ChemPubSoc Europe

DOI: 10.1002/cbic.201600197

CHEMBIOCHEM Full Papers

Exploring the Potential of Norbornene-Modified Mannosamine Derivatives for Metabolic Glycoengineering

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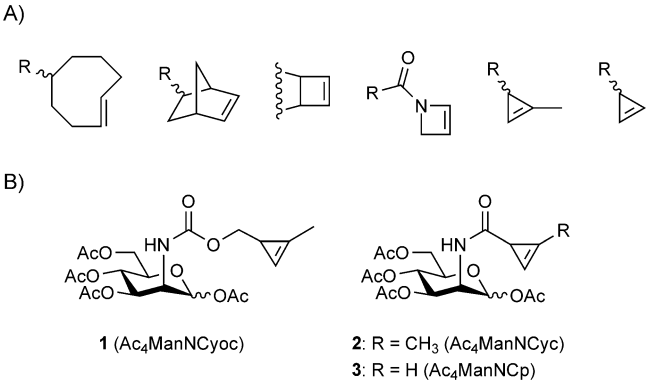
Metabolic glycoengineering (MGE) allows the introduction of unnaturally modified carbohydrates into cellular glycans and their visualization through bioorthogonal ligation. Alkenes, for example, have been used as reporters that can react through inverse-electron-demand Diels–Alder cycloaddition with tetrazines. Earlier, norbornenes were shown to be suitable dienophiles; however, they had not previously been applied for MGE. We synthesized two norbornene-modified mannosamine derivatives that differ in the stereochemistry at the norbornene (*exo/endo* linkage). Kinetic investigations revealed that the *exo* derivative reacts more than twice as rapidly as the *endo* derivative. Through derivatization with 1,2-diamino-4,5-methylenedioxybenzene (DMB) we confirmed that both derivatives are accepted by cells and incorporated after conversion to a sialic acid. In further MGE experiments the incorporated sugars were ligated to a fluorophore and visualized through confocal fluorescence microscopy and flow cytometry.

Introduction

Inverse-electron-demand Diels–Alder (DAinv) cycloaddition represents a well-established class of bioorthogonal ligation reactions. In particular, 1,2,4,5-tetrazines have been shown to react well with a variety of alkenes.[1] The initial product of this [2+4] cycloaddition reacts instantly with release of nitrogen in a retro Diels–Alder reaction, thus making the reaction irreversible.[2] This is just one of the advantages that make it a suitable ligation reaction; in addition, the DAinv reaction can be performed under very mild conditions, such as in aqueous media, at physiological pH, and without addition of a catalyst.[3] As expected for a $LUMO^{diene}$–$HOMO^{dienophile}$-controlled reaction, it proceeds efficiently with electron-rich alkenes as dienophiles.[4] Alternatively, high reaction rates can also be achieved by using strained alkenes.[4–5] As examples, *trans*-cyclooctenes,[1c,6] norbornenes,[1d,6c,e,7] cyclobutenes,[1b] acylazetines,[8] and (methyl)-cyclopropenes[9] have been applied in DAinv reactions (Scheme 1 A).

Out of its wide scope of applications, our group is interested in the use of the DAinv reaction for metabolic glycoengineering (MGE). MGE is a powerful technique that allows the visualization of carbohydrates in cells and even animals.[10] Thus, it contributes to the elucidation of the function of glycosylation. This approach exploits the fact that the enzymes involved in sugar metabolism are promiscuous with regard to unnaturally modified carbohydrates and, thus, will also incorporate carbohydrates that bear chemical reporter groups. Through the employment of a bioorthogonal ligation reaction, the reporter group can then be attached to a probe for visualization or purification. Popular bioorthogonal ligation reactions for MGE are the Staudinger ligation[11] and azide–alkyne cycloaddition (AAC).[12] Additionally, tetrazine-based ligation reactions—the DAinv reaction, for example—have been developed and employed for MGE. To this end, sugars have been successfully modified with terminal alkenes[13] and isonitriles,[14] as well as with cyclopropenes.[9b–g] Because tetrazine ligations can be orthogonal to AAC, it is possible to label two different reporters in the same experiment.[9c–e,13a,14b] Scheme 1 B depicts the cyclopropene-modified mannosamine derivatives reported in the past.



Scheme 1. A) Strained alkenes used in the DAinv reaction. B) Strained alkenes applied as dienophiles in MGE.

So far, cyclopropenes were the only strained alkenes to have been employed for MGE. Even though norbornenes show high DAinv reactivity, they had not previously been applied for MGE, probably because it was assumed that they are too bulky

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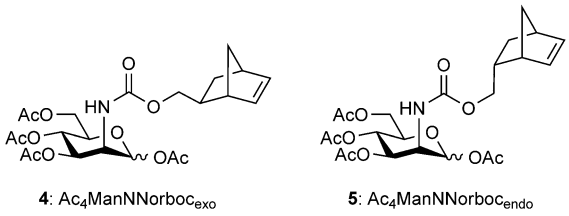
Supporting information for this article can be found under http://dx.doi.org/10.1002/cbic.201600197.

to be metabolically incorporated.[9b,13a] Nevertheless, we hypothesized that, even if the incorporation rate was very low, their high reactivity in the DAinv reaction[5] could make norbornenes an interesting modification for mannosamine derivatives. In addition, norbornenes are stable,[1d] synthetically readily accessible, and commercially available. Here we present two new norbornene-modified mannosamine derivatives that we have kinetically evaluated and applied for MGE.

Results and Discussion

Design of norbornene derivatives

Recently, the laboratories of Carell and Knall investigated the reactivity of several norbornene derivatives,[15] of which (bicyclo[2.2.1]hept-5-en-2-yl)methanol appeared the most promising for our application because it reacts rapidly and the hydroxymethyl group allows functionalization of the norbornene. Because the stereochemistry of the norbornene has been shown to influence its reactivity (the *exo* derivative reacts about three times more rapidly than the *endo* derivative[15a]) we investigated *exo* and *endo* derivatives separately. As monosaccharide we chose mannosamine. In cells, *N*-acetylmannosamine is converted into *N*-acetylneuraminic acid, which is found as a terminal structure of glycoproteins and is thus readily accessible for metabolic labeling, especially on cell surfaces. We therefore designed the two mannosamine derivatives $Ac_4ManNNorboc_{exo}$ (**4**, Scheme 2) and $Ac_4ManNNorboc_{endo}$ (**5**). The hydroxy group of the norbornene allows attachment of



4: $Ac_4ManNNorboc_{exo}$ **5**: $Ac_4ManNNorboc_{endo}$

Scheme 2. Mannosamine derivatives **4** and **5** with norbornene units attached through carbamate linkages.

the norbornene to the sugar through a carbamate linkage. We and others have previously been able to show that alkenes and alkynes attached through carbamate linkages are accepted by the cells' enzymatic machinery[9d–f,13,16] and can even provide improved reaction kinetics.[9a]

Synthesis

A commercially available mixture of *exo*- and *endo*-bicyclo[2.2.1]hept-5-ene-2-carbaldehyde (*exo*-**6** and *endo*-**6**) was separated by silica gel column chromatography, and the isomers were reduced to *exo*-(bicyclo[2.2.1]hept-5-en-2-yl)methanol (**7**) and its *endo* isomer **8**, respectively (Scheme 3).[17] Both **7**[7f] and **8** were then activated by treatment with disuccinimidyl carbonate (**9**). The corresponding activated carbonates **10** and **11** were treated with mannosamine hydrochloride (**12**), and the sugars were peracetylated for facilitated purification as well as increased cell permeability, yielding $Ac_4ManNNorboc_{exo}$ (**4**) and $Ac_4ManNNorboc_{endo}$ (**5**). For kinetic studies and the aldolase reaction, both sugars were deacetylated by treatment with $EtNMe_2$, resulting in $ManNNorboc_{exo}$ (**13**) and $ManNNorboc_{endo}$ (**14**).[1]

