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Supporting Information

Triple Orthogonal Labeling of Glycans by Applying Photoclick Chemistry

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Author Contributions

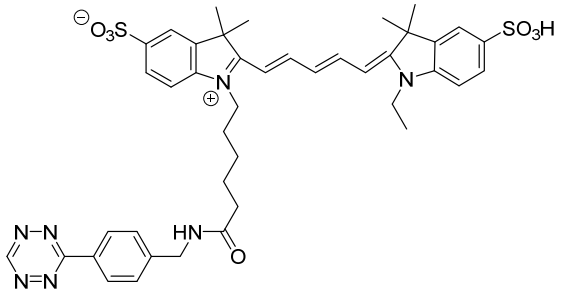
V.S. Conceptualization: Lead; Investigation: Lead; Writing – original draft: Lead; Writing – review & editing: Equal
J.H. Investigation: Equal; Validation: Lead; Writing – original draft: Equal; Writing – review & editing: Equal
A.S. Conceptualization: Lead; Writing – original draft: Equal; Writing – review & editing: Equal
J.D. Investigation: Supporting; Writing – review & editing: Supporting
R.F. Investigation: Supporting; Writing – review & editing: Equal
V.W. Conceptualization: Lead; Funding acquisition: Lead; Supervision: Lead; Writing – review & editing: Lead.

Supporting Information

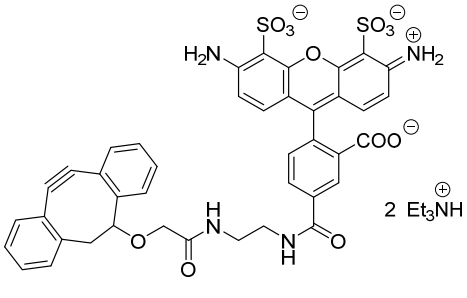
Contents

General Methods

Chemicals were purchased from *Aldrich*, *Acros Organics*, *Fluka*, *TCI* and *Dextra* and used without further purification. 3-(*p*-Benzylamino)-1,2,4,5-tetrazine-Cy5 (tetrazine-Cy5) was purchased from *Jena Bioscience*. Streptavidin-AlexaFluor® 555, AlexaFluor® 488 DIBO alkyne (DIBO-AF488) and Hoechst 33342 were purchased from *Invitrogen*. Technical solvents were distilled prior to use. Thin layer chromatography was performed on silica gel 60 F254 coated aluminum sheets (*Merck*) with detection by UV light (λ = 254 nm or 366 nm). Additionally, the sheets were stained by dipping in acidic ethanolic *p*-anisaldehyde solution, basic $KMnO_4$ solution or ninhydrin solution followed by gentle heating. Preparative flash column chromatography (FC) was performed with an MPLC-*Reveleris* system from *Grace*. Nuclear magnetic resonance (NMR) spectra were recorded at rt on Avance III 400 instruments from *Bruker*. Chemical shifts are reported relative to solvent signals ($CDCl_3$: δ_H = 7.26 ppm, δ_C = 77.16 ppm; DMSO-d_6: δ_H = 2.50 ppm, δ_C = 39.52 ppm). Signals were assigned by two-dimensional $^1H,^1H$ and $^1H,^{13}C$ correlation spectroscopy (COSY, HSQC). High-resolution mass spectrometry (HRMS) was carried out on a micrOTOF II instrument from *Bruker Daltonics*. Semi-preparative high performance liquid chromatography (RP-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* under the following conditions: Column: Eurosphere 100 C18 from *Knauer* (16 x 250 nm), flow: 9 mL min^{-1}; mobile phase: gradient of acetonitrile with 0.1% formic acid (solvent B) in water with 0.1% formic acid (solvent A). Analytic high performance liquid chromatography with mass spectra (HPLC-MS) was performed on a *Shimadzu* system LCMS2020 (pumps LC-20AD, autosampler SIL-20AT HAS, column oven CTO-20AC, UV/Vis detector SPD-20A, RF-20A prominescence fluorescence detector (ex 372 nm, em 456 nm), controller CBM-20A, ESI detector, and software LCMC Solution) under the following conditions: Column: EC125/4 Nucleodur C18 from *Macherey-Nagel*, flow 0.4 mL min^{-1}; mobile phase: gradient of acetonitrile with 0.1% formic acid (solvent B) in water with 0.1% formic acid (solvent A). Fluorescence and absorbance was measured using a *Tecan* infinite M200 reader. For UV-irradiation a hand-held UV-lamp (UVM-18EI Series UV Lamp, 8 Watt, 302 nm) from *UVP* was used. Fluorescence microscopy was performed using a point laser scanning confocal microscope (*Zeiss* LSM 880 Meta) equipped with a highly sensitive GaAsP-detector for spectral imaging. Analysis of the obtained data was performed using ImageJ software version 1.47v.

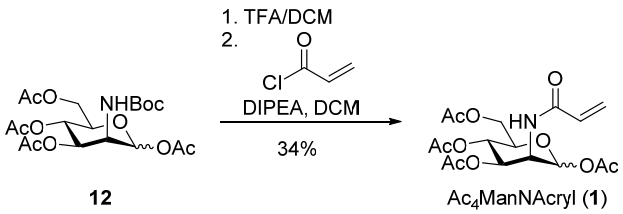


tetrazine-Cy5



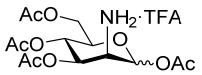
DIBO-AF488

Chemical Synthesis



Scheme S1: Synthesis of Ac_4ManNAcryl (**1**).

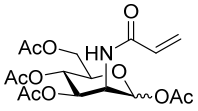
1,3,4,6-Tetra-*O*-acetyl-α/β-D-mannosamine (Ac_4ManNH_2·TFA)



$C_{14}H_{21}NO_9$
347.32 g/mol

Ac_4ManNBoc **12**[1] (1.16 g, 2.59 mmol, 1 equiv) was dissolved in DCM (2.2 mL) and TFA (2.2 mL) was added. The mixture was stirred for 75 min at rt before the solvent was removed. The crude mixture was coevaporated with toluene, followed by EtOAc and PE and used without further purification.

1,3,4,6-Tetra-*O*-acetyl-*N*-acryl-α/β-D-mannosamine (Ac_4ManNAcryl, 1)

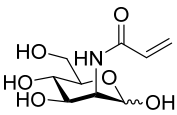


$C_{17}H_{23}NO_{10}$
401.37 g/mol

Ac_4ManNH_2·TFA was dissolved in dry DCM (25 mL) and DIPEA (0.7 mL, 5.70 mmol, 2.2 equiv) was added. The mixture was stirred at rt for 5 min and cooled to 0°C. Acryloyl chloride (0.4 mL, 4.41 mmol, 1.7 equiv) was added dropwise and the mixture was stirred. After 85 min sat. $NaHCO_3$ (20 mL) was added. The aqueous layer was extracted with DCM (3x 30 mL) and the combined organic layers were dried ($MgSO_4$). The crude product was purified using the Grace Reveleris System (PE:EtOAc = 10 - 40%) yielding 34% over two steps as α/β mixture (4:1). R_f (PE:EtOAc, 1:1) = 0.2; **α-anomer: ^{1}H NMR** (400 MHz, $CDCl_3$): δ = 6.34 (dd, J = 17.0, 1.4 Hz, 1H, CH_2a), 6.17 (dd, J = 16.9, 10.2 Hz, 1H, CH), 6.07 (d, J = 1.8 Hz, 1H, H-1), 5.75 (dd, J = 10.2, 1.4 Hz, 2H, NH, CH_2b), 5.37 (dd, J = 10.2, 4.5 Hz, 1H, H-3), 5.19 (t, J = 10.0 Hz, 1H, H-4), 4.73 (ddd, J = 9.3, 4.5, 1.9 Hz, 1H, H-2), 4.32 – 4.23 (m, 1H, H-6a), 4.10 – 4.00 (m, 2H, H-5, H-6b), 2.19, 2.10, 2.07, 2.00 (4x s, 3H, CH_3) ppm; **^{13}C NMR** (101 MHz, $CDCl_3$): δ = 170.7, 170.2, 169.9, 168.2, 165.6 (5x CO), 130.1 (CH), 128.1 (CH_2), 91.8 (C-1), 70.3 (C-5), 69.0 (C-3), 65.7 (C-4), 62.2 (C-6), 49.5 (C-2), 21.0, 20.9, 20.83, 20.76 (4x CH_3) ppm; **β-anomer: ^{1}H NMR** (400 MHz, $CDCl_3$): δ = 6.33 (dd, J = 17.0, 1.4 Hz, 1H, CH_2a), 6.25 – 6.12 (m, 1H, CH), 5.95 (t, J = 9.1 Hz, 1H, NH), 5.88 (d, J = 1.8 Hz, 1H, H-1), 5.73 (dd, J = 10.2, 1.5 Hz, 1H, CH_2b), 5.14 – 5.03 (m, 2H, H-3, H-4), 4.86 (ddd, J = 9.1, 3.8, 1.8 Hz, 1H, H-2), 4.32 – 4.23 (m, 1H, H-6a), 4.15 – 4.08 (m, 1H,

H-6b), 3.81 (ddd, *J* = 9.2, 5.2, 2.5 Hz, 1H, H-5), 2.09, 2.08, 2.03, 1.99 (4x s, CH_3) ppm; **^{13}C NMR** (101 MHz, $CDCl_3$): *δ* = 170.6, 170.3, 169.8, 168.5, 166.1 (5x CO), 130.4 (CH), 127.7 (CH_2), 90.8 (C-1), 73.6, 71.5, 65.5, 62.1, 49.7 (C-2, C-3, C-4, C-5, C-6), 21.2, 20.9, 20.80, 20.78 (4x CH_3) ppm; **HRMS** (ESI-IT): *m/z* calcd for $C_{17}H_{23}NO_{10}$ +H^+: 402.1395 [*M* + H]$^+$; found: 402.1403.

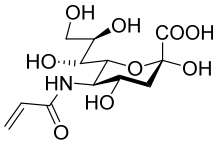
***N*-Acryl-α/β-D-mannosamine (ManNAcryl)**



$C_9H_{15}NO_6$
233.22 g/mol

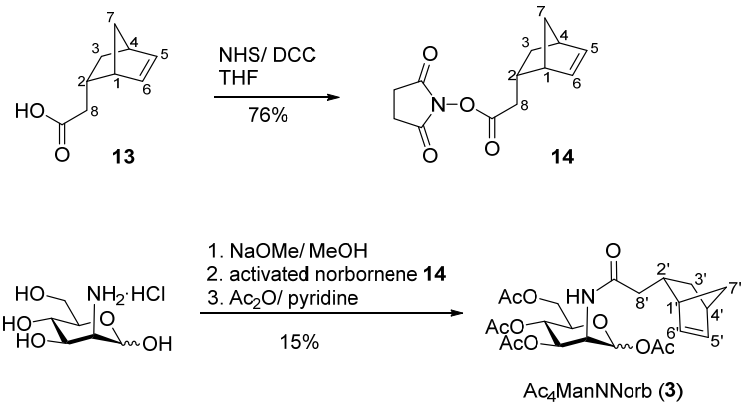
Ac_4ManNAcryl (**1**, 174 mg, 0.43 mmol) was dissolved under nitrogen atmosphere in dry MeOH (12 mL). NaOMe (0.5 M in MeOH, 0.14 mL, 0.07 mmol, 0.17 equiv) was added and the mixture was stirred at rt overnight. Amberlite IR120 was added for neutralization, filtered off after 5 min and the solvent was removed. After lyophilization, the product was obtained as colorless solid in 82% yield (82 mg, 0.35 mmol).

***N*-Acryl-neuraminic acid (Neu5Acryl)**



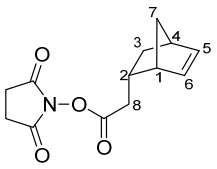
$C_{12}H_{19}NO_9$
321.28 g mol^{-1}

ManNAcryl (23 mg, 0.099 mmol) and sodium pyruvate (163.5 mg, 1.48 mmol) were dissolved in potassium phosphate buffer (1.01 mL, 100 mM, pH 7.1). Sialic acid aldolase (3 U) was added, and the mixture was stirred at rt for 11 days. The mixture was evaporated and the product was dissolved in EtOH (5 mL). The solution was filtered with cotton and evaporated. The product was purified by RP-HPLC (0.1 - 1% B in 20 min, R_t = 4.6 min). The product was concentrated and obtained as a colorless solid (3 mg, 9 %). **HRMS** (ESI-IT): *m/z* calcd for $C_{12}H_{19}NO_9$ +H^+: 320.0976 [*M* + H]$^+$; found: 320.0986.



Scheme S2: Synthesis of Ac$_4$ManNNorb (**3**).

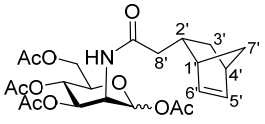
2,5-Dioxopyrrolidin-1-yl 2-((1*S*,2*S*,4*S*)-bicyclo[2.2.1]hept-5-en-2-yl)acetate (NHS-activated *endo*-norbornene 14)



$C_{13}H_{15}NO_4$
249.27 g/mol

(1*S*,2*S*,4*S*)-Bicyclo[2.2.1]hept-5-en-2-ylacetic acid (**13**, 400 mg, 2.63 mmol, 1 equiv) and NHS (423.5 mg, 3.7 mmol, 1.4 equiv) were dissolved in anhydrous THF (14 mL). DCC (652 mg, 3.2 mmol,1.2 equiv) dissolved in THF (8 mL) was added and the mixture was stirred at rt overnight. The mixture was filtered to remove the urea and the filtrate was evaporated. The crude product was purified by column chromatography (PE:EtOAc = 2:1) to yield the activated ester **14** as a white solid (500 mg, 2 mmol, 76%). R_f (PE:EtOAc, 1:1) = 0.5; **^{1}H NMR** (400 MHz, $CDCl_3$): δ = 6.22 (dd, J = 5.8, 3.1 Hz, 1H, H-5 or H-6), 6.00 (dd, J = 5.8, 2.9 Hz, 1H, H-5 or H-6), 2.98 – 2.93 (m, 1H, H-1), 2.88 – 2.78 (m, 5H, 4x NHS and H-4), 2.59 – 2.49 (m, 1H, H-2), 2.46 – 2.32 (m, 2H, H-8), 2.03 – 1.98 (m, 1H, H-7a), 1.48 (dq, J = 8.4, 2.1 Hz, 1H, H-3a), 1.33 – 1.28 (m, 1H, H-3b), 0.66 (ddd, J = 11.8, 4.2, 2.6 Hz, 1H, H-7b) ppm; **^{13}C NMR** (101 MHz, $CDCl_3$): δ = 169.3, 168.3 (C=O), 138.4, 132.1 (C-5, C-6), 49.8 (C-3), 45.7 (C-1), 42.8 (C-4), 36.1 (C-8), 35.1 (C-2), 32.2 (C-7), 25.8 (NHS) ppm.

