ChemPubSoc Europe

DOI: 10.1002/chem.201404716

CHEMISTRY
A European Journal
Full Paper

Bioorthogonal Chemistry

Terminal Alkenes as Versatile Chemical Reporter Groups for Metabolic Oligosaccharide Engineering

Anne-Katrin Späte, Verena F. Schart, Sophie Schöllkopf, Andrea Niederwieser, and Valentin Wittmann*[a]

Abstract: The Diels–Alder reaction with inverse electron demand (DAinv reaction) of 1,2,4,5-tetrazines with electron rich or strained alkenes was proven to be a bioorthogonal ligation reaction that proceeds fast and with high yields. An important application of the DAinv reaction is metabolic oligosaccharide engineering (MOE) which allows the visualization of glycoconjugates in living cells. In this approach, a sugar derivative bearing a chemical reporter group is metabolically incorporated into cellular glycoconjugates and subsequently derivatized with a probe by means of a bioorthogonal ligation reaction. Here, we investigated a series of new mannosamine and glucosamine derivatives with carbamate-linked side chains of varying length terminated by alkene groups and their suitability for labeling cell-surface glycans. Kinetic investigations showed that the reactivity of the alkenes in DAinv reactions increases with growing chain length. When applied to MOE, one of the compounds, peracetylated *N*-butenyloxycarbonylmannosamine, was especially well suited for labeling cell-surface glycans. Obviously, the length of its side chain represents the optimal balance between incorporation efficiency and speed of the labeling reaction. Sialidase treatment of the cells before the bioorthogonal labeling reaction showed that this sugar derivative is attached to the glycans in form of the corresponding sialic acid derivative and not epimerized to another hexosamine derivative to a considerable extent.

Introduction

Visualization of biomolecules is an important prerequisite for fundamental biological as well as medical research. A popular approach is the direct installation of fluorescent tags onto biomolecules.[1] However, since fluorescence dyes can be rather bulky and might influence the properties of the target molecule, the chemical reporter strategy[2] has gained more and more importance to monitor biomolecules. In this strategy, a small functional group, the chemical reporter, is (often metabolically) incorporated into the biomolecule and in a subsequent step derivatized with the fluorescence probe by means of a bioorthogonal ligation reaction.[3] Ideally, the chemical reporter is small and does not alter the function and properties of the biomolecule (e.g., protein, DNA or carbohydrate). Frequently employed bioorthogonal ligation reactions include the Staudinger ligation[4] and the azide–alkyne cycloaddition[5] (often referred to as "click" reaction). Within the last years, the field of bioorthogonal ligation reactions has been greatly advanced by the development of the inverse-electron-demand Diels–Alder reaction (DAinv reaction) of 1,2,4,5-tetrazines and electron rich or strained alkenes.[6]

The DAinv reaction does not require metal catalysts and proceeds fast and in high yields, especially under aqueous conditions.[7] Furthermore, the reaction was shown to be bioorthogonal and can be orthogonal to the azide–alkyne cycloaddition which enables dual-labeling strategies.[8] In the past, a number of dienophiles have been employed for DAinv reactions with 1,2,4,5-tetrazines,[9] among them *trans*-cyclooctenes,[6a,8b,10] norbornenes,[6b,8c,10d,11] cyclobutenes,[6c,12] and cyclooctynes.[10c,d,13] Beside protein modification the reaction has been used in a whole range of applications[6e] including DNA modification[11b,14] and microarray preparation.[11d,15]

The DAinv reaction and related tetrazine ligations have also been appreciated for labeling metabolically engineered oligosaccharides. Metabolic oligosaccharide engineering (MOE) is a valuable technique to visualize cellular glycoconjugates, which has been challenging for a long time. MOE relies on the promiscuity of cellular enzymes that can accept and thus incorporate unnatural sugar derivatives into glycoconjugates.[16] If derivatized with a chemical reporter group, the incorporated sugar can be ligated to a probe.[17] Reporter groups suitable for MOE do not only need to be metabolically stable but must also not exceed a certain size limit in order to be accepted by the enzymes. In the past, a plethora of investigations has been carried out with azides and alkynes but recently, also tetrazine ligations were used. Since the fast-reacting *trans*-cyclooctene-

[a] *A.-K. Späte,[+] V. F. Schart,[+] S. Schöllkopf, Dr. A. Niederwieser, 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

[+] *These authors contributed equally to this work.*

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201404716.

and norbornene-based dienophiles are expected to be too bulky for application in MOE, smaller probes, such as cyclopropenes[8d,f,18] and isonitriles[8g,19] have been developed. Recently, we have shown that terminal alkenes are suitable reporter groups for MOE, and ManNAc derivatives **1** and **2** (Figure 1) with a pentenoyl and hexenoyl side chain, respectively, are accepted by the biosynthetic pathway of HEK 293T and HeLa S3 cells.[8e] Together with an azide-labeled second sugar we were able to employ Ac_4ManNPtl **1** in a dual-labeling strategy. Furthermore, we could show that carbamate derivatives, such as Ac_4ManNPeoc **5** are also well tolerated by the enzymatic machinery[8e] which is in line with observations by others.[18b,20] Although the rate of the DAinv reaction of pentenoyl and hexenoyl groups with a phenyl-(pyrimidin-2-yl)-tetrazine does not reach the value of the reaction of cyclopropenes and isonitriles, it is still one order of magnitude higher than the typical rate constant[21] of the Staudinger ligation. In addition, terminal alkenes are expected to be more stable than strained cyclic alkenes and they are easier accessible. Thus, they might be preferred probes in cases where fast reaction rates are not required.

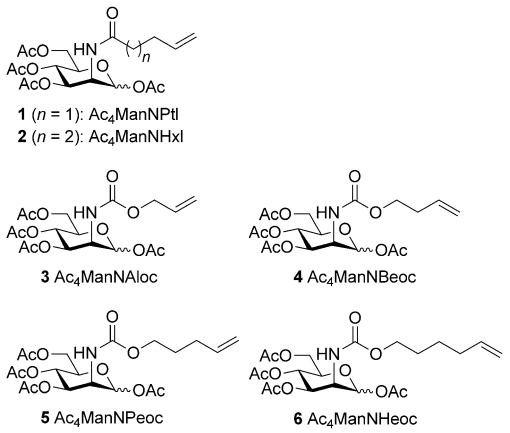


Figure 1. Mannosamine derivatives for MOE. Aloc = allyloxycarbonyl, Beoc = butenyloxycarbonyl, Peoc = pentenyloxycarbonyl, Heoc = hexenyloxycarbonyl.