Kinetics

Firstly, we investigated the reactivity of the new derivatives in the DAinv reaction. An excess of sugar **13** or **14** was treated with the water-soluble tetrazine Tz-PEG-OH (**15**)[13a] in acetate buffer in a cuvette (Scheme 4). The reaction was monitored in each case by following the decrease of the tetrazine absorbance, which has a maximum at 522 nm. We determined pseudo-first-order rate constants (k_{obs}) for different sugar concentrations (Figure 1), and second-order rate constants (k_2) were calculated from these as previously described.[9c,d] In a

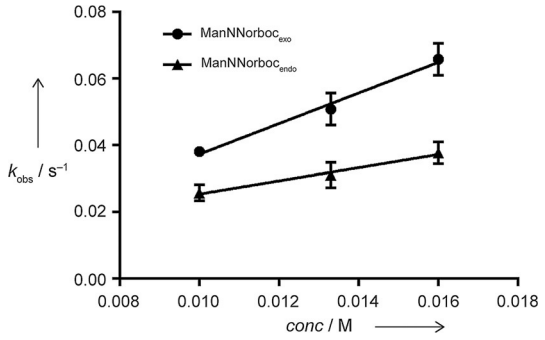


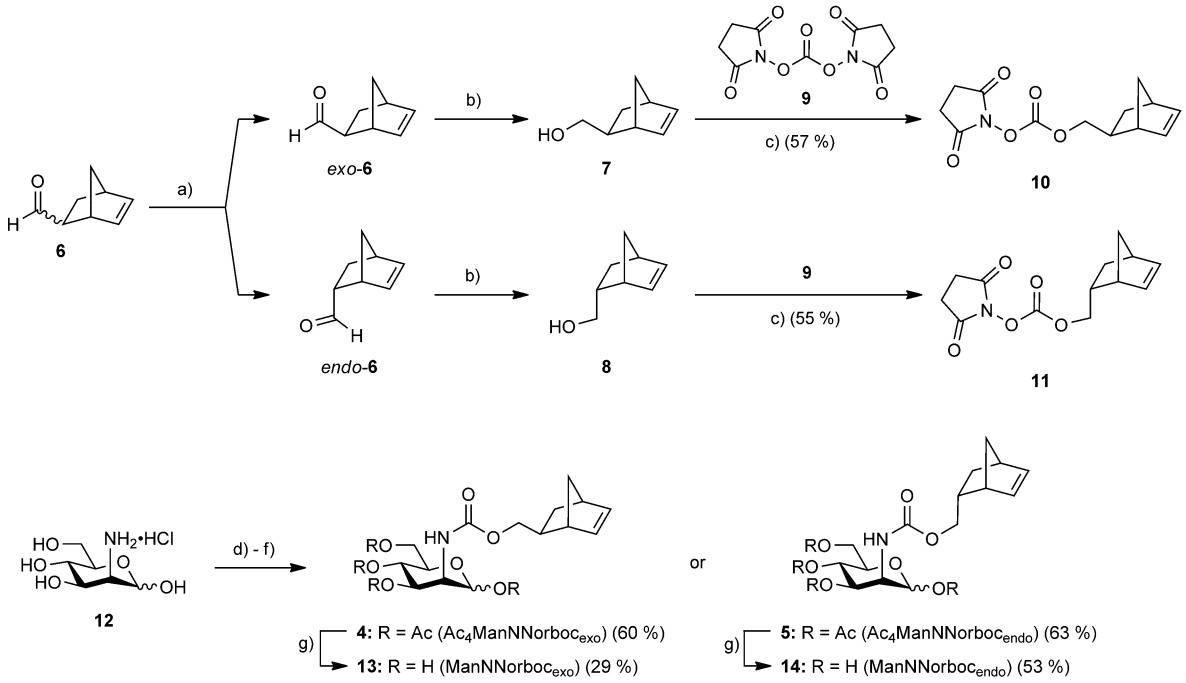
Figure 1. Pseudo-first-order rate constants (k_{obs}) for the reactions between Tz-PEG-OH (**15**) and either $ManNNorboc_{exo}$ (**13**) or $ManNNorboc_{endo}$ (**14**) at different sugar concentrations. From these data second-order rate constants were determined as previously reported.[9c,d]

manner similar to that seen in the results of Vrabel et al.[15a] with the free alcohols **7** and **8**, the *exo* derivative **13** [$k_2 = (4.6 \pm 0.5)\ M^{-1}s^{-1}$] reacts more than twice as rapidly as the *endo* derivative **14** [$k_2 = (2.0 \pm 0.3)\ M^{-1}s^{-1}$]. In comparison with sugars bearing terminal alkenes, and showing rate constants in the range from 0.02 to 0.07 $M^{-1}s^{-1}$,[13] the norbornenes display approximately 100-fold increased reactivity. DAinv reactivity is also improved in comparison with deacetylated $Ac_4ManNCyoc$ (**1**, Scheme 1, $k_2 = 1.0\ M^{-1}s^{-1}$).[9d]

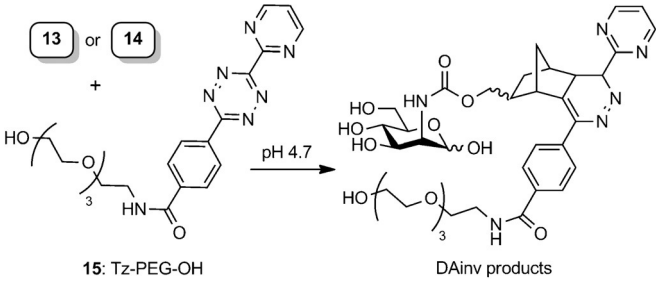
Metabolic glycoengineering

Having ensured that the norbornene derivatives react rapidly in the DAinv reaction, we investigated their suitability for visu-

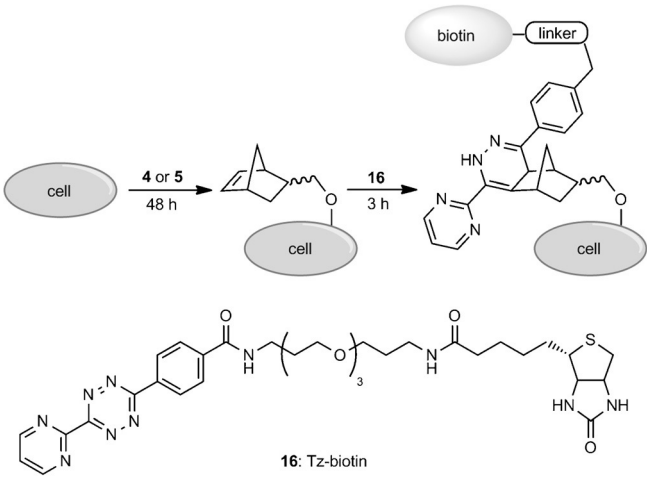
[1] *The two norbornenes* exo-**6** *and* endo-**6** *were each employed as a racemic mixture. Thus, upon coupling to the sugar, two diastereoisomers (each with* α- *and* β*-configuration at the sugar) are obtained. For simplification, we depict only one of these isomers throughout the schemes of this manuscript. All obtained stereoisomers are shown in Figure S1 in the Supporting Information.*



Scheme 3. Synthesis of norbornene-functionalized mannosamine derivatives. a) column chromatography; b) $NaBH_4$, NaOH, MeOH; c) Et_3N, CH_3CN; d) NaOMe, MeOH; e) **10** or **11**; f) Ac_2O, pyr; g) $EtNMe_2$.



Scheme 4. Reaction of norbornene derivatives **13** or **14** with Tz-PEG-OH (**15**) to determine rate constants.



Scheme 5. Strategy for MGE experiments. Cells were fed with sugars **4** or **5** and then treated with Tz-biotin (**16**).

alization of cell-surface glycans. To this end, HEK 293T cells were grown for 48 h in presence either of $Ac_4ManNNorboc_{exo}$ (**4**) or of $Ac_4ManNNorboc_{endo}$ (**5**). As negative control, cells were treated with DMSO only. Cells were then incubated with Tz-biotin (**16**)[13a] and subsequently treated with streptavidin-Alexa Fluor-555 to allow their visualization (Scheme 5). The results showed that labeling both with $Ac_4ManNNorboc_{exo}$ (**4**, Figure 2A) and with $Ac_4ManNNorboc_{endo}$ (**5**, Figure 2B) leads to membrane staining whereas only negligible background staining occurs in the negative control (Figure 2C). That implies that both derivatives were incorporated into cell-surface glycoconjugates and could be visualized with the aid of the DAinv reaction.

