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Rapid Labeling of Metabolically Engineered Cell-Surface Glycoconjugates with a Carbamate-Linked Cyclopropene Reporter

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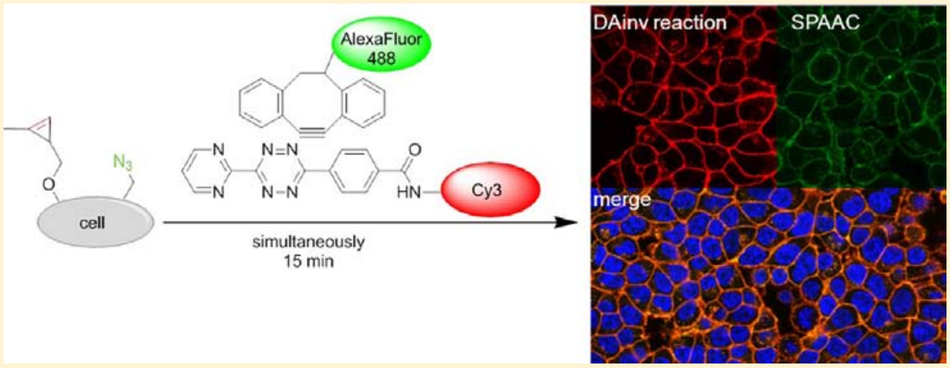
Supporting Information



ABSTRACT: Metabolic oligosaccharide engineering is a valuable tool to monitor cellular carbohydrates. Here, we report the synthesis of a novel *N*-acyl-mannosamine derivative bearing a methylcyclopropene tag that is attached to the sugar via a carbamate moiety. This derivative undergoes rapid Diels–Alder reaction with inverse electron demand. We demonstrate that the cell's biosynthetic machinery incorporates this non-natural mannosamine derivative into glycoconjugates that can, subsequently, be labeled within less than 10 min with a new sulfo-Cy3–tetrazine conjugate. Using this tetrazine-dye conjugate for the detection of the methylcyclopropene-tagged mannosamine derivative, we could achieve dual labeling of two different metabolically incorporated sugars combining a Diels–Alder reaction with inverse electron demand and a strain-promoted azide–alkyne cycloaddition which are carried out simultaneously in a single step.

INTRODUCTION

The development of metabolic oligosaccharide engineering (MOE) as an important tool to visualize glycoconjugates in vitro and in vivo has significantly advanced glycobiology research during the past decade.[1−3] In this approach, cells are grown in the presence of non-natural monosaccharides that contain a functional group with a unique reactivity (a chemical reporter), but are nevertheless accepted by the cell's biosynthetic machinery. For instance, non-natural *N*-acetyl-D-mannosamine (ManNAc) analogues can be fed to cells and are incorporated into membrane sialoglycoconjugates.[4] After successful incorporation of the modified sugar into the glycoconjugate, different ligation reactions[5,6] can be performed with the reporter group to label the glycan. Well established ligation reactions in this context are the ketone-hydrazide ligation,[7] Staudinger ligation,[8] and azide–alkyne [3 + 2] cycloaddition (copper-catalyzed[9,10] or strain-promoted,[11,12] often referred to as click reaction). The Diels–Alder reaction with inverse electron demand (DAinv reaction) has also proven to be a valuable ligation reaction[13−18] that can be orthogonal to azide–alkyne cycloaddition,[19,20] a feature that allows dual labeling of two sugars within one MOE experiment.[21−23] Dienophiles for a DAinv reaction that have been reacted with 1,2,4,5-tetrazines[24] and successfully employed in MOE are terminal alkenes **1**,[21] isonitriles **2**,[23,25] and cyclopropenes **3**[22,26] (Chart 1).

Terminal alkenes **1** are small and undergo DAinv reaction with tetrazines of type **4** with second-order rate constants of up to $k = 0.04\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$.[21] The reaction rate of isonitriles **2** with tetrazines of type **5** is higher (up to $k = 0.57\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$), but not all isonitriles lead to stable ligation products after the DAinv reaction.[27] MOE was also performed using strained cyclopropenes **3** that are small enough to be accepted by cellular enzymes.[22,26] The Devaraj group determined a second-order rate constant of $k = 0.137\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$ for the reaction of **3** with tetrazines of type **6** at 37 °C.[28] They also reported that methylcyclopropene carbamate **7** reacts 100-fold faster ($k = 13\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$). However, **7** has so far not been used for MOE. Both cyclopropenes of types **3** and **7** have been shown to be stable in aqueous solution in the presence of biological nucleophiles.[26,28]

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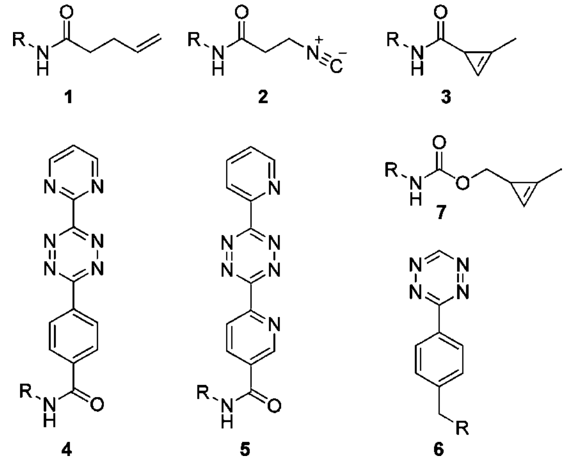
Chart 1. Dienophiles and 1,2,4,5-Tetrazines for DAinv Reactions



We and others could recently demonstrate that ManNAc analogues bearing a carbamate moiety are accepted by the cell's biosynthetic machinery.[21,29] Combining that knowledge with the fact that methylcyclopropene carbamates **7** react significantly faster than the corresponding amides **3**, we hypothesized that a methylcyclopropene attached to the sugar via a carbamate is metabolized, offering the option of rapid sugar labeling by a DAinv reaction. Here we report the synthesis of a novel *N*-acylmannosamine derivative that bears such a tag and reacts instantly with tetrazines, as well as its application in MOE. For dual labeling of cells two different sugars (one azide-labeled, one methylcyclopropene carbamate-labeled) were metabolically incorporated. Incubation with a mixture of an AlexaFluor488-labeled dibenzocyclooctyne (DIBO-488) and a novel sulfo-Cy3-labeled tetrazine (Tz-Cy3) allowed dual labeling within minutes in a single step.

