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

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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 β 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 tetrazines²⁴ 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 $k = 0.04 \text{ M}^{-1} \text{ s}^{-1}$.²¹ The reaction rate of isonitriles 2 with tetrazines of type 5 is higher (up to $k = 0.57 \text{ M}^{-1} \text{ s}^{-1}$), 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 $k = 0.137 \text{ M}^{-1} \text{ s}^{-1}$ 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 ($k = 13 \text{ M}^{-1} \text{ s}^{-1}$). 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}

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* acyl mannosamine 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 ($\lambda = 254$ 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 : $\delta_{\text{H}} = 7.26$ ppm, $\delta_{\text{C}} = 77.16$ 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, HMB, 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 $\times$ 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 $^\circ\text{C}$ 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, $=\text{CH}$), 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_2CH); ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) = 155.92 (C_{quart}), 152.81 (C_{quart}), 145.44 (C_{quart}), 125.42 (C_{arH}), 121.95 (C_{arH}), 120.32 (C_{quart}), 101.83 ($=\text{CH}$), 77.53 (CH_2), 16.79 (CH_3), 11.80 (CH_2CH).

Ac₄ManNCyoc (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 Ac₄ManNCyoc **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, $=\text{CH}$), 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_2CH); ^{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 ($=\text{CH}$), 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 μ L, 0.42 mmol) and *N* Boc hexylenediamine (94 μ L, 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. ^1H 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 μ L) 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. ^1H 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 μ g mL $^{-1}$ streptomycin. All cells were incubated in a 5% carbon dioxide, water saturated incubator at 37 $^{\circ}\text{C}$.

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 μ M 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 μ M) for 15 min at 37 $^{\circ}\text{C}$. After two washes with PBS, cells were incubated with AlexaFluor647 labeled streptavidin (6.6 μ g mL $^{-1}$) and Hoechst 33342 (10 μ g 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 $\times$ 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 μ M 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 μ M) for 5–15 min at 37 $^{\circ}\text{C}$. Cells were washed twice with PBS and nuclei were stained with Hoechst 33342 (10 μ g 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 μ M Ac $_4$ ManNCyoc 13 and 50 μ M 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 μ M) and DIBO 488 24 (50 μ M) for 15 min at 37 $^{\circ}\text{C}$. Cells were washed twice with PBS and nuclei were stained with Hoechst 33342 (10 μ g 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 $^{\circ}\text{C}$) 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 Ac₄ManNCyoc 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 .

for the following experiments (Scheme 2). In acetate buffer (pH 4.7, 20 °C), tetrazine tri(ethylene glycol) conjugate 15 (Tz PEG)²¹ was allowed to react with an excess of ManNCyoc 14,

Chart 2. Structure of Tz–Biotin 17

Figure 2. HEK 293T cells grown with 100 μM Ac₄ManNCyoc 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 .

and pseudo first order rate constants were determined measuring the decrease of the absorption of 15 at $\lambda_{\text{max}} = 522 \text{ nm}$ (Figure 1A). From these values the second order rate constant k of dienophile 14 was determined to be $0.99 \pm 0.1 \text{ M}^{-1} \text{ s}^{-1}$ (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 Ac₄ManNCyoc 13 into

Scheme 3. Synthesis of Tz Cy3 22

Figure 3. HEK 293T cells were grown with 100 μ M Ac₄ManNCyoc **13** (left) or without labeled sugar (right) for 48 h, followed by incubation with 25 μ M 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 μ m.

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 Ac₄ManNCyoc **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 Ac₄ManNCyoc 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 μ M $\text{Ac}_4\text{ManNCyoc}$ 13 and 50 μ M Ac_4GlcNAz 23, (B) 100 μ M $\text{Ac}_4\text{ManNCyoc}$ 13, (C) 50 μ M Ac_4GlcNAz 23, and (D) without addition of non natural sugar for 48 h and incubated with Tz Cy3 22 (25 μ M) and DIBO 488 24 (20 μ M) for 15 min at 37 $^{\circ}$ C. Nuclei were stained with Hoechst33342. Scale bar: 30 μ m.

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 μ M and the time of incubation with Tz-biotin 17 from 6 h to 15 min by using $\text{Ac}_4\text{ManNCyoc}$ 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 Ac₄ManNCyoc **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 Ac₄ManNCyoc **13 and Ac₄GlcNAz **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 (Ac₄GlcNAz) **23** that has been reported to be incorporated into different glycoconjugates³¹ and undergoes rapid SPAAC with DIBO **488 24** (Scheme 4). Since both the DAinv reaction between Ac₄ManNCyoc **13** and Tz Cy3 **22** and SPAAC between Ac₄GlcNAz **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 Ac₄ManNCyoc **13** was fed, a significant membrane staining is only visible in the DAinv channel (Figure 4B), while cells that had been fed with Ac₄GlcNAz **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 Ac₄ManNCyoc **13** is orthogonal to the SPAAC of Ac₄GlcNAz **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 (Ac₄GalNAz **25**) in combination with Ac₄ManNCyoc **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 (Ac₄ManNCyoc **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 Ac₄ManNCyoc **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.

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Notes

The authors declare no competing financial interest.

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