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Supporting Information

Metabolic Glycoengineering with Azide- and Alkene-Modified Hexosamines: Quantification of Sialic Acid Levels

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Author Contributions

J.D. Conceptualization:Supporting; Data curation:Equal; Investigation:Lead; Writing – original draft:Lead; Writing – review & editing:Supporting
V.W. Conceptualization:Lead; Data curation:Equal; Funding acquisition:Lead; Supervision:Lead; Writing – original draft:Supporting; Writing – review & editing:Lead

Supporting Information

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Additional Figures

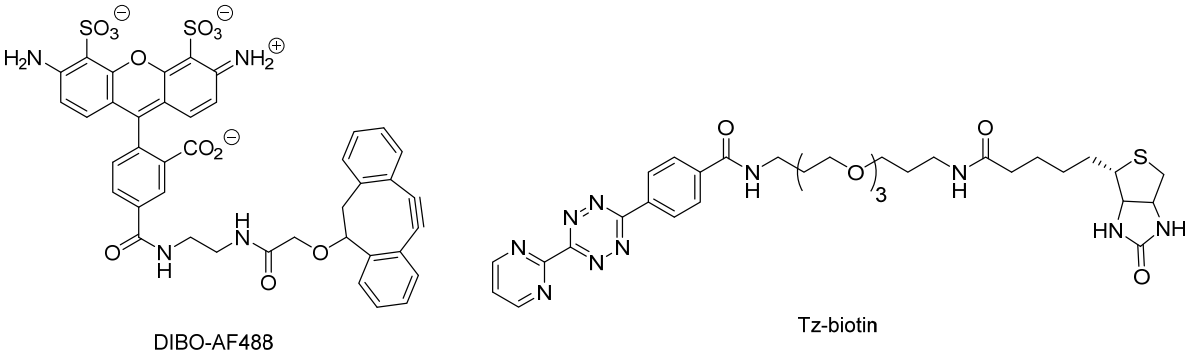


Figure S1. Reagents used in bioorthogonal labeling reactions.

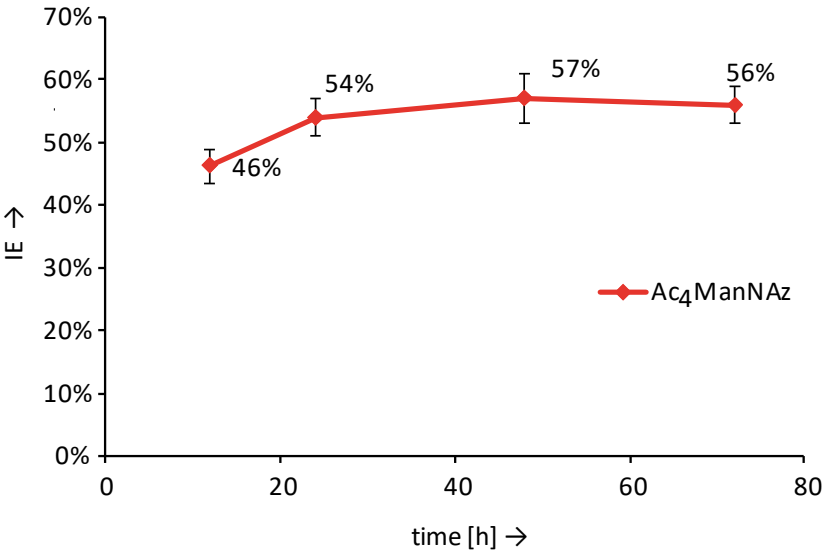


Figure S2. Time-dependency of the incorporation efficiency after MGE with Ac_4ManNAz. HEK 293T cells were incubated with 100 µM Ac_4ManNAz for 12 h, 24 h, 48 h, or 72 h and the culture medium was replaced every 24 hours by fresh culture medium containing 100 µM modified sugar. The sialic acids were released with HOAc, reacted with DMB, and quantified by RP-HPLC.

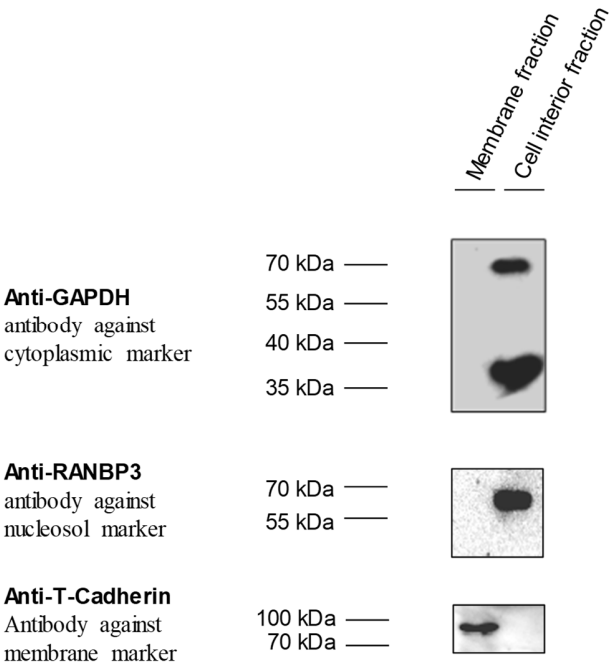


Figure S3. Quality control of the cell fractionation protocol. A Western blot confirms the complete separation of membrane (left lane) and cell interior fraction (right lane). Used antibodies: anti-GAPDH (cytoplasm marker), anti-RANBP3 (nucleus marker), and anti-T-Cadherin (membrane marker).

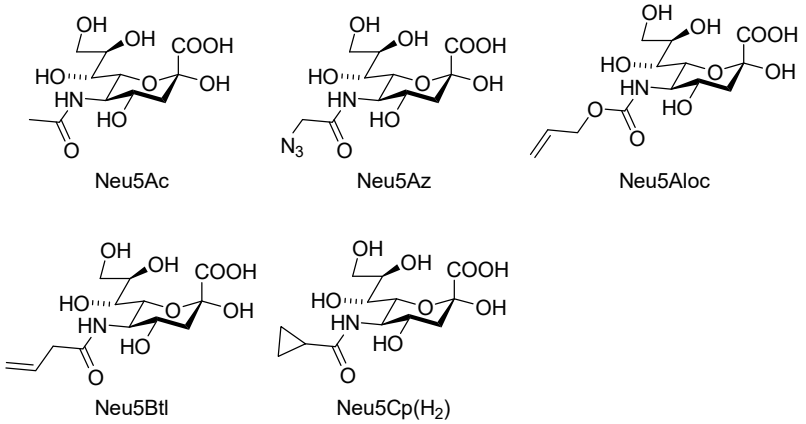
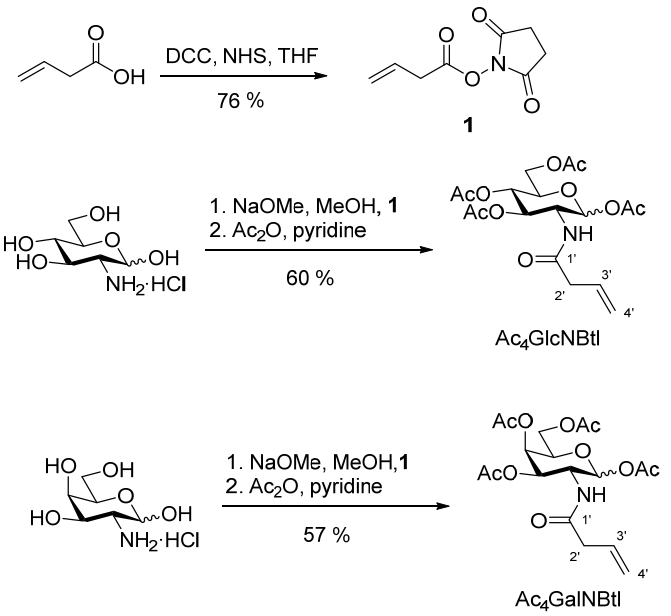


Figure S4. Sialic acids used to produce DMB-labeled reference compounds. Neu5Az was purchased from Chemily Glycoscience and Neu5Ac from Sigma Aldrich. Neu5Aloc,[1] Neu5Btl,[1] and Neu5Cp(H_2)[2] were synthesized according to published procedures.