■ EXPERIMENTAL SECTION

General Methods. All chemicals were purchased from Aldrich, Fluka, and Dextra and used without further purification. AlexaFluor647-labeled streptavidin, AlexaFluor488-DIBO, and Hoechst 33342 were purchased from Invitrogen. Technical solvents were distilled prior to use. All reactions were carried out in dry solvents, purchased from Aldrich. All reactions were monitored by TLC on silica gel 60 F254 (Merck) with detection by UV light (λ = 254 nm). Additionally, acidic ethanolic *p*-anisaldehyde solution followed by gentle heating was used for visualization. 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 ($CDCl_3$: δ_H = 7.26 ppm, δ_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). ESI-MS spectra were recorded on an Esquire 3000 plus instrument from Bruker Daltonics. High-resolution ESI-TOF mass spectra were recorded on a micrOTOF II instrument from Bruker. LC-MS analyses were conducted on a LCMS2020 instrument from Shimadzu (pumps LC-20 AD, autosampler SIL-20AT HAT, column oven CTO-20AC, UV−vis detector SPD-20A, controller CBM-20, ESI detector, and software LCMS-solution) with an EC 125/4 Nucleodur C18, 3 μM column (Machery-Nagel). A binary gradient of acetonitrile (with 0.1% formic acid) in water (with 0.1% formic acid) was used at a flow rate of 0.4 mL min^{-1}. Semipreparative high performance liquid chromatography (HPLC) was conducted on a LC-20A prominence system (pumps LC-20AT, auto sampler SIL-20A, column oven CTO-20AC, diode array detector SPD-M20A, ELSD-LT II detector, controller CBM-20A, and software LC-solution) from Shimadzu. For reversed-phase HPLC an Eurospher 100 C18 column from Knauer (16 × 250 mm, flow 8 mL min^{-1}) was used as stationary phase and a gradient of acetonitrile in water with 0.1% formic acid was used as mobile phase. 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.

Activated Cyclopropene 11. (2-Methyl-3-(trimethylsilyl)-cycloprop-2-en-1-yl)methanol **8**[26,28] (200 mg, 1.2 mmol) was dissolved in THF (6 mL) under nitrogen, treated with Bu_4NF in THF (1.4 M solution, 1 mL), and stirred at room temperature for 2 h. The reaction mixture was cooled to 4 °C and pyridine (5 mL) and *p*-nitrophenyl chloroformate **10** (700 mg, 3.2 mmol) were added. The reaction mixture was stirred and allowed to warm to room temperature overnight. The reaction mixture was concentrated and purified by FC (silica, 0−5% ethyl acetate in petroleum ether) to yield **11** as a colorless oil (250 mg, 83%). ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.35−8.19 (m, 2H, H_{ar}), 7.46−7.32 (m, 2H, H_{ar}), 6.61 (t, *J* = 1.4 Hz, 1H, =CH), 4.26−4.02 (m, 1H, CH_2), 2.17 (d, *J* = 1.2 Hz, 3H, CH_3), 1.77 (td, *J* = 5.3, 1.6 Hz, 1H, CH_2**CH**); ^{13}C NMR (101 MHz, $CDCl_3$): δ (ppm) = 155.92 (C_{quart}), 152.81 (C_{quart}), 145.44 (C_{quart}), 125.42 ($C_{ar}H$), 121.95 ($C_{ar}H$), 120.32 (C_{quart}), 101.83 (=CH), 77.53 (CH_2), 16.79 (CH_3), 11.80 (CH_2CH).

Ac_4ManNCyoc (13). To a solution of mannosamine hydrochloride (**12**) (1 g, 4.6 mmol) in MeOH (10 mL) was added NaOMe (0.5 M in MeOH) (9 mL, 4.6 mmol) under nitrogen. After stirring for 90 min at room temperature, the solution was added to activated cyclopropene **11** (1 g, 4.8 mmol). After stirring for 48 h at room temperature the solvent was evaporated under reduced pressure. The residue was dissolved in pyridine (10 mL) and acetic anhydride (5 mL) was added. After stirring for 24 h at room temperature, the solvents were evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (125 mL), washed with 10% aq. $KHSO_4$ (100 mL), sat. aq. $NaHCO_3$ (100 mL), and brine (100 mL). The organic layer was dried ($MgSO_4$) and the solvent was evaporated under reduced pressure. The residue was purified by FC (silica, 0−7% ethyl acetate in petroleum ether) to afford Ac_4ManNCyoc **13** together with its β-anomer. Further purification by HPLC allowed separation of the α-anomer **13** (200 mg, 10%). ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) = 6.58 (s, 1H, =CH), 6.09 (d, *J* = 1.2 Hz, 1H, H-1), 5.31 (dd, *J* = 10.2, 4.3 Hz, 1H, H-3), 5.20 (t, *J* = 10.0 Hz, 1H, H-4), 5.02 (d, *J* = 9.3 Hz, 1H, NH), 4.34 (ddd, *J* = 9.2, 4.2, 1.2 Hz, 1H, H-2), 4.26 (dd, *J* = 12.2, 4.3 Hz, 1H, H6a), 4.11−3.99 (m, 2H, H-6b, H5), 3.95 (d, *J* = 5.1 Hz, 2H, CH_2), 2.17 (s, 3H, OAc), 2.14 (s, 3H, CH_3), 2.11 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.69−1.64 (m, 1H, CH_2**CH**); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm) = 170.75 (C_{quart}), 170.23 (C_{quart}), 169.74 (C_{quart}), 168.29 (C_{quart}), 156.41 (C_{quart}), 120.56 (C_{quart}), 102.39 (=CH), 92.12 (C-1), 73.48 (CH_2), 70.33 (C-5), 69.31 (C-3), 65.52 (C-4), 62.10 (C-6), 51.25 (C-2), 21.03 (C_{quart}), 20.90 (C_{quart}), 20.78 (C_{quart}), 17.20 (CH_2CH), 11.80

