): δ = 5.91 (ddt, *J* = 17.0, 10.2, 6.7 Hz, 1H; H-3´), 5.43 – 5.20 (m, 2H; 2H-4´), 3.38 (dt, *J* = 6.7, 1.5 Hz, 2H; 2H-2´), 2.82 (s, 4H;

2H-3, 2H-4) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 169.0 (C2, C5), 166.5 (C1´), 127.4 (C3´), 120.6 (C4´), 35.5 (C2´), 25.6 (C3, C4) ppm.

1,3,4,6-Tetra-*O*-acetyl-2-((but-3-enoyl)amino)-2-deoxy-D-mannopyranose (Ac_4ManNBtl, 11): Mannosamine hydrochloride (**29,** 983 mg, 4.56 mmol) was dissolved in dry MeOH (28 mL) under nitrogen atmosphere. 0.5 M NaOMe solution (9.1 mL) was added and the mixture was incubated at room temperature for 1.5 h. **30** (867 mg, 4.74 mmol) was dissolved in dry MeOH (28 mL), added to the reaction mixture, and stirred for 16 h at room temperature. After complete removal of the solvent the residual syrup was dissolved in dry pyridine (12 mL), treated with acetic anhydride (4.2 mL), and stirred overnight at room temperature. The solvent was removed and the product was purified by FC (silica, CH_2Cl_2/ methanol 5:1) to yield **11** as a white solid (290 mg, 25%) as an anomeric mixture (α/β 2:1). A small amount of the mixture was purified by semi-preparative RP-HPLC (36%-39% B in 20 min) to yield mainly the α-anomer (t_R = 11.8 min). TLC R_f = 0.2 (CH_2Cl_2/MeOH 5:1); α-Anomer: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.02 – 5.90 (m, 2H; NH, H-3´), 5.85 (d, *J* = 1.7 Hz, 1H; H-1), 5.35 – 5.24 (m, 2H; 2H-4´), 5.14 – 5.00 (m, 2H; H-3, H-4), 4.75 (ddd, *J* = 9.1, 3.9, 1.7 Hz, 1H; H-2), 4.25 (dd, *J* = 12.5, 4.9 Hz, 1H; H-6a), 4.10 (dd, *J* = 12.5, 2.5 Hz, 1H; H-6b), 3.79 (ddd, *J* = 9.5, 4.9, 2.5 Hz, 1H; H-5), 3.09 (dt, *J* = 7.1, 1.3 Hz, 2H; 2H-2´), 2.09 (m, 6H; 2OAc), 2.05 (s, 3H; OAc), 2.00 (s, 3H; OAc) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 171.2 (C=0), 170.6 (C=0), 170.2 (C=0), 169.7 (C=0), 168.4 (C=0), 131.2 (C3´), 120.1 (C4´), 90.7 (C1), 73.5 (C5), 71.5 (C3), 65.3 (C4), 61.9 (C6), 49.7 (C2), 41.6 (C2´), 24.8 – 20.2 (m; 4C(O)CH_3) ppm; β-Anomer: ^{13}C NMR (101 MHz, $CDCl_3$): δ = 171.2 (C=O), 170.6 (C=O), 170.2 (C=O), 169.7 (C=O), 168.4 (C=O), 131.0 (C3'), 120.1 (C4'), 90.8 (C1), 70.3 (C5), 69.1 (C3), 65.4 (C4), 62.0 (C6), 49.40 (C2), 41.5 (C2'), 21.0 – 20.8 (m, 4C(O)CH3) ppm; HRMS: *m*/*z* calcd for $C_{18}H_{25}NO_{10}$: 416.1551 [M+H]+; found: 416.1532.

2-((But-3-enoyl)amino)-2-desoxy-D-mannopyranose (ManNBtl, 12): Ac_4ManNBtl (**11**, 31 mg, 0.075 mmol, 0.035 M) was dissolved under nitrogen atmosphere in dry MeOH/*N*,*N*-dimethylethylamine (4.5:1). The mixture was stirred at room temperature for 7 days and the solvent was removed to yield ManNBtl as a yellow solid (18 mg, 97%). This material was used without further purification for kinetic measurements.