Chemical Synthesis



Scheme S1: Synthesis of Ac_4GlcNBtl and Ac_4GalNBtl.

2,5-Dioxopyrrolidin-1-yl but-3-enoate (1)

The title compound was prepared according to a published procedure.[1]

1,3,4,6-Tetra-*O*-acetyl-*N*-(but-3-enoyl)-glucosamine (Ac_4GlcNBtl)

Glucosamine hydrochloride (1.14 g, 5.25 mmol) was dissolved in dry MeOH (30 mL) under N_2-atmosphere and 0.5 M NaOMe (10.22 mL, 5.25 mmol) was added. The reaction mixture was stirred for 2.25 h at RT. 2,5-Dioxopyrrolidin-1-yl but-3-enoate **1** (1.00 g, 5.46 mmol) was dissolved in dry MeOH (30 mL) and added to the reaction mixture. After stirring overnight, the solvent of the slightly brownish solution was removed under reduced pressure. The obtained brownish foam was diluted in pyridine (14 mL) under N_2-atmosphere, treated with acetic anhydride (4.82 mL) and stirred for five days. The solvent was removed under reduced pressure, the residue diluted with DCM (140 mL) and washed with saturated $KHSO_4$ (150 mL), saturated $NaHCO_3$ (150 mL) and brine (150 mL). The organic layer was dried over $MgSO_4$ and purified by flash chromatography (gradient elution 18-20 % ethyl acetate in petroleum ether) to yield Ac_4GlcNBtl as a colorless solid (1.302 g, 60 %) as anomeric mixture (α/β, 1:2). The α-anomer was separated by HPLC (40-70% B in A over 30 min).

^{1}H NMR (400 MHz, $CDCl_3$) (α-anomer): δ (ppm) = 6.07 (d, *J* = 3.7 Hz, 1H; H1 α), 5.91 (d, *J* = 9.0 Hz, 1H; NH), 5.78 - 5.68 (m, 1H; H3‘), 5.17 – 5.04(m, 4H; H3, H4, 2H-4‘), 4.36 (ddd, *J* = 10.5, 9.1 3.7 Hz, 1H, H2), 4.14 (dd *J* = 12.3, 4.0 Hz, 1H; H6a) 4.02 – 3.90 (m, 2H; H5, H6b), 2.84 – 2.82 (m, 2H; 2H-2‘), 2.02 (s, 3H; OAc), 1.97 (s, 3H; OAc), 1.94 (s, 3H; OAc), 1.92 (s, 3H; OAc); **^{13}C-NMR** (101 MHz, $CDCl_3$) (α-anomer): δ (ppm) = 171.22 (O=C), 170.59 (O=C), 170.50 (O=C) 169.03 (O=C), 168.56 (O=C), 130.70 (C3‘), 119.56 (C4‘), 90.39 (C1 α), 70.32 (C4), 69.64 (C5), 67.49 (C3), 61.49 (C6), 50.93 (C2), 40.08 (C2‘), 20.89 (CH_3), 20.68 (CH_3), 20.53 (CH_3), 20.42 (CH_3); **HRMS**: m/z calcd for $C_{18}H_{25}NO_{10}$: 438.1370 [*M*+Na]$^+$, found: 438.1360.

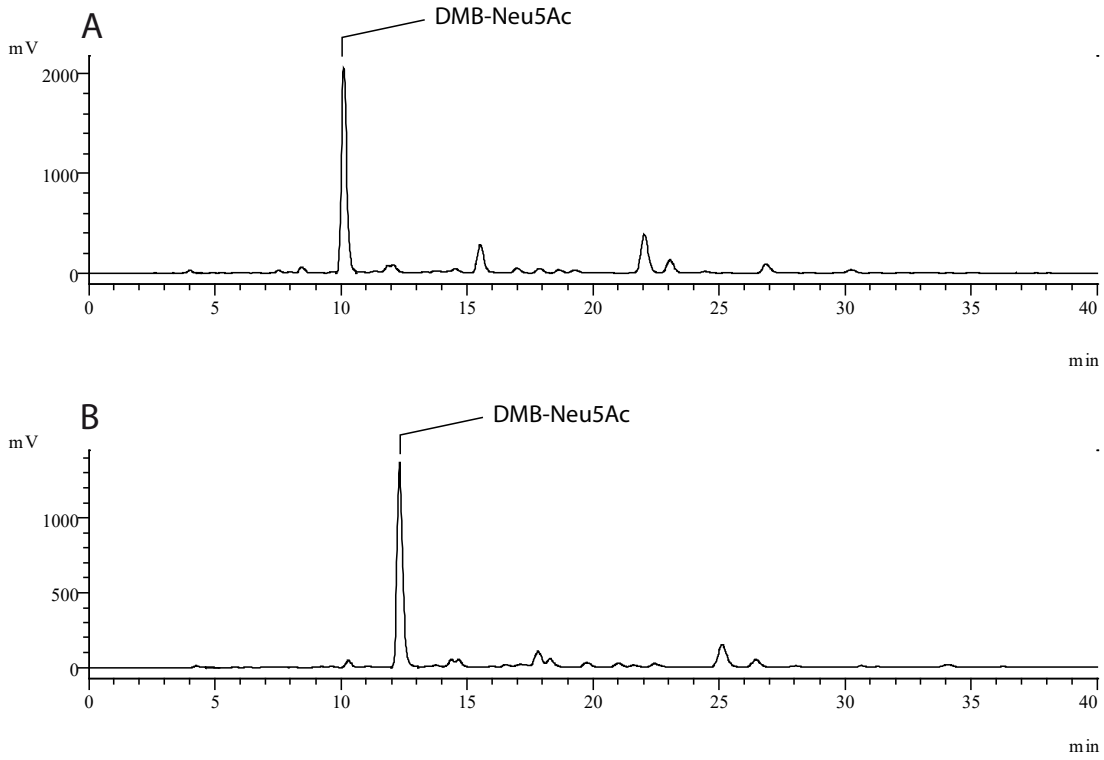
1,3,4,6-Tetra-*O*-acetyl-*N*-(but-3-enoyl)-galactosamine (Ac_4GalNBtl)

Galactosamine hydrochloride (1.14 g, 5.25 mmol) was dissolved in dry MeOH (30 mL) under N_2-atmosphere and 0.5 M NaOMe (10.2 mL, 5.25 mmol) was added. The reaction mixture was stirred for 1.5 h at rt. 2,5-Dioxopyrrolidin-1-yl but-3-enoate **1** (1.0 g, 5.46 mmol) was dissolved in dry MeOH (30 mL) and added to the reaction mixture. After stirring overnight, the solvent of the slightly brownish solution was removed under reduced pressure. The obtained brownish foam was diluted in pyridine (14 mL), treated with acetic anhydride (4.82 mL) and stirred for around three days. The solvent was removed under reduced pressure, the residue diluted with DCM (140 mL) and washed with saturated $KHSO_4$ (150 mL), saturated $NaHCO_3$ (150 mL) and brine (150 mL). The organic layer was dried over $MgSO_4$ and purified by flash chromatography (gradient elution 18-20 % ethyl acetate in petroleum ether) to yield Ac_4GalNBtl as a colorless solid (1.236 g, 57 %) as an anomeric mixture (α/β, 1:2).