Microscopy experiments had already indicated brighter membrane staining for the *exo* derivative than for the *endo* derivative. For a quantitative analysis of staining intensities we used flow cytometry. HEK 293T cells were grown with the sugar derivatives $Ac_4ManNNorboc_{exo}$ (**4**) or $Ac_4ManNNorboc_{endo}$ (**5**) or with DMSO only. For fluorescence labeling, cells were treated with Tz-biotin (**16**, 100 μM, 3 h at 37 °C) followed by streptavidin-Alexa Fluor-647. Because streptavidin-Alexa Fluor-647 is not cell-permeable, flow cytometry measurements allow results limited to fluorescence staining of cell-surface glycoconjugates to be obtained. The flow cytometry results confirm that both sugar derivatives show fluorescence staining relative to cells that were grown without modified sugar, with use of the *exo* derivative **4** giving a significantly higher labeling effi-

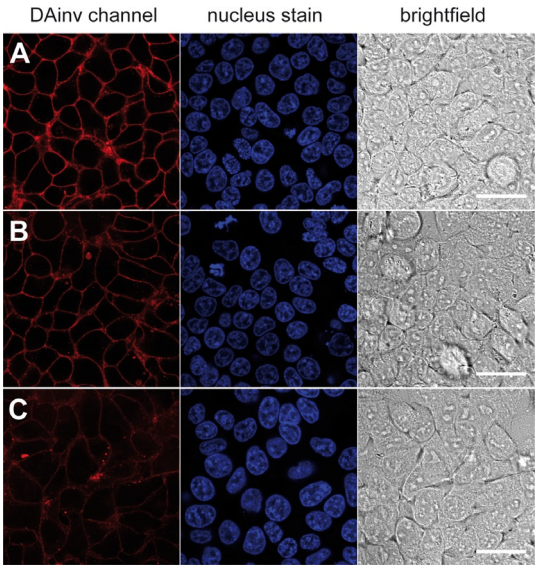


Figure 2. HEK 293T cells were grown with A) 100 μM $Ac_4ManNNorboc_{exo}$ (**4**), B) 100 μM $Ac_4ManNNorboc_{endo}$ (**5**), or C) DMSO for 48 h. Subsequently, cells were incubated with Tz-biotin (**16**, 100 μM, 3 h, 37 °C) and then streptavidin-Alexa Fluor-555. Nuclei were stained with Hoechst 33342. Scale bar: 30 μm.

ciency than that of the *endo* derivative **5** (Figure 3). This might be due to the higher DAinv reactivity of the *exo* derivative **4**. Comparison of different sugar concentrations revealed that cells grown with 250 μM of modified sugars displayed even more intensive membrane staining than cells grown with 100 μM of unnatural sugar (Figure 3B).

To include cytoplasmic proteins in our investigation as well, we performed a western blot analysis of metabolically labeled cells. Cells grown in the presence of sugar derivatives **4** or **5** or with DMSO as negative control were lysed, and the soluble fractions were incubated with Tz-biotin (**16**, 100 μM, 2 h, room temperature). These experiments show that the norbornene derivatives are incorporated into glycoconjugates and can be visualized with the aid of the DAinv reaction (Figure S2). Thus, both norbornene reporters can also be applied to label soluble glycoproteins.

Determination of incorporation efficiencies

Next, we wanted to ensure that the norbornene-modified ManNAc derivatives would be incorporated into sialic acid derivatives. Furthermore, we wanted to investigate whether the incorporation rates would differ for the *exo* and the *endo* derivatives or if the different fluorescence intensities observed in the flow cytometry experiment were solely due to different reaction rates of the *exo* and the *endo* derivatives. For this purpose we employed a method that allows quantification of incorporated sialic acids. In this approach, sialic acids are released from glycoproteins by mild acid treatment and subsequently allowed to react with 1,2-diamino-4,5-methylenedioxybenzene (DMB, **17**). DMB selectively reacts with α-keto acids and can thus be applied to fluorescent labeling of sialic acids.[18]

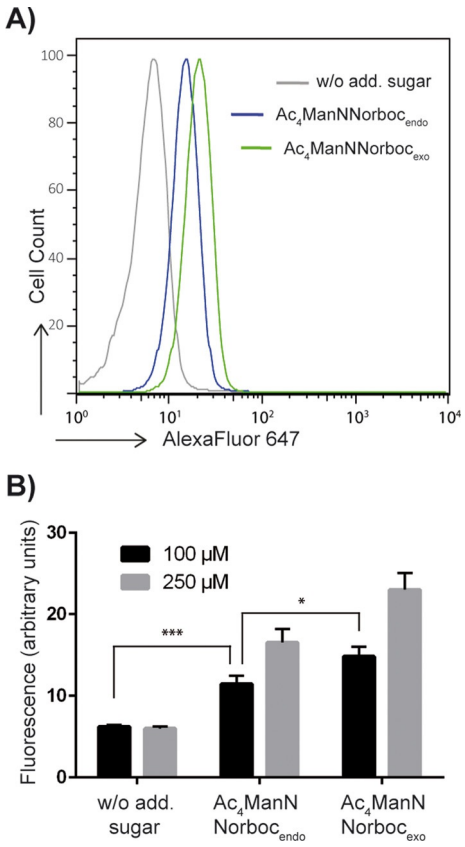
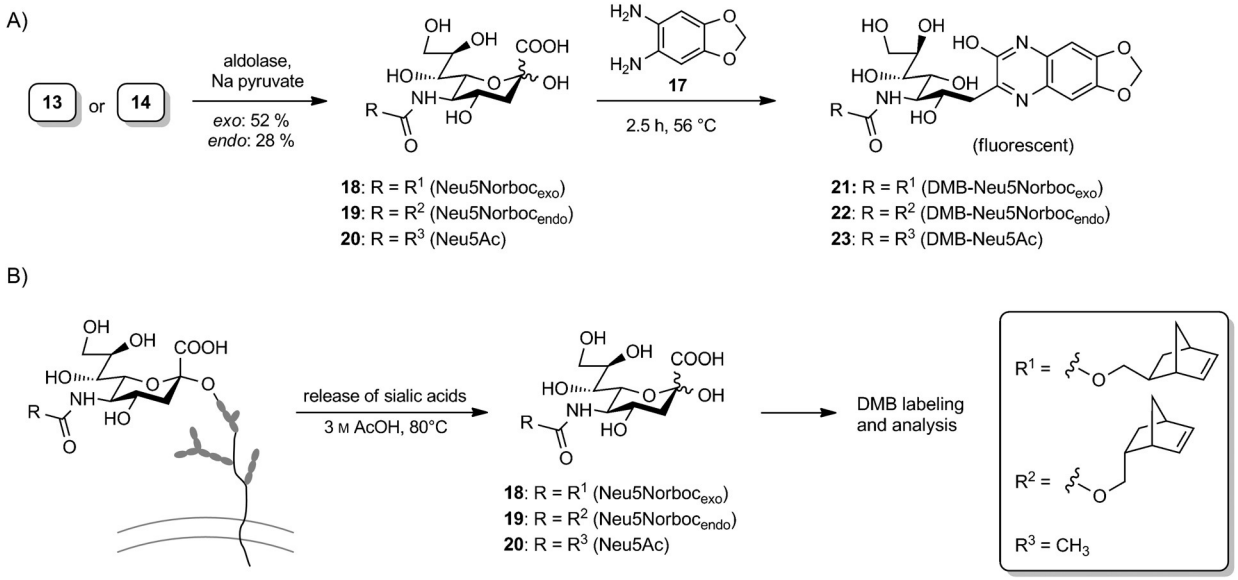