(C_{quart}); ESI-MS: m/z calcd. for $C_{20}H_{27}NO_{11}$: 480.15 $[M + Na]^+$, found: 480.00.

Cy3-amido-hexylamine 20. The triethylammonium salt of Cy3-NHS **18** (220 mg, 0.26 mmol) was dissolved in dry DMF (4 mL). EtN(*i*-Pr)$_2$ (73 μL, 0.42 mmol) and *N*-Boc-hexylenediamine (94 μL, 0.42 mmol) were added. The solution was stirred overnight at room temperature. Ethyl acetate (20 mL) was added to the solution to precipitate Cy3 conjugate **19**. The product was collected by centrifugation, washed with additional 20 mL of ethyl acetate, and purified by RP-HPLC. Without further analysis, **19** was dissolved in DMF (2 mL) and trifluoroacetic acid (2 mL) was added. The solution was stirred for 5 h at room temperature and then concentrated under reduced pressure to remove the volatile acid. Ethyl acetate (20 mL) was added to precipitate the product which was collected by centrifugation and washed with additional 20 mL of ethyl acetate. The precipitate was dissolved in water (9 mL) and purified by RP-HPLC to give **20** (67 mg, 30%) as a magenta oil. ^{1}H NMR (400 MHz, D_2O): δ (ppm) = 8.52 (t, J = 13.5 Hz, 1H), 7.96−7.82 (m, 4H), 7.42−7.33 (m, 2H), 6.40 (dd, J = 13.3 Hz, 4.2 Hz, 2H), 4.20−4.07 (m, 4H), 3.00−2.90 (m, 4H), 2.20 (t, J = 6.9 Hz, 2H), 1.92−1.82 (m, 2H), 1.74 (s, 12H), 1,69−1.52 (m, 4H), 1.42−1.35 (m, 2H), 1.25−1.12 (m, 4H); HR-ESI-MS m/z calcd. for $[C_{37}H_{51}N_4O_7S_2]^-$: 727.3194, found: 727.3191.

Tz-Cy3 22. Cy3-amido-hexylamine **20** (59 mg, 0.081 mmol) was dissolved in DMSO/pyridine 9/1 (5 mL). Tz-succinimidyl ester **21**[30] (61 mg, 0.162 mmol) and EtN(*i*-Pr)$_2$ (14 μL) were added. The solution was stirred overnight, and the product was precipitated by addition of ethyl acetate (60 mL), collected by centrifugation, and washed with ethyl acetate (20 mL). The solid was dissolved in water (7 mL) and purified by RP-HPLC to give the triethylammonium salt of **22** (59 mg, 68%) as a violet powder. ^{1}H NMR (400 MHz, MeOH-d_4): δ (ppm) = 9.11 (d, J = 4.9 Hz, 2H), 8.69 (d, J = 8.3 Hz, 2H), 8.52 (t, J = 13.5 Hz, 1H), 8.07 (d, J = 8.4 Hz, 2H), 8.00−7.82 (m, 6H), 7.77 (t, J = 4.9 Hz, 1H), 7.43−7.36 (m, 2H), 6.53 (t, J = 13.5 Hz, 2H), 4.32−4.10 (m, 5H), 3.40 (t, J = 7.2 Hz, 2H), 3.18−3.08 (m, 2H), 2.25−2.15 (m, 2H), 1.91−1.36 (m, overlapping signals, 28H); HR-ESI-MS m/z calcd. for $[C_{50}H_{57}N_{10}O_8S_2]^-$: 989.3808, found: 989.3800.

Cell Growth Conditions. HEK 293T cells were grown in Dulbecco's Modified Essential Medium (DMEM) supplemented with 5% FBS, 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. All cells were incubated in a 5% carbon dioxide, water saturated incubator at 37 °C.

Fluorescence Microscopy with Tz−Biotin. HEK 293T cells (6500−7500 cells/cm^2) were seeded in 8-well ibiTreat μ-Slides (ibidi) and allowed to attach for 12 h. Cells were then incubated with 100 μM Ac$_4$ManNCyoc **13** for 48 h. No sugar was added as negative control. Cells were washed two times with phosphate-buffered saline (PBS) and then treated with Tz−biotin **17** (25 μM) for 15 min at 37 °C. After two washes with PBS, cells were incubated with AlexaFluor647-labeled streptavidin (6.6 μg mL^{-1}) and Hoechst 33342 (10 μg mL^{-1}) for 20 min at room temperature in the dark. Cells were washed twice with PBS and DMEM was added for microscopy. A Zeiss 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 *ImageJ* software version 1.45 s.