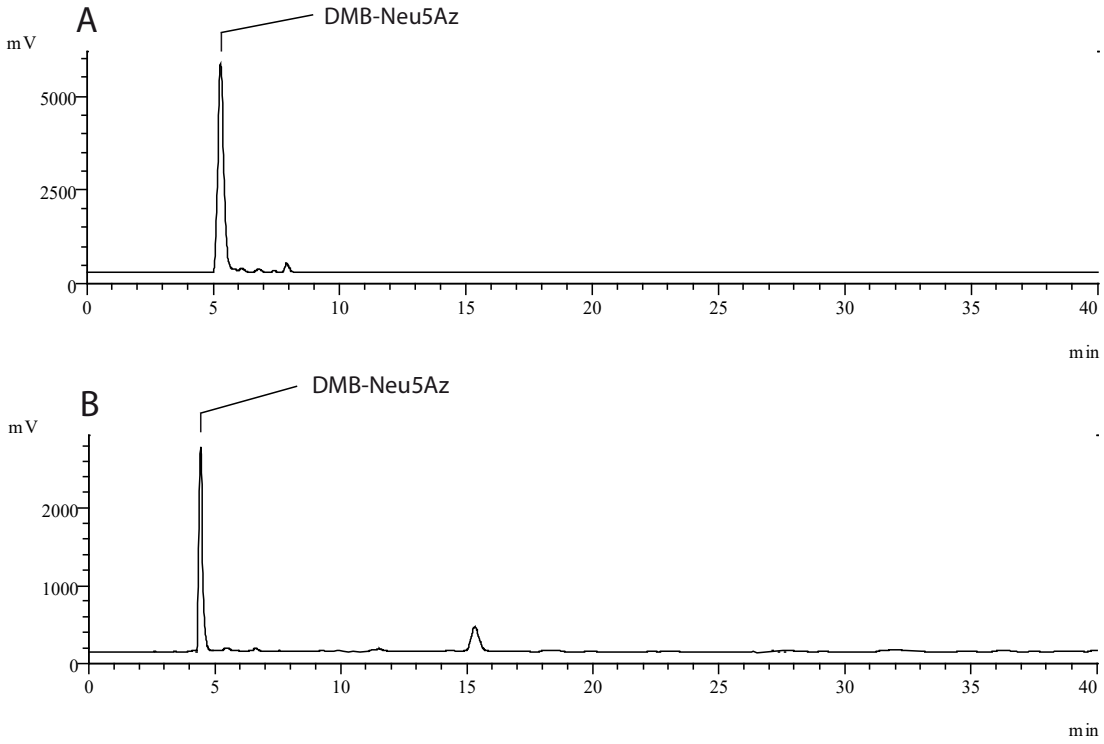
^{1}H-NMR (600 MHz, $CDCl_3$) (α- and β-anomer): δ (ppm) = 6.25 – 6.11 (m, 1H; H1α and H1β), 5.89 – 5.75 (m, 1H; H3´), 5.70 (dd, *J* = 9.3, 4.1 Hz, 1H; NH), 5.38 (dd, *J* = 3.3, 1.4 Hz, 0.65H; H4β), 5.28 (dd, *J* = 8.9, 7.1 Hz, 0.35H; H3α), 5.26 – 5.09 (m, 3.3H; 2H4´, H3β, H4α), 4.72 – 4.59 (m, 1H; H2α, H2β), 4.25 – 4.10 (m, 1.5H; H5α, H5β, H6α), 4.10 – 3.96 (m, 2H; 2H6β, H6α), 2.99 – 2.85 (m, 2H; 2H2´), 2.13 (s, 2H; CH_3β), 2.11 (s, 2H; CH_3β), 2.07 (s, 1H; CH_3α), 2.06 (s, 1H; CH_3α), 2.05 (s, 1H; CH_3α), 2.01 (s, 1H; CH_3α), 1.99 (s, 2H; CH_3β), 1.97 (s, 2H; CH_3β); **^{13}C-NMR** (151 MHz, $CDCl_3$) (α- and β-anomer): δ (ppm) = 171.06 (C=O), 170.90 (C=O), 170.87 (C=O), 170.80 (C=O), 170.48 (C=O), 170.42 (C=O), 170.25 (C=O), 169.98 (C=O), 169.10 (C=O), 168.86 (C=O), 130.88 (C3´β), 130.69 (C3´α), 120.18 (C4´α), 119.93 18 (C4´β), 94.02 (C1α), 91.19 (C1β), 78.70 (H5α), 73.90 (C3α), 70.36 (C4α), 68.63 (C5β), 67.73 (C3β), 66.72 (C4β), 62.13 (C6α), 61.33 (C6β), 56.30 (C2α), 47.00 (C2β), 41.40 (C2´β), 41.16 (C2´α), 21.07 (CH3), 20.90 (CH3), 20.83 (CH3), 20.75 (CH3), 20.74 (CH3), 20.72 (CH3), 20 .69 (CH3), 20.67 (CH3); **HRMS**: m/z calcd for $C_{18}H_{25}NO_{10}$: 438.1370 [*M*+Na]$^+$, found: 438.1358.

RP-HPLC Chromatograms of DMB-Labeled Sialic Acid Reference Compounds

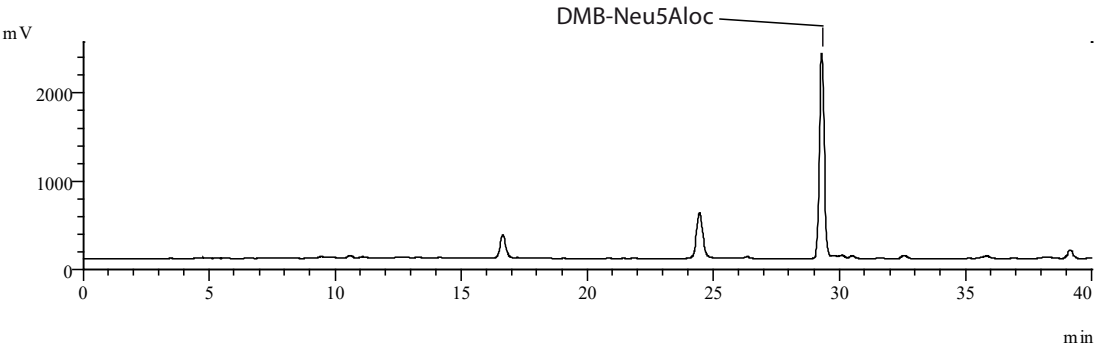
Conditions used in the following experiments: Mobile phase: gradient of acetonitrile with 0.1% formic acid (solvent B) in water with 0.1% formic acid (solvent A). Used columns: Nucleodur C18 Gravity, 3 µm, 125 x 4 mm from Macherey-Nagel, flow: 0,4 mL min^{-1}; Kinetex C18, 2.6 µm, 100 Å, 150 x 4.6 mm from Phenomenex, flow: 0,4 mL min^{-1}. Fluorescence detector (excitation 372 nm, emission 456 nm).