Figure 3. A) Fluorescence intensity distributions of HEK 293T cells grown with 250 μM $Ac_4ManNNorboc_{exo}$ (**4**, green) or with 250 μM $Ac_4ManNNorboc_{endo}$ (**5**, blue) or without addition of unnatural sugar (gray) and labeled with Tz-biotin (**16**, 100 μM, 3 h, 37 °C) followed by addition of streptavidin-Alexa Fluor-647. B) Column diagram of fluorescence of HEK 293T cells grown with 100/250 μM $Ac_4ManNNorboc_{exo}$ (**4**) or with 100/250 μM $Ac_4ManNNorboc_{endo}$ (**5**) or without addition of unnatural sugar. Cells were then incubated with Tz-biotin (**16**, 100 μM, 3 h, 37 °C) and then with streptavidin-Alexa Fluor-647. Data were statistically analyzed by means of a t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Firstly, reference compounds were chemoenzymatically prepared (Scheme 6A). To this end the norbornene-modified mannosamine derivatives **13** and **14** were enzymatically converted by treatment with sialic acid aldolase[19] to afford the corresponding sialic acids, which were purified by RP-HPLC to yield $Neu5Norboc_{exo}$ (**18**) and $Neu5Norboc_{endo}$ (**19**) in 52 and 28% yield, respectively. Treatment of **18** or **19** or of unmodified *N*-acetylneuraminic acid (**20**) with fluorogenic DMB (**17**) gave fluorescent products **21**–**23**. Formation of **21**–**23** was monitored by RP-HPLC with use of a fluorescence detector (excitation 372 nm, emission 456 nm) and confirmed by use of an ESI-MS detector (Figures S3–S5).

To release sialic acids, metabolically engineered cells were treated with 3 M acetic acid for 1.5 h at 80 °C (Scheme 6B). Subsequently, the cleaved sialic acids were fluorescently labeled by treatment with DMB (**17**), and the labeled sialic acids **21**–**23** could be identified by RP-HPLC analysis with the aid of a fluorescence detector (Figures S6–S8) and correlated to the reference compounds. This confirmed the incorporation of the norbornene-modified mannosamines into sialic acid analogues.



Scheme 6. A) Chemoenzymatic preparation of reference compounds with sialic acid aldolase followed by DMB labeling reaction and analysis. B) Release of cellular sialic acids with 3 M acetic acid followed by DMB labeling and RP-HPLC analysis.

Incorporation efficiencies of the norbornene sugars were determined from the ratios of the fluorescence integrals of DMB-Neu5AcNorboc (**21** or **22**) to that of the labeled natural sialic acid DMB-Neu5Ac (**23**). This revealed comparable incorporation rates of about 1% for both the *exo* and the *endo* derivative. The more intense labeling of Ac_4ManNNorboc$_{exo}$ (**4**) after the DAinv reaction relative to the *endo* derivative **5** is therefore likely due to its faster DAinv reactivity.

Dual labeling

Previously, it had been shown that the DAinv reaction and strain-promoted azide–alkyne cycloaddition (SPAAC) can be used in the same experiment.[7d,9b–e,13a,14b,20] To establish that the norbornene sugar Ac_4ManNNorboc$_{exo}$ (**4**) is suitable for a dual labeling experiment, we employed **4** in combination with peracetylated azidoacetylglucosamine[21] (Ac_4GlcNAz). HEK 293T cells were grown in the presence of Ac_4ManNNorboc$_{exo}$ (**4**) and Ac_4GlcNAz for 48 h. Subsequently, the cells were incubated with Tz-biotin (**16**) and then with a mixture of streptavidin-Alexa Fluor-555 (to visualize norbornene-labeled sialic acids), DIBO-Alexa Fluor-488 (to visualize metabolized GlcNAz by SPAAC), and Hoechst 33342 (to visualize nuclei, Figure S9). Under the fluorescence microscope, cells showed distinct membrane staining both in the DAinv and in the SPAAC channel (Figure 4A). Control experiments, in which only one sugar was applied but both ligation reactions were performed, only showed staining in the channel corresponding to the sugar (Figure 4B and C). Cells grown without modified sugar did not show any membrane staining (Figure 4D). This experiment confirmed that the DAinv reaction with a norbornene-modified carbohydrate can be performed together with SPAAC of an azide-modified carbohydrate in the same experiment.

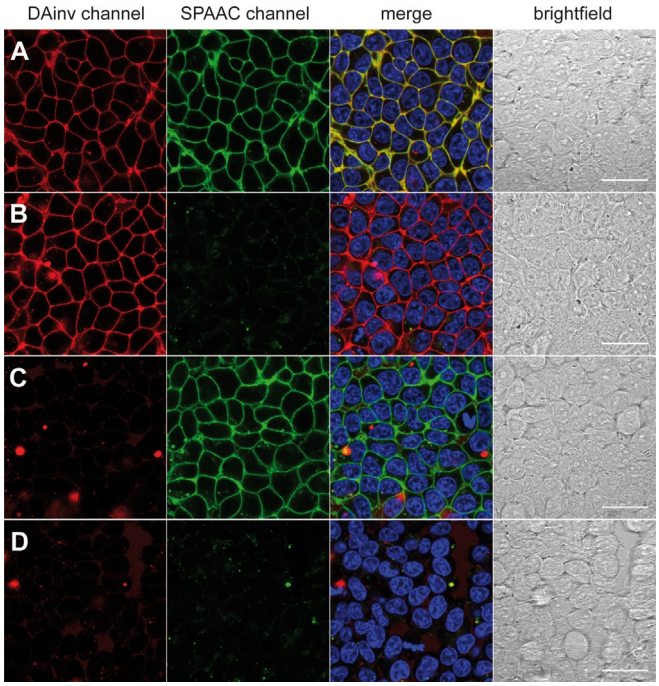


Figure 4. HEK 293T cells were grown with A) 100 μM Ac_4ManNNorboc$_{exo}$ (**4**) and 50 μM Ac_4GlcNAz, B) 100 μM Ac_4ManNNorboc$_{exo}$ (**4**), C) 50 μM Ac_4GlcNAz, and D) without addition of unnatural sugar for 48 h. Cells were incubated with Tz-biotin (**16**, 100 μM, 3 h, 37 °C) and then with a mixture of streptavidin-Alexa Fluor-555 (6.6 μg mL^{-1}), DIBO-Alexa Fluor-488 (20 μM), and Hoechst 33342 (10 μg mL^{-1}) for 30 min at 37 °C. Scale bar: 30 μm.

Even though the norbornenes display high reaction rates, a rather long labeling time of 3 h for the DAinv reaction was required in order to obtain significant staining. This is most likely due to the low incorporation efficiency. In comparison with previously published terminal-alkene-modified sugars,

which required labeling times of 6 h, we consider the norbornene sugars superior, due to their faster reactivity, although the labeling efficiency does not reach the level achievable with cyclopropene-modified sugars. Norbornene-modified sugars, however, are synthetically better accessible than cyclopropene-modified sugars and more stable.

Conclusion

In summary, we have synthesized two new mannosamine derivatives bearing norbornene reporters for use in metabolic glycoengineering. In each of the derivatives the norbornene unit is attached to the sugar through a carbamate linkage. The compounds differ in the stereochemistry at the norbornene unit (*exo* or *endo* configuration). Kinetic studies showed that both derivatives react rapidly in the DAinv reaction with a 1,2,4,5-tetrazine, the *exo* derivative ($k_2 = 4.6\ M^{-1}s^{-1}$) more than twice as rapidly as the *endo* derivative ($k_2 = 2.0\ M^{-1}s^{-1}$). Performing DMB labeling experiments, we found the metabolic incorporation efficiencies to be comparable for both isomers but, due to its higher reactivity, $Ac_4ManNNorboc_{exo}$ (**4**) gives more distinct membrane staining in labeling experiments. Compound **4** can also be combined with a strained azide-modified sugar by copper-free click chemistry, allowing labeling of two different carbohydrates in the same experiment. In conclusion, we have been able to expand the toolbox of sugars available for MGE with a rapidly reacting derivative that is easily synthetically accessible and, despite its bulkiness, is accepted by the cellular metabolism.