Fluorescence Microscopy with Tz-Cy3. HEK 293T cells (6500−7500 cells/cm^2) were seeded in 8-well ibiTreat μ-Slides (ibidi) and allowed to attach for 12 h. Cells were then incubated with 100 μM Ac$_4$ManNCyoc **13** for 48 h. No sugar was added as negative control. Cells were washed two times with PBS and then treated with Tz-Cy3 **22** (25 μM) for 5−15 min at 37 °C. Cells were washed twice with PBS and nuclei were stained with Hoechst 33342 (10 μg mL^{-1}) for 20 min at room temperature in the dark. Cells were washed twice with PBS, and DMEM was added for microscopy. Microscopy was performed as described above.

Fluorescence Microscopy with Tz-Cy3 for Dual Labeling. HEK 293T cells (7500 cells/cm^2) were seeded in 8-well ibiTreat μ-Slides (ibidi) and allowed to attach for 12 h. Cells were then incubated with 100 μM Ac$_4$ManNCyoc **13** and 50 μM Ac$_4$GlcNAz **23** for 48 h. No sugar or only one sugar was added as negative control. Cells were washed two times with PBS and then treated with Tz-Cy3 **22** (25 μM) and DIBO-488 **24** (50 μM) for 15 min at 37 °C. Cells were washed twice with PBS and nuclei were stained with Hoechst 33342 (10 μg mL^{-1}) for 20 min at room temperature in the dark. Cells were washed twice with PBS, and DMEM was added for microscopy. Microscopy was performed as described above.

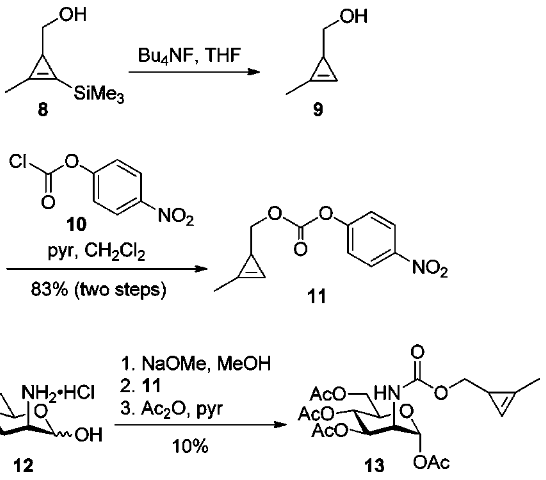
Kinetic Measurements. For kinetic studies, Ac$_4$ManNCyoc **13** was first deacetylated with MeOH/EtNMe$_2$ 5/1 to give ManNCyoc **14**. Stock solutions of Tz-PEG **15**[21] and ManNCyoc **14** were made in acetate buffer (pH 4.8, 20 °C) and mixed in a quartz cuvette for final concentrations of 1 mM Tz-PEG **15** and 10, 13.3, and 16.6 mM, respectively, ManNCyoc **14**. The reaction was monitored by the decreasing absorption of the tetrazine at 522 nm. Pseudo-first-order rate constants were determined for every concentration of ManNCyoc **14** by plotting $\ln(A_0/A_t)$ versus time. For the determination of A_0, a 1 mM solution of only Tz-PEG **15** 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 versus the corresponding ManNCyoc concentration, followed by linear regression. All measurements were carried out in triplicate. Sufficient stability of Tz-PEG **15** was verified by measuring the absorption at 522 nm of a solution of Tz-PEG **15** in acetate buffer[21] and PBS (Figure S2). Product formation was confirmed by RP-HPLC and ESI-MS analysis (Figure S3).

RESULTS AND DISCUSSION

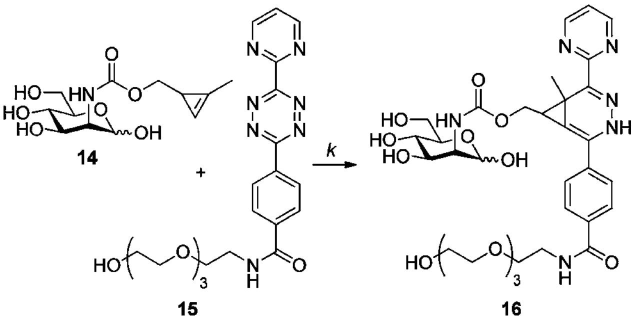
Synthesis of Ac$_4$ManNCyoc 13. Ac$_4$ManNCyoc **13** with a methylcyclopropene attached as a carbamate was a promising dienophile-modified sugar that was expected to have an increased reaction rate in DAinv reactions compared to previously described mannosamine derivatives. Starting from silyl-protected methylcyclopropene **8**,[26,28] we first synthesized activated carbonate **11** in two steps (Scheme 1). Since methylcyclopropenyl methanol **9** is prone to polymerization upon concentration,[26] we carried out the deprotection and activation step as a one-pot reaction. Removal of the trimethylsilyl group of **8** was achieved with tetra-*n*-butylammonium fluoride. Subsequent addition of *p*-nitrophenyl chloroformate **10** and pyridine yielded **11** in 83% yield over two steps. Carbonate **11** was then coupled to the amino group of mannosamine that was obtained by neutralization of mannosamine hydrochloride (**12**) with sodium methoxide. Subsequent acetylation gave Ac$_4$ManNCyoc **13**.