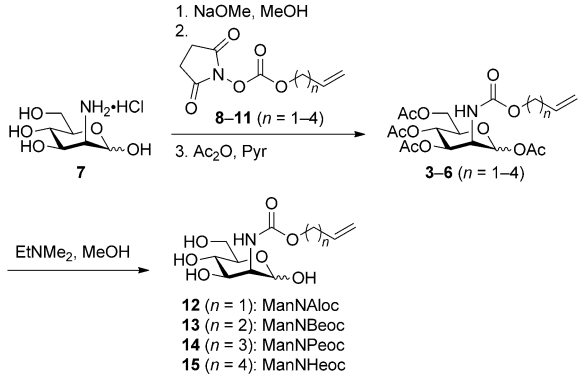
DAinv reactions are controlled by a $LUMO^{diene}$–$HOMO^{dienophile}$ interaction in the transition state.[22] Efficient reactions require a high HOMO energy of the dienophile which arises from electron-donating substituents at the double bond. Electron-withdrawing substituents, on the other hand, lower the HOMO energy and, thus, decrease the DAinv reaction rate with a given diene. It can be expected that carbamates of type **3**–**6** show a higher DAinv reactivity with an increasing distance between the double bond and the carbamate group with its inductive (−I) effect (i.e., with increasing chain length). On the other hand, an important feature of a sugar derivative suitable for use in MOE is its acceptance by the cells' biosynthetic machinery, and it was shown that ManNAc derivatives with longer acyl chains than the natural acetyl group are metabolized with reduced efficiency.[23] Taken together, varying the length of the acyl chain is expected to have two opposite effects on the suitability of a ManNAc derivative for glycan labeling after MOE and the optimal length is not easily predictable.

Here, we present the series **3**–**6** of ManNAc derivatives featuring carbamate-linked terminal alkenes with varying chain length and their suitability for labeling cell-surface glycans. Butenyloxycarbonyl-derivatized mannosamine **4** turned out to have the perfect balance between metabolic incorporation efficiency and DAinv reactivity for labeling cell-surface sialic acids and leads to significantly improved labeling efficiency compared to previously reported ManNAc derivative **5**.

Results and Discussion

Synthesis of Mannosamine Derivatives

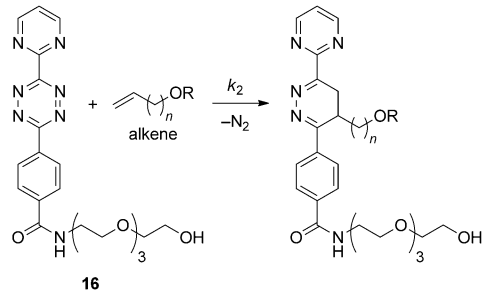
The synthesis of mannosamine derivatives **3**–**6** is depicted in Scheme 1. Mannosamine hydrochloride **7** was neutralized with NaOMe solution and reacted with alkenyl succinimidyl carbonates **8**–**11**. The crude products were peracetylated to yield mannosamine derivatives **3**–**6** in yields between 50 and 80%. For subsequent kinetic studies in acetate buffer, the compounds were de-*O*-acetylated using ethyldimethylamine in methanol to give carbohydrates **12**–**15**.



Scheme 1. Synthesis of mannosamine derivatives **3**–**6** and deacetylation to **12**–**15**.

Kinetic Studies

To determine second-order rate constants of different terminal alkenes in DAinv reactions, we selected water-soluble phenyl-(pyrimidin-2-yl)-tetrazine **16**[8e] as reaction partner (Scheme 2) because this type of tetrazine had been proven to be suited for a number of applications[6c,10d,11b,d] including MOE[8e] due to its reactivity and, at the same time, stability in aqueous media. For the measurements we exploited the fact that tetrazine **16** has a characteristic absorption maximum at 522 nm. Thus, the DAinv reaction could be followed by measuring the decrease of absorbance of **16** over time. Second-order rate constants k_2 were calculated as reported earlier[8e] (see Figure 2 for representative data points and Table 1 for calculated rate constants).



Scheme 2. DAinv reaction between terminal alkenes (alkenols or mannosamine derivatives **12**–**15**) and tetrazine **16**. Only one isomer of the product is shown.

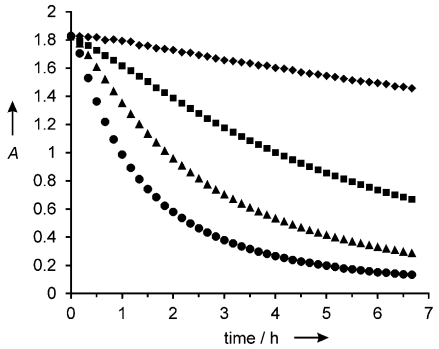


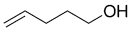
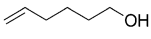
Figure 2. Kinetics of reaction of alkenols (5 mM) with tetrazine **16** (5 mM) in 100 mM acetate buffer (pH 4.8) monitored by measuring the decrease of absorption at 522 nm of **16** over time. A = absorbance at 522 nm, ◆ = allyl alcohol, ■ = butenol, ▲ = pentenol, ● = hexenol.

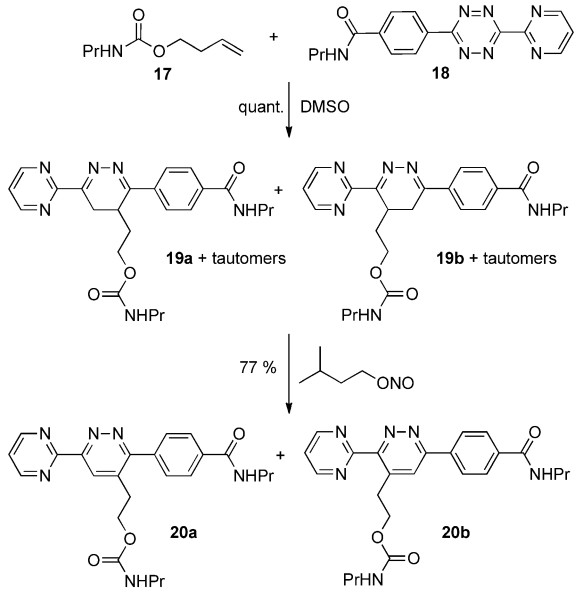
In orienting experiments, we first determined the second-order rate constants k_2 of four alkenols (allyl alcohol, butenol, pentenol, and hexenol) with tetrazine **16** (Table 1, entries 1–4). As expected, the rate constant increases with growing distance between the alkene and the electron-withdrawing oxygen corresponding to a higher HOMO energy of the dienophile. The biggest increase of k_2 by a factor of 5.5 is observed between allyl alcohol and butenol. Further elongation of the carbon chain to pentenol and hexenol leads to a stepwise increase of k_2 by factors of 3.1 and 2.4, respectively. The same trend is observed for the series of mannosamine derivatives **12**–**15** (entries 5–8). In this case k_2 increases by an factor of 9.3 when going from the Aloc (entry 5) to the Beoc derivative (entry 6). Further chain elongation ends up in a rate constant of 0.074 $M^{-1}s^{-1}$ for ManNHeoc **15** (entry 8) comparable to the k_2 value of hexenol of 0.08 $M^{-1}s^{-1}$ (entry 4).