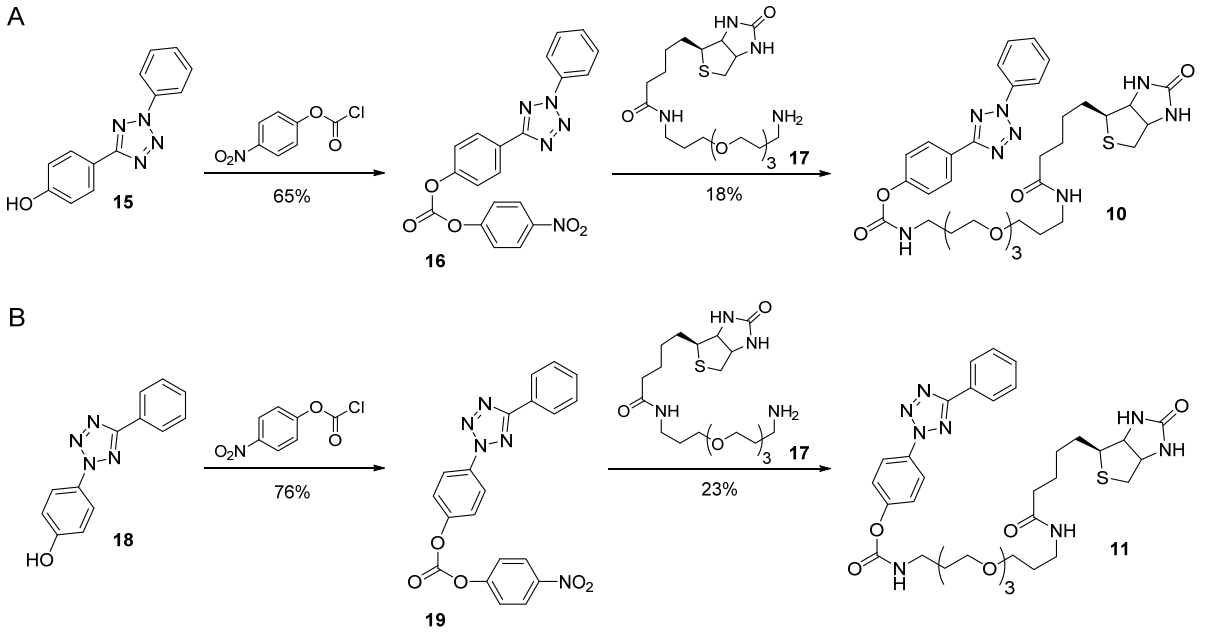
1,3,4,6-Tetra-*O*-acetyl-*N*-(3-(2-(bicyclo[2.2.1]hept-5-en-2-yl)-α/β-D-mannosamine (Ac$_4$ManNNorb, 3)



$C_{23}H_{31}NO_{10}$
481.50 g/mol

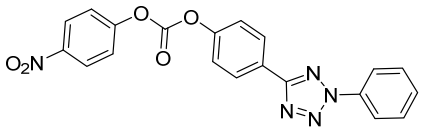
Mannosamine hydrochloride (360 mg, 1.7 mmol, 1 equiv) was dissolved in dry methanol (10 mL) and neutralized by addition of NaOMe (0.5 M, 3.5 mL) and stirred at rt for 75 min. NHS-activated *endo*-norbornene (**14**, 498 mg, 2 mmol, 1.2 equiv) was suspended in MeOH (10 mL) and added. The

mixture was allowed to stir overnight before the solvent was evaporated. The crude product was dissolved in pyridine (1.6 mL), Ac_2O (1.6 mL, 17 mmol, 10 equiv) was added and the mixture was stirred overnight at rt. The mixture was concentrated, diluted with DCM (50 mL) and washed with 10% $KHSO_4$ solution (50 mL), saturated $NaHCO_3$ (50 mL) and brine (50 mL). The organic layer was dried ($MgSO_4$) and the solvent was evaporated. The crude product was purified by flash column chromatography (PE:EtOAc = 1:1) to yield Ac_4ManNNorb (**3**) as a white foam (0.24 mmol, 120 mg, 15%). R_f (PE:EtOAc, 1:1) = 0.2; **α-anomer: ^{1}H NMR** (400 MHz, $CDCl_3$): δ = 6.19 (dt, J = 5.6, 2.8 Hz, 1H, H-5' or H-6'), 6.06 – 5.93 (m, 2H, H-1, H-5' or H-6'), 5.68 – 5.59 (m, 1H, NH), 5.36 – 5.27 (m, 1H, H-3), 5.25 – 5.07 (m, 1H, H-4), 5.02 (dt, J = 9.9, 4.1 Hz, 1H, H-3), 4.64 (dtd, J = 9.1, 4.5, 1.9 Hz, 1H, H-2), 4.33 – 4.21 (m, 1H, H-6a), 4.16 – 3.98 (m, 1H, H-6b), 3.79 (ddq, J = 9.8, 5.0, 2.5 Hz, 1H, H-5), 2.93 – 2.74 (m, 2H, H-1', H-4'), 2.55 – 2.40 (m, 1H, H-2'), 2.23 – 1.79 (m, 15H, H-7'a, H-8', 4x CH_3), 1.51 – 1.38 (m, 1H, H-3'a), 1.31 – 1.21 (m, 1H, H-3'b), 0.58 (tdd, J = 11.7, 4.3, 2.6 Hz, 1H, H-7'b) ppm; **β-anomer: ^{1}H NMR** (400 MHz, $CDCl_3$): δ = 6.19 (dt, J = 5.6, 2.8 Hz, 1H, H-5' or H-6'), 6.06 – 5.93 (m, 1H, H-5' or H-6'), 5.85 (t, J = 1.5 Hz, 1H, H-1), 5.76 – 5.68 (m, 1H, NH), 5.25 – 5.07 (m, 1H, H-4), 5.02 (dt, J = 9.9, 4.1 Hz, 1H, H-3), 4.76 (ddt, J = 9.0, 3.6, 1.6 Hz, 1H, H-2), 4.33 – 4.21 (m, 1H, H-6a), 4.16 – 3.98 (m, 2H, H-5, H-6b), 2.93 – 2.74 (m, 2H, H-1', H-4'), 2.55 – 2.40 (m, 1H, H-2'), 2.23 – 1.79 (m, 15H, H-7'a, H-8', 4x CH_3), 1.51 – 1.38 (m, 1H, H-3'a), 1.31 – 1.21 (m, 1H, H-3'b), 0.58 (tdd, J = 11.7, 4.3, 2.6 Hz, 1H, H-7'b) ppm; **both anomers: ^{13}C NMR** (101 MHz, $CDCl_3$): δ = 173.4, 172.9, 172.8, 170.6, 170.2, 170.14, 170.09, 169.8, 169.7, 168.3 (10 x CO), 138.2, 138.1, 132.2, 132.1 (C-5', C-6'), 91.8, 90.8 (C-1), 73.5, 71.6, 70.2, 69.2, 65.4, 65.2 (C-3, C-4, C-5), 62.1, 62.0 (C-6), 49.9 (C-3'), 49.5, 49.1 (C-2), 45.8, 42.9, 41.9, 35.8, 32.0 (C-1', C-2', C-4', C-7', C-8'), 21.0, 20.94, 20.90, 20.88, 20.85, 20.82, 20.80, 20.7 (8x CH_3) ppm; **HRMS** (ESI-IT): *m/z* calcd for $C_{23}H_{31}NO_{10}$ +H^+: 482.2021 [M + H]$^+$; found: 482.2008.



Scheme S3: Synthesis of tetrazole-biotin **10** (A) and **11** (B).

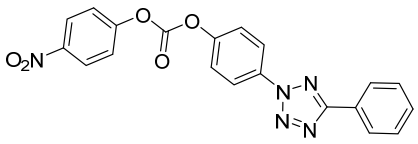
4-Nitrophenyl (4-(2-phenyl-2*H*-tetrazol-5-yl)phenyl) carbonate (16)



$C_{20}H_{13}N_5O_5$
403.35 g/mol

Tetrazole-OH **15**[2] (456 mg, 1.91 mmol, 1 equiv) and *para*-nitrophenyl choroformate (1.01 g, 4.97 mmol, 2.6 equiv) were dissolved in dry pyridine (15 mL) and cooled to 0°C under nitrogen conditions. The mixture was stirred for 22 h while warming up to rt. The solvent was removed and the crude mixture was purified by column chromatography (PE:EtOAc, 1:1 to EtOAc) yielding the product **16** (620 mg, 1.54 mmol, 81%) as light yellow solid. R_f (PE:EtOAc, 1:1) = 0.6; **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 8.42 – 8.35 (m, 2H), 8.32 – 8.27 (m, 2H), 8.21 – 8.15 (m, 2H), 7.78 – 7.60 (m, 7H) ppm; **^{13}C NMR** (101 MHz, DMSO-d_6): δ = 163.9 (CO), 139.6 (NCN), 130.2, 128.2, 126.1, 125.5, 122.72, 122.67, 122.4, 120.0, 115.8 ppm.

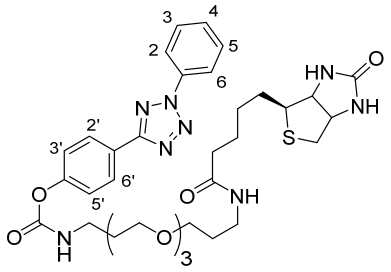
4-Nitrophenyl (4-(5-phenyl-2*H*-tetrazol-2-yl)phenyl) carbonate (19)



$C_{20}H_{13}N_5O_5$
403.35

Tetrazole-OH **18**[2] (178 mg, 0.75 mmol, 1 equiv) and *para*-nitrophenyl chloroformate (392 mg, 1.94 mmol, 2.6 equiv) were dissolved in dry pyridine (6 mL) and cooled to 0°C under nitrogen conditions. The mixture was stirred for 19 h while warming up to rt. The solvent was removed and the crude mixture was purified by column chromatography (PE:EtOAc, 2:1 to 1:2) yielding the activated tetrazole **19** (230 mg, 0.57 mmol, 76%) as light yellow solid. R_f (PE:EtOAc, 1:1) = 0.7; **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 8.38 (dd, J = 9.4, 2.8 Hz, 2H), 8.33 – 8.26 (m, 2H), 8.19 (dd, J = 7.4, 2.2 Hz, 2H), 7.81 – 7.71 (m, 4H), 7.66 – 7.58 (m, 3H) ppm; **^{13}C NMR** (101 MHz, DMSO-d_6): δ = 154.9 (CO), 145.5 (NCN), 129.4, 126.7, 126.1, 125.5, 123.1, 122.70, 122.66, 121.6, 115.8 ppm.

13-Biotinylamido-4,7,10-trioxatridecan-1-(4-(2-phenyl-tetrazol-5-yl)phenoxy)carbamate (tetrazole-biotin, 10)

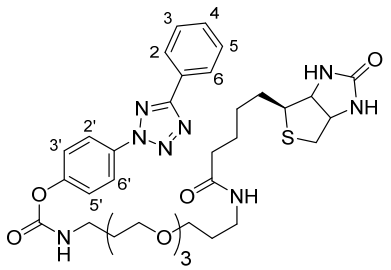


$C_{34}H_{46}N_8O_7S$
710.84 g/mol

Activated tetrazole **16** (100 mg, 0.25 mmol, 1 equiv) and biotin-PEG-NH_2[3] (**17**, 133 mg, 0.3 mmol, 1.2 equiv) were dissolved under nitrogen conditions in dry DMF (5 mL) and pyridine (0.5 mL). The mixture was stirred at rt for 64 h and evaporated. The oily yellow residue was dissolved in DCM (30 mL) and washed with water (2x 15 mL). The combined aqueous layers were extracted with DCM (2x 15 mL) and the combined organic layers were dried ($MgSO_4$) and evaporated. The resulting yellow oil was purified by column chromatography (DCM:MeOH, 8:1) to give tetrazole-biotin **10** as colorless oil (32.2 mg, 0.045 mmol, 18%). R_f (DCM:MeOH, 10:1) = 0.1; **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 8.21 – 8.13 (m, 4H, H-2, H-6, H-3‘ and H-5‘ or H-2‘ and H-6‘), 7.87 (t, J = 5.7 Hz, 1H, NHCO), 7.76 – 7.66 (m, 3H, NHCO, H-3, H-5), 7.66 – 7.59 (m, 1H, H-4), 7.39 – 7.32 (m, 2H, H-2‘ and H-6‘ or H-3‘ and H-5‘), 6.40 (s, 1H, NH), 6.34 (s, 1H, NH), 4.32 – 4.26 (m, 1H, NHC<u>H</u>CH_2), 4.15 – 4.09 (m, 1H, NHC<u>H</u>CH), 3.56 – 3.43 (m, 10H, 5x CH_2O), 3.40 (q, J = 6.3 Hz, 2H, CH_2O), 3.18 – 3.12 (m, 2H, CONHC<u>H</u>$_2$), 3.11 – 2.98 (m, 3H, CHS, CONHC<u>H</u>$_2$), 2.81 (dd, J = 12.4, 5.1 Hz, 1H, CH_{exo}), 2.57 (d, J = 12.4 Hz, 1H, CH_{endo}), 2.04 (t, J = 7.4 Hz, 2H, CH_2), 1.73 (p, J = 6.6 Hz, 2H, CH_2), 1.66 – 1.54 (m, 4H, 2x CH_2), 1.54 – 1.38 (m, 2H, CH_2), 1.30 (dq, J = 15.3, 8.3, 7.5 Hz, 2H, CH_2) ppm; **^{13}C NMR** (101 MHz, DMSO-d_6): δ = 171.9, 164.1, 162.7, 153.9, 153.1, 136.2 (C_{quart}), 130.3, 130.2, 127.8 (5x C_{Ar}), 123.0 (C_{quart}), 122.6,

120.0 (4x C_{Ar}), 69.8, 69.6, 69.55, 68.1, 67.9 (5x CH_2), 61.1 (NH<u>C</u>HCH), 59.2 (NH<u>C</u>HCH_2), 55.4 (CHS), 40.3 (SCH_2), 37.9, 35.7 (2x CH_2NH), 35.2, 29.4, 28.2, 28.0, 25.3 (5x CH_2) ppm; **HRMS** (ESI-IT): *m/z* calcd for $C_{34}H_{46}N_8O_7S+H^+$: 711.3283 [*M* + H]$^+$; found: 711.3266.