***N*-(Allyloxycarbonyl)-neuraminic acid (Neu5Aloc, 19):** ManNAloc (**4**, 78 mg, 0.3 mmol), sodium pyruvate (490 mg, 4.46 mmol), and sialic acid aldolase (2 U) were reacted in phosphate buffer (3 mL) for 7 days according to GP1. Semi-preparative RP-HPLC (3%-5% B in 20 min, t_R = 6.8 min) gave Neu5Aloc (**19**) as a white solid (30 mg, 38%) as an anomeric mixture (α/β 1:10). TLC: R_f = 0.2 (EtOAc/MeOH/EtOH/H_2O/AcOH 2:1:1:1:0.1); ^{1}H NMR (400 MHz, D_2O), *β*-Anomer: δ = 6.03 (m, 1H; H-2'), 5.56 – 5.18 (m, 2H; 2H-3'), 4.65 (m, 2H; 2H-1'), 4.11 (m, 2H; H-4, H-6), 4.02 – 3.87 (m, 1H; H-5), 3.87 – 3.63 (m, 3H; H-9a, H-9b, H-8,), 3.62 – 3.38 (m, 1H; H-7), 2.36 (dd, *J* = 13.0, 4.9 Hz, 1H; H-3eq), 1.93 ("t", *J* = 12.3 Hz, 1H; H-3ax) ppm; ^{13}C NMR (101

MHz, D_2O), *β*-Anomer: *δ* = 173.7 (C1), 158.3 (NC=O), 132.8 (C2´), 117.3 (C3´), 95.4 (C2), 70.6 (C6), 70.4 (C8), 68.3 (C7), 66.9 (C4), 66.0 (C1´), 63.3 (C9), 53.5 (C5), 39.0 (C3) ppm; HRMS: *m*/*z* calcd for $C_{13}H_{21}NO_{10}$: 350.1082 $[M-H]^-$; found: 350.1083.

***N*-(But-3-en-1-yl-oxycarbonyl)-neuraminic acid (Neu5Beoc, 20):** ManNBeoc (**6**, 20 mg, 0.09 mmol), sodium pyruvate (110 mg, 1.38 mmol), and sialic acid aldolase (2 U) were reacted in phosphate buffer (800 µL) for 7 days according to GP1. Semi-preparative RP-HPLC (5%-25% B in 20 min, t_R = 12.5 min) gave Neu5Beoc (**20**) as a white solid (3.4 mg, 13%) as an anomeric mixture (α/β 1:10). TLC: R_f = 0.3 (EtOAc/MeOH/EtOH/H_2O/AcOH 2:1:1:1:0.1); ^{1}H NMR (600 MHz, D_2O), *β*-Anomer: *δ* = 5.82 (ddt, J = 17.0, 10.2, 6.7 Hz, 1H; H-3'), 5.14 – 5.03 (m, 2H; H-4'), 4.29 (m, 2H; H-4, H-6), 3.94 (t, J = 9.9 Hz, 2H; 2H-1'), 3.79 (dd, J = 11.9, 2.7 Hz, 1H; H-9a), 3.70 (ddd, J = 9.2, 6.5, 2.7 Hz, 1H; H-8), 3.62 – 3.52 (m, 3H; H-7, H-5, H-9b), 2.34 (q, J = 6.4 Hz, 2H; 2H-2'), 2.18 (dd, J = 13.3, 4.9 Hz, 1H; H-3eq), 1.78 ("t", J = 12.3 Hz, 1H; H-3ax) ppm; ^{13}C NMR (151 MHz, D_2O), *β*-Anomer: *δ* = 176.1 (C1), 158.6 (C=O), 134.8 (C3´), 117.0 (C4´), 96.1 (C2), 70.4 (C6), 68.4 (C8), 67.28 (C7), 64.5 (C1´), 63.25 (C9), 53.5 (C5), 39.2 (C3), 32.9 (C2´) ppm; HRMS: *m*/*z* calcd for $C_{14}H_{23}NO_{10}$: 364.1238 $[M-H]^-$; found: 364.1244.

***N*-(Pent-4-en-1-yl-oxycarbonyl)-neuraminic acid (Neu5Peoc, 21):** ManNPeoc (**8**, 28 mg, 0.10 mmol), sodium pyruvate (158 mg, 1.44 mmol), and sialic acid aldolase (2 U) were reacted in phosphate buffer (0.98 mL) for 14 days according to GP1. Semi-preparative RP-HPLC (5%-15% B in 30 min, t_R = 16.6 min) gave Neu5Peoc (**21**) as a white solid (5.6 mg, 15%) as an anomeric mixture (α/β 1:12.5). TLC: R_f = 0.28 (EtOAc/MeOH/EtOH/H_2O/AcOH 2:1:1:1:0.1); ^{1}H NMR (600 MHz, D_2O), *β*-Anomer: *δ* = 5.88 (ddt, *J* = 17.0, 10.2, 6.7 Hz, 1H; H-4'), 5.10 – 4.98 (m, 2H; 2H-5'), 4.13 – 3.97 (m, 4H; 2H-1', H-4, H-6), 3.89 – 3.78 (m, 1H; H-9a), 3.73 (ddd, *J* = 9.3, 6.6, 2.6 Hz, 1H; H-8), 3.67 – 3.55 (m, 3H; H-9b, H-5, H-7), 2.24 (dd, *J* = 13.0, 4.9 Hz, 1H; H-3eq), 2.12 ("q", *J* = 7.3 Hz, 2H; 2H-3'), 1.83 ("t", *J* = 12.3 Hz, 1H; H-3ax), 1.72 ("p", *J* = 7.1 Hz, 2H; 2H-2') ppm; ^{13}C NMR (151 MHz, D_2O / CD_3OD), *β*-Anomer: *δ* = 174.9 (C1), 158.8 (NC=O), 138.5 (C4'), 114.9 (C5'), 95.7 (C2), 70.5 (C6), 70.3 (C8), 68.4 (C7), 67.1 (C4), 65.1 (C1'), 63.2 (C9), 53.4 (C5), 39.1 (C3), 29.3 (C3'), 27.4 (C2') ppm; HRMS: *m*/*z* calcd for $C_{15}H_{25}NO_{10}$: 378.1395 $[M-H]^-$; found: 378.1392.