DAinv Reaction in Preparative Scale

To characterize the product and reaction yield of a DAinv reaction with a carbamate-modified terminal alkene, we choose model carbamate **17** and tetrazine **18**[11d] as reaction partners and performed a DAinv reaction in DMSO (Scheme 3). During this reaction, two isomeric dihydropyridazines **19a**/**b**, that exist in several tautomeric forms, were formed. LC-MS analysis revealed that these products were partially oxidized to the corresponding pyridazines **20a**/**b** (Figure S1). To facilitate NMR characterization, the oxidation was completed by the addition of isopentyl nitrite to give the pyridazines **20a**/**b** in a yield of 77%.

Table 1. Second-order rate constants k_2 of DAinv reaction of tetrazine **16** with terminal alkenes.

Entry	Alkene	k_2 [$M^{-1}s^{-1}$]
1		0.002 ± 0.0002
2		0.011 ± 0.001
3		0.034 ± 0.003
4		0.080 ± 0.006
5	ManNAloc **12**	0.0015 ± 0.0001
6	ManNBeoc **13**	0.014 ± 0.003
7	ManNPeoc **14**	0.017 ± 0.002[8e]
8	ManNHeoc **15**	0.074 ± 0.013



Scheme 3. DAinv reaction of alkene **17** and tetrazine **18**[11d] followed by oxidation to yield pyridazines **20a**/**b**.

Detection of Cell-Surface Oligosaccharides

To examine the suitability of the alkene-labeled mannosamine derivatives **3**–**6** for labeling cell-surface oligosaccharides, the sugars were applied for MOE. HEK 293T cells were grown in the presence of one of the monosaccharides **3**–**6**. Subsequently, the cells were incubated with tetrazine-biotin (Tz-biotin) **21**[8e] (Figure 3) in order to react incorporated alkenes. After labeling with streptavidin-AlexaFluor647, confocal fluorescence laser-scanning microscopy showed cell membrane staining for all mannosamine derivatives (Figure 4B–E) while no staining was detected when only DMSO was added to the cells (Figure 4A). Interestingly, Ac_4ManNAloc **3** showed a weak membrane staining even though its reactivity in the DAinv reaction is rather low (Figure 4B). For Ac_4ManNBeoc **4** the most intense

labeling was obtained (Figure 4C), which was significantly brighter than the one for both Ac_4ManNPeoc **5** (Figure 4D) and Ac_4ManNHeoc **6** (Figure 4E) even though **5** and **6** react faster in the DAinv reaction. A plausible explanation for this observation is a more efficient incorporation of Beoc derivative **4** having a shorter side chain than derivatives **5** and **6** that overcompensates its lower reactivity. Within the series of sugar derivatives **3**–**6**, clearly Ac_4ManNBeoc **4** is best suited for labeling cell-surface sialic acids.

Since monosaccharides can be isomerized in cells by action of several epimerases,[20,24] we wanted to confirm that the incorporated mannosamine derivative **4** is indeed converted into a sialic acid and attached to glycans rather than being incorporated as glucosamine or galactosamine derivate. Thus, HEK 293T cells that had been cultivated with 100 μM Ac_4ManNBeoc **4** for 48 h were incubated with sialidase (10 mU mL^{-1}, 1 h, 37 °C) prior to staining. Since no cell-membrane staining was detected after sialidase treatment (Figure 5B), we conclude, that epimerization of compound **4** to the corresponding glucosamine or galactosamine derivatives had not taken place to a considerable extent (Figure 5).

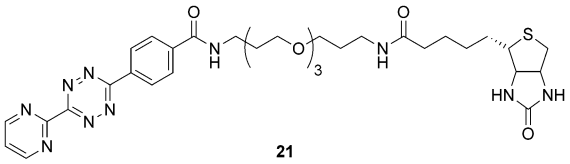


Figure 3. Structure of Tz-biotin **21**.

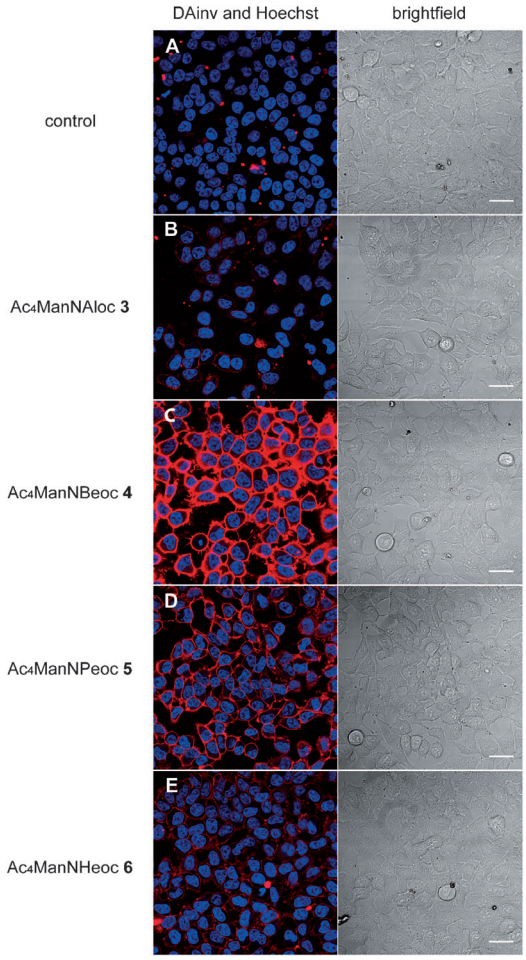


Figure 4. HEK 293T cells were grown with DMSO (A) or with 100 μM Ac_4ManNAloc **3** (B), Ac_4ManNBeoc **4** (C), Ac_4ManNPeoc **5** (D), and Ac_4ManNHeoc **6** (E), respectively, for 48 h. For visualization, cells were incubated with Tz-biotin **21** (1 mM, 6 h, 37 °C) followed by streptavidin-AlexaFluor647 (20 min, 37 °C). Nuclei were stained with Hoechst33342. Scale bar: 30 μm.