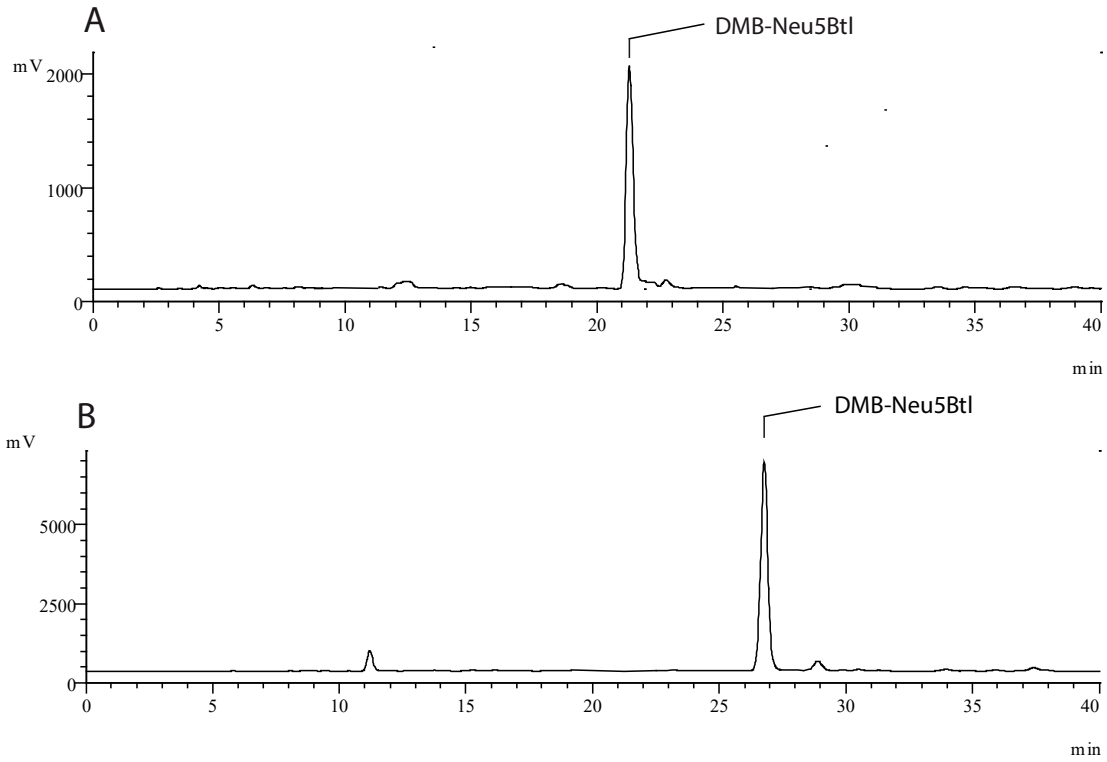
DMB-Neu5Ac: Analytical RP-HPLC with (A) 10-20% B over 40 min: t_R = 10.5 min (Kinetex C18); (B) 10-25% B over 40 min: t_R = 12.2 min (Nucleodur C18 Gravity).



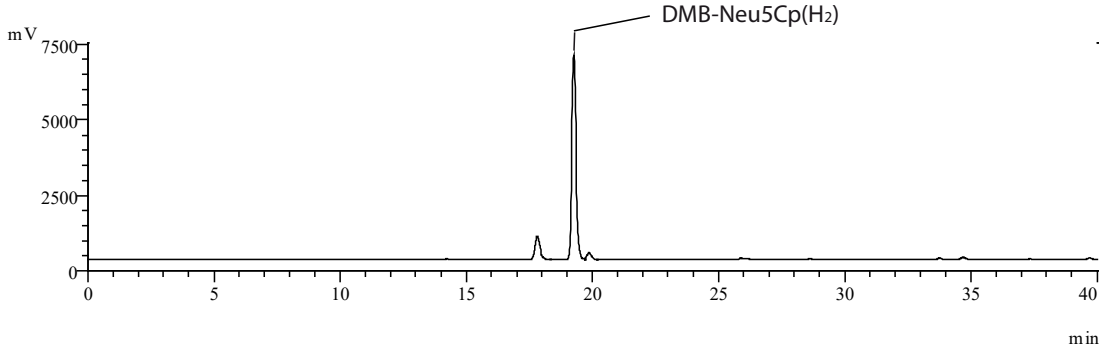
DMB-Neu5Az: Analytical RP-HPLC with (A) 10-20% B over 40 min: t_R = 5.1 min (Kinetex C18); (B) 10-25% B over 40 min: t_R = 4.8 min (Nucleodur C18 Gravity).



DMB-Neu5Aloc: Analytical RP-HPLC (10-20% B over 40 min): t_R = 29.5 min (Kinetex C18).

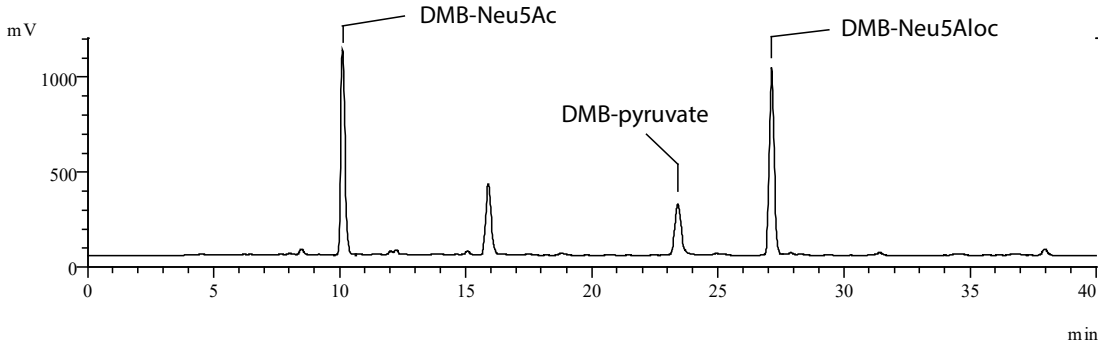


DMB-Neu5Btl: Analytical RP-HPLC with (A) 10-20% B over 40 min: t_R = 21.5 min (Kinetex C18); (B) 10-25% B over 40 min: t_R = 26.9 min (Nucleodur C18 Gravity).

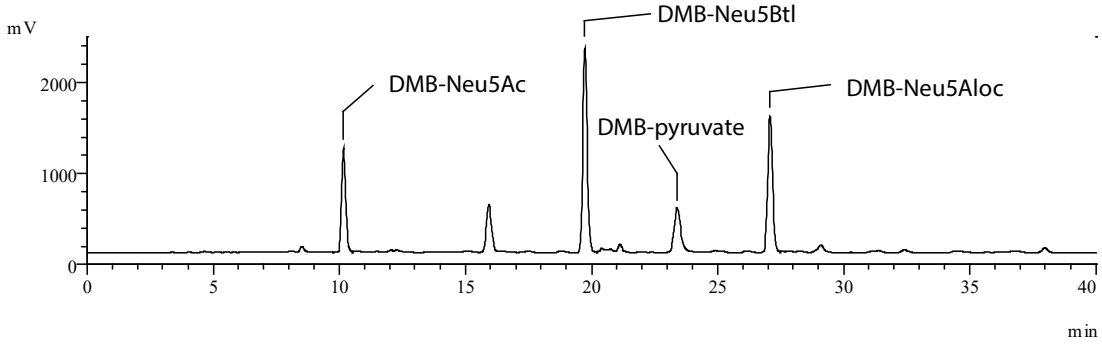


DMB-Neu5Cp(H_2): Analytical RP-HPLC (10-20% B over 40 min): t_R = 19.2 min (Kinetex C18).

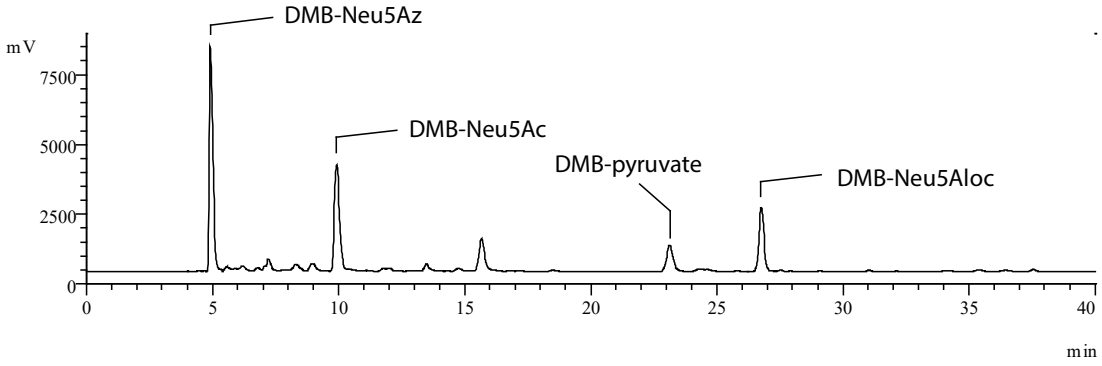
Selected RP-HPLC Chromatograms of DMB-Labeled Sialic Acids from Whole Cell Lysates of HEK 293T Cells