***N*-(Hex-5-en-1-yl-oxycarbonyl)-neuraminic acid (Neu5Heoc, 22):** ManNHeoc (**10**, 30 mg, 0.1 mmol), sodium pyruvate (162 mg, 1.47 mmol), and sialic acid aldolase (2.5 U) were reacted in phosphate buffer (1 mL) for 8 days according to GP1. Semi-preparative RP-HPLC (5%-20% B in 20 min, t_R = 20 min) gave Neu5Heoc (**22**) as a white solid (5 mg, 14%) as an anomeric mixture (α/β 1:14). TLC**:** R_f = 0.32 (EtOAc /MeOH/EtOH/H_2O/AcOH 2:1:1:1:0.1); ^{1}H NMR (600 MHz, D_2O), *β*-Anomer: *δ* = 5.94 (ddt, *J* = 17.0, 10.2, 6.6 Hz, 1H; H-5'), 5.14 – 5.01 (m, 2H; 2H-6'), 4.21 – 4.01 (m, 4H; H-4, H-6, 2-H-1'), 3.88 (dd, *J* = 12.0, 2.7 Hz, 1H; H-9a), 3.79 (ddd, *J* = 9.2, 6.5, 2.7 Hz, 1H; H-8), 3.72 – 3.62 (m, 3H; H-5, H-7, H-9b), 2.31 (dd, *J* = 13.1, 5.0 Hz, 1H;

H-3eq), 2.13 (“q”, *J* = 7.2 Hz, 2H; 2H-4’), 1.90 (“t”, *J* = 12.3 Hz, 1H; H-3ax), 1.68 (quint, *J* = 7.0 Hz, 2H; 2H-2’), 1.55 – 1.45 (m, 2H; 2H-3’) ppm; ^{13}C NMR (151 MHz, D_2O), *β*-Anomer: *δ* = 174.35 (C1), 158.9 (NC=O), 139.6 (C5’), 114.43 (C6’), 95.6 (C2), 70.6 (C6), 70.4 (C8), 68.4 (C7), 67.0 (C1’), 65.7 (C9), 63.3 (C4), 53.5 (C5), 39.1 (C3), 32.6 (C4’), 27.7 (C2’), 24.4 (C3’) ppm; HRMS: *m*/*z* calcd for $C_{16}H_{27}NO_{10}$: 392.1551 $[M-H]^-$; found: 392.1550.

***N*-(But-3-enoyl)-neuraminic acid (Neu5Btl, 23):** ManNBtl (**12**, 70.7 mg, 0.28 mmol), sodium pyruvate (474 mg, 4.3 mmol), and sialic acid aldolase (8 U) were reacted in phosphate buffer (2.9 mL) for 20 days according to GP1. Semi-preparative RP-HPLC (3%-5% B in 20 min, t_R = 7.4 min) gave Neu5Btl (**23**) as a white solid (20 mg, 21%) as an anomeric mixture (α/β 1:14). TLC: R_f = 0.27 (EtOAc /MeOH/EtOH/H_2O/AcOH 2:1:1:1:0.1); ^{1}H NMR (400 MHz, D_2O), *β*-Anomer: *δ* = 5.95 (ddt, *J* = 17.1, 10.1, 6.9 Hz, 1H; H3’), 5.35 – 5.21 (m, 2H; 2H-4’), 4.17 – 4.04 (m, 2H; H-4, H-6), 3.96 (t, *J* = 10.2 Hz, 1H; H-5), 3.86 (dd, *J* = 11.8, 2.7 Hz, 1H; H-9a), 3.77 (ddd, *J* = 9.0, 6.3, 2.5 Hz, 1H; H-8), 3.63 (dd, *J* = 11.8, 6.3 Hz, 1H; H-9b), 3.60 – 3.50 (m, 1H; H-7), 3.13 (d, *J* = 6.8 Hz, 2H; 2H-2’), 2.34 (dd, *J* = 13.0, 4.9 Hz, 1H; H-3eq), 1.90 (“t”, *J* = 12.3 Hz, 1H; H-3ax) ppm; ^{13}C NMR (101 MHz, D_2O), *β*-Anomer: *δ* = 175.3 (C1), 173.5 (C1’), 130.9 (C3’), 119.3 (C4’), 95.4 (C2), 70.4 (C6), 70.2 (C8), 68.3 (C7), 66.6 (C4), 63.2 (C9), 52.1 (C5), 40.7 (C2’), 39.0 (C3) ppm; HRMS: *m*/*z* calcd for $C_{13}H_{21}NO_9$: 334.1133 $[M-H]^-$; found: 334.1139.