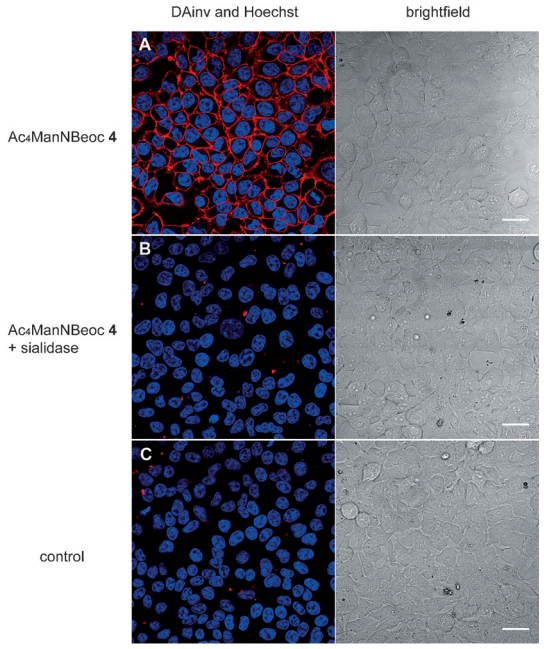


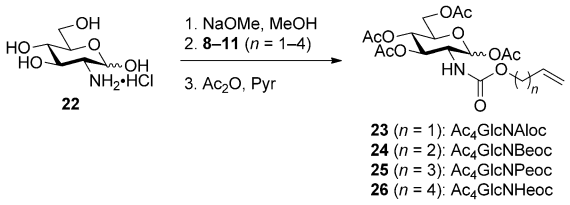
Figure 5. HEK 293T cells were grown with 100 μM Ac_4ManNBeoc **4** (A, B) or with DMSO (C) for 48 h. For removal of the sialic acids, cells were treated with sialidase (10 mU mL^{-1}, 1 h, 37 °C) (B). Staining was achieved by incubation with Tz-biotin **21** (1 mM, 6 h, 37 °C) followed by addition of streptavidin-AlexaFluor647. Nuclei were stained with Hoechst33342. Scale bar: 30 μm.

Synthesis of Alkene-Modified Glucosamine Derivatives

Previously, it has been shown[25] that *N*-acetylglucosamine derivatives with a reporter group in the acyl chain, such as *N*-azidoacetylglucosamine, are suitable probes to label *O*-GlcNAcylated intracellular proteins[26] employing MOE. Thus, to extend possible applications of terminal alkenes, we prepared the series **23**–**26** of carbamate-modified glucosamine derivatives with varying chain length starting from glucosamine hydrochloride **22** (Scheme 4). We employed all four glucosamine derivatives for MOE, however, we experienced cell toxicity for all compounds at the concentrations necessary for detection of cell-surface glycans.

Conclusion

In summary, we present a series of new mannosamine and glucosamine derivatives with carbamate-linked side chains of



Scheme 4. Synthesis of glucosamine derivatives **23**–**26**.

varying length terminated by alkene groups. Kinetic investigations showed that the reactivity of the alkenes in DAinv reactions increases with growing chain length. When applied to MOE, all mannosamine derivatives could be employed to label cell-surface carbohydrates on living HEK 293T cells, though with substantially differing intensity as detected by confocal fluorescence microscopy. Whereas the compound with the shortest side chain (Ac_4ManNAloc **3**) gave only a faint staining of the plasma membrane, probably due to its low reactivity in the DAinv labeling reaction, the compounds Ac_4ManNHeoc **6** and Ac_4ManNPeoc **5** with longer side chains resulted in somewhat more intense labeling. Ac_4ManNBeoc **4**, however, clearly gave the brightest staining. Obviously, the length of its side chain represents the optimal balance between incorporation efficiency and speed of the labeling reaction. Sialidase treatment of the cells before the bioorthogonal labeling reaction showed that Ac_4ManNBeoc **4** is attached to the glycans in form of the corresponding sialic acid derivative and not epimerized to another hexosamine derivative to a considerable extent.

Experimental Section

General Methods

Chemicals were purchased from Aldrich, Acros Organics, Fluka, and Dextra and used without further purification. AlexaFluor 647-labeled streptavidin and Hoechst33342 were purchased from Invitrogen. Technical solvents were distilled prior to use. All reactions were carried out in dry solvents. Thin-layer chromatography (TLC) was performed on silica gel 60 F254 coated aluminum sheets (Merck) with detection by UV light ($\lambda = 254$ nm). Additionally, the sheets were stained by dipping in acidic ethanolic *p*-anisaldehyde solution or basic $KMnO_4$ solution followed by gentle heating. Preparative flash column chromatography (FC) was performed with an MPLC-Reveleris system from Grace. Nuclear magnetic resonance (NMR) spectra were recorded at room temperature on Avance III 400 and Avance III 600 instruments from Bruker. Chemical shifts are reported relative to solvent signals ($CDCl_3$: $\delta_H = 7.26$ ppm, $\delta_C = 77.16$ ppm). Signals were assigned by first-order analysis and, when feasible, assignments were supported by two-dimensional $^1H,^1H$ and $^1H,^{13}C$ correlation spectroscopy (COSY, HMBC and HSQC). High-resolution mass spectrometry (HRMS) was carried out on a micrOTOF II instrument from Bruker Daltonics. UV/Vis Absorption was measured using a Carry 50 instrument from Varian and software scanning kinetics. Microscopy was performed using a point laser scanning confocal microscope (Zeiss LSM 510 Meta) equipped with Meta detector for spectral imaging.