13-Biotinylamido-4,7,10-trioxatridecan-1-(4-(5-phenyl-tetrazol-2-yl)phenoxy)carbamate (tetrazole-biotin 11)



$C_{34}H_{46}N_8O_7S$
710.85 g/mol

Activated tetrazole **19** (766 mg, 1.9 mmol, 1 equiv) and biotin-PEG-NH_2[3] (**17**, 1.1 g, 2.46 mmol, 1.2 equiv) were dissolved under nitrogen conditions in dry DMF (45 mL) and pyridine (4 mL). The mixture was stirred at rt for 41 h and evaporated. The oily yellow residue was dissolved in DCM (50 mL) and washed with water (5x 50 mL). The combined aqueous layers were extracted with DCM (5x 50 mL) and the combined organic layers were dried ($MgSO_4$) and evaporated. The resulting yellow oil was purified by column chromatography (DCM:MeOH, 10:1) to give tetrazole-biotin **11** as colorless solid (307 mg, 0.44 mmol, 23%). R_f (DCM:MeOH, 10:1) = 0.6; **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 8.24 – 8.09 (m, 4H, H-2, H-6 and H-2‘, H-6‘ or H-3‘, H-5‘), 7.91 (t, *J* = 5.7 Hz, 1H, NHCO), 7.73 (t, *J* = 5.6 Hz, 1H, NHCO), 7.68 – 7.51 (m, 3H, H-3, H-5, H-4), 7.49 – 7.40 (m, 2H, H-2‘, H-6‘ or H-3‘, H-5‘), 6.40 (s, 1H, NH), 6.34 (s, 1H, NH), 4.35 – 4.24 (m, 1H, NHC<u>H</u>), 4.11 (ddt, *J* = 7.9, 5.2, 2.7 Hz, 1H, NHC<u>H</u>), 3.61 – 3.43 (m, 10H, 5x C<u>H</u>$_2$CO), 3.38 (td, *J* = 6.3, 2.0 Hz, 2H, C<u>H</u>$_2$CO), 3.20 – 3.12 (m, 2H, NHC<u>H</u>$_2$), 3.07 (dq, *J* = 7.3, 5.6 Hz, 3H, CHS, CNC<u>H</u>$_2$), 2.81 (dd, *J* = 12.4, 5.0 Hz, 1H, CH_2S_{exo}), 2.57 (d, *J* = 12.5 Hz, 1H, CH_2S_{endo}), 2.04 (t, *J* = 7.4 Hz, 2H, CH_2), 1.73 (p, *J* = 6.6 Hz, 2H, CH_2), 1.66 – 1.54 (m, 4H, 2x CH_2), 1.53 – 1.39 (m, 2H, CH_2), 1.29 (hept, *J* = 8.0, 7.3 Hz, 2H, CH_2) ppm; **^{13}C NMR** (101 MHz, DMSO-d_6): δ = 171.9, 164.5, 162.7, 153.8, 152.2, 131.0 (6x C_{quart}), 129.4, 126.7 (4x C_{Ar}), 126.5 (C_{quart}), 123.3, 121.2 (5x C_{Ar}), 69.8, 69.58, 69.55, 68.1, 67.9 (6x CH_2O), 61.1, 59.2 (2x NHCH), 55.4 (CHS), 40.5 (CH_2S), 37.9, 35.7 (2x CH_2NH), 35.2, 29.4, 28.2, 28.1, 25.3 (6x CH_2) ppm; **HRMS** (ESI-IT): *m/z* calcd for $C_{34}H_{46}N_8O_7S+H^+$: 711.3283 [*M* + H]$^+$; found: 711.3263.

Cell Culture

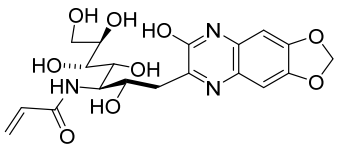
Human embryonic kidney 293T (HEK 293T) cells were grown in Dulbecco's modified Eagle medium (DMEM, *Gibco*) supplemented with 10% fetal calf serum (FCS), 100 units mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (*Gibco*) at 37°C and 5% CO_2. Mycoplasma contamination was negatively tested using the Venor®GeM Classic Kit (*Minerva Biolabs*).

Metabolic Glycoengineering

Wells were coated with 0.01% poly-L-lysine (*Sigma*) in phosphate-buffered saline (PBS) for 1 h at 37 °C (or overnight at 4°C) and rinsed with PBS. 42 000 HEK 293T cells/well were seeded in 4-well ibiTreat µ-Slides (*ibidi*) and allowed to attach overnight. Cells were then incubated with the mentioned concentration of the corresponding ManNAc derivative(s) (using stock solutions of 100 mM in DMSO) for 48 h. The same amount of pure DMSO was added as solvent control.

DMB Labeling of Sialic Acids

Preparation and characterization of DMB-Neu5Acryl as reference compound for DMB labeling experiments



$C_{19}H_{23}N_3O_9$
437.41 g mol^{-1}

Neu5Acryl (0.1 mg, 0.23 μmol) was dissolved in DMB solution (265 μL; 5.3 mM DMB*2 HCl, 16 mM $Na_2S_2O_4$, 40 mM TFA in MilliQ water). 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, 25 µL). The obtained solution was directly used for analytical RP-HPLC-MS measurements. For fluorescence detection, the mixture was diluted with MilliQ water (1:400). A gradient of 10 - 25% B in 40 min was used. The chromatogram is shown in Figure S1. $\boldsymbol{R_t}$ = 14 min; **HRMS** (ESI-IT): *m/z* calcd for $C_{19}H_{23}N_3O_9$+H^+: 438.1502 [*M* + H]$^+$, found: 438.1507.

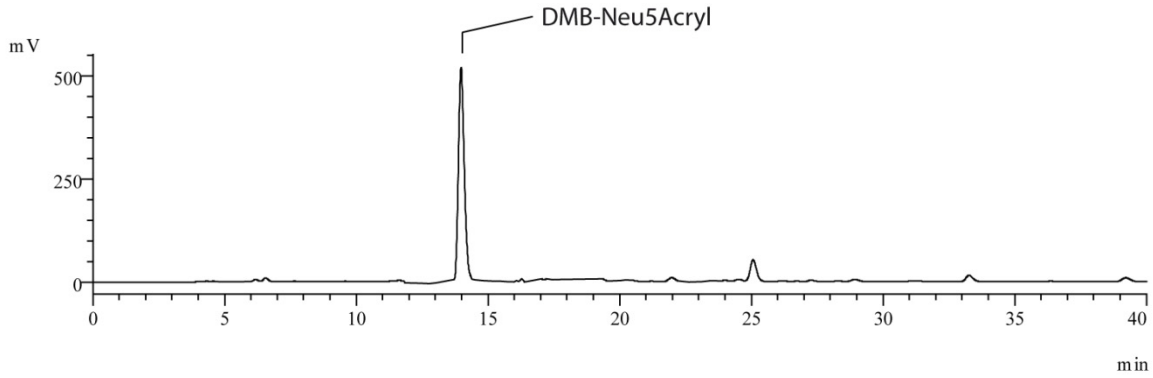


Figure S1: Analysis of DMB-Neu5Acryl by RP-HPLC (10 – 25% B in 40 min) with a fluorescence detector (excitation 372 nm, emission 456 nm).

Preparation and characterization of DMB-Neu5Ac as reference compound for DMB labeling experiments

Derivatisation of Neu5Ac was carried out as previously reported.[4] DMB-Neu5Ac: $\boldsymbol{R_t}$ = 9.6 min.

DMB labeling of cellular sialic acids after metabolic glycoengineering

Experiments were performed similar to the previously reported protocol.[4] HEK 293T cells were seeded (600.000 cells in a 6 cm dish) and allowed to attach for 24 h. The cells were then incubated with Ac_4ManNAcryl (**1**, 100 µM) in DMEM (4 mL) for 48 h. DMSO was added as solvent control. Cells were trypsinated and re-suspended in DMEM (5 mL) and pelleted by centrifugation (5 min, 400 g). The supernatant was discarded and the pellet was washed with PBS (3 times). Cells were transferred to

Eppendorf tubes (500 000 cells per tube) and pelleted by centrifugation (5 min, 400 g). The supernatant was discarded and the pellet was suspended in AcOH (3 M, 300 µL), and incubated at 80°C for 90 min in a thermomixer (300 rpm). Ammonia (25% in H_2O, 20 µL) was added and the solvent was removed using a SpeedVac. The resulting pellet was suspended in EtOH (200 µL) and concentrated again (2 times). The pellet was re-suspended in DMB solution [DMB * 2HCl (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 the dark in a thermomixer (300 rpm) followed by cooling on ice (10 min) and neutralization with NaOH (0.5 M, 25 µL). Analysis was performed by analytical RP-HPLC with a gradient of 10-25% B in 40 min (Figures S2, S3). The incorporation efficiency *IE* of the acryl amide modified sugar was calculated from the integrals *I* of the RP-HPLC signals of DMB-labeled Neu5Ac (I_{Neu5Ac}) and DMB-labeled Neu5Acryl ($I_{Neu5Acryl}$) according to the formula $IE = I_{Neu5Acryl} (I_{Neu5Acryl} + I_{Neu5Ac})^{-1} * 100\%$. It was found to be 43±8%. Three independent experiments were performed with three replicates each.

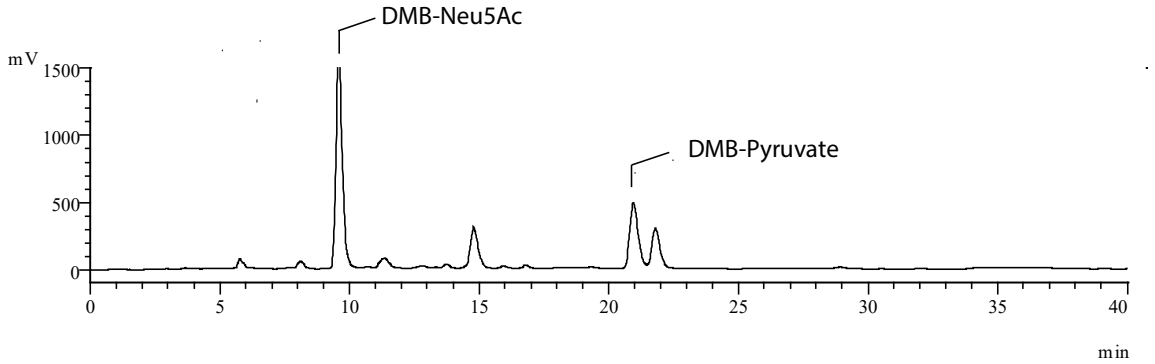


Figure S2: RP-HPLC analysis (10 - 25% B in 40 min) with a fluorescence detector (excitation 372 nm, emission 456 nm) of DMB-labeled sialic acids released from cells grown without added sugar (solvent control).

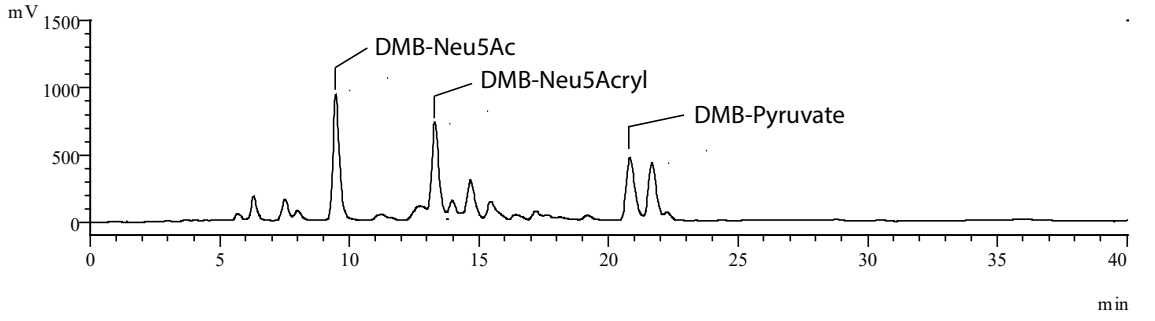


Figure S3: RP-HPLC analysis (10 - 25% B in 40 min) with a fluorescence detector (excitation 372 nm, emission 456 nm) of DMB-labeled sialic acids released from cells grown with Ac_4ManNAcryl (**1**).