***N*-(Pent-4-enoyl)-neuraminic acid (Neu5Ptl, 24):** ManNPtl (**14**, 100 mg, 0.38 mmol), sodium pyruvate (631 mg, 5.74 mmol), and sialic acid aldolase (6 U) were reacted in phosphate buffer (3.9 mL) for 26 days according to GP1. Semi-preparative RP-HPLC (18%-20% B in 30 min, t_R = 4.8 min) gave Neu5Ptl (**24**) as a with solid (10 mg, 8%) as an anomeric mixture (α/β 1:7). TLC: R_f = 0.30 (EtOAc /MeOH/EtOH/H_2O/AcOH 2:1:1:1:0.1); ^{1}H NMR (600 MHz, D_2O), *β*-Anomer: *δ* = 5.85 (ddt, *J* = 16.5, 10.3, 6.2 Hz, 1H; H-4’), 5.15 – 4.98 (m, 2H, 2H-5’), 4.04 – 3.95 (m, 2H; H-4, H-6), 3.89 (t, *J* = 10.2 Hz, 1H, H-5), 3.85 – 3.78 (m, 1H; H-9a), 3.73 (ddd, *J* = 9.2, 6.6, 2.6 Hz, 1H; H-8), 3.56 (dd, *J* = 11.8, 6.7 Hz, 1H, H-9b), 3.51 (dd, *J* = 9.1, 1.2 Hz, 1H; H-7), 2.42 – 2.29 (m, 4H; 2H-2’,2H-3’), 2.22 (dd, *J* = 13.0, 4.9 Hz, 1H; H-3eq), 1.81 (dd, *J* = 13.0, 11.5 Hz, 1H; H-3ax) ppm; ^{13}C NMR (151 MHz, D_2O), *β*-Anomer: *δ* = 176.8 (C1), 175.5 (C1’), 137.0 (C4’), 115.7 (C5’), 96.0 (C2), 70.3 (C8), 70.2 (C6), 68.5 (C7), 66.9 (C4), 63.2 (C6), 52.0 (C5), 39.2 (C3), 35.2 (C2’), 29.3 (C3’) ppm; HRMS: *m*/*z* calcd for $C_{14}H_{23}NO_9$: 348.1289 $[M-H]^-$; found: 348.1292.

***N*-(Hex-5-enoyl)-neuraminic acid (Neu5Hxl, 25):** ManNHxl (**16**, 32 mg, 0.12 mmol), sodium pyruvate (203 mg, 1.84 mmol), and sialic acid aldolase (2 U) were reacted in phosphate buffer (1.25 mL) for 20 days according to GP1. Semi-preparative RP-HPLC (3%-5% B in 30 min, t_R = 19.7 min) gave Neu5Hxl (**25**) as a white solid (14 mg, 31%) as an anomeric mixture (α/β 1:7.6). TLC: R_f = 0.28 (EtOAc /MeOH/EtOH/H_2O/AcOH 2:1:1:1:0.1); ^{1}H NMR (400 MHz, D_2O), *β*-Anomer: *δ* = 5.94 (ddt, *J* = 17.1, 10.3, 6.6 Hz, 1H; H-5’), 5.20 – 5.04 (m, 2H; 2H-6’), 4.13 – 4.02 (m, 2H; H-4, H-6), 3.99 (dd, *J* = 10.3, 9.3 Hz, 1H; H-5), 3.94 – 3.85 (m, 1H; H-9a), 3.85 –