General Procedure for the Synthesis of Sugar Carbamates

The hexosamine hydrochloride (1 equiv) was dissolved in MeOH, and NaOMe (0.5 M in MeOH, 1 equiv) was added under nitrogen. The reaction mixture was stirred at room temperature for 2 h. A solution of the alkenyl succinimidyl carbonate (1.2 equiv) in MeOH was added and the reaction mixture was stirred for 16 h. The solvent was evaporated and the residual oil was dissolved in pyridine (10 equiv) and acetic anhydride (10 equiv), and the reaction mixture was stirred for 16 h. Then, the solvent was removed and the residual oil was dissolved in CH_2Cl_2 (100 mL) and washed with 10% aq. $KHSO_4$ (2×100 mL), sat. aq. $NaHCO_3$ (2×100 mL), and brine (100 mL). The organic layer was dried ($MgSO_4$) and the solvent evaporated. The crude product was purified by FC.

1,3,4,6-Tetra-*O*-acetyl-2-((allyloxycarbonyl)amino)-2-deoxy-D-mannopyranose (Ac_4ManNAloc) (3): Mannosamine hydrochloride **7** (500 mg, 2.31 mmol) and allyl succinimidyl carbonate **8** (574 mg, 2.88 mmol) were reacted in MeOH (20 mL) according to the general procedure. The crude product was purified by FC (petroleum ether/ethyl acetate 1:1). To remove remaining *N*-hydroxysuccinimide (NHS), the combined product fractions were evaporated, redissolved in CH_2Cl_2 and extracted three times with 1 N NaOH. Ac_4ManNAloc **3** was obtained as mixture of anomers (0.5 g, 50%, α/β 2:1). $R_f = 0.46$ (petroleum ether/ethyl acetate 1:1). **α-Anomer**: ^1H NMR (400 MHz, $CDCl_3$): $\delta = 6.09$ (d, $J = 1.9$ Hz, 1H, H-1), 5.93 (ddt, $J = 16.6$, 11.5, 5.9 Hz, 1H, CH), 5.39–5.10 (m, 4H, H-4, NH, $CHCH_2$), 5.09–4.98 (m, 1H, H-3), 4.58 (dt, $J = 5.7$, 1.5 Hz, 2H, CH_2), 4.35 (dd, $J = 9.8$, 4.3 Hz, 1H, H-2), 4.30–4.20 (m, 1H, H-6a), 4.17–3.96 (m, 2H, H-5, H-6b), 2.18 (s, 3H, CH_3), 2.10 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 2.02 (s, 3H, CH_3). **β-Anomer**: ^1H NMR (400 MHz, $CDCl_3$): $\delta = 5.93$ (ddt, $J = 16.6$, 11.5, 5.9 Hz, 1H, CH), 5.85 (d, $J = 1.8$ Hz, 1H, H-1), 5.39–5.10 (m, 4H, H-4, NH, $CHCH_2$), 5.09–4.98 (m, 1H, H-3), 4.58 (dt, $J = 5.7$, 1.5 Hz, 2H, CH_2), 4.48 (dd, $J = 8.2$, 3.4 Hz, 1H, H-2), 4.30–4.20 (m, 1H, H-6a), 4.17–3.96 (m, 1H, H-6b), 3.78 (ddd, $J = 9.8$, 4.9, 2.6 Hz, 1H, H-5), 2.12 (s, 3H, CH_3), 2.10 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 2.02 (s, 3H, CH_3). **α/β-Anomers**: ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 170.7$, 170.7, 170.2, 170.2, 169.7, 168.2, 155.8 (CO α,β), 132.7 ($CHCH_2\beta$), 132.5 ($CHCH_2\alpha$), 118.5 ($CHCH_2\alpha$), 118.1 ($CHCH_2\beta$), 92.0 (C-1α), 90.8 (C-1β), 73.5, 71.7, 70.3, 69.3, 66.3, 66.2, 65.4, 65.3 (C-3α,β, C-4α,β, C-5α,β, CH_2α,β), 62.0 (C-6α), 61.9 (C-6β), 51.3 (C-2α,β), 21.0, 21.0, 20.9, 20.9, 20.8, 20.8 (4x CH_3α,β); HRMS: *m/z* calcd for $C_{18}H_{25}NO_{11}$: 454.13198 $[M+Na]^+$, found: 454.13046.

1,3,4,6-Tetra-*O*-acetyl-2-((but-3-en-1-yl-oxycarbonyl)amino)-2-deoxy-D-mannopyranose (Ac_4ManNBeoc) (4): Mannosamine hydrochloride **7** (840 mg, 3.9 mmol) and but-3-en-1-yl succinimidyl carbonate **9** (1 g, 4.7 mmol) were reacted in MeOH (30 mL) according to the general procedure. The crude product was purified by FC (petroleum ether/ethyl acetate 1:2). To remove remaining NHS, the combined product fractions were evaporated, redissolved in CH_2Cl_2 and extracted three times with 2 N NaOH. Ac_4ManNBeoc **4** was obtained as mixture of anomers (1.4 g, 80%, α/β 2:1). $R_f = 0.37$ (petroleum ether/ethyl acetate 1:1). **α-Anomer**: ^1H NMR (400 MHz, $CDCl_3$): $\delta = 6.09$ (d, $J = 1.5$ Hz, 1H, H-1), 5.83–5.72 (m, 1H, CH), 5.31 (dd, $J = 10.2$, 4.3 Hz, 1H, H-3), 5.23–5.05 (m, 3H, H-4, $CHCH_2$), 5.02 (d, $J = 10.1$ Hz, 1H, NH), 4.34 (dd, $J = 9.6$, 3.0 Hz, 1H, H-2), 4.30–4.20 (m, 1H, H-6a), 4.20–4.06 (m, 3H, H-6b, OCH_2), 4.04–3.97 (m, 1H, H-5), 2.46–2.31 (m, 2H, CH_2), 2.18 (s, 3H, CH_3), 2.10 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 2.03 (s, 3H, CH_3). **β-Anomer**: ^1H NMR (400 MHz, $CDCl_3$): $\delta = 5.84$ (d, $J = 1.9$ Hz, 1H, H-1), 5.83–5.72 (m, 1H, CH), 5.24–5.05 (m, 4H, H-3, H-4, $CHCH_2$), 5.03 (d, $J = 9.7$ Hz, 1H, NH), 4.47 (d, $J = 9.0$ Hz, 1H, H-2), 4.30–4.20 (m, 1H, H-6a), 4.20–4.06 (m, 3H, H-6b, OCH_2), 3.78 (ddd, $J = 9.6$, 4.9, 2.5 Hz, 1H, H-5), 2.46–2.31 (m, 2H, CH_2), 2.12 (s, 3H, CH_3), 2.10 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 2.04 (s, 3H, CH_3). **α/β-Anomers**: ^{13}C NMR (101 MHz, $CDCl_3$):