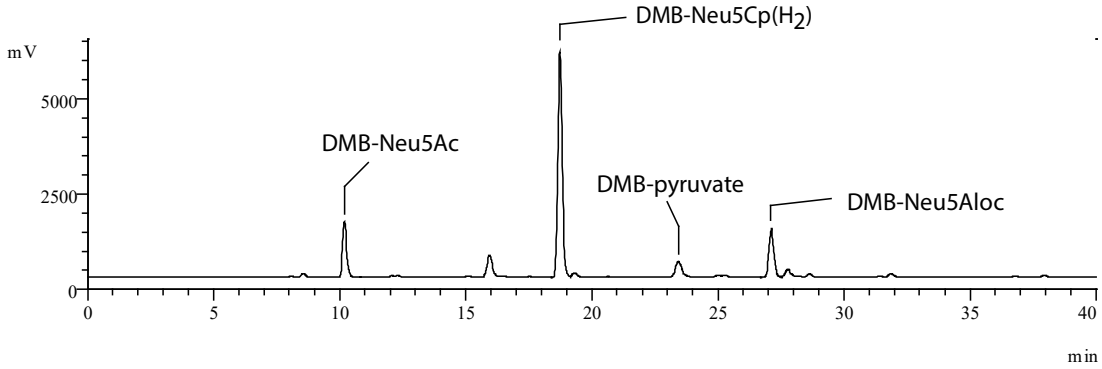
HEK 293T cells were grown without addition of unnatural sugar for 48 h. Sialic acids were cleaved off by treatment with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 µL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).



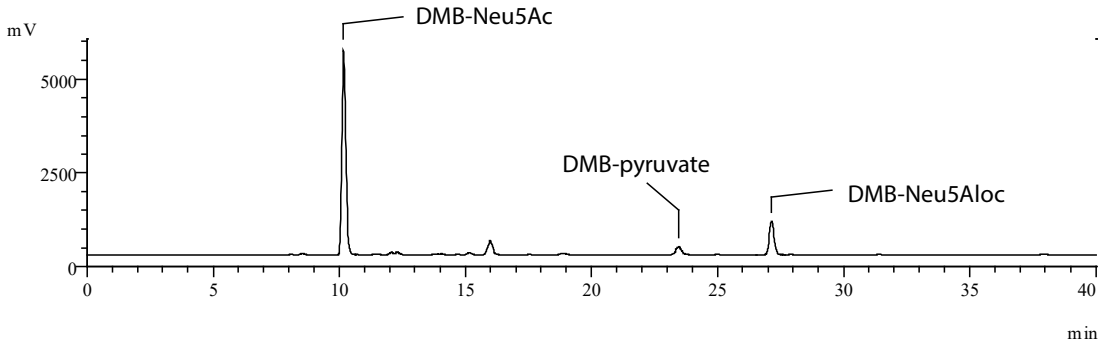
HEK 293T cells were grown with 100 µM **Ac_4ManNBtl** in the cell medium for 48 h. Sialic acids were cleaved off by treatment with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 µL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).



HEK 293T cells were grown with 100 μM **Ac_4ManNAz** in the cell medium for 48 h. Sialic acids were cleaved off by treatment with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 μL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).

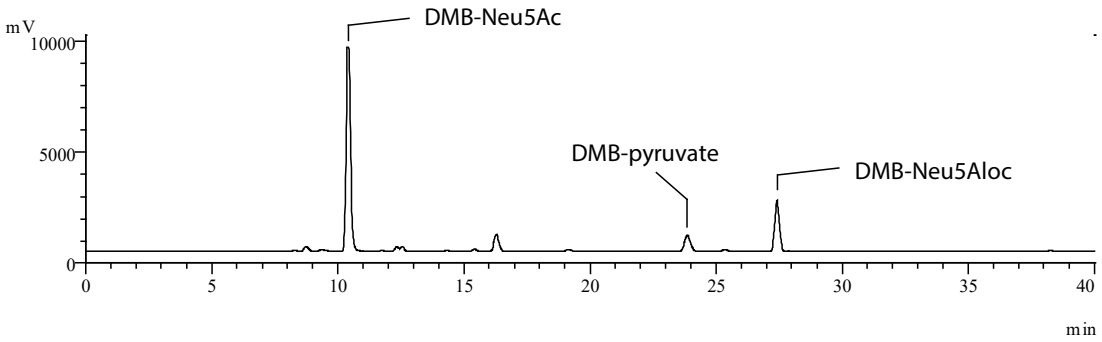


HEK 293T cells were grown with 100 μM **Ac_4ManNCp(H_2)** in the cell medium for 48 h. Sialic acids were cleaved off by treatment with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 μL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).

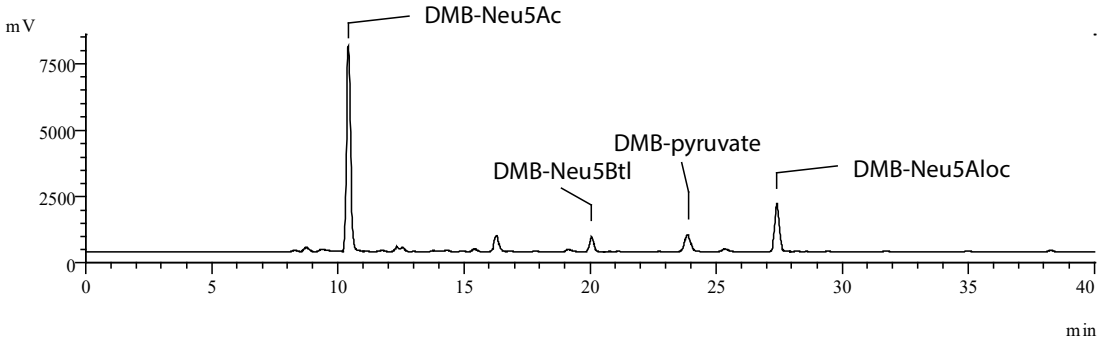


HEK 293T cells were grown with 100 µM **Ac_4ManNAc** in the cell medium for 48 h. Sialic acids were cleaved off by treatment with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 µL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).

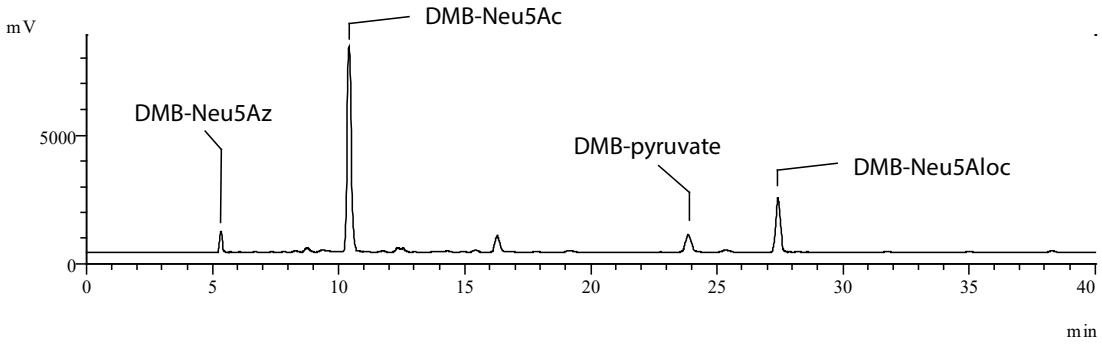
Selected RP-HPLC Chromatograms of DMB-Labeled Sialic Acids from Whole Cell Lysates of HeLa S3 Cells



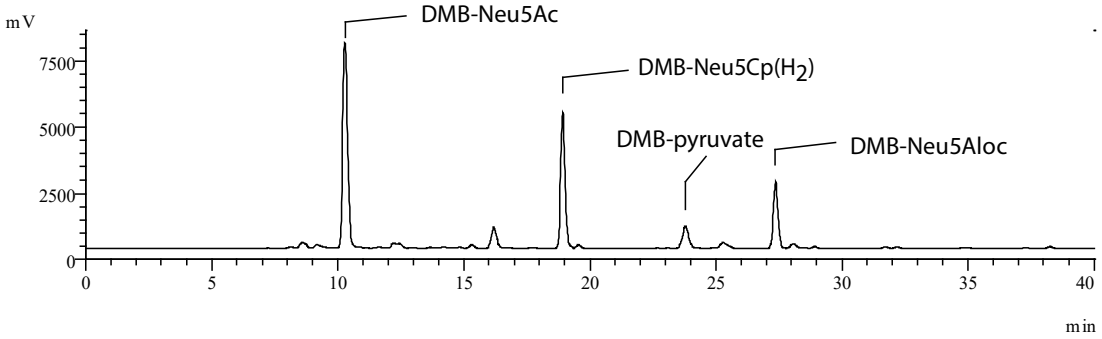
HeLa S3 cells were grown without addition of unnatural sugar for 48 h. Sialic acids were cleaved off by treatment with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 µL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).