3.78 (m, 1H; H-8), 3.70 – 3.62 (m, 1H; H-9b), 3.57 (d, *J* = 9.0 Hz, 1H; H-7), 2.38 (t, *J* = 7.3 Hz, 2H; 2H-2'), 2.29 (dd, *J* = 12.9, 4.8 Hz, 1H; H-3eq), 2.20 – 2.07 (m, 2H; 2H-4'), 1.90 ("t", *J* = 12.1 Hz, 1H; H-3ax), 1.77 (quint, *J* = 7.4 Hz, 2H; 2H-3') ppm; ^{13}C NMR (151 MHz, D_2O), ***β***-Anomer: ***δ*** = 177.7 (C1), 160.5 (C1'), 138.6 (C5'), 115.1 (C6'), 90.9 (C2), 70.3 (C6), 68.6 (C8), 67.0 (C7), 63.2 (C4), 59.3 (C9), 52.1 (C5), 39.3 (C3), 35.3 (C2'), 32.4 (C4'), 24.6 (C3') ppm; HRMS: *m*/*z* calcd for $C_{15}H_{25}NO_9$: 362.1446 $[M-H]^-$; found: 362.1449.

N*-(But-3-en-1-yl-aminocarbonyl)-neuraminic acid (Neu5Beac, 26):** ManNBeac (**18**, 41 mg, 0.15 mmol), sodium pyruvate (245 mg, 2.22 mmol), and sialic acid aldolase (4 U) were reacted in phosphate buffer (1.5 mL) for 15 days according to GP1. Semi-preparative RP-HPLC (3%-8% B in 30 min, t_R = 18 min) gave Neu5Beac (**26**) as a white solid (0.3 mg, 5%). TLC: R_f = 0.2 (EtOAc /MeOH/EtOH/H_2O/AcOH 2:1:1:1:0.1); ^{1}H NMR (400 MHz, D_2O), ***β-Anomer: ***δ*** = 5.91 (m, 1H; H-3'), 5.26 – 5.09 (m, 2H; 2H-4'), 4.15 – 3.95 (m, 2H; H-4, H-6), 3.95 – 3.70 (m, 3H; H-9a, H-5, H-8), 3.70 – 3.54 (m, 2H; H-7, H-9b), 3.31 ("t", *J* = 6.8 Hz, 2H; 2H-1'), 2.64 ("t", *J* = 6.6 Hz, 2H; 2H-2'), 2.38 – 2.25 (m, 1H; H-3eq), 1.98 – 1.82 (m, 1H; H-3ax) ppm; HRMS: *m*/*z* calcd for $C_{14}H_{24}N_2O_9$: 363.1398 $[M-H]^-$; found: 363.1456.

Kinetic measurements: Kinetic measurements were carried out as previously described (Figure S1).[7b] Briefly, solutions of ManNPeoc (**8**), ManNBtl (**12**), or ManNBeac (**18**) (each 10 mM) and Tz-PEG-OH (**1**) (10 mM) in acetate buffer (pH 4.8) were mixed in a quartz cuvette to give final concentrations of each 5 mM. The reaction was monitored over time by measuring the decrease of absorption at 522 nm which is characteristic for Tz-PEG-OH (**1**). Second-order rate constants k_2 were determined by plotting $[\text{Tz-PEG-OH}]^{-1}$ against the time, followed by analysis via linear regression. The measurements were carried out in triplicates. The stability of Tz-PEG-OH (**1**) was published before.

Cell growth conditions: HEK 293T (human embryonic kidney) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing fetal bovine serum (FBS, 10%), penicillin (100 U mL^{-1}) and streptomycin (100 U mL^{-1}). The cells were incubated at 37 °C and 5 % carbon dioxide in a water-saturated incubator. The cells were diluted every third day by washing with PBS buffer and detaching with trypsin and EDTA.

Sugar stock solutions: The sugars were prepared as stock solutions (100 mM) in DMSO and stored at –20 °C. They were freshly diluted into media on the day of the experiment.

MGE and fluorescence microscopy: Similar to previously described experiments,[7b] HEK 293T cells (18,000 cells cm^{-2}) were seeded in 4-well ibiTreat µ-Slides (ibidi) Ph+ coated with poly-L-lysine (0.0025%, 1 h, 37 °C). After one day, cells were incubated with the mannosamine derivatives Ac_4ManNBtl (**11**), Ac_4ManNPtl (**13**), Ac_4ManNHxl (**15**), Ac_4ManNBeoc (**5**) or

Ac_4ManNBeac (**17**) (100 or 250 μM) for 48 h. As negative control the corresponding volume of DMSO only was added. Cells were washed twice with PBS and then incubated with Tz-biotin (**2,** 1 mM in media) for 6 h at 37 °C. After washing twice with PBS, the cells were incubated with streptavidin-AlexaFluor-555 (6.6 μg mL^{-1}) and Hoechst 33342 (10 μg mL^{-1}) for 20 min at 37 °C. After three washes with PBS, media (DMEM) was added for fluorescence microscopy.