Kinetic Studies. To determine the performance of the labeled mannosamine derivative in DAinv reactions, sugar **13** was deacetylated using *N*,*N*-dimethylethylamine in methanol yielding ManNCyoc **14**. Within the cell, that process is performed by nonspecific esterases. Deprotection also ensured water solubility

Scheme 1. Synthesis of Ac_4ManNCyoc 13



Scheme 2. Reaction of ManNCyoc 14 with Tz-PEG 15 to Determine Rate Constants



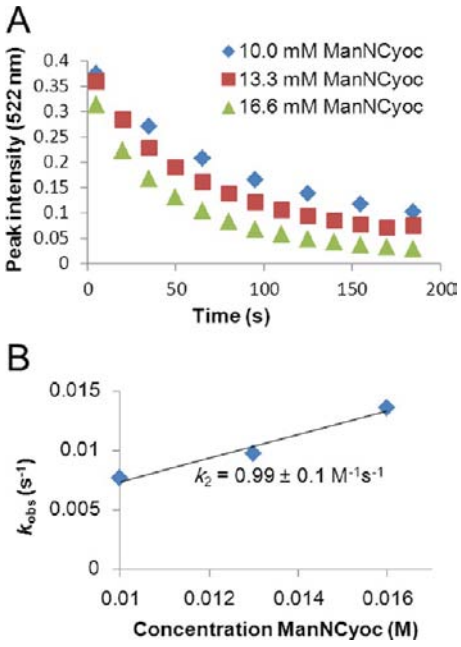
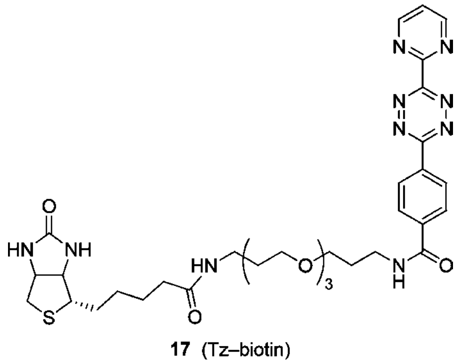


Figure 1. (A) Decrease of tetrazine absorbance at 522 nm over time for the reaction of Tz-PEG **15** (1 mM) with ManNCyoc **14** (10 mM, 13.3 mM, 16.6 mM) and (B) resulting second-order rate constant *k*.

Chart 2. Structure of Tz–Biotin 17



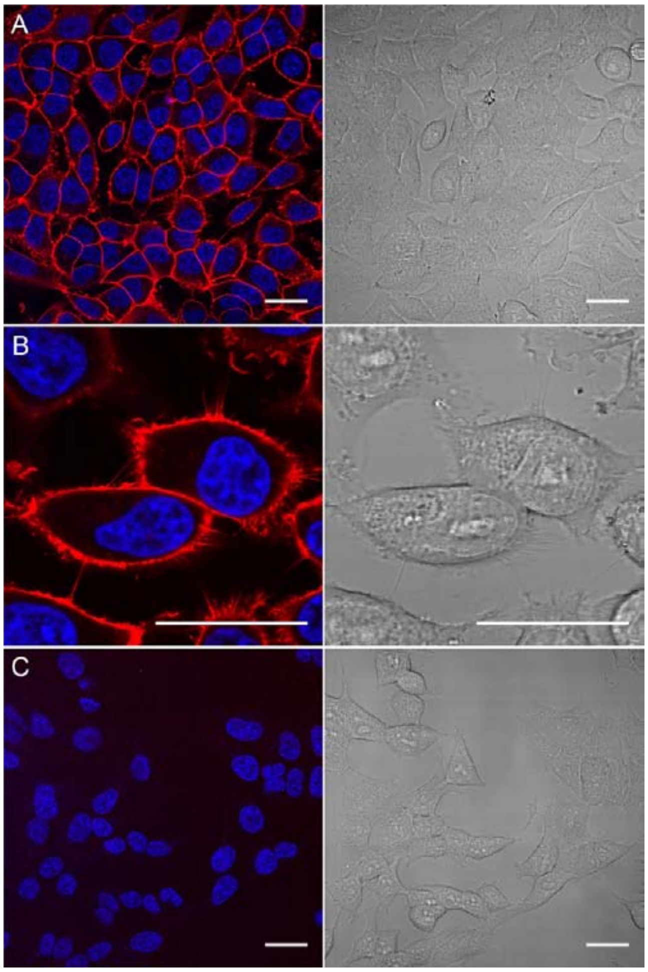
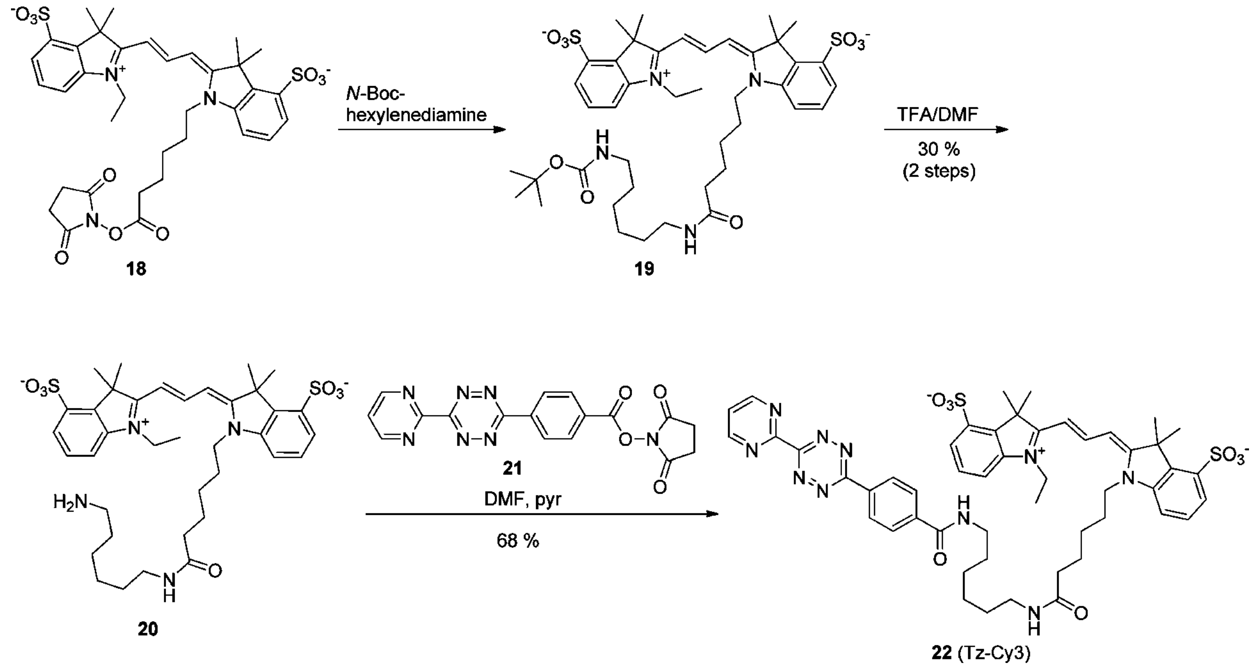