UV and Fluorescence Spectra

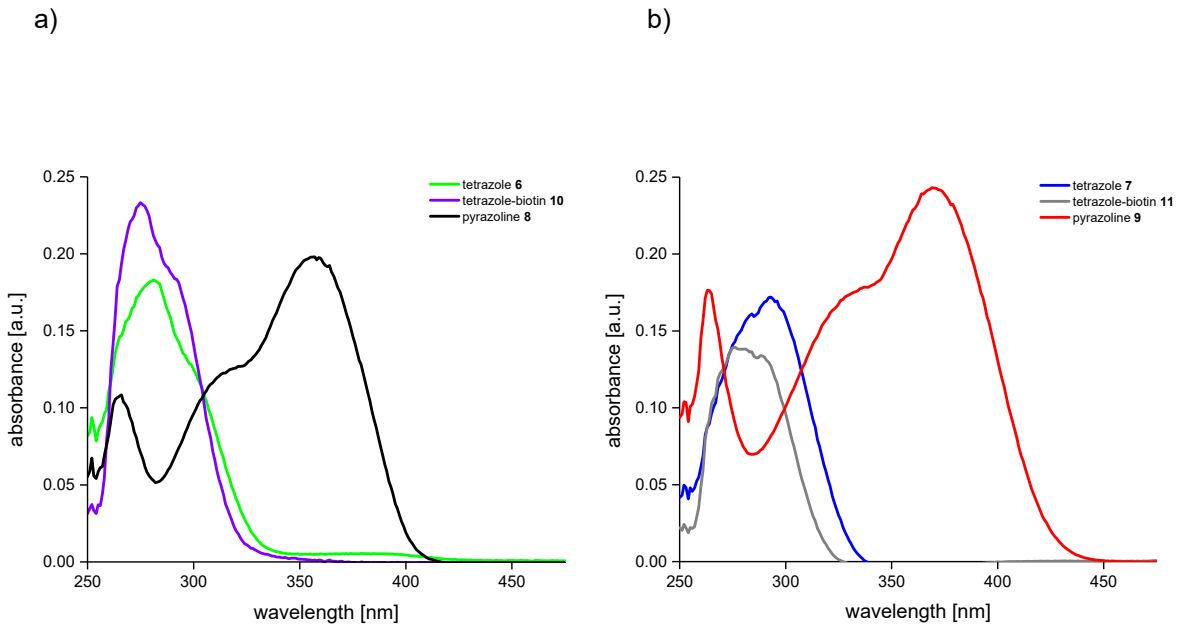


Figure S4: a) Absorbance spectra of tetrazole **6**, tetrazole-biotin **10** and pyrazoline **8**. All compounds were measured in solutions of 10 µM in DMSO with solvent correction. b) Absorption spectra of tetrazole **7**, tetrazole-biotin **11** and pyrazoline **9**. All compounds were measured in solutions of 10 µM in DMSO with solvent correction.

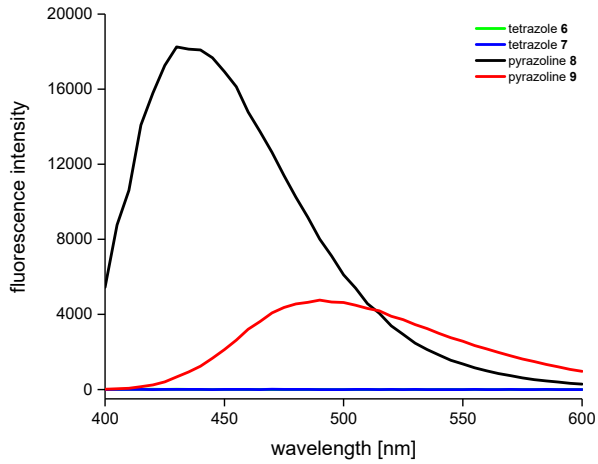


Figure S5: Fluorescence spectra of tetrazoles **6** and **7** and pyrazolines **8** and **9**. All compounds were measured in solutions of 100 µM in DMSO. Samples were excited at 360 nm.

Plate Reader Assay

Stock solutions of tetrazoles (2 mM) and alkenes (200 µM) in ethanol were mixed 1:1 in a 96-well plate (*Corning*, 96 flat bottom black polystyrol). The plate was irradiated (302 nm) with a hand held UV-lamp which was directly placed on top of the plate for the depicted time. Fluorescence *F* was measured using a *Tecan* infinite M200 reader (ex: 360 nm, em (tetrazoles **6** and **10**): 445 nm, em (tetrazoles **7** and **11**): 485 nm). Standard deviations were calculated from at least two replicates.

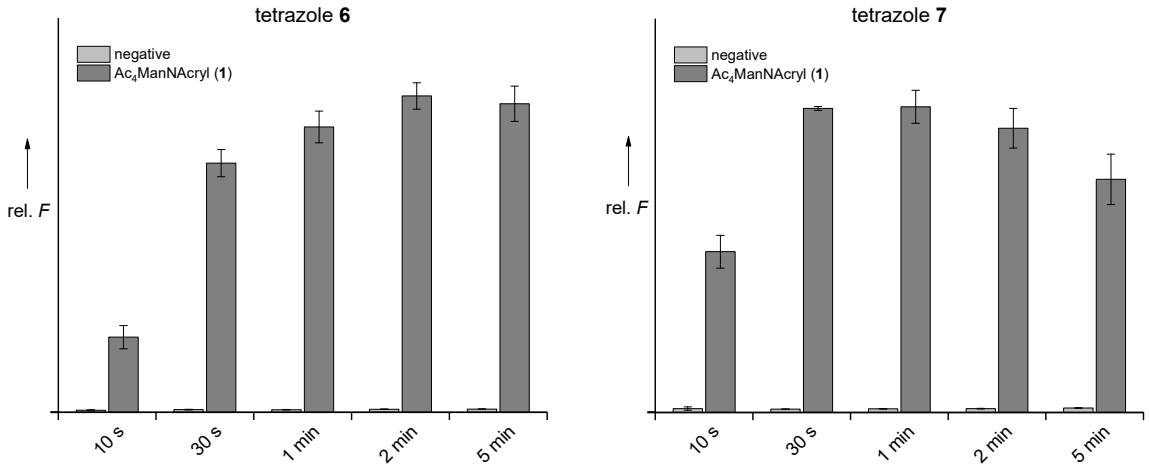


Figure S6: Results of the plate reader assay with different irradation times. Solutions containing tetrazole and Ac_4ManNAcryl in ethanol were irradiated for 10 s to 5 min (302 nm). As negative control only ethanol was added.

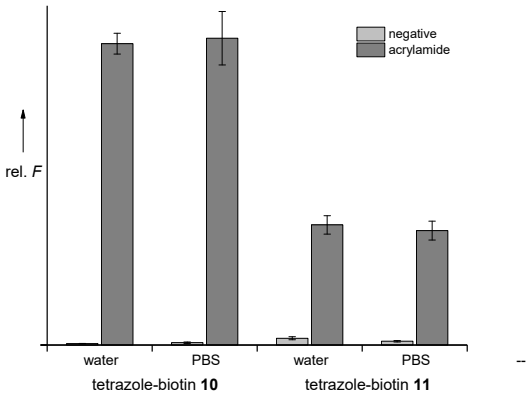


Figure S7: Plate reader assay investigating PBS as solvent. Solutions containing tetrazole-biotin and acrylamide in water or PBS were irradiated for 5 min (302 nm). As negative control only solvent was added.

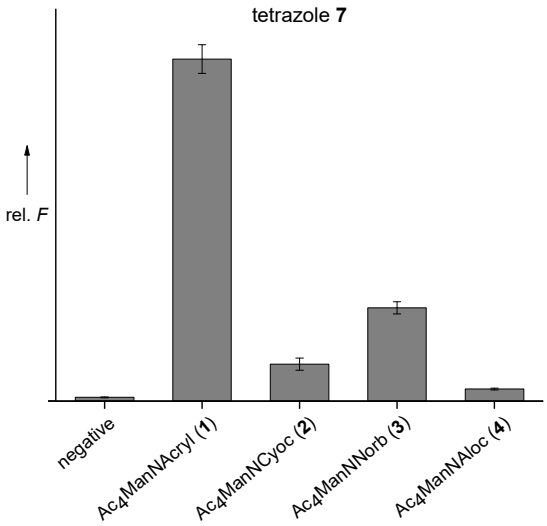
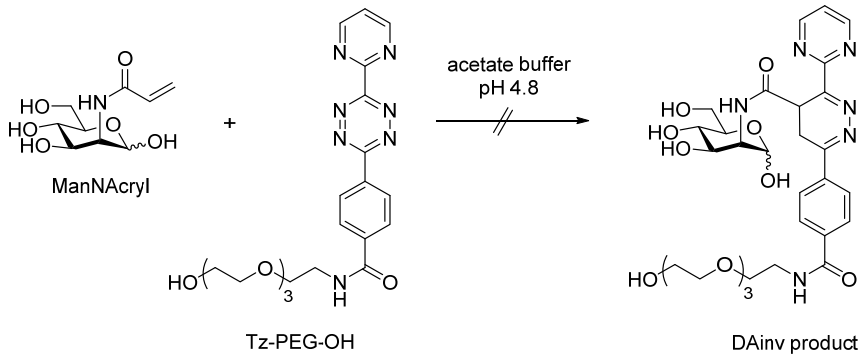


Figure S8: Results of the plate reader assay with different alkenes. Solutions containing tetrazole **7** and sugars in ethanol were irradiated for 1 min (302 nm). As negative control only ethanol was added.

ManNAcryl Does Not React in a DAinv Reaction

A



B

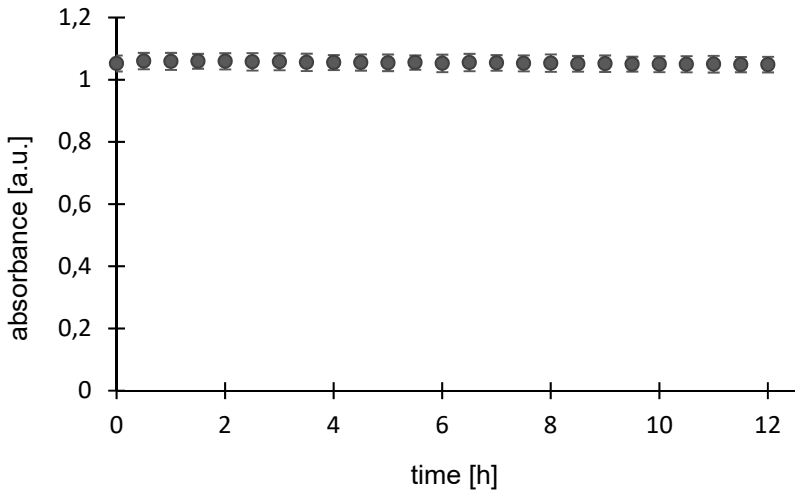


Figure S9: A) Possible reaction of ManNAcryl with Tz-PEG-OH. The absorbance of the tetrazine was monitored at λ=522 nm over time. B) Plot of the absorbance of Tz-PEG-OH at 522 nm against time. The fact, that the absorbance remains constant confirms that ManNAcryl does not react in the DAinv reaction.

Staining of Cell-Surface Glycans by the Photoclick Reaction

Cells were washed two times with PBS and then treated with tetrazole-biotin (stock: 100 mM in DMSO, 100 µM in PBS). Cells were irradiated for 1 min with a hand held UV-lamp (302 nm) which was directly placed on top of the *ibidi*. Cells were incubated for 15 min at rt before they were washed twice with PBS and incubated with AlexaFluor555®-labeled streptavidin (6.6 µg mL^{-1}) and Hoechst 33342 (10 µg mL^{-1}) for 20 min at rt in the dark. Cells were washed twice with PBS, and DMEM was added for microscopy.

Dual Labeling Experiments

Cells were washed two times with PBS and then treated with tetrazine-Cy5 (stock: 5 mM in DMSO, 40 µM in DMEM, 1 h, 37°C) or DIBO-AF488 (stock: 5 mM in DMSO, 20 µM in DMEM, 1 h, 37°C). After two washes with PBS, cells were treated with tetrazole-biotin **10** (stock: 100 mM in DMSO, 100 µM in PBS). Cells were irradiated for 1 min with a hand held UV-lamp (302 nm) which was directly placed on top of the *ibidi*. Cells were incubated for 15 min at rt before they were washed twice with PBS and incubated with AlexaFluor555®-labeled streptavidin (6.6 µg mL^{-1}) and Hoechst 33342 (10 µg mL^{-1}) for 20 min at rt in the dark. Cells were washed twice with PBS, and DMEM was added for microscopy.