Preparation of reference compounds for DMB labeling experiments: For the synthesis of the DMB-labeled reference components the neuraminic acid derivative (Neu5Aloc (**19**), Neu5Beoc (**20**), Neu5Peoc (**21**), Neu5Heoc (**22**), Neu5Btl (**23**), Neu5Ptl (**24**), Neu5Hxl (**25**) or Neu5Beac (**26**), 0.032 mmol) was dissolved in 265 μL DMB solution (5.3 mM DMB · 2 HCl, 16 mM $Na_2S_2O_4$, 40 mM TFA in MilliQ water). The mixture was incubated for 2.5 h at 56 °C in a thermomixer (300 rpm) in the dark. After the incubation the mixture was cooled on ice for 10 min and neutralized with aqueous NaOH (0.5 M, 21 μL). The solution was analyzed by analytical RP-HPLC-MS measurements. For fluorescence detection (excitation 372 nm, emission 456 nm) a dilution was necessary (1:400).

DMB-Neu5Aloc (31): analytical RP-HPLC (10%-30% B in 40 min): t_R = 22.5 min; HRMS: *m*/*z* calcd for $C_{20}H_{25}N_3O_{10}$: 468.1613 $[M+H]^+$; found: 468.1613.

DMB-Neu5Beoc (32): analytical RP-HPLC (10%-30% B in 40 min): t_R = 24.9 min, (10%-25% in 40 min): t_R = 36.3 min; HRMS: *m*/*z* calcd for $C_{21}H_{27}N_3O_{10}$: 482.1769 $[M+H]^+$; found: 482.1748.

DMB-Neu5Peoc (33): analytical RP-HPLC (10%-30% B in 40 min): t_R = 34.5 min; HRMS: *m*/*z* calcd for $C_{22}H_{29}N_3O_{10}$: 496.1926 $[M+H]^+$; found: 496.1926.

DMB-Neu5Heoc (34): analytical RP-HPLC (10%-30% B in 40 min): t_R = 41.0 min; HRMS: *m*/*z* calcd for $C_{23}H_{31}N_3O_{10}$: 510.2082 $[M+H]^+$; found: 510.2059.

DMB-Neu5Btl (35): analytical RP-HPLC (10%-18% B in 85 min): t_R = 29.3 min; HRMS: *m*/*z* calcd for $C_{20}H_{25}N_3O_9$: 452.1664 $[M+H]^+$; found: 452.1651.

DMB-Neu5Ptl (36): analytical RP-HPLC (10%-18%B in 85 min): t_R = 48.1 min; HRMS: *m*/*z* calcd for $C_{21}H_{27}N_3O_9$: 466.1820 $[M+H]^+$; found: 466.1805.

DMB-Neu5Hxl (37): analytical RP-HPLC (10%-18% B in 85 min): t_R = 75.5 min, (10%-25% in 40 min): t_R = 37.9 min; HRMS: *m*/*z* calcd for $C_{22}H_{29}N_3O_9$: 480.1977 $[M+H]^+$; found: 480.1953.

DMB-Neu5Beac (38): analytical RP-HPLC (10%-25% B in 40 min): t_R = 24.1 min; HRMS: *m*/*z* calcd for $C_{21}H_{28}N_4O_9$: 481.1881 $[M-H]^+$; found: 481.2292.

DMB labeling of sialic acids released from engineered cells: HEK 293T cells were seeded (450,000 cells in a 5 cm dish) and incubated for 24 h with 4 mL media. The medium was exchanged with media (4 mL) containing 100 μM of the modified sugar (Ac_4ManNAloc (**3**), Ac_4ManNBeoc (**5**), Ac_4ManNPeoc (**7**), Ac_4ManNHeoc (**9**), Ac_4ManNBtl (**11**), Ac_4ManNPtl (**13**), Ac_4ManNHxl (**15**), or Ac_4ManNBeac (**17**)). As a solvent control the corresponding volume of pure DMSO was added. The cells were cultured for 48 h with or without added sugar. The cells were trypsinated, resuspended in media and pelleted by centrifugation (5 min, 500 g). The pellets were washed 3 times with PBS Buffer (5 mL) and transferred to Eppendorf tubes (400,000 cells per tube). The cells were pelleted again (5 min 500 g) and the supernatant was discarded. The cells were resuspended in AcOH (3 M, 300 μL), and the mixture was incubated at 80 °C for 90 min in a thermomixer (300 rpm). The solution was diluted with MilliQ water (400 μL) and neutralized with a solution of NH_3 (25%) in water (20 μL). The solvent was removed in a SpeedVac, and the pellet was washed 3 times with EtOH (200 μL) and concentrated after every step. Then the pellet was dissolved in 265 μL DMB-labeling solution (5.3 mM DMB · 2 HCl, 16 mM $Na_2S_2O_4$, 40 mM TFA in MilliQ water). The mixture was incubated at 56 °C for 2.5 h in a thermomixer (300 rpm) in darkness. The mixture was cooled on ice for 10 min, neutralized with aqueous NaOH (0.5 M, 25 μL) and analyzed by analytical RP-HPLC-MS (for gradients and chromatograms see Figures S10–S22).