Figure 2. HEK 293T cells grown with 100 μM Ac_4ManNCyoc **13** (A, B) or without addition of **13** (C) for 48 h and incubated with Tz–biotin **17** (25 μM, 15 min, 37 °C) followed by incubation with streptavidin–AlexaFluor647. Nuclei were stained with Hoechst33342. Scale bar: 30 μm.

for the following experiments (Scheme 2). In acetate puffer (pH 4.7, 20 °C), tetrazine-tri(ethylene glycol)-conjugate **15** (Tz-PEG)[21] was allowed to react with an excess of ManNCyoc **14**, and pseudo-first-order rate constants were determined measuring the decrease of the absorption of **15** at λ_{max} = 522 nm (Figure 1A). From these values the second-order rate constant *k* of dienophile **14** was determined to be 0.99 ± 0.1 M^{-1} s^{-1} (Figure 1B). Formation of the ligation product **16** was verified by LC-MS (Figure S3).

Labeling Metabolized ManNCyoc with Tz–Biotin/Streptavidin–AlexaFluor647 (two-step labeling). To monitor metabolic incorporation of Ac_4ManNCyoc **13** into

Scheme 3. Synthesis of Tz-Cy3 22



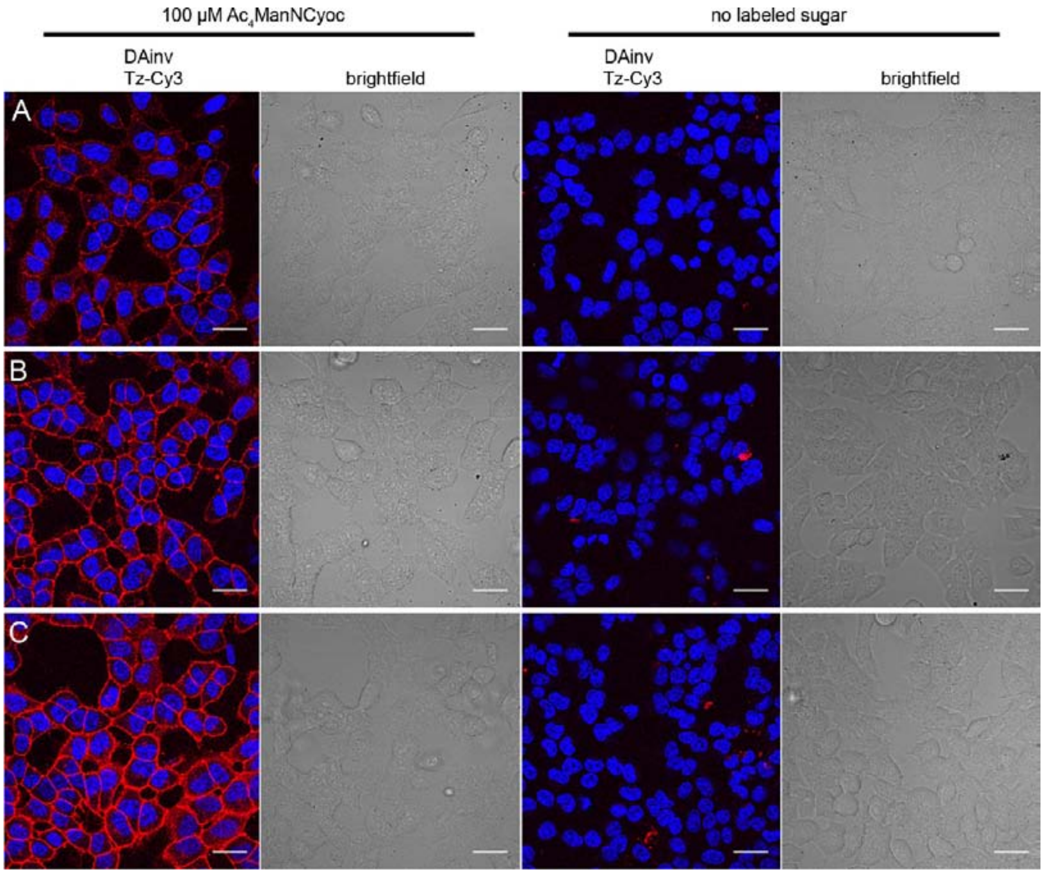
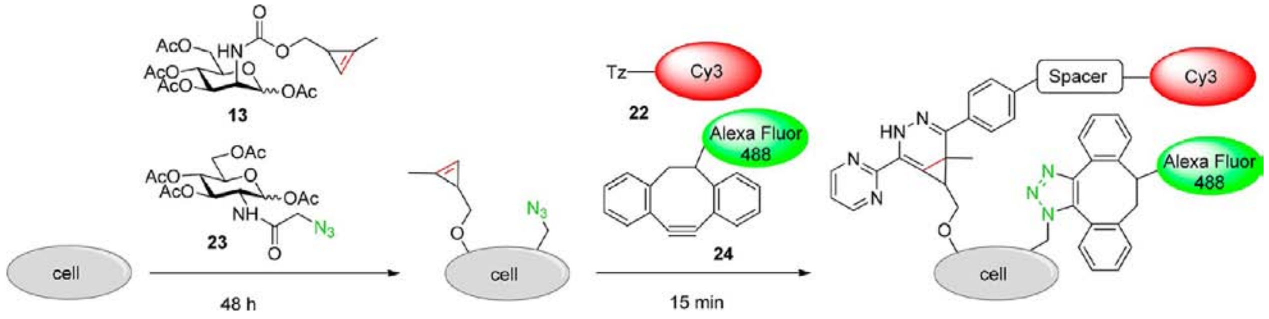