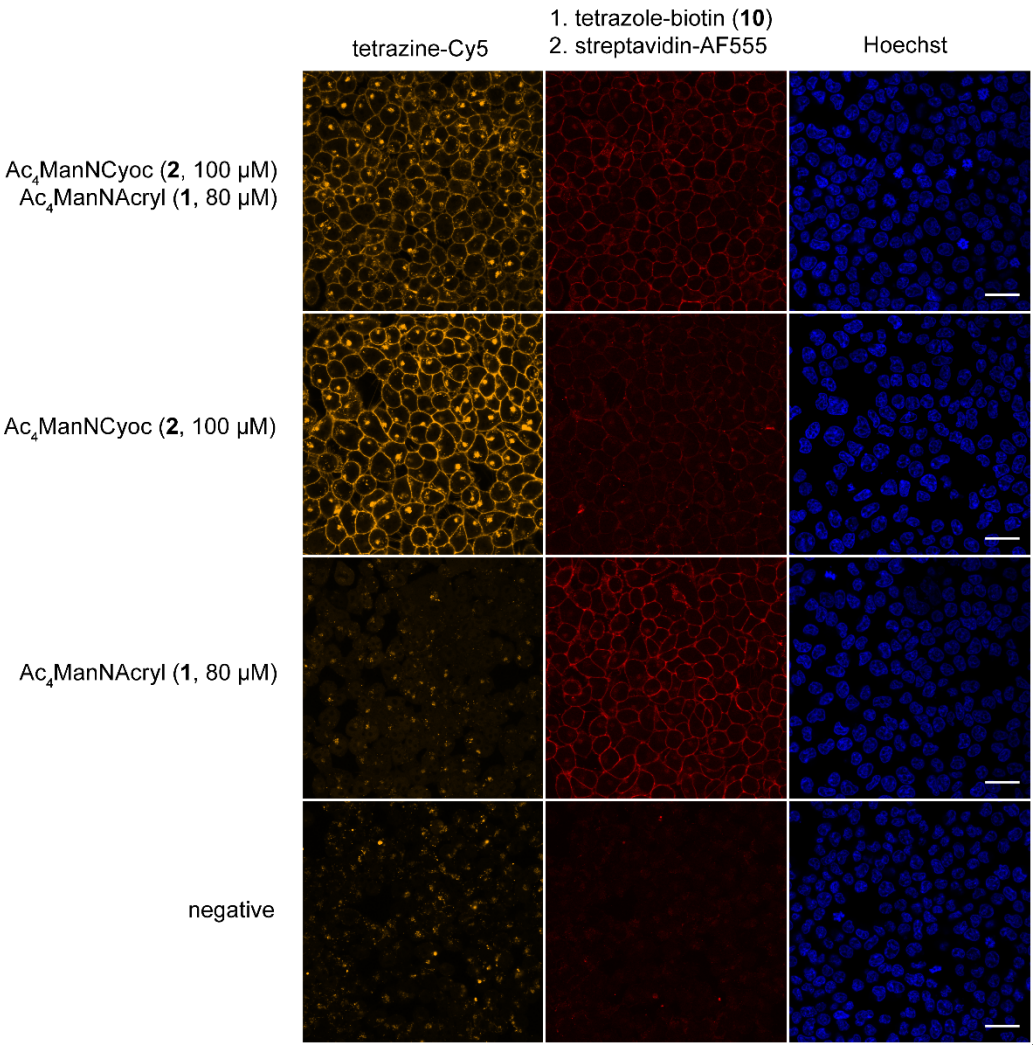


Figure S10: Dual labeling using the photoclick reaction and the inverse-electron-demand Diels-Alder reaction. HEK 293T cells were incubated with Ac_4ManNCyoc (**2**, 100 µM) and Ac_4ManNAcryl (**1**, 80 µM) for 48 h. As controls only one sugar or DMSO only (negative control) was added. For labeling, cells were reacted with tetrazine-Cy5 (40 µM, 1 h, 37°C) followed by tetrazole-biotin **10** (30 s, 302 nm; 15 min, rt) and streptavidin-AF555. Nuclei were stained with Hoechst 33342. Scale bar 30 µm.

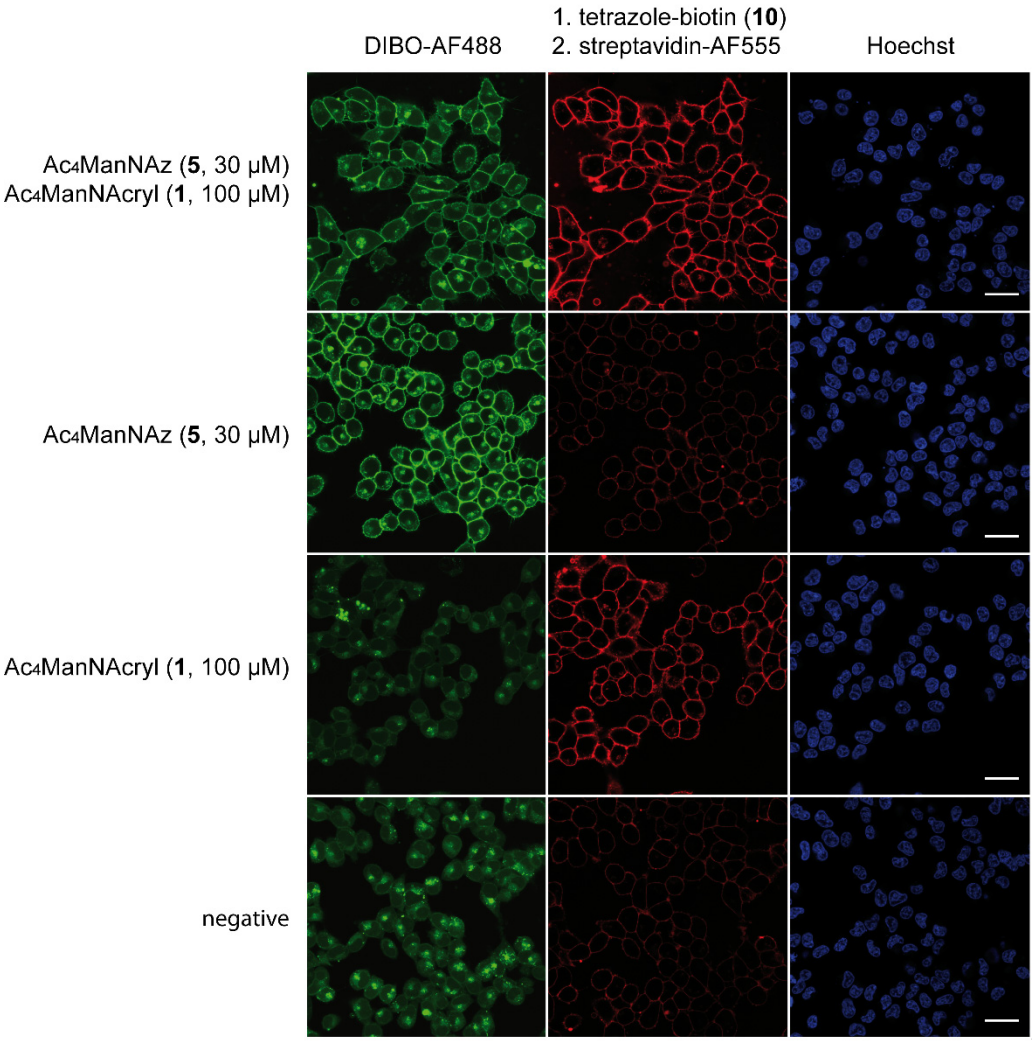


Figure S11: Dual labeling using the photoclick reaction and the strain promoted Click reaction. HEK 293T cells were incubated with Ac_4ManNAz (**5**, 30 μM) and Ac_4ManNAcryl (**1**, 100 μM) for 48 h. As controls only one sugar or DMSO only (negative control) was added. For labeling cells were reacted with DIBO-AF488 (20 μM, 1 h, 37°C) followed by tetrazole-biotin **10** (1 min, 302 nm; 15 min, rt) and streptavidin-AF555. Nuclei were stained with Hoechst 33342. Scale bar 30 μm.

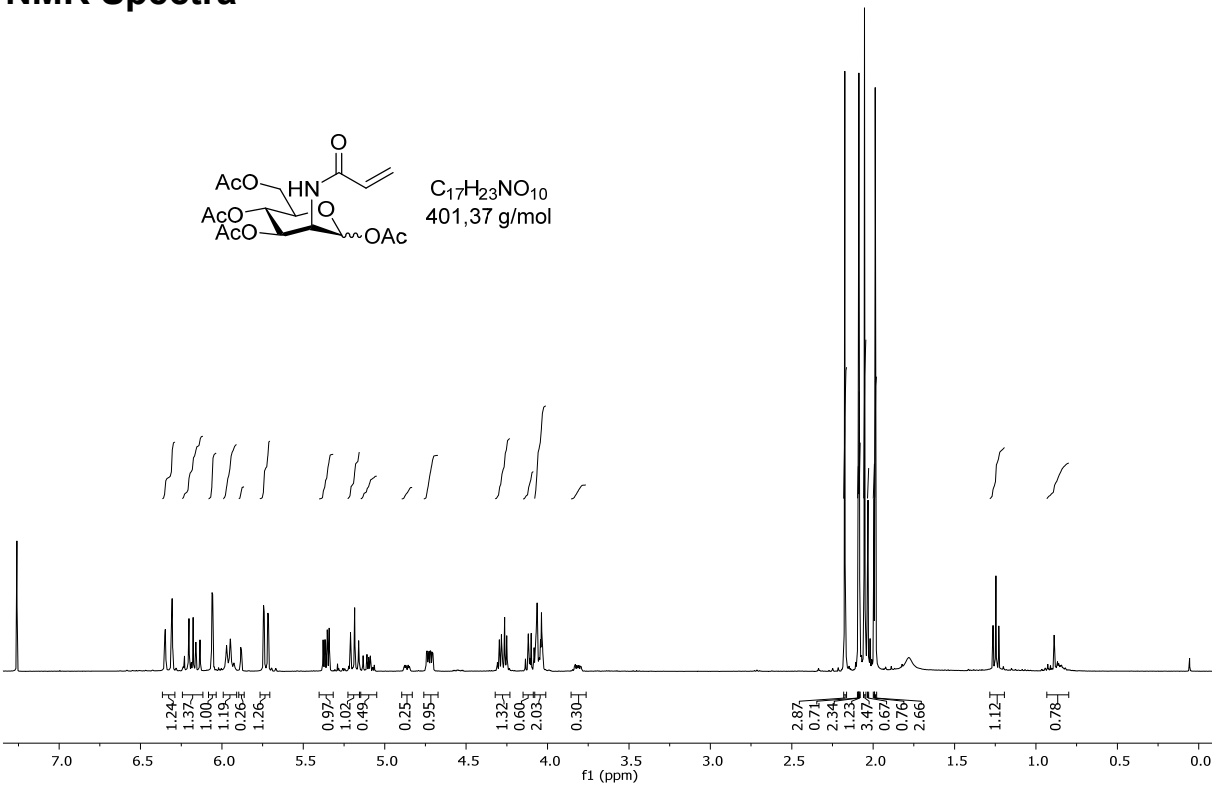
Triple Labeling Experiments

Cells were washed two times with PBS and then treated with tetrazine-Cy5 (stock: 5 mM in DMSO, 40 µM in DMEM, 1 h, 37°C) and DIBO-AF488 (stock: 5 mM in DMSO, 20 µM in DMEM, 1 h, 37°C). After two washes with PBS, cells were treated with tetrazole-biotin **10** (stock: 100 mM in DMSO, 100 µM in PBS). Cells were irradiated for 1 min with a hand held UV-lamp (302 nm) which was directly placed on top of the *ibidi*. Cells were incubated for 15 min at rt before they were washed twice with PBS and incubated with AlexaFluor555®-labeled streptavidin (6.6 µg mL^{-1}) and Hoechst 33342 (10 µg mL^{-1}) for 20 min at rt in the dark. Cells were washed twice with PBS, and DMEM was added for microscopy.

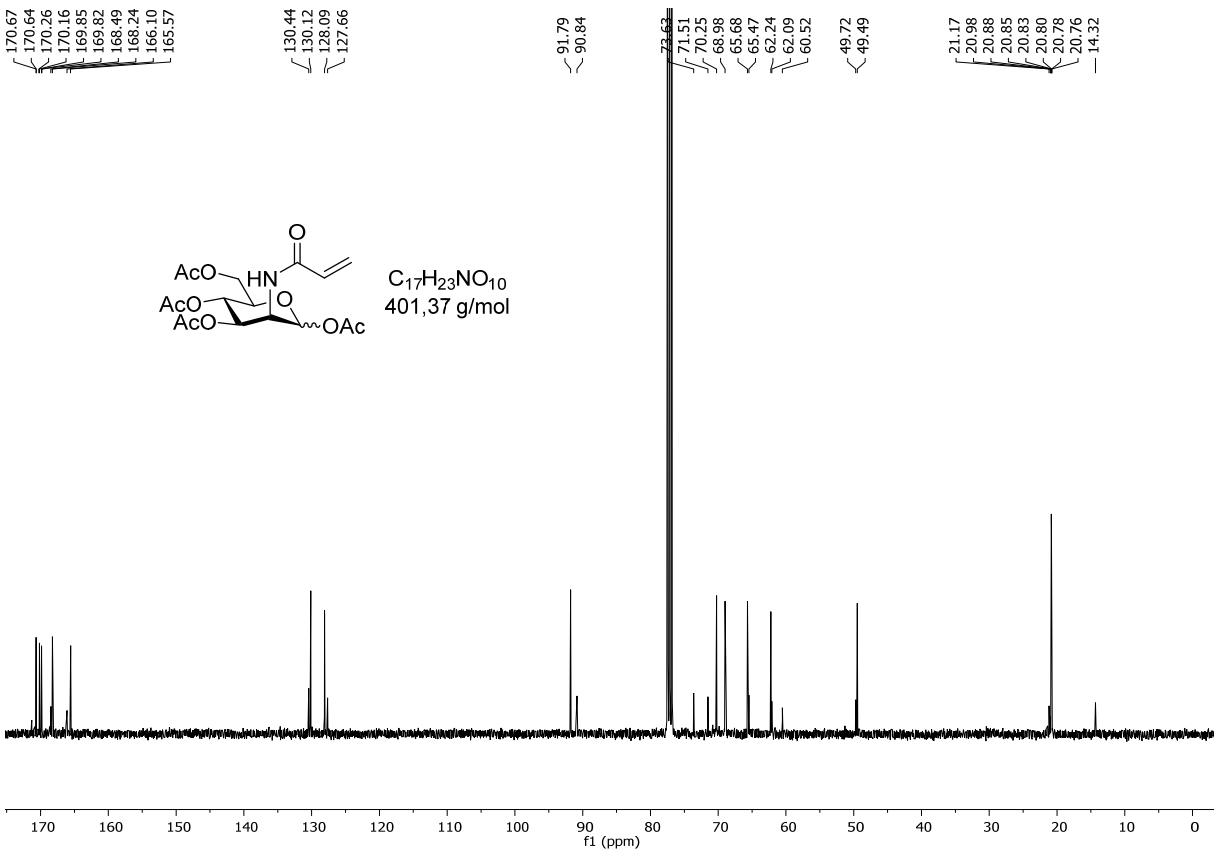
References

[1] C. M. Cole, J. Yang, J. Šečkutė, N. K. Devaraj, *ChemBioChem* **2013**, *14*, 205-208.
[2] W. Song, Y. Wang, Z. Yu, C. I. R. Vera, J. Qu, Q. Lin, *ACS Chem. Biol.* **2010**, *5*, 875-885.
[3] N. Charvet, P. Reiss, A. Roget, A. Dupuis, D. Grunwald, S. Carayon, F. Chandezon, T. Livache, *J. Mater. Chem.* **2004**, *14*, 2638-2642.
[4] A.-K. Späte, J. E. G. A. Dold, E. Batroff, V. F. Schart, D. E. Wieland, O. R. Baudendistel, V. Wittmann, *ChemBioChem* **2016**, *17*, 1374-1383.