Experimental Section

General methods: All chemicals were purchased from Sigma–Aldrich, Apollo, Fluka, Dextra, and Carbosynth and used without further purification. Sialidase was purchased from Carbosynth (MS110801004). Alexa Fluor-555-labeled streptavidin, DIBO-Alexa Fluor-488, and Hoechst 33342 were purchased from Invitrogen. $Ac_4GlcNAz$ was synthesized according to a published procedure.[22] Technical solvents were distilled prior to use. All reactions were carried out in dry solvents. Reactions were monitored by TLC on silica gel 60 F254 (Merck) with detection under UV light ($\lambda = 254$ nm). Additionally, acidic ethanolic *p*-anisaldehyde solution or basic $KMnO_4$ solution followed by gentle heating were used for visualization. Preparative flash column chromatography (FC) was performed with an MPLC-Reveleris 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 ($CDCl_3$: $\delta_H = 7.26$ ppm, $\delta_C = 77.16$ ppm; CD_3OD: $\delta_H = 4.87$ ppm, $\delta_C = 49.00$ ppm; D_2O: $\delta_H = 4.73$ ppm). Signals were assigned by first-order analysis; when feasible, assignments were supported by two-dimensional $^1H,^1H$ and $^1H,^{13}C$ correlation spectroscopy (COSY, HMBC, and HSQC). Numbering of norbornene derivatives is given in the Supporting Information. High-resolution mass spectra (HRMS) were recorded with a micrOTOF II instrument from Bruker Daltonics. Semipreparative RP-HPLC was conducted with a LC-20A prominence system (high-pressure pumps LC-20 AT, autosampler SIL-20A, column oven CTO-20AC, detector SPD-M20A and ELSD-LT II, fraction collector FRC-10 A, controller CBM-20A and LC Software Solution) from Shimadzu under the following conditions. The column was a Eurosphere 100 C18 from Knauer (16× 250 mm), the flow was 9 mL min^{-1}, and the mobile phase was a 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 with a Shimadzu system LCMS2020 (pumps LC-20 AD, autosampler SIL-20AT HAS, column oven CTO-20AC, UV/Vis detector SPD-20 A, fluorescence detector RF-20A, controller CBM-20, ESI detector, LCMS Software Solution). The column was a Nucleodure C_{18} Gravity 3 μm from Macherey–Nagel (125×4 mm), the flow was 0.4 mL min^{-1}, and the mobile phase was a 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.

***exo*-(Bicyclo[2.2.1]hept-5-en-2-yl)methanol (7) and *endo*-(bicyclo[2.2.1]hept-5-en-2-yl)methanol (8)**: These compounds have in the meantime become commercially available (FCH Group Reagents for Synthesis and Sigma–Aldrich). We started from an *exo*/*endo* mixture of bicyclo[2.2.1]hept-5-ene-2-carbaldehyde (*exo*/*endo*-**6**; each isomer as a racemic mixture) and separated the diastereomers by column chromatography[17] (petrol ether/diethyl ether) to obtain *exo*-**6** and *endo*-**6**, each as a racemic mixture. The isomers were reduced with $NaBH_4$ by a procedure previously described by Blanco et al.[17]

exo-(Bicyclo[2.2.1]hept-5-en-2-yl)methyl succinimidyl carbonate (**10**) was synthesized as described previously by Neumaier et al.[7f]

***endo*-(Bicyclo[2.2.1]hept-5-en-2-yl)methyl succinimidyl carbonate (11)**: *endo*-(Bicyclo[2.2.1]hept-5-en-2-yl)methanol (**8**, 1.04 g, 8.3 mmol) was stirred in dry acetonitrile (30 mL) and triethylamine (3.3 mL, 24.2 mmol) under nitrogen at RT. After 30 min disuccinimidyl carbonate (3.5 g, 13.8 mmol) was added to the solution. The resulting mixture was stirred overnight and then concentrated under vacuum. The product was purified by FC (silica, CH_2Cl_2) to yield **11** as a white solid (1.21 g, 55%). ^1H NMR (400 MHz, $CDCl_3$): $\delta = 6.27$–6.13 (m, 1H; H-2), 6.04–5.90 (m, 1H; H-3), 4.13–4.06 (m, 1H; OCH_2CH), 3.97–3.89 (m, 1H; OCH_2CH), 2.99–2.91 (m, 1H; H-4), 2.88–2.73 (m, 5H; $CH_2CH_2^{succinimide}$, H-1), 2.51 (m, 1H; H-5), 1.89 (ddd, $J = 11.9$, 9.3, 3.8 Hz, 1H; H-6), 1.49 (dd, $J = 8.4$, 2.2 Hz, 1H; H-7), 1.35–1.17 (m, 1H; H-7), 0.58 ppm (ddd, $J = 11.8$, 4.5, 2.6 Hz, 1H; H-6); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 168.8$ (C=O), 151.7 (C=O), 138.2 (C-2), 132.2 (C-3), 74.9 (C-8), 49.6 (C-7), 43.9 (C-4), 42.3(C-1), 37.8(C-5), 28.9 (C-6), 25.6 ppm ($CH_2CH_2^{succinimide}$).

1,3,4,6-Tetra-*O*-acetyl-2-[(*exo*-bicyclo[2.2.1]hept-5-en-2-yl)methoxycarbonylamino]-2-deoxymannopyranose ($Ac_4ManNNorboc_{exo}$, 4): Mannosamine hydrochloride (**12**, 306 mg, 1.42 mmol) was suspended in dry MeOH (5.0 mL), neutralized with NaOMe in MeOH (3.2 mL, 1.42 mmol), and stirred for 1 h at RT under nitrogen. A solution of **10** (392 mg, 1.48 mmol) in dry MeOH (5 mL) was added and the reaction mixture was stirred overnight at RT until TLC indicated complete reaction [$ManNNorboc_{exo}$: $R_f = 0.39$ (CH_2Cl_2/MeOH 5:1)]. After complete removal of the solvent, the residual syrup was dissolved in dry pyridine (4.5 mL), treated with acetic anhydride (1.5 mL), and stirred for two days at RT. The solvent was evaporated, and the residue was diluted with 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, petroleum ether/ethyl acetate 1:1) to deliver **4** as a white solid (426 mg, 60%, α/β mixture). TLC: $R_f = 0.56$ (petroleum ether/ethyl acetate 1:1); ^1H NMR (400 MHz, $CDCl_3$): α-anomer: $\delta = 6.20$–6.02 (m, 3H; H-1, H-2′, H-3′), 5.32 (dd, $J = 10.2$, 4.3 Hz, 1H; H-3), 5.20 (t, $J = 10.0$ Hz, 1H; H-4), 4.99 (d, $J = 9.4$ Hz, 1H; NH), 4.39–4.30 (m, 1H; H-2), 4.26 (dd, $J = 12.3$, 4.4 Hz, 1H; H-6), 4.20–4.11 (m, 1H; H-8′), 4.10–3.92 (m, 3H; H-6, H-5, H-8′), 2.84 (br s, 1H; H-1′ or H-4′),