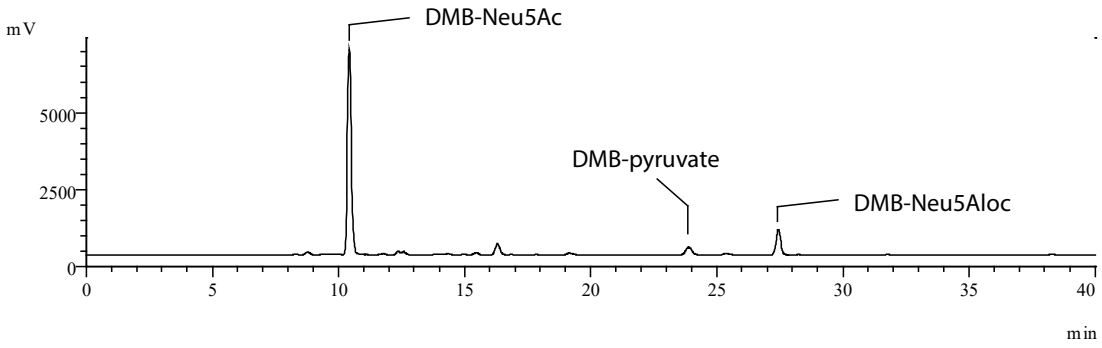
HeLa S3 cells were grown with 100 µM **Ac_4ManNBtl** in the cell medium for 48 h. Sialic acids were cleaved off by treatment with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 µL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).



HeLa S3 cells were grown with 100 µM **$Ac_4ManNAz$** in the cell medium for 48 h. Sialic acids were cleaved off by treatment with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 µL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).

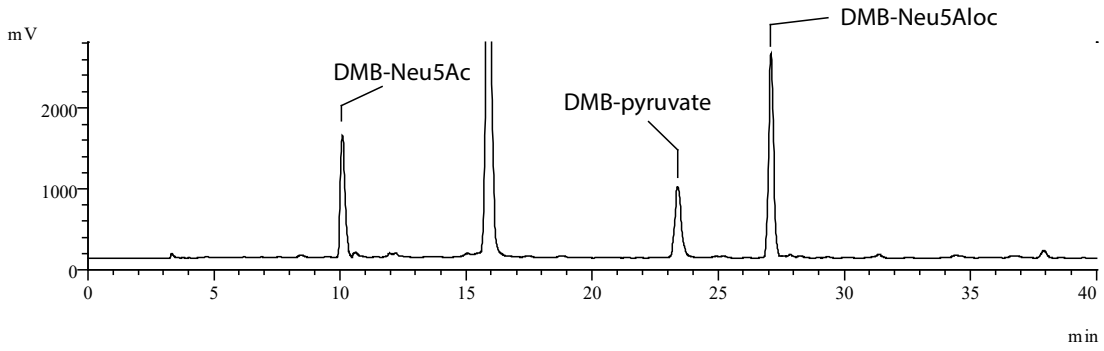


HeLa S3 cells were grown with 100 µM **$Ac_4ManNCp(H_2)$** in the cell medium for 48 h. Sialic acids were cleaved off by treatment with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 µL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).

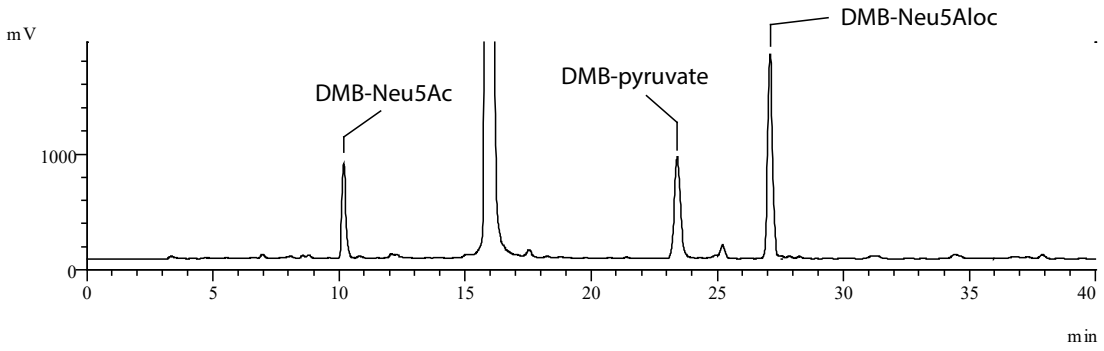


HeLa S3 cells were grown with 100 µM **Ac_4ManNAc** in the cell medium for 48 h. Sialic acids were cleaved off by treatment with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 µL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).

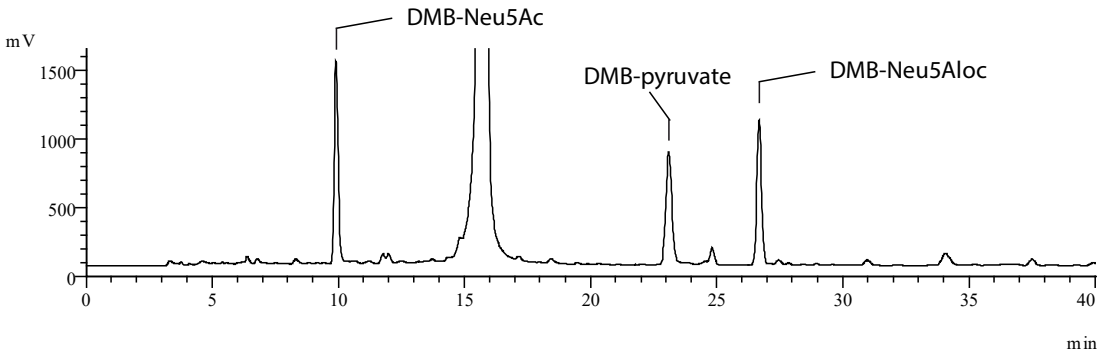
Selected RP-HPLC Chromatograms of DMB-Labeled Sialic Acids after Cell Fractionation



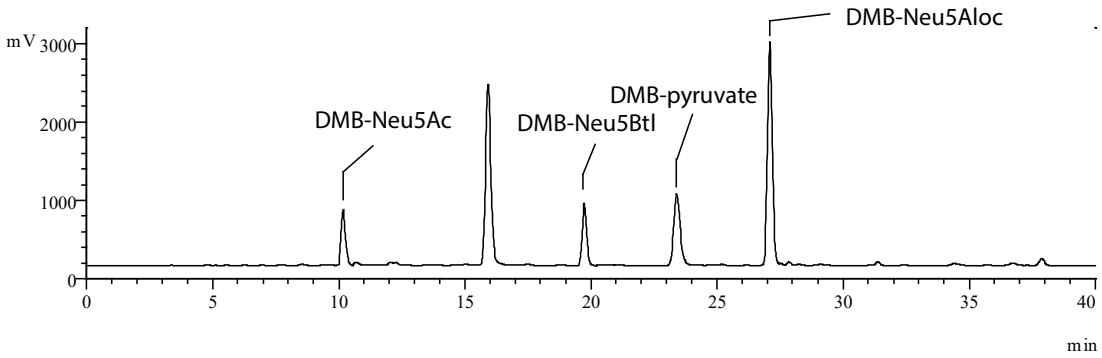
HEK 293T cells were grown without addition of unnatural sugar for 48 h. The cells were fractionated and the sialic acids of the **membrane fraction** were cleaved off by treatment with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 µL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).