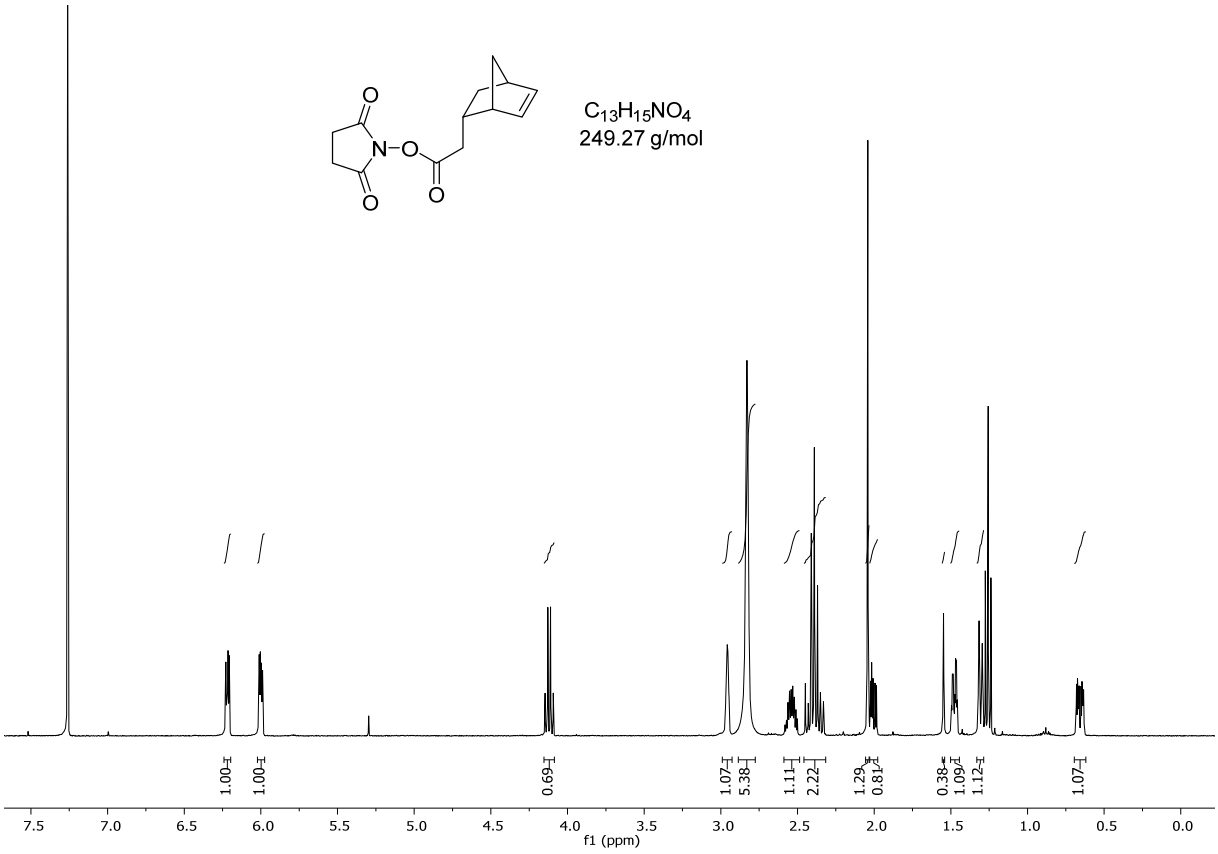
NMR Spectra



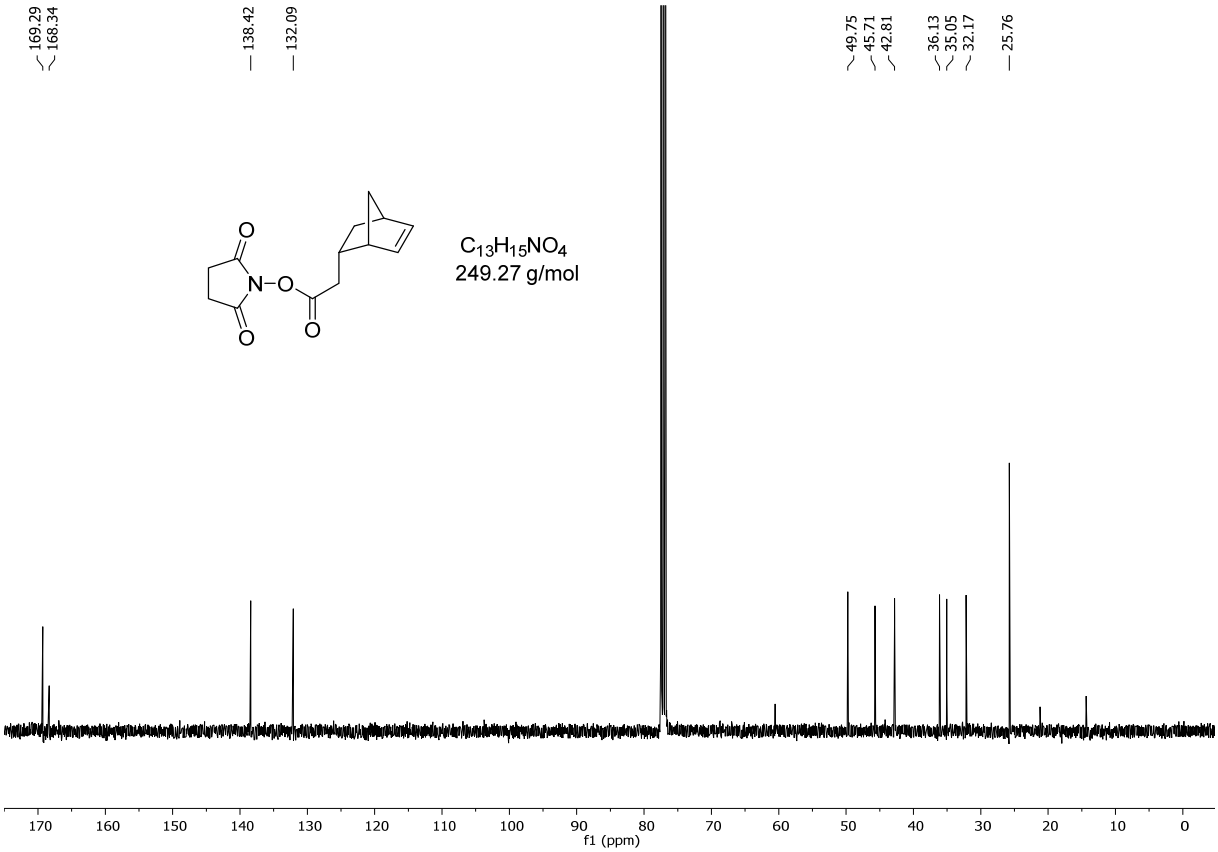
^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of the α/β mixture of Ac_4ManNAcryl (**1**).



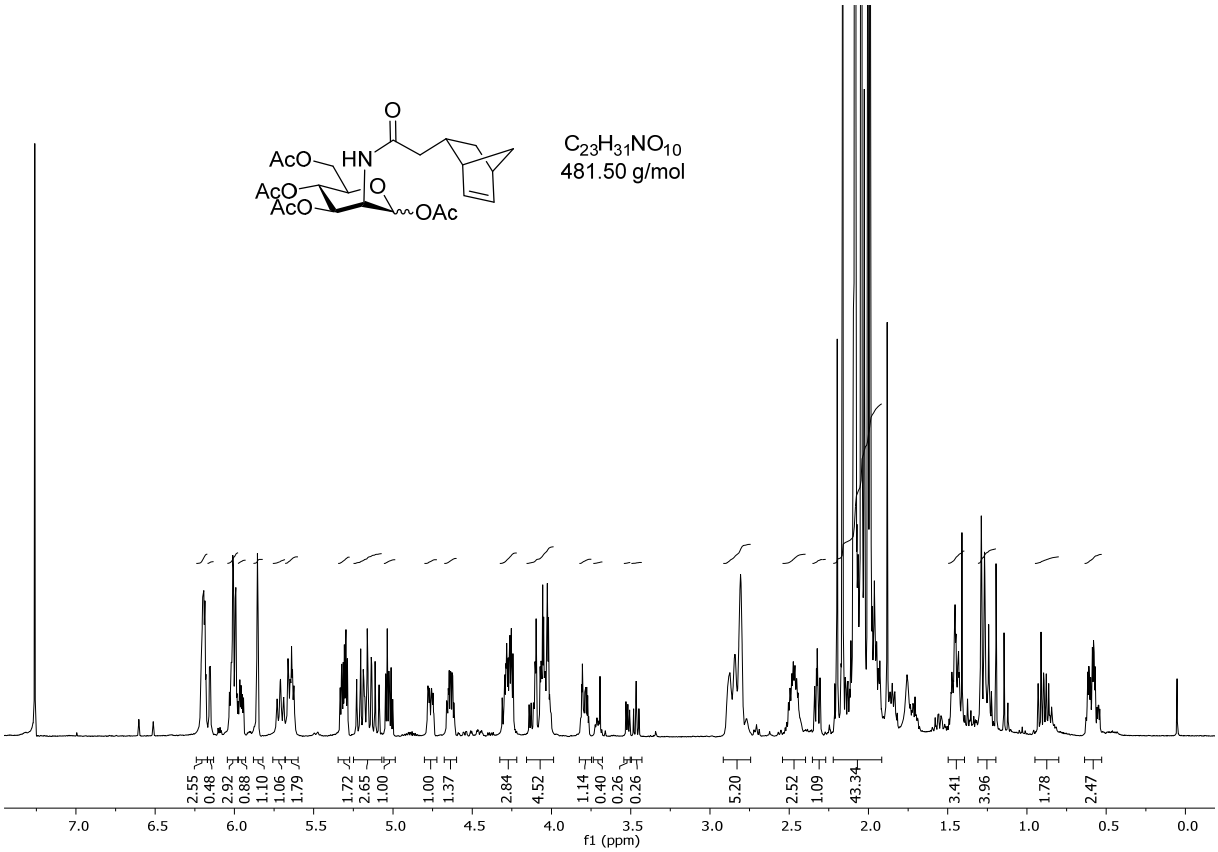
^{13}C NMR spectrum ($CDCl_3$, 101 MHz) of the α/β mixture of Ac_4ManNAcryl (**1**).



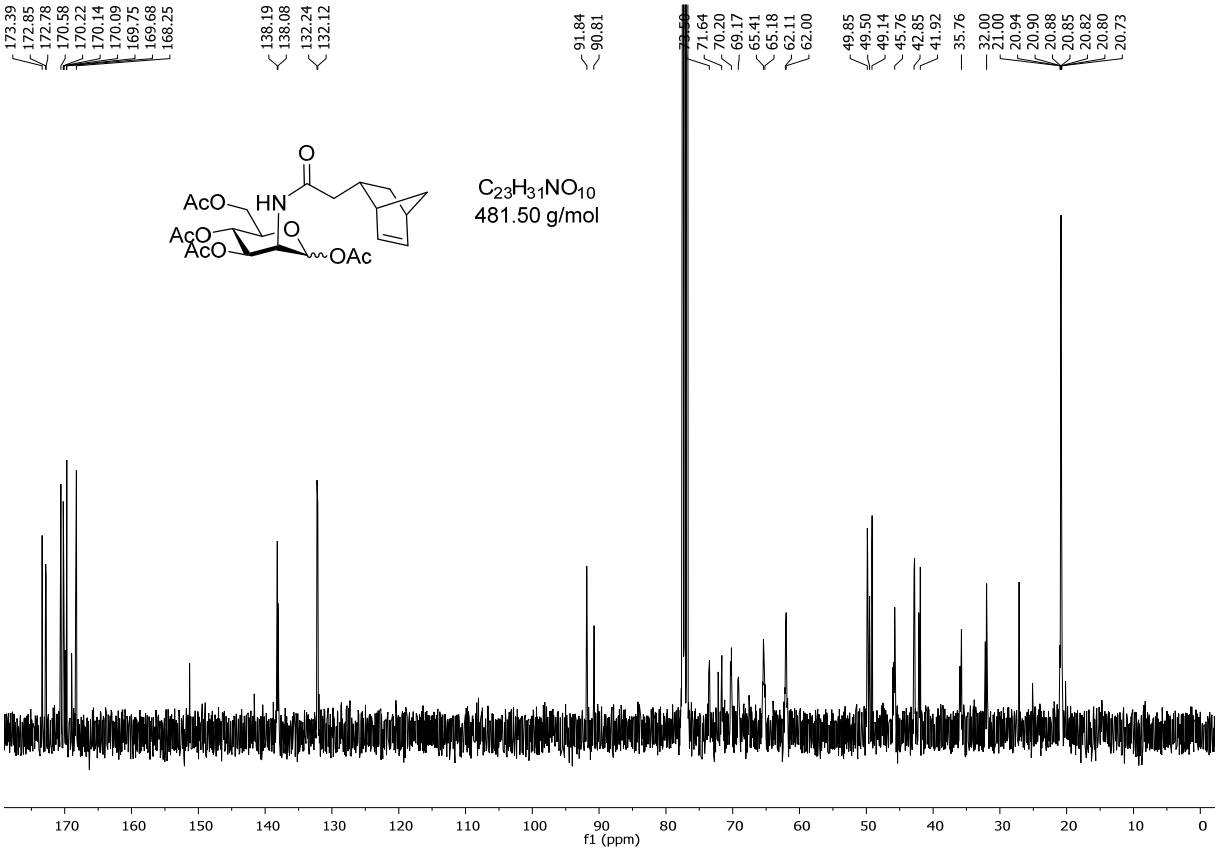
^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of activated norbornene.