2.73 (brs, 1H; H-4′ or H-1′), 2.18 (s, 3H; OAc), 2.11 (s, 3H; OAc), 2.06 (s, 3H; OAc), 2.02 (s, 3H; OAc), 1.73 (brs, 1H; H-5′), 1.43–1.11 ppm (m, 4H; H-7′, H-6′); β-anomer: δ = 6.18–6.00 (m, 2H; H-2′, H-3′), 5.85 (d, J = 1.7 Hz, 1H; H-1), 5.14 (dd, J = 9.7, 2.7 Hz, 1H; H-3), 5.11–5.07 (m, 1H; H-4), 5.05 (d, J = 3.9 Hz, 1H; NH), 4.48 (d, J = 9.4 Hz, 1H; H-2), 4.26 (dd, J = 12.2, 4.4 Hz, 1H; H-6), 4.21–3.93 (m, 3H; H-6, H-8′a, H-8′b), 3.78 (ddd, J = 9.5, 5.0, 2.5 Hz, 1H; H-5), 2.84 (brs, 1H; H-1′ or H-4′), 2.73 (brs, 1H; H-4′ or H-1′), 2.19 (s, 3H; OAc), 2.12 (s, 3H; OAc), 2.09 (s, 3H; OAc), 2.03 (s, 3H; OAc), 1.73 (brs, 1H; H-5′), 1.42–1.22 (m, 3H; H-7′ab, H-6′), 1.20–1.08 ppm (m, 1H; H-6′); ^{13}C NMR (101 MHz, $CDCl_3$): α- and β-anomer: δ = 170.7 (C=O), 170.2 (C=O), 169.8 (C=O), 168.3 (C=O), 156.3 (CON), 137.1 (C-2′ or C-3′), 136.3 (C-3′ or C-2′), 92.0 (C-1α), 90.9 (C-1β), 73.6 (C-5β), 71.7 (C-3β), 70.3 (C-5α), 69.8 (C-8′), 69.3 (C-3α), 65.5 (C-4), 62.1 (C-6), 51.5 (C-2β), 51.3 (C-2α), 45.1 (C-7′), 43.7 (C-4′ or C-1′), 41.7 (C-1′ or C-4′), 38.4 (C-5′), 29.6 (C-6′), 21.0 (OAc), 21.0 (OAc), 20.9 (OAc), 20.8 ppm (OAc); HRMS: *m/z* calcd for $C_{23}H_{31}NO_{11}$: 498.1970 $[M+H]^+$; found: 498.1974.

1,3,4,6-Tetra-*O*-acetyl-2-[(*endo*-bicyclo[2.2.1]hept-5-en-2-yl)methoxycarbonylamino]-2-deoxymannopyranose (Ac_4ManNNorboc$_{endo}$, 5): Mannosamine hydrochloride (**12**, 375 mg, 1.73 mmol) was suspended in dry MeOH (6.0 mL), neutralized with NaOMe (3.9 mL, 1.73 mmol), and stirred for 1 h at RT under nitrogen. A solution of **11** (480 mg, 1.8 mmol) in dry MeOH (5.0 mL) was added and the reaction mixture was stirred overnight at RT until TLC indicated complete reaction [ManNNorboc$_{endo}$: R_f = 0.34 (CH_2Cl_2/MeOH 5:1)]. After complete removal of the solvent the residual syrup was dissolved in dry pyridine (5.5 mL), treated with acetic anhydride (1.8 mL), and stirred overnight at RT. 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, petroleum ether/ethyl acetate 1:1) to yield **5** as a white solid (542 mg, 63%). TLC: R_f = 0.45 (petroleum ether/ethyl acetate 1:1); ^{1}H NMR (600 MHz, $CDCl_3$): α-anomer: δ = 6.10–6.05 (m, 1H; H-2′), 6.03–5.98 (m, 1H; H-1), 5.92–5.83 (m, 1H; H-3′), 5.22 (dd, J = 10.1, 4.1 Hz, 1H; H-3), 5.15–5.08 (m, 1H; H-4), 4.88 (d, J = 9.4 Hz, 1H; NH), 4.25 (dd, J = 8.5, 4.6 Hz, 1H; H-2), 4.18 (dd, J = 12.0, 5.1 Hz, 1H; H-6), 4.02 (dd, J = 12.5, 2.6 Hz, 1H; H-6), 3.94 (ddd, J = 10.1, 4.6, 2.5 Hz, 1H; H-5), 3.82–3.72 (m, 1H; H-8′), 3.63–3.53 (m, 1H; H-8′), 2.81 (brs, 1H; H-4′), 2.74 (brs, 1H; H-1′), 2.38–2.27 (m, 1H; H-5′), 2.15–1.88 (m, 12H; OAc), 1.79–1.72 (m, 1H; H-6′), 1.42–1.32 (m, 1H; H-7′), 1.21–1.13 (m, 1H; H-7′), 0.53–0.38 ppm (m, 1H; H-6′); β-anomer: δ = 6.10–6.05 (m, 1H; H-2′), 5.92–5.83 (m, 1H; H-3′), 5.76 (app.t, J = 1.6 Hz, 1H; H-1), 5.06 (dt, J = 9.6, 4.8 Hz, 1H; H-4), 4.97 (d, J = 9.7 Hz, 1H; NH), 4.94 (dd, J = 10.9, 3.8 Hz, 1H; H-3), 4.41–4.35 (m, 1H; H-2), 4.18 (dd, J = 12.0, 5.1 Hz, 1H; H-6), 3.98 (dd, J = 12.4, 2.5 Hz, 1H; H-6), 3.82–3.72 (m, 1H; H-8′), 3.70 (ddd, J = 9.3, 5.2, 2.6 Hz, 1H; H-5), 3.63–3.53 (m, 1H; H-8′), 2.81 (brs, 1H; H-4′), 2.74 (brs, J = 3.3 Hz, 1H; H-1′), 2.38–2.27 (m, 1H; H-5′), 2.15–1.88 (m, 12H; OAc), 1.79–1.72 (m, 1H; H-6′), 1.42–1.32 (m, 1H; H-7′), 1.21–1.13 (m, 1H; H-7′), 0.53–0.38 ppm (m, 1H; H-6′); ^{13}C NMR (151 MHz, $CDCl_3$) α- and β-anomers: δ = 170.59 (C=O), 170.08 (C=O), 169.65 (C=O), 168.15 (C=O) 156.78 (N–C=O), 156.08 (N–C=O), 137.71 (C-2′), 132.24 (C-3′), 91.91 (C-1α), 90.76 (C-1β), 73.38 (C-5α), 71.57 (C-3β), 70.21 (C-5β), 69.04 (C-3α), 65.22 (C-8′), 62.03 (C-6), 51.30 (C-2β), 51.08 (C-2α), 49.42 (C-7′), 43.86 (C-4′), 42.23 (C-1′), 38.08 (C-5′), 28.92 (C-6′), 20.90 (C(O)CH_3), 20.84 (C(O)CH_3), 20.76 (C(O)CH_3), 20.66 ppm (C(O)CH_3); HRMS: *m/z* calcd for $C_{23}H_{31}NO_{11}$: 498.1970 $[M+H]^+$; found: 498.1976.

2-[(*exo*-Bicyclo[2.2.1]hept-5-en-2-yl)methoxycarbonylamino]-2-deoxymannopyranose (ManNNorboc$_{exo}$, 13): Ac_4ManNNorboc$_{exo}$ (**4**, 550 mg, 1.11 mmol) was dissolved in dry MeOH (14 mL) under nitrogen. $EtNMe_2$ (3 mL, 26 mmol) was added to the solution, and the mixture was stirred at RT for four days. Additional $EtNMe_2$ (1.5 mL, 13 mmol) was added, and the mixture was stirred at RT for another four days. The solvent was evaporated, and the crude product was purified by FC (silica, 0–10% MeOH in CH_2Cl_2 in 10 min followed by 10% MeOH in CH_2Cl_2 for 10 min). Product **13** was obtained as a white solid (105 mg, 29%).

2-[(*endo*-Bicyclo[2.2.1]hept-5-en-2-yl)methoxycarbonylamino]-2-deoxymannopyranose (ManNNorboc$_{endo}$, 14): Ac_4ManNNorboc$_{endo}$ (**5**, 320 mg, 0.64 mmol) was dissolved in dry MeOH (28 mL) under nitrogen. $EtNMe_2$ (6 mL, 52 mmol) was added to the solution, and the mixture was stirred at RT for four days. Additional $EtNMe_2$ (3 mL, 26 mmol) was added, and the mixture was stirred at RT for another four days. The solvent was evaporated, and the crude product was purified by FC (silica, 0–10% MeOH in CH_2Cl_2 in 20 min). Product **15** was obtained as a white solid (112 mg, 53%).