δ = 170.6, 169.9, 168.4, 168.1, 156.6 (COα,β), 134.0 (*C*HCH$_2$), 117.3 (CH*C*H$_2$), 91.9 (C-1α), 90.7 (C-1β), 73.4 (C-5β), 71.6 (C-3β), 70.2 (C-5α), 69.1 (C-3α), 65.3, 65.2 (C-4α,β), 64.5 (OCH$_2$), 61.9, 61.8 (C-6α,β), 51.3 (C-2α,β), 33.4 (OCH$_2$*C*H$_2$), 20.9 (CH$_3$), 20.8 (CH$_3$), 20.8 (CH$_3$), 20.7 (CH$_3$), 20.7 (CH$_3$), 20.6 (CH$_3$); HRMS: *m/z* calcd for $C_{19}H_{27}NO_{11}$: 468.14763 $[M + Na]^+$, found: 468.14524.

1,3,4,6-Tetra-*O*-acetyl-2-((hex-5-en-1-yl-oxycarbonyl)amino)-2-deoxy-D-mannopyranose (Ac_4ManNHeoc) (6): Mannosamine hydrochloride **7** (740 mg, 3.45 mmol) and hex-5-en-1-yl succinimidyl carbonate **11** (1 g, 4.14 mmol) were reacted in MeOH (30 mL) according to the general procedure. The crude product was purified by FC (petroleum ether/ethyl acetate 1:1). To remove remaining NHS, the combined product fractions were evaporated, redissolved in CH_2Cl_2 and extracted three times with 2 N NaOH. Ac_4ManNHeoc **6** was obtained as mixture of anomers (1.06 g, 64%, α/β 4:3). R_f = 0.84 (petroleum ether/ethyl acetate 1:1). **α-Anomer**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.08 (s, 1H, H-1), 5.81–5.70 (m, 1H, C*H*CH$_2$), 5.30 (dd, *J* = 10.6, 3.9 Hz, 1H, H-3), 5.22–5.11 (m, 1H, H-4), 5.10–4.94 (m, 3H, NH, CHC*H*$_2$), 4.33 (dd, *J* = 9.8, 4.2 Hz, 1H, H-2), 4.28–4.22 (m, 1H, H-6a), 4.13–3.98 (m, 4H, H-6b, H-5, OCH$_2$), 2.17 (s, 3H, CH$_3$), 2.11 (s, 3H, CH$_3$), 2.10–2.06 (m, 2H, C*H*$_2$CHCH$_2$), 2.05 (s, 6H, 2x CH$_3$), 1.70–1.58 (m, 2H, OCH$_2$C*H*$_2$CH$_2$), 1.46 (q, *J* = 7.1, 6.5 Hz, 2H, OCH$_2$CH$_2$C*H*$_2$). **β-Anomer**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.84 (s, 1H, H-1), 5.81–5.70 (m, 1H, C*H*CH$_2$), 5.22–5.11 (m, 1H, H-4), 5.10–4.94 (m, 4H, NH, H-3, CHC*H*$_2$), 4.46 (dd, *J* = 9.3, 3.5 Hz, 1H, H-2), 4.28–4.22 (m, 1H, H-6a), 4.13–3.98 (m, 3H, H-6b, OCH$_2$), 3.77 (ddd, *J* = 9.6, 5.0, 2.5 Hz, 1H, H-5), 2.10–2.06 (m, 8H, 2x CH$_3$, C*H*$_2$CHCH$_2$), 2.02 (s, 3H, CH$_3$), 2.01 (s, 3H, CH$_3$), 1.70–1.58 (m, 2H, OCH$_2$C*H*$_2$CH$_2$), 1.46 (q, *J* = 7.1, 6.5 Hz, 2H, OCH$_2$CH$_2$C*H*$_2$). **α/β-Anomers**: ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.7, 170.2, 169.7, 168.2, 156.9 (COα,β), 138.4 (*C*HCH$_2$), 115.0 (CH*C*H$_2$), 92.0, 90.8 (C-1α,β), 73.5, 71.7, 70.3, 69.3 (C-3α,β, C-5α,β), 65.7, 65.5, 62.1, 62.0 (C-4α,β, OCH$_2\alpha$,β), 51.4, 51.2 (C-2α,β), 33.43, 33.40 (*C*H$_2$CHCH$_2\alpha$,β), 28.51, 28.46 (OCH$_2$CH$_2$CH$_2\alpha$,β), 25.20, 25.18 (OCH$_2$CH$_2$CH$_2\alpha$,β), 21.0, 20.94, 20.87, 20.85, 20.79, 20.76 (4x CH$_3\alpha$,β); HRMS: *m/z* calcd for $C_{21}H_{31}NO_{11}$: 496.17893 $[M+Na]^+$, found: 496.17665.

Cell Growth Conditions

HEK 293T cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. All cells were incubated with 5% carbon dioxide in a water-saturated incubator at 37 °C. Wells were coated with 0.01% poly-L-lysine (Sigma) and 25 μg mL^{-1} fibronectin (Sigma) in phosphate-buffered saline (PBS) for 1 h at 37 °C and rinsed with PBS prior to cell seeding.

Fluorescence Microscopy

HEK 293T cells (22000 cells per well) were seeded in 4-well ibiTreat μ-Slides (ibidi) and allowed to attach for 12 h. Cells were then incubated with 100 μM of the corresponding mannosamine derivative (**3**–**6**) for 48 h. The sugars were prepared as stock solutions in DMSO (100 mM) and diluted into media. DMSO only was added as negative control. Cells were washed two times with PBS and then treated with Tz-biotin **21** (6 h, 1 mM) at 37 °C. After two washes with PBS, cells were incubated with AlexaFluor 647-labeled streptavidin (6.6 μg mL^{-1}) and Hoechst 33342 (10 μg mL^{-1}) for 20 min at 37 °C in the dark. Cells were washed twice with PBS, and DMEM was added for microscopy. A Zeiss microscope LSM 510 Meta equipped with a 40×1.3 NA Plan-Neofluar oil DIC immersion objective was employed for imaging. Analysis of the obtained data was performed using Image J software version 1.45S.