Acknowledgements

We are thankful to the late Professor Werner Reutter whose research laid the basis for the current investigations. We will not forget the many inspiring discussions we had during our collaboration. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 969 and SPP 1623), the University of Konstanz, and the Konstanz Research School Chemical Biology. We thank Stefan Bitter for support with synthesis, the Bioimaging Center of the University of Konstanz for providing the fluorescence microscopy instrumentation and the NMR Core Facility for providing the NMR instrumentation.

Keywords: carbohydrates • Diels-Alder reaction • metabolic engineering • sialic acids • DMB labeling

[1] a) *Essentials of Glycobiology*, 2nd ed. (Eds.: A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart, M. E. Etzler), Cold Spring Harbor Laboratory Press, Plainview, NY, **2009**; b) R. Schauer, *Zoology* **2004**, *107*, 49-64.

[2] a) B. Cheng, R. Xie, L. Dong, X. Chen, *ChemBioChem* **2016**, *17*, 11-27; b) P. R. Wratil, R. Horstkorte, W. Reutter, *Angew. Chem., Int. Ed.* **2016**, *55*, 9482-9512; c) T. J. Sminia, H. Zuilhof, T. Wennekes, *Carbohydr. Res.* **2016**, *435*, 121-141; d) S. T. Laughlin, C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 12-17.

[3] H. Kayser, R. Zeitler, C. Kannicht, D. Grunow, R. Nuck, W. Reutter, *J. Biol. Chem.* **1992**, *267*, 16934-16938.

[4] a) E. M. Sletten, C. R. Bertozzi, *Angew. Chem., Int. Ed.* **2009**, *48*, 6974-6998; b) J. A. Prescher, C. R. Bertozzi, *Nat. Chem. Biol.* **2005**, *1*, 13-21.

[5] E. Saxon, C. R. Bertozzi, *Science* **2000**, *287*, 2007-2010.

[6] a) C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057-3064; b) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem., Int. Ed.* **2002**, *41*, 2596-2599; c) N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046-15047; d) X. Ning, J. Guo, Margreet A. Wolfert, G.-J. Boons, *Angew. Chem., Int. Ed.* **2008**, *47*, 2253-2255.

[7] a) D. M. Patterson, L. A. Nazarova, B. Xie, D. N. Kamber, J. A. Prescher, *J. Am. Chem. Soc.* **2012**, *134*, 18638-18643; b) A. Niederwieser, A.-K. Späte, L. D. Nguyen, C. Jüngst, W. Reutter, V. Wittmann, *Angew. Chem., Int. Ed.* **2013**, *52*, 4265-4268; c) C. M. Cole, J. Yang, J. Šečkutė, N. K. Devaraj, *ChemBioChem* **2013**, *14*, 205-208; d) A.-K. Späte, H. Bußkamp, A. Niederwieser, V. F. Schart, A. Marx, V. Wittmann, *Bioconjugate Chem.* **2014**, *25*, 147-154; e) D. M. Patterson, K. A. Jones, J. A. Prescher, *Mol. BioSyst.* **2014**, *10*, 1693-1697; f) A.-K. Späte, V. F. Schart, J. Häfner, A. Niederwieser, T. U. Mayer, V. Wittmann, *Beilstein J. Org. Chem.* **2014**, *10*, 2235-2242; g) A.-K. Späte, V. F. Schart, S. Schöllkopf, A. Niederwieser, V. Wittmann, *Chem. Eur. J.* **2014**, *20*, 16502-16508; h) D.-C. Xiong, J. Zhu, M.-J. Han, H.-X. Luo, C. Wang, Y. Yu, Y. Ye, G. Tai, X.-S. Ye, *Org. Biomol. Chem.* **2015**, *13*, 3911-3917; i) A.-K. Späte, J. E. G. A. Dold, E. Batroff, V. F. Schart, D. E. Wieland, O. R. Baudendistel, V. Wittmann, *ChemBioChem* **2016**, *17*, 1374-1383.