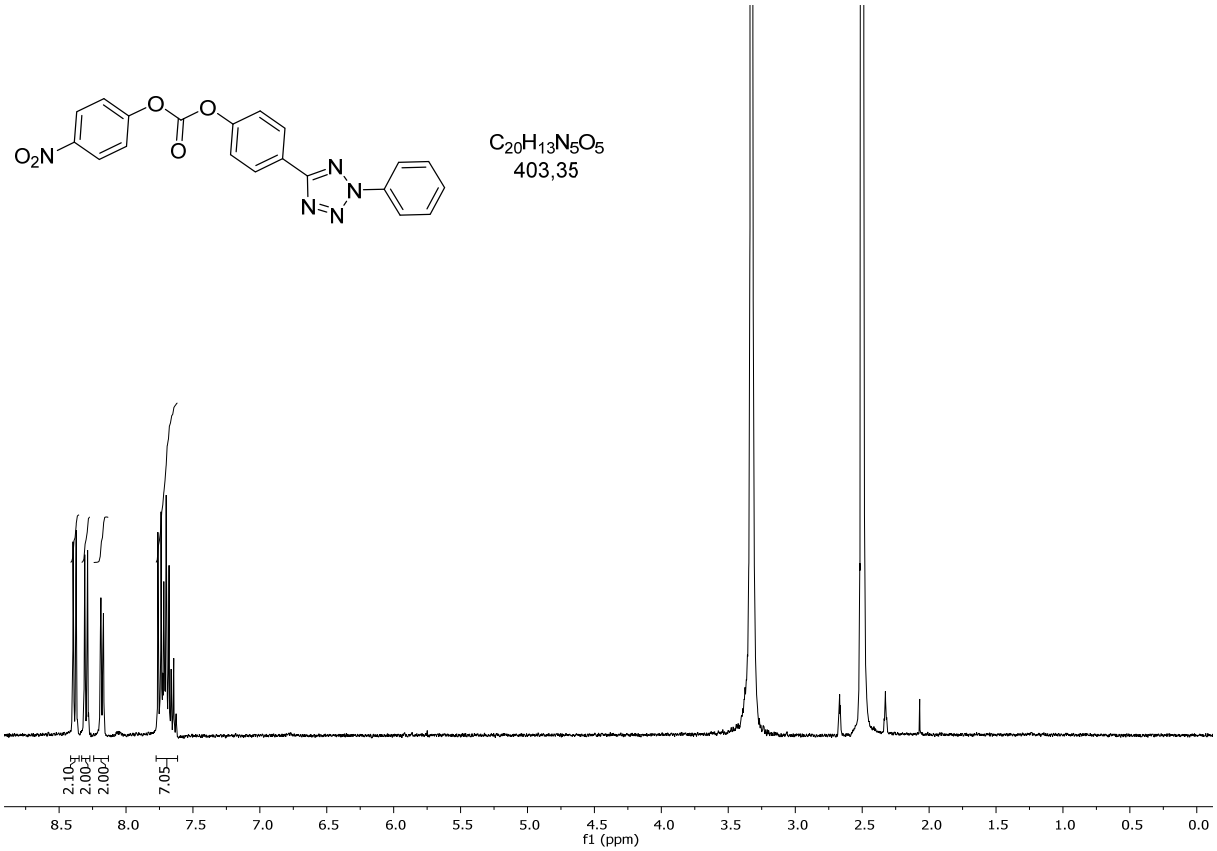
^{13}C NMR spectrum ($CDCl_3$, 101 MHz) of activated norbornene.



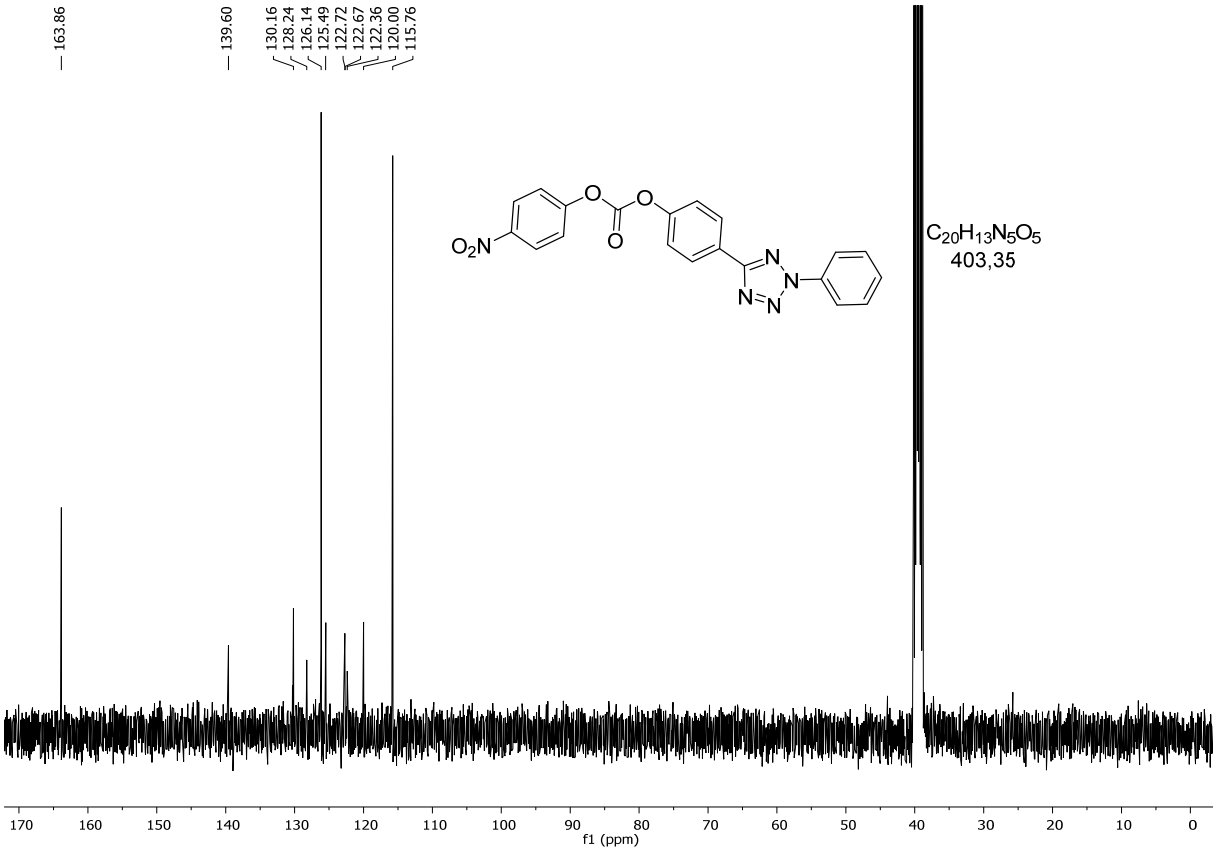
^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of Ac_4ManNNorb (**3**).



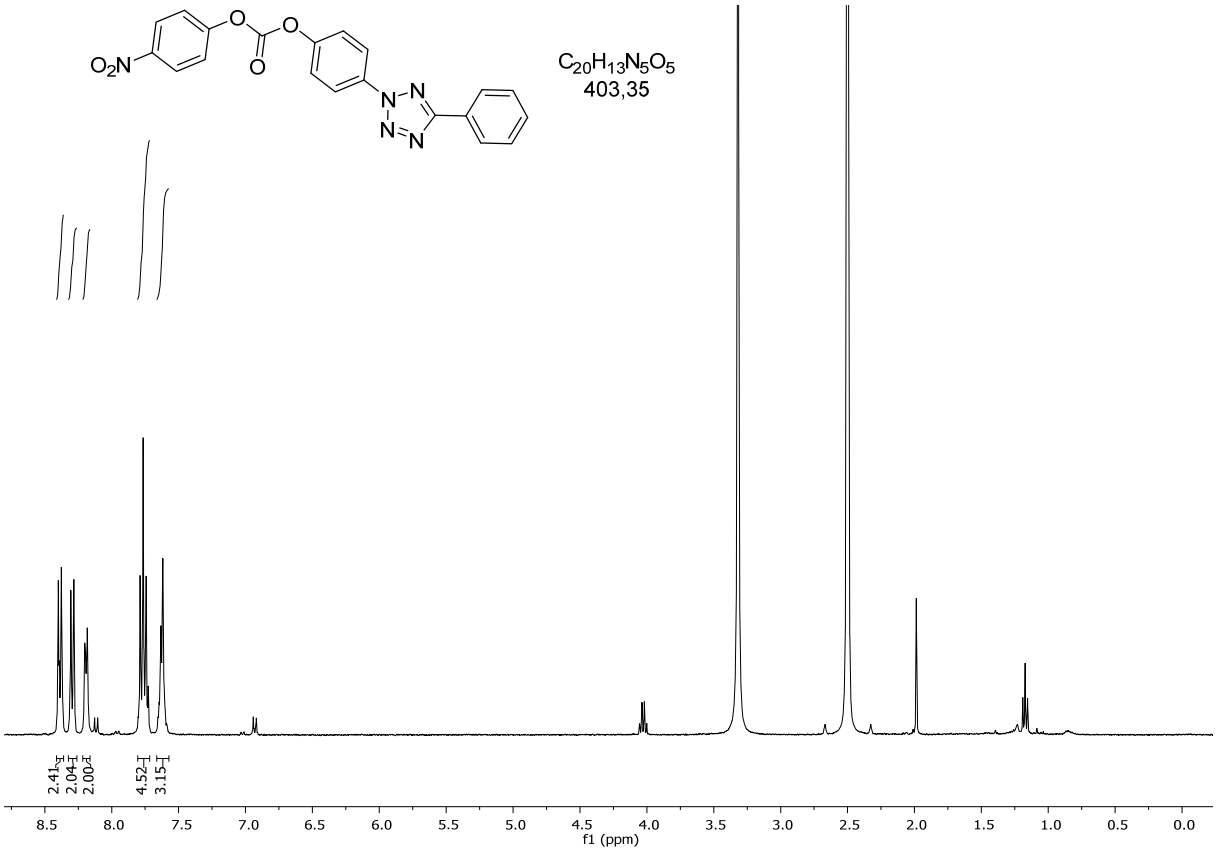
^{13}C NMR spectrum ($CDCl_3$, 101 MHz) of Ac_4ManNNorb (**3**).



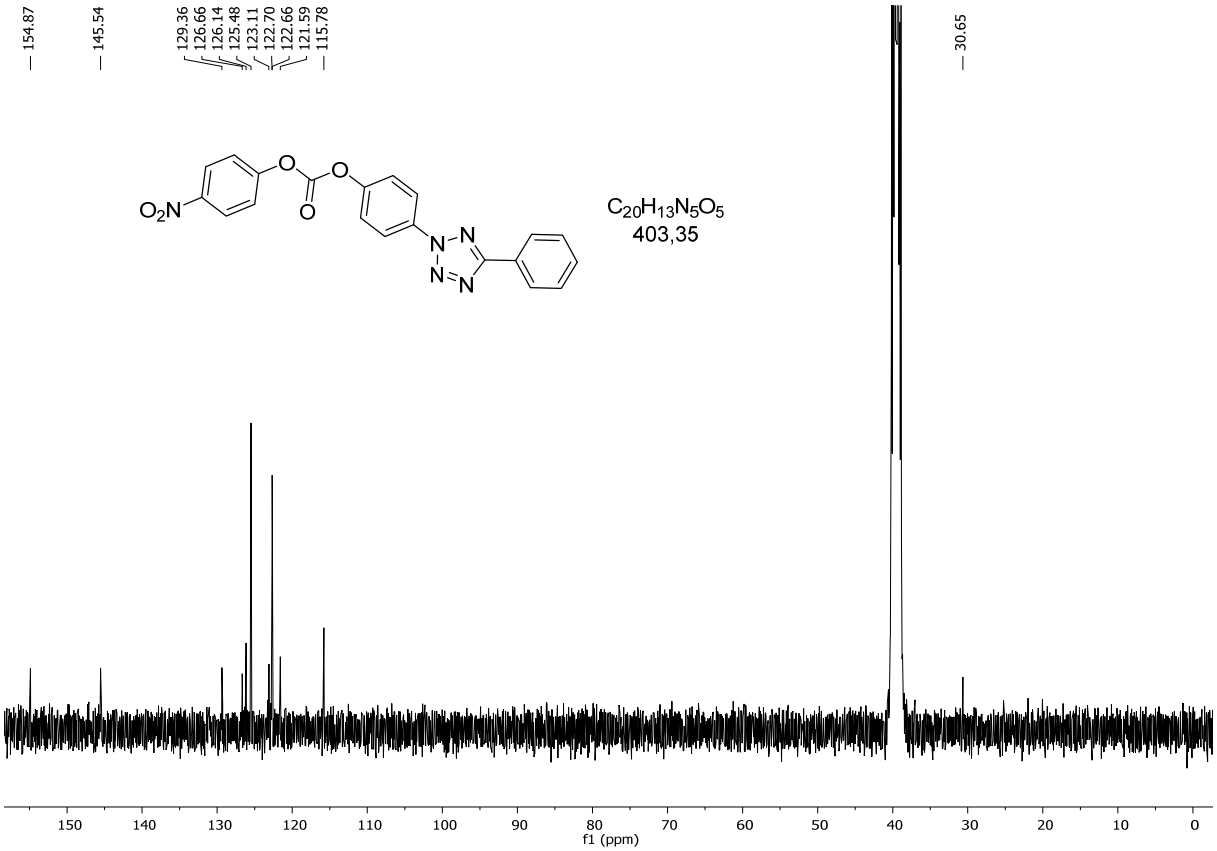
^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of activated tetrazole **16**.