Sialidase Experiments

HEK 293T cells were treated in the same way as for fluorescence microscopy. Prior to incubation with Tz-biotin **21**, sialidase (0.5 U mL^{-1} in Opti-MEM, Roche) was added for 1 h at 37 °C. Cells were washed two times with PBS and labeled as described previously.

Kinetic Measurements

For deacetylation of **3**–**6**, the respective mannosamine derivative (0.5 g) was dissolved in dry MeOH (28 mL), and EtNMe$_2$ (6 mL) was added. The reaction mixture was stirred for 8 d at RT during which additional EtNMe$_2$ (3 mL) was added two times (day 2 and 4). The solvent was evaporated and the brown residue was purified by FC (CH_2Cl_2/MeOH 10:1) yielding free sugars **12**--**15**. For kinetic measurements, stock solutions (10 mM in 100 mM acetate buffer, pH 4.8) of alkenols, mannosamine derivatives **12**–**15**, and tetrazine **16** were prepared. The reaction partners were mixed in a quartz cuvette immediately before the measurement. To monitor the reaction over time, the absorption at λ_{max} = 522 nm was measured and the tetrazine concentration was calculated using Beer–Lambert law. The second-order rate constant was determined by plotting the inverse tetrazine concentration versus time followed by linear regression analysis as reported earlier.[8e]

Acknowledgements

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 the Bioimaging Center of the University of Konstanz for providing the fluorescence microscopy instrumentation.

Keywords: bioorthogonal chemistry · carbohydrates · cycloaddition · metabolic oligosaccharide engineering · tetrazines

[1] G. T. Hermanson, *Bioconjugate Techniques*, 3edrd edAcademic Press, New York, **2013**.
[2] J. A. Prescher, C. R. Bertozzi, *Nat. Chem. Biol.* **2005**, *1*, 13–21.
[3] E. M. Sletten, C. R. Bertozzi, *Angew. Chem. Int. Ed.* **2009**, *48*, 6974–6998; *Angew. Chem.* **2009**, *121*, 7108–7133.
[4] E. Saxon, C. R. Bertozzi, *Science* **2000**, *287*, 2007–2010.
[5] a) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2002**, *41*, 2596–2599; *Angew. Chem.* **2002**, *114*, 2708–2711; b) C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057–3064; c) N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046–15047; d) X. Ning, J. Guo, M. A. Wolfert, G.-J. Boons, *Angew. Chem. Int. Ed.* **2008**, *47*, 2253–2255; *Angew. Chem.* **2008**, *120*, 2285–2287.
[6] a) M. L. Blackman, M. Royzen, J. M. Fox, *J. Am. Chem. Soc.* **2008**, *130*, 13518–13519; b) N. K. Devaraj, R. Weissleder, S. A. Hilderbrand, *Bioconjugate Chem.* **2008**, *19*, 2297–2299; 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) J. Šečkutė, N. K. Devaraj, *Curr. Opin. Chem. Biol.* **2013**, *17*, 761–767; e) A.-C. Knall, C. Slugovc, *Chem. Soc. Rev.* **2013**, *42*, 5131–5142.
[7] J. W. Wijnen, S. Zavarise, J. B. F. N. Engberts, *J. Org. Chem.* **1996**, *61*, 2001–2005.
[8] a) M. R. Karver, R. Weissleder, S. A. Hilderbrand, *Angew. Chem. Int. Ed.* **2012**, *51*, 920–922; *Angew. Chem.* **2012**, *124*, 944–946; b) J. Schoch, M. Staudt, A. Samanta, M. Wiessler, A. Jäschke, *Bioconjugate Chem.* **2012**, *23*, 1382–1386; c) L. I. Willems, N. Li, B. I. Florea, M. Ruben, G. A. van der

Marel, H. S. Overkleeft, *Angew. Chem. Int. Ed.* **2012**, *51*, 4431 – 4434; *Angew. Chem.* **2012**, *124*, 4507 – 4510; d) D. M. Patterson, L. A. Nazarova, B. Xie, D. N. Kamber, J. A. Prescher, *J. Am. Chem. Soc.* **2012**, *134*, 18638 – 18643; e) A. Niederwieser, A.-K. Späte, L. D. Nguyen, C. Jüngst, W. Reutter, V. Wittmann, *Angew. Chem. Int. Ed.* **2013**, *52*, 4265 – 4268; *Angew. Chem.* **2013**, *125*, 4359 – 4363; f) C. M. Cole, J. Yang, J. Šečkutė, N. K. Devaraj, *ChemBioChem* **2013**, *14*, 205 – 208; g) Y. A. Wainman, A. A. Neves, S. Stairs, H. Stöckmann, H. Ireland-Zecchini, K. M. Brindle, F. J. Leeper, *Org. Biomol. Chem.* **2013**, *11*, 7297 – 7300.

[9] J. Sauer, D. K. Heldmann, J. Hetzenegger, J. Krauthan, H. Sichert, J. Schuster, *Eur. J. Org. Chem.* **1998**, 2885 – 2896.

[10] a) N. K. Devaraj, S. Hilderbrand, R. Upadhyay, R. Mazitschek, R. Weissleder, *Angew. Chem. Int. Ed.* **2010**, *49*, 2869 – 2872; *Angew. Chem.* **2010**, *122*, 2931 – 2934; b) R. Rossin, P. Renart Verkerk, S. M. van den Bosch, R. C. M. Vulders, I. Verel, J. Lub, M. S. Robillard, *Angew. Chem. Int. Ed.* **2010**, *49*, 3375 – 3378; *Angew. Chem.* **2010**, *122*, 3447 – 3450; c) K. Lang, L. Davis, S. Wallace, M. Mahesh, D. J. Cox, M. L. Blackman, J. M. Fox, J. W. Chin, *J. Am. Chem. Soc.* **2012**, *134*, 10317 – 10320; d) T. Plass, S. Milles, C. Koehler, J. Szymański, R. Mueller, M. Wießler, C. Schultz, E. A. Lemke, *Angew. Chem. Int. Ed.* **2012**, *51*, 4166 – 4170; *Angew. Chem.* **2012**, *124*, 4242 – 4246; e) M. T. Taylor, M. L. Blackman, O. Dmitrenko, J. M. Fox, *J. Am. Chem. Soc.* **2011**, *133*, 9646 – 9649.

