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Supporting Information

69451 Weinheim, Germany

Two-Color Glycan Labeling of Live Cells by a Combination of Diels–Alder and Click Chemistry**

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Supporting Information

Contents

General Methods

All chemicals were purchased from *Aldrich*, *Fluka*, and *Dextra* and used without further purification. AlexaFluor®647-labeled streptavidin and AlexaFluor®488-DIBO were purchased from *Invitrogen*. Technical solvents were distilled prior to use. For all reactions, dry solvents were purchased from *Fluka* and *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 or cerium reagent (5 g molybdatophosphoric acid, 2.5 g ceric sulfate tetrahydrate, 25 mL sulfuric acid, 225 mL water) followed by gentle heating were used for visualization. Preparative flash column chromatography was performed on silica gel Geduran 60 (40-60 µm, *Merck)* with solvent systems specified. 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, NOESY 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*. Elemental analyses were performed on a vario EL instrument from *Elementar*. 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}. Preparative 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 normal-phase HPLC a Nucleodur 100-5 VP column from *Machery-Nagel* (10 × 250 mm, flow 6 mL min^{-1}) was used as stationary phase and a mixture of ethyl acetate and *n*-hexane was used as mobile phase. For reversed-phase HPLC a Eurospher 100 C18 column from *Knauer* (16 × 250 nm, 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.

Chemical Synthesis

1,3,4,6-Tetra-*O*-acetyl-*N*-4-pentenoylmannosamine (Ac_4ManNPtl) (2)

To a solution of mannosamine hydrochloride (506 mg, 2.3 mmol) in MeOH (17 mL) were added 4.6 mL NaOMe (0.5 M) (2.3 mmol) under N_2 atmosphere. After stirring for 90 min at room temperature, a solution of succinimidyl pent-4-enoate[1] (532 mg, 2.7 mmol) in MeOH (2 mL) was added. Completion of the reaction was monitored by TLC and the solvent was evaporated under reduced pressure. The residue was purified by a short silica gel chromatography (CH_2Cl_2/MeOH 5:1) to afford **1** (348 mg, 58 %). 118 mg thereof were dissolved in pyridine (3 mL) and acetic anhydride (0.6 mL) was added. After stirring for 3 h at room temperature the solvents were removed in vacuum. The residue was dissolved in CH_2Cl_2 (25 mL) and washed with 10 % $KHSO_4$ solution (20 mL), saturated $NaHCO_3$ solution (20 mL) and brine (20 mL). The organic phase was dried ($MgSO_4$) and the solvent was evaporated under reduced pressure. The residue was purified by silica gel chromatography (petroleum ether/ethyl acetate 1:1) to afford **2** as mixture of anomers (α/β 1.4:1) (143 mg, 74 %). **TLC**: $R_f = 0.2$ (petroleum ether/ethyl acetate 1:1); **CHN analysis** (in %): found C 52.98, H 5.91, N 3.39 (calcd. C 53.14, H 6.34, N 3.26). A sample of the mixture was subjected to normal phase HPLC (45 % ethyl acetate in *n*-hexane) to separate the anomers.

2α: **^{1}H NMR** (400.1 MHz, $CDCl_3$): $\delta = 6.01$ (d, $J = 1.8$ Hz, 1H; H-1), 5.93-5.79 (m, 1H; =CH), 5.72 (d, $J = 9.2$ Hz, 1H; NH), 5.32 (dd, $J = 10.2$, 4.4 Hz, 1H; H-3), 5.21-5.06 (m, 3H; H-4 and $=CH_2$), 4.65 (ddd, $J = 9.3$, 4.4, 1.9 Hz, 1H; H-2), 4.27 (dd, $J = 12.5$, 4.8 Hz, 1H; H-6b), 4.10-3.99 (m, 2H; H-6a and H-5), 2.46-2.33 (m, 4H; CH_2CH_2), 2.17 (s, 3H; OAc), 2.10 (s, 3H; OAc), 2.06 (s, 3H; OAc), 2.00 (s, 3H; OAc) ppm; **^{13}C NMR** (100.6 MHz, $CDCl_3$): δ = 172.3, 170.5, 170.0, 169.6, 168.1 (C=O), 136.7 (=CH), 116.3 ($=CH_2$), 91.8 (C-1), 70.2 (C-5), 68.9 (C-3), 65.5 (C-4), 62.1 (C-6), 49.2 (C-2), 35.8 (CH_2), 29.5 (CH_2), 20.9, 20.8, 20.8, 20.7 (C(O)$\underline{C}H_3$) ppm.