Figure 3. HEK 293T cells were grown with 100 μM Ac$_4$ManNCyoc **13** (left) or without labeled sugar (right) for 48 h, followed by incubation with 25 μM Tz-Cy3 **22** at 37 °C for (A) 5 min, (B) 10 min, and (C) 15 min. Nuclei were stained with Hoechst 33342. Scale bar: 30 μm.

glycoconjugates, HEK 293T cells were grown in the presence of **13** for two days, reacted with Tz–biotin **17** (Chart 2), and then labeled with streptavidin–AlexaFluor647. Confocal laser scanning microscopy showed a distinct labeling of the cell membrane of cells that had been cultivated with Ac$_4$ManNCyoc **13** (Figure 2A,B). The negative control, in which cells had not been treated with sugar **13**, did not show any membrane staining (Figure 2C). This confirms that Ac$_4$ManNCyoc can be employed to label

Scheme 4. Strategy for Dual Labeling with Two Different Metabolically Incorporated Monosaccharides and a One-Step-Labeling Procedure



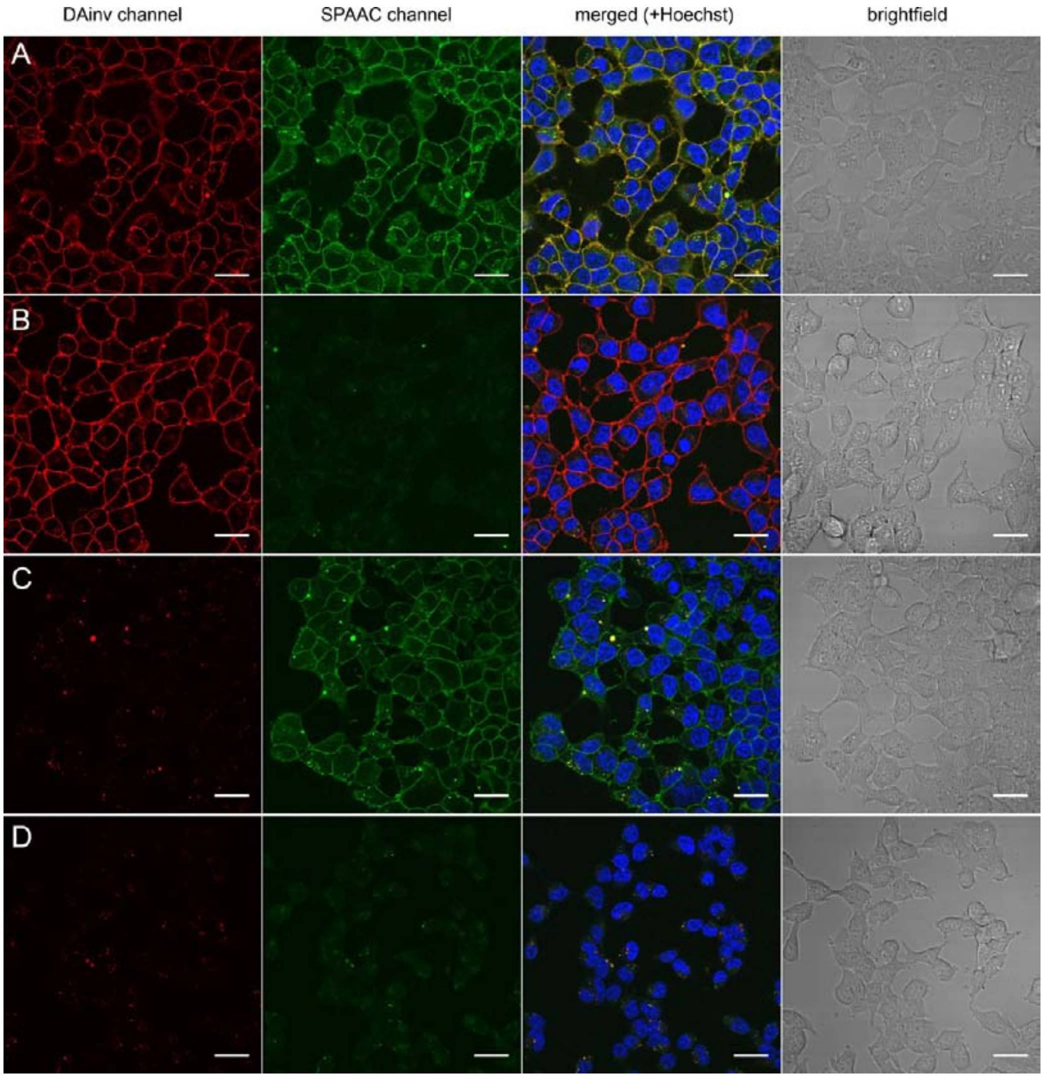


Figure 4. HEK 293T cells were grown with (A) 100 μM Ac_4ManNCyoc **13** and 50 μM Ac_4GlcNAz **23**, (B) 100 μM Ac_4ManNCyoc **13**, (C) 50 μM Ac_4GlcNAz **23**, and (D) without addition of non-natural sugar for 48 h and incubated with Tz-Cy3 **22** (25 μM) and DIBO-488 **24** (20 μM) for 15 min at 37 °C. Nuclei were stained with Hoechst33342. Scale bar: 30 μm.

glycoconjugates. Looking at the cells with higher magnification (Figure 2B), the staining allows detailed detection of membrane structures. In comparison to the DAinv reaction of terminal alkenes, we could significantly reduce the concentration of Tz−biotin **17** from 1 mM to 25 μM and the time of incubation with Tz−biotin **17** from 6 h to 15 min by using Ac_4ManNCyoc **13**.