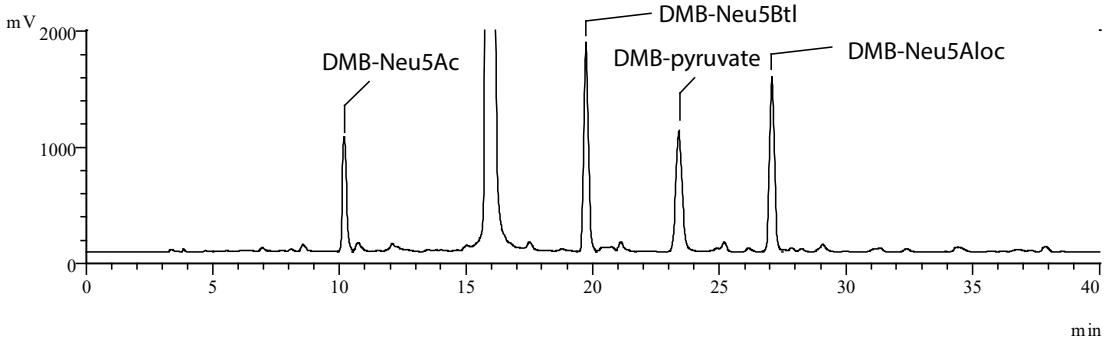
HEK 293T cells were grown without addition of unnatural sugar for 48 h. The cells were fractionated and the sialic acids of the **cell interior fraction** were treated with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 µL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).



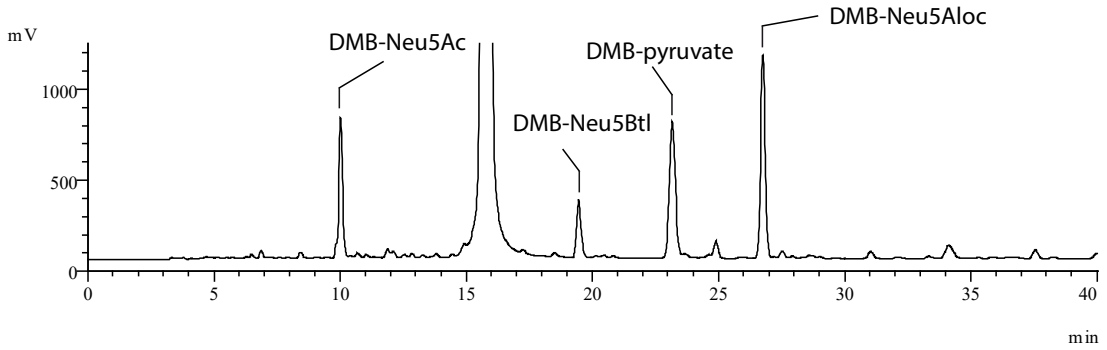
HEK 293T cells were grown without addition of unnatural sugar for 48 h. The cells were fractionated and the **cell interior fraction** was treated with 0.2 M sodium borohydride overnight at 4 °C to reduce the free sialic acids. After the **reduction step**, the excess $NaBH_4$ was quenched with concentrated TFA and the mixture concentrated. Anomerically modified sialic acids were cleaved with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 µL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).



HEK 293T cells were grown with 100 µM **Ac_4ManNBtl** in the cell medium for 48 h. The cells were fractionated and the sialic acids of the **membrane fraction** were cleaved off by treatment with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 µL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).

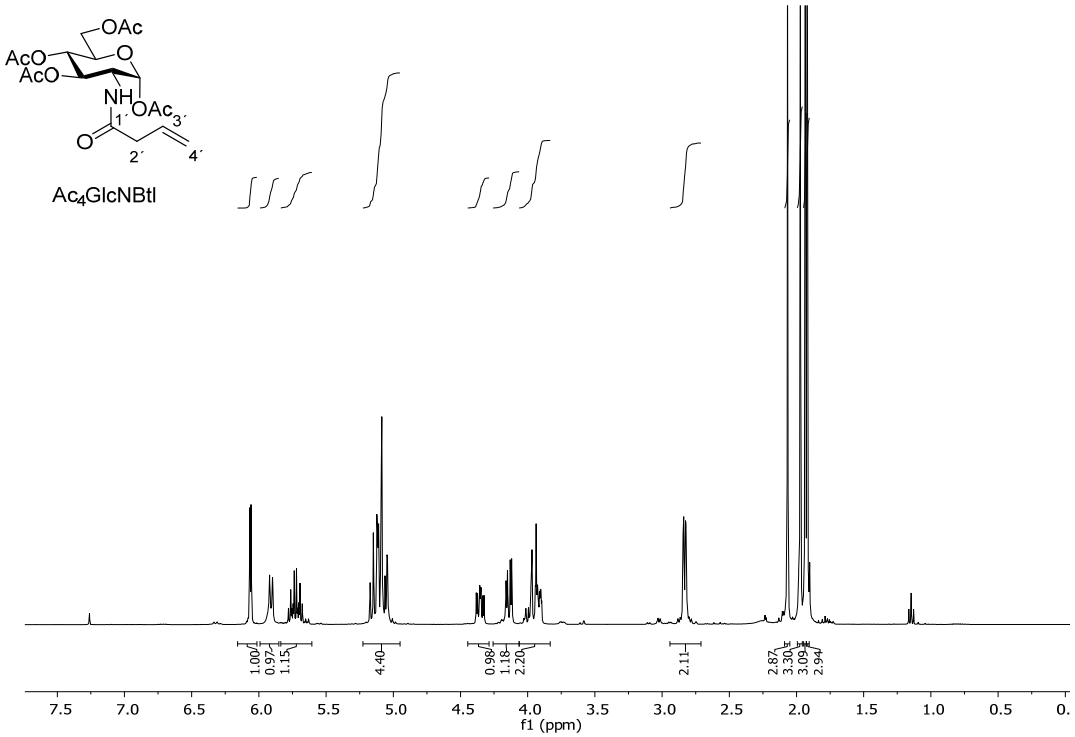


HEK 293T cells were grown with 100 µM **Ac_4ManNBtl** in the cell medium for 48 h. The cells were fractionated and the sialic acids of the **cell interior fraction** were treated with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 µL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).