[8] a) R. A. Carboni, R. V. Lindsey, *J. Am. Chem. Soc.* **1959**, *81*, 4342-4346; b) J. Sauer, D. K. Heldmann, J. Hetzenegger, J. Krauthan, H. Sichert, J. Schuster, *Eur. J. Org. Chem.* **1998**, 2885-2896; c) K. Braun, M. Wiessler, V. Ehemann, R. Pipkorn, H. Spring, J. Debus, B. Didinger, M. Koch, G. Muller, W. Waldeck, *Drug Des. Dev. Ther.* **2008**, *2*, 289-301; d) M. L. Blackman, M. Royzen, J. M. Fox, *J. Am. Chem. Soc.* **2008**, *130*, 13518-13519; e) N. K. Devaraj, R. Weissleder, S. A. Hilderbrand, *Bioconjugate Chem.* **2008**, *19*, 2297-2299; f) J. Schoch, M. Wiessler, A. Jäschke, *J. Am. Chem. Soc.* **2010**, *132*, 8846-8847; g) J. Šečkutė, N. K. Devaraj, *Curr. Opin. Chem. Biol.* **2013**, *17*, 761-767; h) A.-C. Knall, C. Slugovc, *Chem. Soc. Rev.* **2013**, *42*, 5131-5142.

[9] F. Doll, A. Buntz, A.-K. Späte, V. F. Schart, A. Timper, W. Schrimpf, C. R. Hauck, A. Zumbusch, V. Wittmann, *Angew. Chem., Int. Ed.* **2016**, *55*, 2262–2266.

[10] Y.-J. Lee, Y. Kurra, Y. Yang, J. Torres-Kolbus, A. Deiters, W. R. Liu, *Chem. Commun.* **2014**, *50*, 13085-13088.

[11] a) H. Bußkamp, E. Batroff, A. Niederwieser, O. S. Abdel-Rahman, R. Winter, V. Wittmann, A. Marx, *Chem. Commun.* **2014**, *50*, 10827-10829; b) U. Rieder, N. W. Luedtke, *Angew. Chem., Int. Ed.* **2014**, *53*, 9168-9172.

[12] a) R. Sustmann, *Pure Appl. Chem.* **1974**, *40*, 569-593; b) F. Thalhammer, U. Wallfahrer, J. Sauer, *Tetrahedron Lett.* **1990**, *31*, 6851-6854.

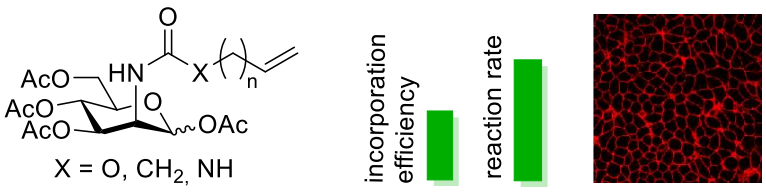
[13] a) O. T. Keppler, P. Stehling, M. Herrmann, H. Kayser, D. Grunow, W. Reutter, M. Pawlita, *J. Biol. Chem.* **1995**, *270*, 1308-1314; b) S. J. Luchansky, S. Argade, B. K. Hayes, C. R. Bertozzi, *Biochemistry* **2004**, *43*, 12358-12366; c) H. Möller, V. Böhrsch, J. Bentrop, J. Bender, S. Hinderlich, C. P. R. Hackenberger, *Angew. Chem., Int. Ed.* **2012**, *51*, 5986-5990; d) K. W. Dehnert, J. M. Baskin, S. T. Laughlin, B. J. Beahm, N. N. Naidu, S. L. Amacher, C. R. Bertozzi, *ChemBioChem* **2012**, *13*, 353-357; e) Z. Zhou, G. Liao, S. Stepanovs, Z. Guo, *J. Carbohydr. Chem.* **2014**, *33*, 395-407.

[14] a) S. Hara, Y. Takemori, M. Yamaguchi, M. Nakamura, Y. Ohkura, *Anal. Biochem.* **1987**, *164*, 138-145; b) M. Nakamura, S. Hara, M. Yamaguchi, Y. Takemori, Y. Ohkura, *Chem. Pharm. Bull.* **1987**, *35*, 687-692; c) S. Hara, M. Yamaguchi, Y. Takemori, K. Furuhata, H. Ogura, M. Nakamura, *Anal. Biochem.* **1989**, *179*, 162-166.

[15] a) D. G. Comb, S. Roseman, *J. Biol. Chem.* **1960**, *235*, 2529-2537; b) M. J. Kim, W. J. Hennen, H. M. Sweers, C. H. Wong, *J. Am. Chem. Soc.* **1988**, *110*, 6481-6486.

10.1002/cbic.201700002

Entry for the table of contents



Impact of chain length and linker moiety on glycan visualization: Eight different *N*-acylmannosamine derivatives including carbamates, amides, and a urea were evaluated for their suitability for metabolic glycoengineering. Terminal alkenes served as chemical reporter groups for bioorthogonal ligation with an inverse-electron-demand Diels-Alder reaction. The overall labeling efficiency on cell surfaces is determined by the metabolic acceptance of the carbohydrate and its reactivity in the ligation reaction.