***N*-[(*exo*-Bicyclo[2.2.1]hept-5-en-2-yl)methoxycarbonyl]neuraminic acid (Neu5Norboc$_{exo}$, 18)**: ManNNorboc$_{exo}$ (**13**, 29 mg, 0.09 mmol) and sodium pyruvate (154 mg, 1.44 mmol) were dissolved in phosphate buffer (0.89 mL, 100 mM, pH 7.1). Sialic acid aldolase (2 U) was added, and the mixture was incubated at RT and 300 rpm for 17 days. The mixture was lyophilized, and the product was purified by RP-HPLC (18–26% B in 20 min, t_R = 12.5 min) to yield **18** as a white solid (19 mg, 52%) and in the form of an anomeric mixture (α/β 1:20). 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 = 6.25–5.94 (m, 2H; H-2′, H-3′), 4.22–4.10 (m, 1H; H-8′a), 4.09–3.96 (m, 3H; H-8′b, H-4, H-6), 3.89–3.82 (m, 1H; H-9a), 3.75 (ddd, J = 9.2, 6.5, 2.7 Hz, 1H; H-8), 3.70–3.59 (m, 3H; H-5, H-7, H-9b), 2.85 (brs, 1H; H-1′), 2.72 (d, J = 11.3 Hz, 1H; H-4′), 2.28 (dd, J = 13.0, 4.9 Hz, 1H; H-3$_{eq}$), 1.86 (app.t, J = 12.3 Hz, 1H; H-3$_{ax}$), 1.80–1.66 (m, 1H; H-5′), 1.38–1.27 (m, 2H; H-7′), 1.27–1.16 ppm (m, 2H; H-6′); ^{13}C NMR (151 MHz, D_2O) β-anomer: δ = 174.4 (C-1), 158.8 (C-9′), 137.3 (C-2′), 136.5 (C-3′), 95.6 (C-2), 70.6 (C-6), 70.3 (C-8), 69.6 (C-8′), 68.3 (C-7), 67.0 (C-4), 63.2 (C-9), 53.4 (C-5), 44.4 (C-7′), 43.3 (C-4′), 41.3 (C-1′), 39.0 (C-3), 37.9 (C-5′), 28.6 ppm (C-6′); HRMS: *m/z* calcd for $C_{18}H_{27}NO_{10}$: 418.1708 $[M+H]^+$; found: 418.1696.

***N*-[(*endo*-Bicyclo[2.2.1]hept-5-en-2-yl)methoxycarbonyl]neuraminic acid (Neu5Norboc$_{endo}$, 19)**: ManNNorboc$_{endo}$ (**14**, 20.5 mg, 0.06 mmol) and sodium pyruvate (109 mg, 0.93 mmol) were dissolved in phosphate buffer (0.63 mL, 100 mM, pH 7.1). Sialic acid aldolase (2 U) was added, and the mixture was incubated at RT and 300 rpm for 26 days. The mixture was lyophilized, and the product was purified by RP-HPLC (18–26% B in 20 min, t_R = 12.5 min) to yield **19** as a white solid (7 mg, 28%) and in the form of an anomeric mixture (α/β 1:20). 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 = 6.23 (dd, J = 5.8, 3.0 Hz, 1H; H-2′), 6.04–5.96 (m, 1H; H-3′), 4.08–3.98 (m, 2H; H-4, H-6), 3.90–3.80 (m, 2H; H-8′a, H-9a), 3.80–3.73 (m, 1H; H-8′), 3.71–3.55 (m, 4H; H-9b, H-8′b, H-7, H-5), 2.89 (brs, 1H; H-4′), 2.83 (d, J = 4.0 Hz, 1H; H-1′), 2.51–2.36 (m, 1H; H-5′), 2.28 (dd, J = 13.0, 4.9 Hz, 1H; H-3$_{eq}$), 1.91–1.78 (m, 2H; H-6′a, H-3$_{ax}$), 1.44–1.36 (m, 1H; H-7′), 1.28 (d, J = 8.3 Hz, 1H; H-7′), 0.59–0.47 ppm (m, 1H; H-6′b); ^{13}C NMR (151 MHz, D_2O): β-anomer: δ = 174.4 (C-1), 158.8 (C-9′), 137.9 (C-2′), 132.2 (C-3′), 95.6 (C-2), 70.6 (C-6), 70.3 (C-8), 69.0 (C-8′), 68.3 (C-7), 67.0 (C-4), 63.2 (C-9), 53.4 (C-5), 48.8 (C-7′), 43.5 (C-4′), 41.9 (C-1′), 39.0 (C-3), 37.5 (C-5′), 28.0 ppm (C-6′); HRMS: *m/z* calcd for $C_{18}H_{27}NO_{10}$: 418.1708 $[M+H]^+$; found: 418.1684.

Kinetic measurements: For kinetic studies, stock solutions of Tz-PEG-OH (**15**),[13a] ManNNorboc$_{exo}$ (**13**), and ManNNorboc$_{endo}$ (**14**)

were prepared in acetate buffer (100 mM, pH 4.7, 20 °C) and mixed in a quartz cuvette to afford final concentrations of 1 mM Tz-PEG-OH (**15**) and 10, 13.3, or 16.6 mM of sugar. The reactions were monitored through the decreasing absorption of the tetrazine at 522 nm. Pseudo-first-order rate constants were determined for every concentration of sugar by plotting $\ln(A_o/A_t)$ over time. For the determination of A_o, a 1 mM solution of only Tz-PEG-OH (**15**) in acetate buffer was used. A_t is the absorption of the reaction mixture at time point *t*. Analysis by linear regression provided pseudo-first-order rate constants. Second-order rate constants were determined by plotting the pseudo-first-order rate constants against the corresponding sugar concentration, followed by linear regression. All measurements were carried out at least in triplicate. Sufficient stability of Tz-PEG-OH (**15**) had previously been verified in our lab by measuring the absorption at 522 nm of a solution of Tz-PEG-OH (**15**) in acetate buffer.[13a]

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%), and penicillin and streptomycin (each 100 U mL^{-1}). Cells were incubated under carbon dioxide (5%) in a water-saturated incubator at 37 °C. The cells were diluted every three days 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: In an approach similar to that used in previously described experiments,[9d,13b] HEK 293T cells (17500 cells cm^{-2}) were seeded in 4-well ibiTreat μ-Slides (ibidi) Ph+ coated with poly-L-lysine (0.0025%, 1 h at 37 °C or o/n at 4 °C). After 12 h, cells were incubated for 48 h with labeled mannosamine [$Ac_4ManNNorboc_{exo}$ (**4**) or $Ac_4ManNNorboc_{endo}$ (**5**), 100 μM]. DMSO only was added as solvent control. Cells were washed twice with PBS and then treated with Tz-biotin (**16**,[13a] 100 μM) for 1–3 h at 37 °C. After two washes with PBS, cells were incubated with streptavidin-Alexa Fluor-555 (6.6 μg mL^{-1}) and Hoechst 33342 (10 μg mL^{-1}) for 20 min at 37 °C in the dark. Cells were washed three times with PBS, and DMEM was added for microscopy. Fluorescence microscopy was performed with a Zeiss LSM 780 instrument equipped with a 40×1.4 NA Plan-Apochromat oil immersion objective and a GaAsP-detector array for spectral imaging. Analysis of the obtained data was performed by use of Image J software version 1.45 S.2.

Dual labeling experiments: HEK 293T cells (17500 cells cm^{-2}) were seeded in 4-well ibiTreat μ-Slides (ibidi) Ph+ coated with poly-L-lysine (0.0025%, 1 h at 37 °C or o/n at 4 °C) and allowed to attach for 16 h. Cells were then incubated with $Ac_4ManNNorboc_{exo}$ (**4**, 100 μM) and $Ac_4GlcNAz$ (50 μM) for 48 h. Either no sugar or only one sugar was added as negative control. Cells were washed twice with PBS and then treated with Tz-biotin (**16**, 100 μM) for 3 h. Unbound Tz-biotin (**16**) was washed away (2× with PBS) and cells were incubated with a mixture of DIBO-Alexa Fluor-488 (20 μM), streptavidin-Alexa Fluor-555 (6.6 μg mL^{-1}), and Hoechst 33342 (10 μg mL^{-1}) for 30 min at 37 °C in the dark. Cells were washed three times with PBS, and DMEM was added for microscopy. Microscopy was performed as described above.