2β: **^{1}H NMR** (400.1 MHz, $CDCl_3$): δ = 5.94-5.82 (m, 1H; =CH), 5.86 (d, $J = 1.8$ Hz, 1H; H-1), 5.78 (d, $J = 9.1$ Hz, 1H; NH), 5.18-5.01 (m, 4H; $=CH_2$, H-3 and H-4), 4.79 (ddd, $J = 9.0$, 3.9, 1.8 Hz, 1H; H-2), 4.28 (dd, $J = 12.5$, 5.1 Hz, 1H; H-6b), 4.10 (dd, $J = 12.5$, 2.5 Hz, 1H; H-6a), 3.80 (ddd, $J = 9.6$, 5.1, 2.5 Hz, 1H; H-5), 2.47-2.34 (m, 4H; CH_2CH_2), 2.10 (s, 6H; 2 OAc), 2.06 (s, 3H; OAc), 2.00 (s, 3H; OAc) ppm; **^{13}C NMR** (150.9 MHz, $CDCl_3$): δ = 173.0, 170.6, 170.2, 169.8, 168.4 (C=O), 136.8 (=CH), 116.2 ($=CH_2$), 90.8 (C-1), 73.6 (C-5), 71.5 (C-3), 65.4 (C-4), 62.0 (C-6), 49.6 (C-2), 36.1 (CH_2), 29.7 (CH_2), 20.93, 20.90, 20.87, 20.83 (C(O)$\underline{C}H_3$) ppm.

Succinimidyl Hex-5-enoate

To a solution of 5-hexenoic acid (364 mg, 3.19 mmol) and *N*-hydroxysuccinimide (514 mg, 4.46 mmol) in THF (12 mL) was added dicyclohexylcarbodiimide (790 mg, 3.83 mmol) dissolved in THF (6 mL). After stirring over night at room temperature the mixture was filtered and concentrated under reduced pressure. The residue was purified by silica gel chromatography (petroleum ether/ethyl acetate 1:1) to afford succinimidyl hex-5-enoate (600 mg, 89%). **TLC**: $R_f = 0.5$ (petroleum ether/ethyl acetate 1:1); **^{1}H NMR** (400.1 MHz, $CDCl_3$): $\delta = 5.80\text{-}5.69$ (m, 1H; =CH, H-2), 5.07-4.97 (m, 2H; $=CH_2$, H-1), 2.77 (s, 4H, CH_2, H-10 und H-11), 2.57 (t, $J = 7.1$ Hz, 2H; CH_2, H-5), 2.14 (q, $J = 7.1$ Hz, 2H; CH_2, H-3), 1.81 (quint., $J = 7.2$ Hz, 2H; CH_2, H-4) ppm; **^{13}C NMR** (100.6 MHz, $CDCl_3$): $\delta = 169.3$, 168.5 (C=O), 136.9 (C-2), 116.1 (C-1), 32.5 (C-3), 30.1 (C-5), 25.6 (C-10 und C-11), 23.7 (C-4) ppm.

1,3,4,6-Tetra-*O*-acetyl-*N*-5-hexenoylmannosamine (Ac_4ManNHxl) (4)

To a solution of mannosamine hydrochloride (527 mg, 2.4 mmol) in MeOH (17 mL) were added 4.8 mL (2.4 mmol) NaOMe (0.5 M) under N_2 atmosphere. After stirring for 90 min at room temperature, a solution of succinimidyl hex-5-enoate (582 mg, 2.7 mmol) in MeOH (0.5 mL) was added. After stirring for 18 h at room temperature the solvents were evaporated under reduced pressure. The residue was dissolved in pyridine (15 mL) and acetic anhydride (3 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) and washed with 10 % $KHSO_4$ solution (100 mL), saturated $NaHCO_3$ solution (100 mL) and brine (100 mL). The organic phase was dried ($MgSO_4$) and the solvent was evaporated under reduced pressure. The residue was purified by silica gel chromatography (petroleum ether/ethyl acetate 1:1) to afford **4** as a mixture of anomers (α/β 2:1) (460 mg, 45 %). **TLC**: $R_f = 0.2$ (petroleum ether/ethyl acetate 1:1); **CHN analysis** (in %): found C 54.14, H 6.63, N 3.30 (calcd. C 54.17, H 6.59, N 3.16). A sample of the mixture was subjected to normal phase HPLC (45 % ethyl acetate in *n*-hexane) to separate the anomers.