Synthesis of Tz-Cy3 (22) for a One-Step Labeling Strategy. While we could reduce the incubation time and concentration of Tz−biotin in comparison to our labeling protocol for terminal alkenes, a one-step labeling is even more elegant. This requires the use of a dye-coupled tetrazine. Tz-Cy3 **22** was chosen to be a suitable tetrazine−dye conjugate as the absorption/emission wavelengths of Cy3 can be combined with those of AlexaFluor488-labeled probes. In addition, Tz-Cy3 **22** is, owing to the sulfo-groups, highly water-soluble resulting in low background staining. Furthermore, using sulfo-Cy3 rather than Cy3 facilitated chemical purification of **22**. Commercially available sulfo-Cy3-succinimidyl ester **18** was reacted with N-Boc-hexylenediamine, and the Boc-protecting group of **19** was removed with trifluoroacetic acid (TFA) in DMF to give **20**. Finally, the free amine was reacted with Tz-succinimidyl ester **21**[30] to yield Tz-Cy3 **22** in 68% yield (Scheme 3).

MOE and Subsequent Labeling with Tz-Cy3 22 (one-step labeling). To test the suitability of the novel Tz-Cy3 **22**, HEK 293T cells were cultured in the presence of Ac_4ManNCyoc **13**. Subsequently, the cells were incubated with Tz-Cy3 **22**. Confocal laser scanning microscopy showed a clear labeling of the cell membrane already after 5 min of incubation with Tz-Cy3 (Figure 3A, left). Prolonging the incubation time to 10 min (Figure 3B, left) increased the intensity significantly, while an even longer incubation time (15 min, Figure 3C, left) only incrementally increased the intensity. Control experiments, in which cells were cultured in the absence of labeled sugar but otherwise treated in the same way, did not show any membrane staining (Figure 3, right).

Dual Labeling with Ac_4ManNCyoc 13 and Ac_4GlcNAz 23. Current interest in the area of MOE has turned to dual labeling strategies employing two different metabolically incorporated monosaccharides, for example, ManNAc and *N*-acetylglucosamine (GlcNAc) derivatives in the same experiment. As the DAinv reaction can be orthogonal to the strain-promoted azide−alkyne cycloaddition (SPAAC), we combined these two ligation reactions for dual labeling. As second sugar derivative, we chose peracetylated *N*-azidoacetylglucosamine (Ac_4GlcNAz) **23** that has been reported to be incorporated into different glycoconjugates[31] and undergoes rapid SPAAC with DIBO-488 **24** (Scheme 4). Since both the DAinv reaction between Ac_4ManNCyoc **13** and Tz-Cy3 **22** and SPAAC between Ac_4GlcNAz **23** and DIBO-488 **24** occur within minutes, labeling of both sugars could be carried out simultaneously in a single step.

HEK 293T cells were grown in the presence of both sugars (**13** and **23**) for 48 h and subsequently stained by incubation with a mixture of Tz-Cy3 **22** and DIBO-488 **24** for 15 min and then investigated by confocal fluorescence microscopy (Figure 4A). In control experiments, cells were grown with only one (Figures 4B,C) or no sugar (Figure 4D). When both sugars were fed, a clear membrane staining was detected in both the DAinv channel (red) and the SPAAC channel (green) indicating that both sugars have been incorporated into membrane glycoconjugates and could be stained. If only Ac_4ManNCyoc **13** was fed, a significant membrane staining is only visible in the DAinv channel (Figure 4B), while cells that had been fed with Ac_4GlcNAz **23** alone only show membrane staining in the SPAAC channel (Figure 4C). When no sugar was present, no membrane staining was obtained (Figure 4D). These experiments show that the DAinv reaction of Ac_4ManNCyoc **13** is orthogonal to the SPAAC of Ac_4GlcNAz **23** and both reactions can be performed at the same time, facilitating the labeling reaction significantly. Dual Labeling was also successfully performed using peracetylated *N*-azidoacetylgalactosamine (Ac_4GalNAz **25**) in combination with Ac_4ManNCyoc **13** (Figure S4). Furthermore, we could show that also the combination of SPAAC and DAinv reactions using Tz-biotin **17** and subsequent labeling with streptavidin-AlexaFluor647 is possible and results in clear staining of membrane glycoproteins (Figure S5).

■ CONCLUSION

In summary, we have developed a fast reacting mannosamine derivative (Ac_4ManNCyoc **13**), bearing a carbamate-linked methylcyclopropene tag that is small enough to be accepted by the cell's metabolism and at the same time highly reactive in the inverse-electron-demand Diels−Alder reaction. We demonstrated that a labeling time of as low as 5 min is sufficient for a distinct membrane staining, making Ac_4ManNCyoc **13** a very valuable mannosamine derivative for MOE experiments. Furthermore, we introduced Tz-Cy3 **22**, a novel tetrazine−dye conjugate that not only allows one-step fluorescent labeling of alkenes, but also stands out by its high water solubility leading to remarkably low background staining. Finally, we reported − to the best of our knowledge − the first procedure in which dual sugar labeling is achieved by simultaneous DAinv and SPAAC reactions in a single step.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional MOE experiments, stability of tetrazine, analytical data of synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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