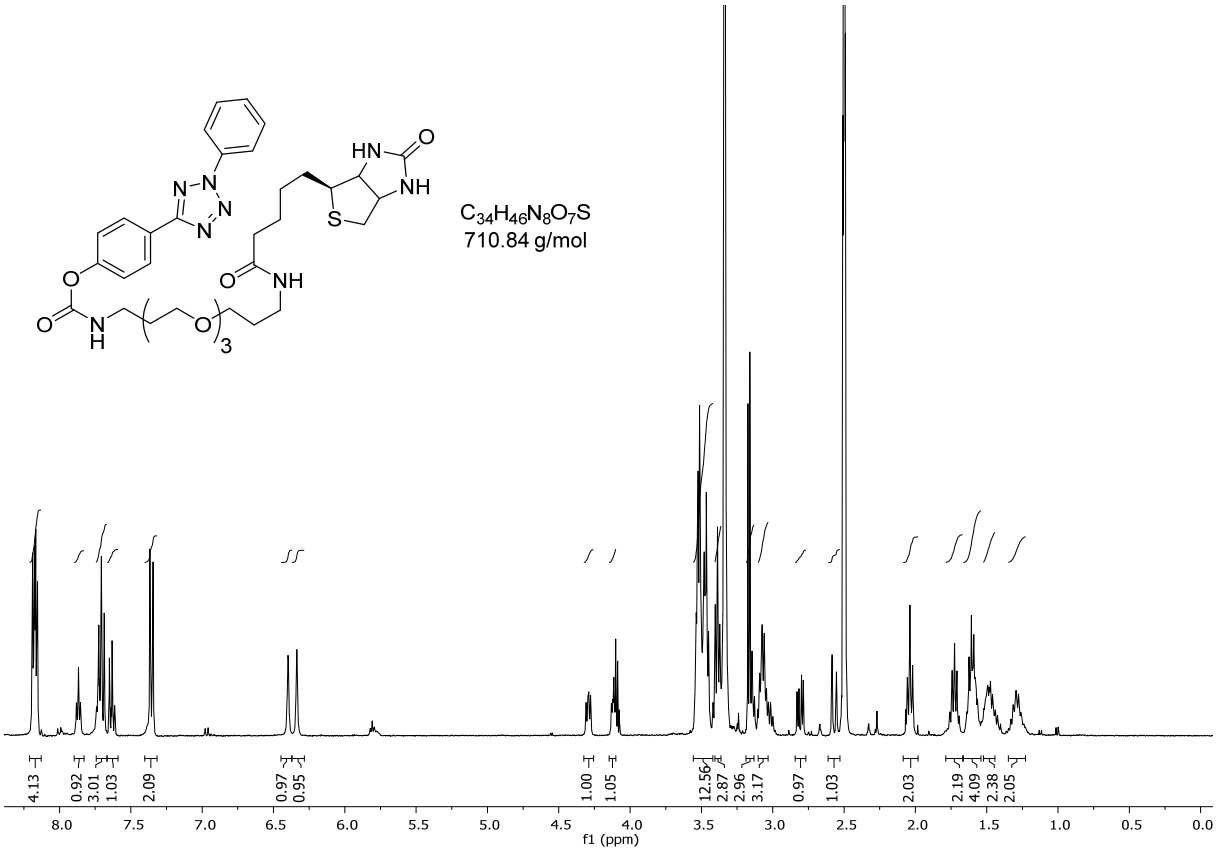
^{13}C NMR spectrum (DMSO-d_6, 101 MHz) of activated tetrazole **16**.



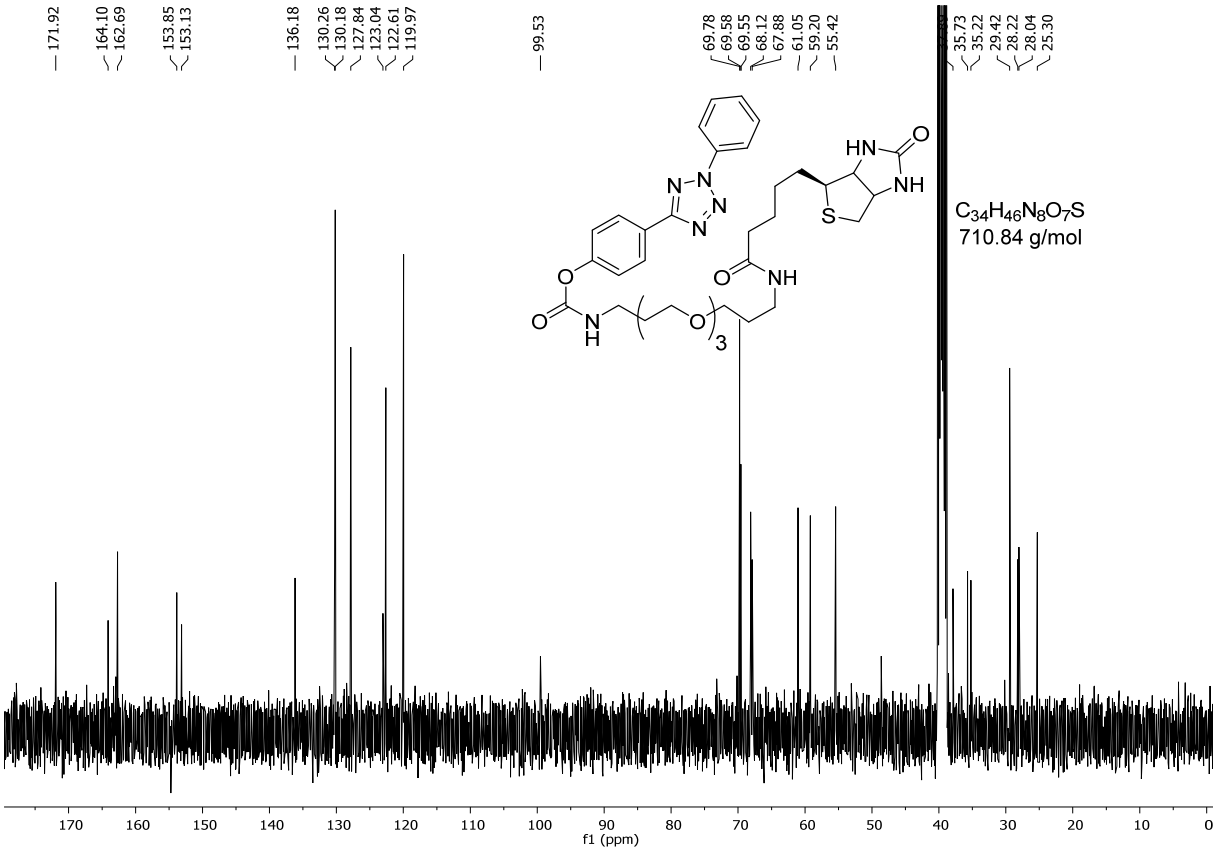
^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of activated tetrazole **17**.



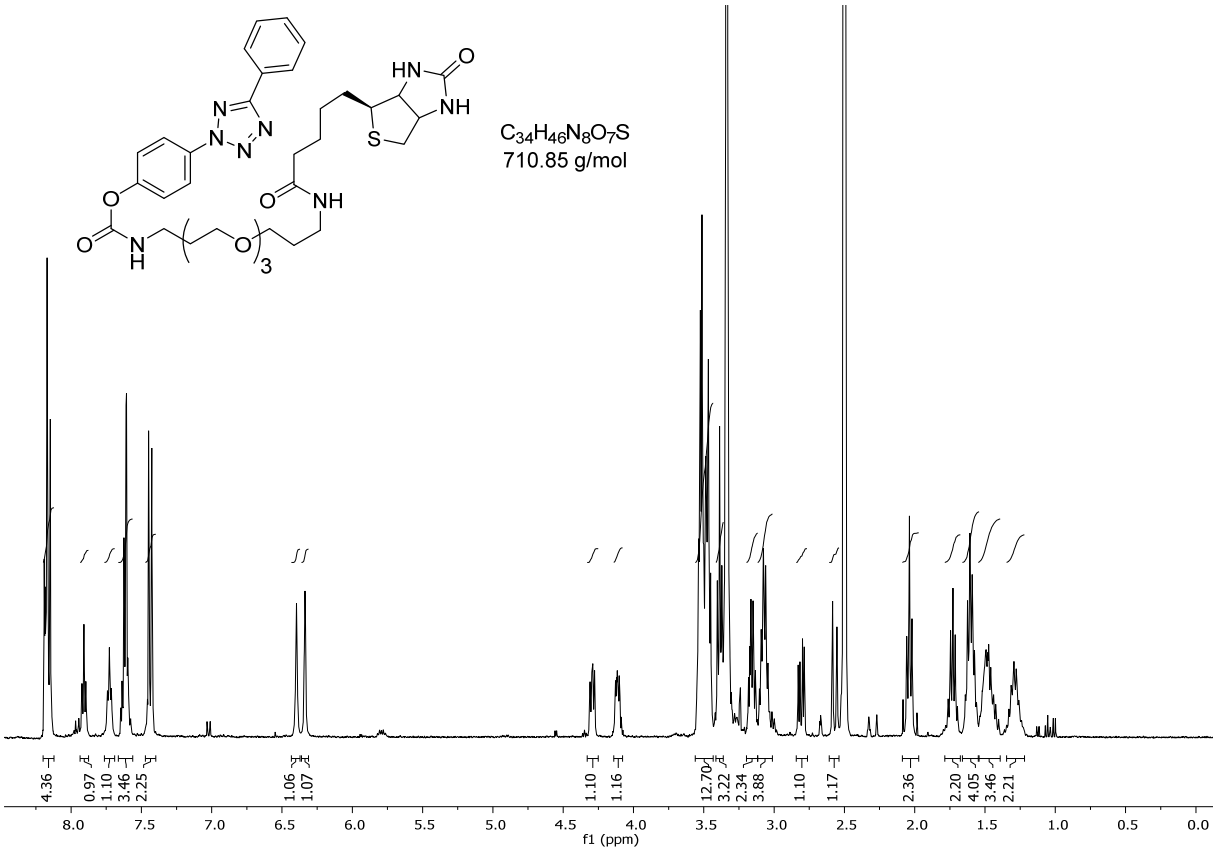
^{13}C NMR spectrum (DMSO-d_6, 101 MHz) of activated tetrazole **17**.



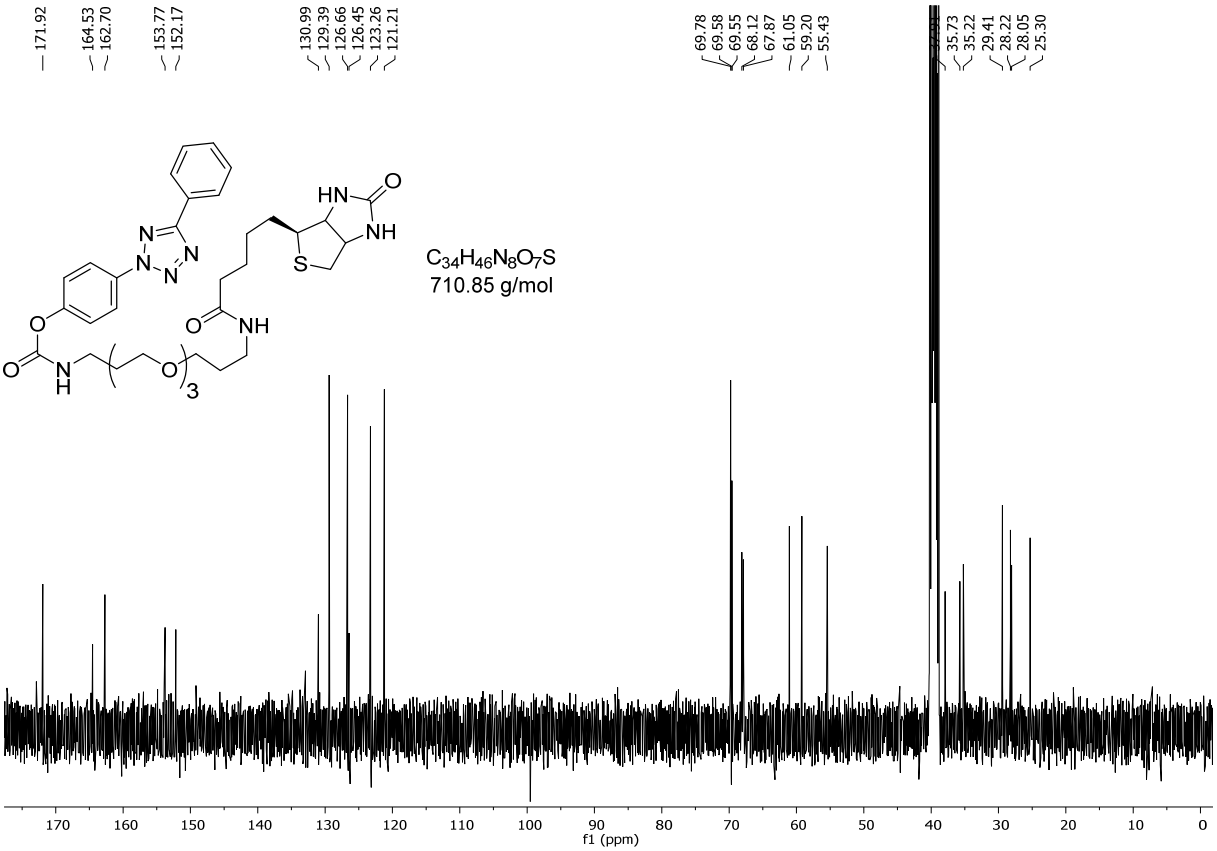
^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of tetrazole-PEG-biotin **10**.



^{13}C NMR spectrum (DMSO-d_6, 101 MHz) of tetrazole-PEG-biotin **10**.



^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of tetrazole-PEG-biotin **11**.



^{13}C NMR spectrum (DMSO-d_6, 101 MHz) of tetrazole-PEG-biotin **11**.