4α: **^{1}H NMR** (400.1 MHz, $CDCl_3$): $\delta = 6.02$ (d, $J = 1.8$ Hz, 1H; H-1), 5.85-5.72 (m, 1H; =CH), 5.65 (d, $J = 9.2$ Hz, 1H; NH), 5.33 (dd, $J = 10.2$, 4.4 Hz, 1H; H-3), 5.17 (t, $J = 10.2$ Hz, 1H; H-4), 5.12-4.99 (m, 2H; $=CH_2$), 4.66 (ddd, $J = 9.3$, 4.5, 1.9 Hz, 1H; H-2), 4.28 (dd, $J = 12.5$, 4.6 Hz, 1H; H-6b), 4.09-4.01 (m, 2H; H-5 and H-6a), 2.26 (t, $J = 7.4$ Hz, 2H; $=CHC\underline{H_2}$), 2.16-2.11 (m, 2H; $C(O)CH_2$), 2.18 (s, 3H; OAc), 2.10 (s, 3H; OAc), 2.06 (s, 3H;

OAc), 2.00 (s, 3H; OAc), 1.84-1.68 (m, 2H; =CHCH$_2$C$\underline{H_2}$) ppm; **^{13}C NMR** (100.6 MHz, $CDCl_3$): δ = 172.9, 170.6, 170.1, 169.8, 168.3 (C=O), 137.8 (=CH), 115.8 (=CH_2), 91.9 (C-1), 70.3 (C-5), 69.1 (C-3), 65.6 (C-4), 62.2 (C-6), 49.3 (C-2), 35.8 (=CH$\underline{C}H_2$), 33.0 (C(O)$\underline{C}H_2$), 24.7 (=CHCH$_2\underline{C}H_2$), 21.0, 20.87, 20.86, 20.78 (C(O)$\underline{C}H_3$) ppm.

4β: **^{1}H NMR** (400.1 MHz, $CDCl_3$): δ = 5.86 (d, J = 1.8 Hz, 1H; H-1), 5.83-5.70 (m, 1H; =CH), 5.73 (d, J = 9.1 Hz, 1H; NH), 5.16-5.00 (m, 4H; =CH_2, H-3 and H-4), 4.79 (ddd, J = 9.0, 3.9, 1.8 Hz, 1H; H-2), 4.29 (dd, J = 12.5, 5.1 Hz, 1H; H-6b), 4.11 (dd, J = 12.5, 2.5 Hz, 1H; H-6a), 3.80 (ddd, J = 9.6, 5.1, 2.5 Hz, 1H; H-5), 2.30 (dt, J = 7.2, 1.2 Hz, 2H; C(O)CH_2), 2.18-2.10 (m, 2H; =CHC$\underline{H_2}$), 2.10 (s, 6H; 2 OAc), 2.06 (s, 3H; OAc), 2.01 (s, 3H; OAc), 1.78 (quint. J = 7.3 Hz, 2H; =CHCH$_2$C$\underline{H_2}$) ppm; **^{13}C NMR** (150.9 MHz, $CDCl_3$): δ = 173.6, 170.6, 170.2, 169.8, 168.4 (C=O), 137.9 (=CH), 115.8 (=CH_2), 90.8 (C-1), 73.6 (C-5), 71.6 (C-3), 65.3 (C-4), 62.0 (C-6), 49.5 (C-2), 36.0 (=CH$\underline{C}H_2$), 32.9 (C(O)$\underline{C}H_2$), 24.9 (=CHCH$_2\underline{C}H_2$), 20.93, 20.89, 20.86, 20.82 (C(O)$\underline{C}H_3$) ppm.