Western blot analysis: Western blot analysis was performed in a modified version of the previously described protocol.[9f] HEK 293T cells were seeded (1200000 cells/10 cm dish), and after 16 h the media was exchanged with media containing labeled sugar [$Ac_4ManNNorboc_{exo}$ (**4**) or $Ac_4ManNNorboc_{endo}$ (**5**), 100 μM]. As a solvent control, DMSO was added instead of the sugar. The cells were cultured for 48 h with or without the added sugar. Cells were trypsinated, resuspended in PBS (5 mL), and pelleted by centrifugation (5 min, 400 *g*). The supernatant was discarded, and the pellet was resuspended in PBS (1 mL) and pelleted by centrifugation (5 min, 400 *g*). The cells were lysed in lysis buffer (250 μL) containing Triton X-100 (0.5%), DNase (30 μg mL^{-1}), RNase (30 μg mL^{-1}), β-glycerophosphate (20 mM), sodium fluoride (20 mM), sodium orthovanadate (0.3 mM), complete X protease inhibitor (Roche; 1×), NaCl (300 mM), Tris·HCl (pH 7.4, 25 mM), EDTA (5 mM), and 2-acetamido-2-deoxy-D-glucopyranosylidenamino *N*-phenylcarbamate [PUGNAc (*O*-GlcNAc-β-*N*-acetylglucosaminidase inhibitor to maintain *O*-GlcNAcylation during lysis), Sigma–Aldrich, 100 μM], and incubation at 4 °C was carried out for 30 min. The lysate was cleared by centrifugation (22000 *g*, 30 min, RT). Tz-biotin (**16**) was added to the lysate to afford a final concentration of 100 μM. The samples were incubated for 2 h at RT, SDS-sample buffer (3×55.5 μL) was added, and the sample was heated at 90 °C for 15 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis with 10% polyacrylamide gels and transferred to nitrocellulose membranes (BioRad). Transfer efficiency was analyzed with Ponceau S staining. The membranes were blocked in milk (5% in PBS-T) for 1 h at RT, followed by incubation with anti-biotin antibody (Abnova, Anti-Biotin mAb clone SB58c, 1:2000 dilution in milk) at 4 °C overnight or anti-α-tubulin antibody (AA4.3, hypridoma supernatant in 1% FCS, 1:200 dilution in milk) for 1 h at RT. The membranes were washed (3×, 10–15 min, PBS-T), incubated with secondary horseradish-peroxidase-conjugated anti-mouse antibody [Dianova, goat anti-mouse igG (H+L)-HRP, 1:50000 dilution in milk, 1 h, RT] and washed again (3×, 10–15 min, PBS-T). Blots were developed by use of an ECL detection system (clarity Western ECL substrate, BioRad) and visualized with a CCD camera (Raytest-1 000, Fujifilm).

Flow cytometry analysis: HEK 293T cells were seeded in 12-well plates (170000 cells/well) coated with poly-L-lysine (0.0025%, 1 h, 37 °C). After 12 h cells were incubated for 48 h with labeled mannosamine [$Ac_4ManNNorboc_{exo}$ (**4**), $Ac_4ManNNorboc_{endo}$ (**5**), 100 or 250 μM] A corresponding amount of DMSO was added as solvent control. Cells were washed with PBS (2×) and then treated with Tz-biotin (**16**,[13a] 100 μM) for 3 h at 37 °C. After two washes with PBS, cells were incubated with streptavidin-Alexa Fluor-647 (6.6 μg mL^{-1}) for 30 min at 37 °C in the dark. Cells were washed with PBS, then blocked with FACS buffer [PBS+ FCS (10%)] for 5 min and again washed three times with PBS. Then cells were released with trypsin-EDTA (100 μL/well) and resuspended in FACS buffer (400 μL/well). 50000 cells were counted per measurement. For flow cytometry analysis BD FACSCalibur was used, and the obtained data were evaluated with FlowJo Software version 8.8.7 and processed with Graphpad prism (the averaging called median was used). Experiments were performed in triplicate. Data were statistically analyzed by means of a t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Preparation of reference compounds for DMB labeling experiments: The sialic acid derivative [$Neu5Norboc_{exo}$ (**18**), $Neu5Norboc_{endo}$ (**19**), or Neu5Ac (**20**), 0.1 mg] was dissolved in DMB solution [DMB·2 HCl (5.3 mM), $Na_2S_2O_4$ (16 mM), TFA (40 mM) in MilliQ water, 265 μL]. The mixture was incubated for 2.5 h at 56 °C in a thermomixer (300 rpm) in the dark. Subsequently, the mixture was cooled on ice for 10 min and neutralized with NaOH (0.5 M, 21 μL). For analytical RP-HPLC-MS measurements, the reaction mixture was diluted with MilliQ water (1:2), filtered, and injected (10 μL). For fluorescence detection ($\lambda_{ex}=372$ nm, $\lambda_{em}=456$ nm) a higher dilution was

necessary (1:400) and only 3 μL were injected. A gradient of 10–30.6% B in 55 min was used. Chromatograms are shown in Figures S3–S5.

Derivatization of Neu5Norboc$_{exo}$ (18): Product DMB-Neu5Norboc$_{exo}$ (**21**); t_R = 51.0 min; HRMS: *m/z* calcd for $C_{25}H_{31}N_3O_{10}$: 534.2082 [*M*+H$^+$]; found: 534.2067.

Derivatization of Neu5Norboc$_{endo}$ (19): Product DMB-Neu5Norboc$_{endo}$ (**22**); t_R = 51.2 min; HRMS: *m/z* calcd for $C_{25}H_{31}N_3O_{10}$: 534.2082 [M+H$^+$]; found: 534.2060.

Derivatization of Neu5Ac (20): Product DMB-Neu5Ac (**23**); t_R = 11.3 min.

DMB labeling of sialic acids released from engineered cells: HEK 293T cells were seeded (450 000 cells in a 5 cm dish) and after 48 h the media was exchanged with medium (4 mL) containing labeled sugar [Ac$_4$ManNNorboc$_{exo}$ (**4**) or Ac$_4$ManNNorboc$_{endo}$ (**5**), 250 μM]. As a solvent control DMSO was added. The cells were cultured for 48 h with or without the additional sugar. Cells were trypsinated and resuspended in media (5 mL) and pelleted by centrifugation (6 min, 400 *g*). The supernatant was discarded and the pellet was resuspended in PBS (5 mL). Cells were transferred to Eppendorf tubes (450 000 cells per tube) and pelleted by centrifugation (6 min, 400 *g*). The supernatant was discarded, the pellet was resuspended in AcOH (3 M, 200 μL), and the mixture was incubated at 80 °C for 90 min in a thermomixer (300 rpm). The solution was diluted with MilliQ H_2O (500 μL) and neutralized through addition of NH_3 solution (25% in H_2O, 20 μL). The solvent was removed with a SpeedVac, and the pellet was suspended in dry EtOH (200 μL) and concentrated again. The EtOH wash was repeated twice. The pellet was resuspended in DMB solution [DMB·2 HCl (5.3 mM), $Na_2S_2O_4$ (16 mM), TFA (40 mM) in MilliQ water, 265 μL]. The mixture was incubated for 2.5 h at 56 °C in a thermomixer (300 rpm). The mixture was cooled to 0 °C, neutralized with NaOH (0.5 M, 25 μL), and analyzed by analytical RP-HPLC-MS (10–30.6% B in 55 min; Figures S6–S8).

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 our "Schwerpunktskurs" students Marcia Maier, Verena Betzler, Rebecca Faißt, and Lisa Haiber for support with synthesis and kinetics, the Bioimaging Center of the University of Konstanz for providing the fluorescence microscopy instrumentation, and the FlowKon facility for the support in flow cytometry analysis.

Keywords: carbohydrates · Diels–Alder reaction · metabolic engineering · norbornenes · sialic acids

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Manuscript received: April 4, 2016

Accepted article published: May 4, 2016

Final article published: June 17, 2016