Supporting Information for

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

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Fluorescence Microscopy with Tz-Cy3 for Dual Labeling with Ac₄GalNAz. HEK 293T cells (7500 cells/cm²) were seeded in 8-well ibiTreat µ-Slides (ibidi) and allowed to attach for 12 h. Cells were then incubated with 100 µM Ac₄ManNCyoc 13 and 50 µM Ac₄GalNAz 25 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 a mixture of Tz-Cy3 22 (25 µM) and AlexaFluor®488-DIBO 24 (20 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.

Fluorescence Microscopy with Tz-Biotin for Dual Labeling with Ac₄GlcNAz. HEK 293T cells (7500 cells/cm²) were seeded in 8-well ibiTreat µ-Slides (ibidi) and allowed to attach for 12 h. Cells were then incubated with 100 µM Ac₄ManNCyoc 13 and 50 µM Ac₄GlcNAz 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 a mixture of Tz-biotin 17 (25 µM) and AlexaFluor®488-DIBO 24 (20 µM) for 15 min at 37 °C. After two washes with PBS, cells were incubated with AlexaFluor®647-labeled streptavidin (6.6 µg mL⁻¹) and Hoechst 33342 (10 µg mL⁻¹) for 20 min at room temperature in the dark. Cells were washed twice with PBS, and DMEM was added for microscopy. Microscopy was performed as described above.

Figure S1. Mechanism of the DAinv reaction.
**Stability of tetrazine 15.** To determine the stability of Tz-PEG 15, solutions of 15 (5 mM) in acetate buffer (pH 4.8)\(^1\) and PBS (pH 7.2) were prepared. Decomposition of 15 was followed at room temperature by measuring its absorption at 522 nm over time (Figure S2).

![Graph showing absorption over time](image)

**Figure S2.** Decrease of the absorption at 522 nm over time of solutions of Tz-PEG 15 (5 mM) in acetate buffer (pH 4.8)\(^1\) and PBS (pH 7.2).

Figure S3. (A) HPLC analysis of Tz-PEG 15 using a gradient of CH$_3$CN in H$_2$O (with 0.1% formic acid, 20-90% in 10 min) and (B) Peak analyzed by ESI-MS. Calcd: [M + H]$^+$: 456.20, [M + Na]$^+$: 478.18 found: [M + H]$^+$: 456.35, [M + Na]$^+$: 478.05. (C) HPLC analysis (gradient as described under (A)) of ligation product 16 after the Diels-Alder reaction in solution for kinetic measurements. All Tz-PEG 15 (retention time: 5.6 min) has reacted. Ligation product formation 16 can be observed (retention time 3.3 min). (D) ESI-MS of 16 calcd. 359.16 [M + 2H]$^{2+}$, 717.31 [M + H]$^+$, 739.29 [M + Na]$^+$, found: 359.00 [M + 2H]$^{2+}$, 717.50 [M + H]$^+$, 739.15 [M + Na]$^+$. 

C

![Chromatogram](image)

D

![MS Spectrum](image)
Figure S4. HEK 293T cells were grown with (A) 100 µM Ac₄ManNCyoc 13 and 50 µM Ac₄GalNAz 25, (B) 100 µM Ac₄ManNCyoc 13, (C) 50 µM Ac₄GalNAz 25, and (D) without non-natural sugar for 48 h and incubated with a mixture of 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.
Figure S5. HEK 293T cells were grown with (A) 100 µM Ac₄ManNCyoc 13 and 50 µM Ac₄GlcNAz 23, (B) 100 µM Ac₄ManNCyoc 13, (C) 50 µM Ac₄GlcNAz 23, and (D) without non-natural sugar for 48 h and incubated with a mixture of Tz-biotin 17 (25 µM) and DIBO-488 24 (20 µM) for 15 min at 37 °C followed by labeling with Streptavidin-AlexaFluor647. Nuclei were stained with Hoechst33342. Scale bar: 30 µm.
Figure S6. $^1$H NMR spectrum (CDCl$_3$, 400.1 MHz) of 11.
**Figure S7.** $^{13}$C NMR spectrum (CDCl$_3$, 100.6 MHz) of 11.
Figure S8. $^1$H NMR spectrum (CDCl$_3$, 400.1 MHz) of 13.
Figure S9. $^{13}$C NMR spectrum (CDCl$_3$, 100.6 MHz) of 13.