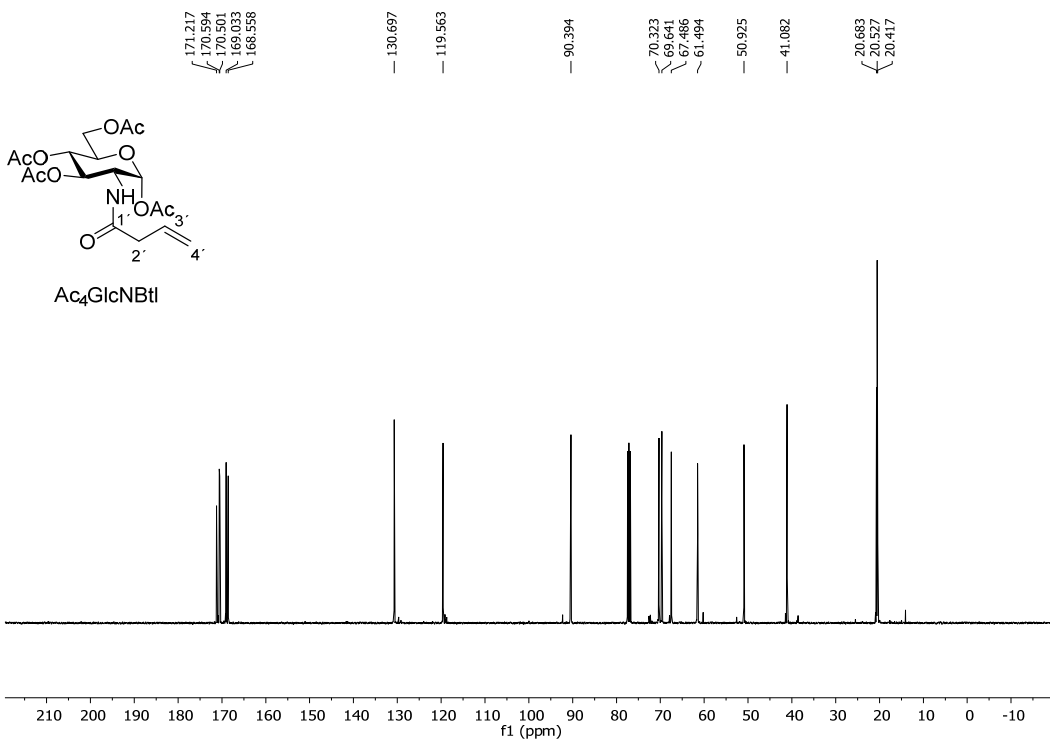


HEK 293T cells were grown with 100 µM **Ac_4ManNBtl** in the cell medium for 48 h. The cells were fractionated and the **cell interior fraction** was treated with 0.2 M sodium borohydride overnight at 4 °C to reduce the free sialic acids. After the **reduction step**, the excess $NaBH_4$ was quenched with concentrated TFA and the mixture concentrated. Anomerically modified sialic acids were cleaved with acidic acid (3 M, 80 °C, 90 min), Neu5Aloc was added as standard (35 µL of 0.0028 mg·mL^{-1} solution) and DMB labeling was carried out. Analytical RP-HPLC (10-20% B over 40 min, Kinetex C18).

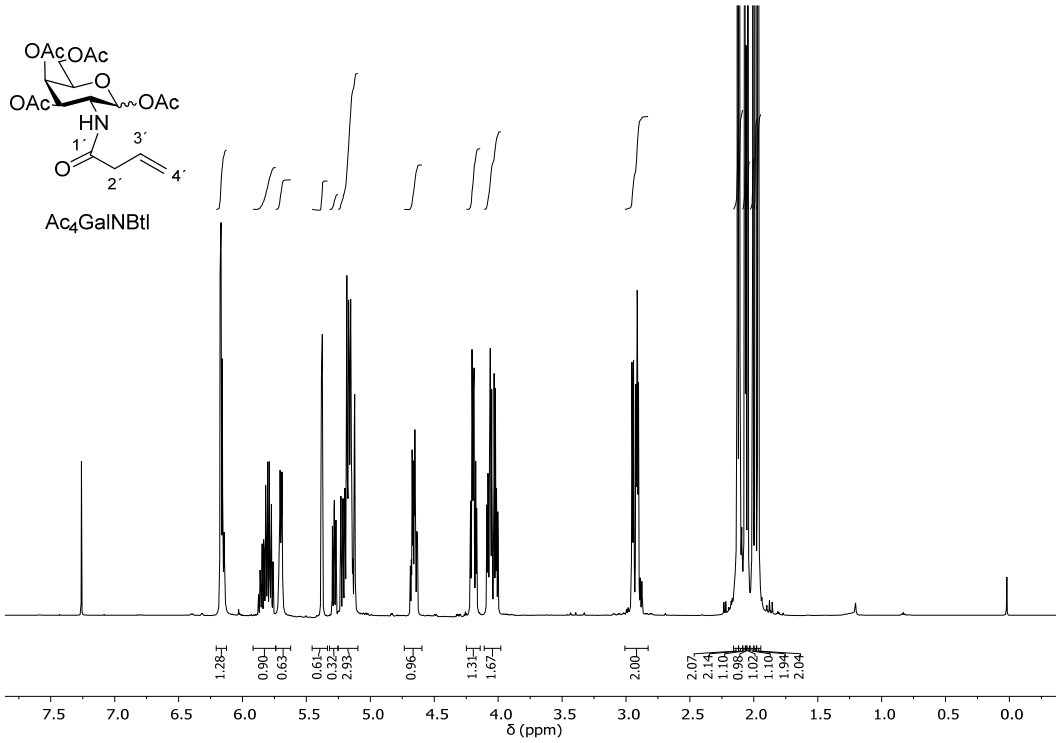
NMR Spectra



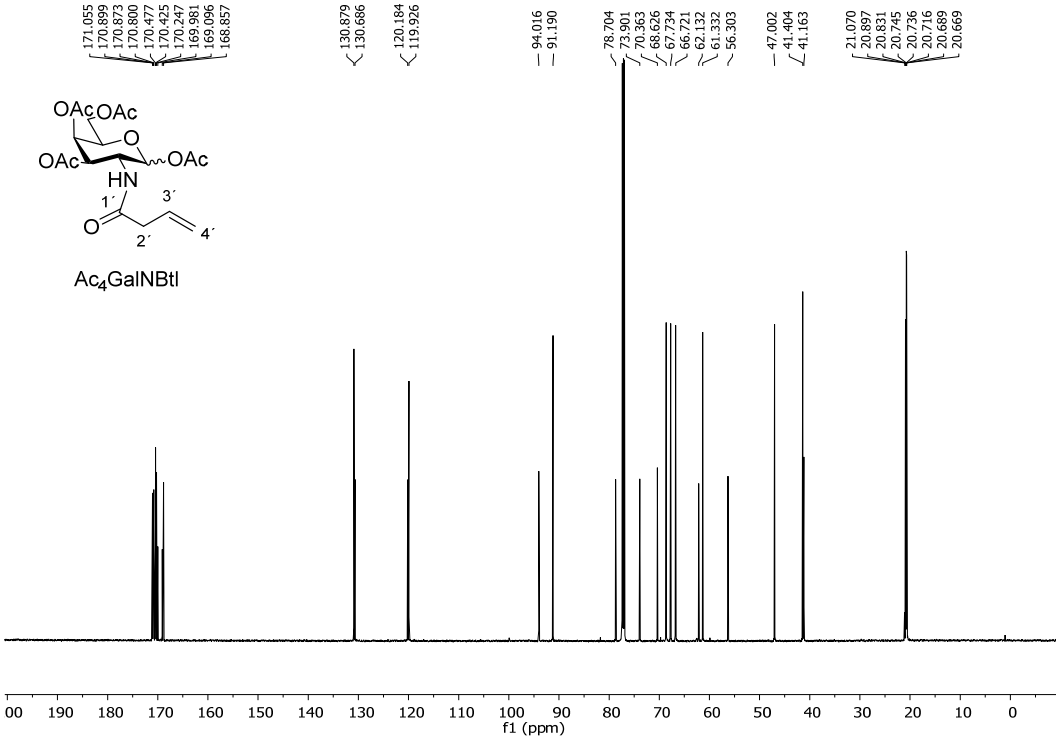
^{1}H NMR-(400 MHz, $CDCl_3$) of the α-anomer of Ac_4GlcNBtl.



^{13}C NMR (101 MHz, $CDCl_3$) of the α-anomer of Ac_4GlcNBtl.



^{1}H NMR (600 MHz, $CDCl_3$) of $Ac_4GalNBtl$.



^{13}C NMR (151 MHz, $CDCl_3$) of $Ac_4GalNBtl$.

References

[1] J. E. G. A. Dold, J. Pfotzer, A.-K. Späte, V. Wittmann, *ChemBioChem* **2017**, *18*, 1242-1250.

[2] J. Hassenrück, V. Wittmann, *Beilstein J. Org. Chem.* **2019**, *15*, 584-601.