[11] a) K. Lang, L. Davis, J. Torres-Kolbus, C. Chou, A. Deiters, J. W. Chin, *Nat. Chem.* **2012**, *4*, 298 – 304; b) J. Schoch, M. Wiessler, A. Jäschke, *J. Am. Chem. Soc.* **2010**, *132*, 8846 – 8847; c) E. Kaya, M. Vrabel, C. Deiml, S. Prill, V. S. Fluxa, T. Carell, *Angew. Chem. Int. Ed.* **2012**, *51*, 4466 – 4469; *Angew. Chem.* **2012**, *124*, 4542 – 4545; d) H. S. G. Beckmann, A. Niederwieser, M. Wiessler, V. Wittmann, *Chem. Eur. J.* **2012**, *18*, 6548 – 6554.

[12] a) W. Waldeck, M. Wiessler, V. Ehemann, R. Pipkorn, H. Spring, J. Debus, B. Didinger, G. Mueller, J. Langowski, K. Braun, *Int. J. Med. Sci.* **2008**, *5*, 273 – 284; b) R. Pipkorn, W. Waldeck, B. Didinger, M. Koch, G. Mueller, M. Wiessler, K. Braun, *J. Pept. Sci.* **2009**, *15*, 235 – 241; c) S. B. Engelsma, L. I. Willems, C. E. van Paaschen, S. I. van Kasteren, G. A. van der Marel, H. S. Overkleeft, D. V. Filippov, *Org. Lett.* **2014**, *16*, 2744 – 2747.

[13] W. Chen, D. Wang, C. Dai, D. Hamelberg, B. Wang, *Chem. Commun.* **2012**, *48*, 1736 – 1738.

[14] 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; Angew. Chem.* **2014**, *126*, 9322 – 9326.

[15] C.-J. Zhang, C. Y. J. Tan, J. Ge, Z. Na, G. Y. J. Chen, M. Uttamchandani, H. Sun, S. Q. Yao, *Angew. Chem. Int. Ed.* **2013**, *52*, 14060 – 14064; *Angew. Chem.* **2013**, *125*, 14310 – 14314.

[16] O. T. Keppler, R. Horstkorte, M. Pawlita, C. Schmidt, W. Reutter, *Glycobiology* **2001**, *11*, 11R – 18R.

[17] D. H. Dube, C. R. Bertozzi, *Curr. Opin. Chem. Biol.* **2003**, *7*, 616 – 625.

[18] a) A.-K. Späte, H. Bußkamp, A. Niederwieser, V. F. Schart, A. Marx, V. Wittmann, *Bioconjugate Chem.* **2014**, *25*, 147 – 154; b) D. M. Patterson, K. A. Jones, J. A. Prescher, *Mol. BioSyst.* **2014**, *10*, 1693 – 1697.

[19] a) A. A. Neves, H. Stöckmann, Y. A. Wainman, J. C. H. Kuo, S. Fawcett, F. J. Leeper, K. M. Brindle, *Bioconjugate Chem.* **2013**, *24*, 934 – 941; b) S. Stairs, A. A. Neves, H. Stöckmann, Y. A. Wainman, H. Ireland-Zecchini, K. M. Brindle, F. J. Leeper, *ChemBioChem* **2013**, *14*, 1063 – 1067.

[20] L. A. Bateman, B. W. Zaro, K. N. Chuh, M. R. Pratt, *Chem. Commun.* **2013**, *49*, 4328 – 4330.

[21] E. M. Sletten, C. R. Bertozzi, *Acc. Chem. Res.* **2011**, *44*, 666 – 676.

[22] R. Sustmann, *Pure Appl. Chem.* **1974**, *40*, 569 – 593.

[23] O. T. Keppler, P. Stehling, M. Herrmann, H. Kayser, D. Grunow, W. Reutter, M. Pawlita, *J. Biol. Chem.* **1995**, *270*, 1308 – 1314.

[24] a) J. Du, M. A. Meledeo, Z. Wang, H. S. Khanna, V. D. P. Paruchuri, K. J. Yarema, *Glycobiology* **2009**, *19*, 1382 – 1401; b) M. Boyce, I. S. Carrico, A. S. Ganguli, S.-H. Yu, M. J. Hangauer, S. C. Hubbard, J. J. Kohler, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 3141 – 3146; c) B. W. Zaro, Y.-Y. Yang, H. C. Hang, M. R. Pratt, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 8146 – 8151.

[25] D. J. Vocadlo, H. C. Hang, E.-J. Kim, J. A. Hanover, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 9116 – 9121.

[26] G. W. Hart, M. P. Housley, C. Slawson, *Nature* **2007**, *446*, 1017 – 1022.

Received: August 3, 2014
Published online on ■■ ▮▮, 0000

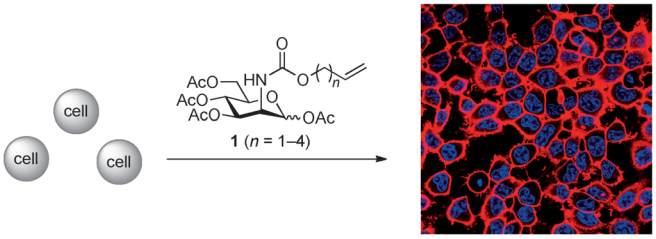
FULL PAPER

Bioorthogonal Chemistry

*A.-K. Späte, V. F. Schart, S. Schöllkopf, A. Niederwieser, V. Wittmann**

■■ – ■■

Terminal Alkenes as Versatile Chemical Reporter Groups for Metabolic Oligosaccharide Engineering



Shine bright: Metabolic oligosaccharide engineering with peracetylated *N*-butenyloxycarbonylmannosamine **1** ($n=2$) leads to cell-surface glycoconjugate staining with higher intensity compared with previously reported terminal alkene reporters. Analogues with shorter ($n=1$) or longer ($n=3$–4) side chains are much less efficient for labeling.

Chemically modified… mannosamine derivatives with side chains of different length terminated by alkenes were applied for metabolic oligosaccharide engineering (MOE). During MOE the sugars are transformed by cells into the corresponding sialic acids and incorporated into cell-surface glycoconjugates (see picture). As terminal alkenes can function as dienophiles during inverse-electron-demand Diels–Alder reactions, the sugars can be fluorescently labeled by ligation to a tetrazine conjugate. The compound with a butenyloxycarbonyl side chain leads to the optimal balance between incorporation efficiency and speed of the ligation reaction resulting in the most intense fluorescence signal. For more details, see the Full Paper by V. Wittmann et al. on p. ■■ ff.