Succinimidyl Pent-4-en-1-yl Carbonate

The title compound was prepared according to a procedure of Lang *et al.*[2] Disuccinimidyl carbonate (1.5 g, 5.8 mmol) was added to a solution of pent-4-en-1-ol (293 mg, 3.4 mmol) and NEt_3 (1.4 mL, 10.3 mmol) in acetonitrile (12 mL). The reaction mixture was stirred overnight and then concentrated under vacuum. The product was purified by silica gel chromatography (CH_2Cl_2) to yield the title compound as a white solid (440 mg, 57 %). **TLC**: R_f = 0.37 (CH_2Cl_2); **^{1}H NMR** (400.1 MHz, $CDCl_3$) δ = 5.79 (ddt, J = 16.9, 10.2, 6.7 Hz, 1H, =CH), 5.11 – 5.00 (m, 2H, =CH_2), 4.34 (t, J = 6.6 Hz, 2H, OCH_2), 2.84 (s, 4H, C(O)CH_2CH_2C(O)), 2.22 – 2.14 (m, 2H, =CHC$\underline{H_2}$), 1.86 (dq, J = 8.4, 6.7 Hz, 2H, OCH_2C$\underline{H_2}$) ppm; **^{13}C NMR** (100.6 MHz, $CDCl_3$) δ = 168.7 (C=O), 136.6 (=CH), 116.0 (=CH_2), 70.8 (O$\underline{C}H_2$), 29.5 (=CH$\underline{C}H_2$), 27.5 (O$CH_2\underline{C}H_2$), 25.5 (C(O)$\underline{C}H_2\underline{C}H_2$C(O)) ppm.

1,3,4,6-Tetra-*O*-acetyl-*N*-5-pentenyloxycarbonylmannosamine (Ac_4ManNPeoc) (6)

Mannosamine hydrochloride (400 mg, 1.85 mmol) was suspended in MeOH (10 mL) and treated with 0.5 M NaOMe solution in MeOH (3.6 mL, 1.85 mmol) under N_2 atmosphere. The reaction mixture was stirred for 2 h. Succinimidyl pent-4-en-1-yl carbonate (440 mg, 1.93 mmol) in MeOH (10 mL) was added and the mixture was stirred at room temperature overnight. The solvent was evaporated and the residual brown oil was dissolved in pyridine

(5 mL), treated with acetic anhydride (1.7 mL), and stirred at room temperature overnight. The mixture was concentrated, diluted with CH_2Cl_2 (50 mL) and washed with 10% $KHSO_4$ solution (50 mL), saturated $NaHCO_3$ solution (50 mL) and brine (50 mL). The organic layer was dried ($MgSO_4$) and the solvent was evaporated. The crude was purified by column chromatography on SiO_2 (petroleum ether/ethyl acetate 1:1) to deliver **6** as an off white solid as mixture of anomers (α/β 1:1.2) (680 mg, 80 %). **TLC**: R_f = 0.53 (petroleum ether/ethyl acetate 1:1); **^{1}H NMR** (400.1 MHz, $CDCl_3$) δ = 6.09 (d, J = 1.7 Hz, 1H; H-1, α), 5.84 (d, J = 1.4 Hz, 1H; H-1, β), 5.82–5.73 (m, 2H; =CH, α and β), 5.30 (dd, J = 10.2, 4.3 Hz, 1H, H-3, α), 5.23–5.13 (m, 2H; H-4, α and β), 5.13–4.95 (m, 7H, $=CH_2$, α and β, NH, α and β, H-3, β), 4.46 (ddd, J = 9.3, 3.8, 1.6 Hz, 1H; H-2, β), 4.33 (ddd, J = 9.5, 4.2, 1.8 Hz, 1H; H-2, α), 4.29–4.20 (m, 2H, OCH_2), 4.16–3.95 (m, 7H, OCH_2, H-5, α, H-6a/b, α and β), 3.77 (ddd, J = 9.6, 4.9, 2.5 Hz, 1H, H-5, β), 2.33 (s, 3H, OAc, α), 2.17 (m, 4H, $CH_2CH_2C\underline{H}_2$, α and β), 2.11 (s, 3H, OAc, β), 2.09 (s, 6H, OAc, α and β), 2.05 (s, 6H, OAc, α and β), 2.02 (s, 3H, OAc, β), 2.01 (s, 3H, OAc, α), 1.73 (m, 4H, $OCH_2C\underline{H}_2$, α and β,) ppm; **^{13}C NMR** (150.9 MHz, $CDCl_3$) δ = 170.93 (C=O), 170.91 (C=O), 170.5 (C=O), 170.4 (C=O), 170.0 (C=O), 168.8 (C=O), 168.5 (2 C=O), 157.1 (C=O), 156.4 (C=O), 137.8 (=CH), 137.8 (=CH), 115.7 ($=CH_2$), 115.7 ($=CH_2$), 92.2 (C-1), 91.0 (C-1), 73.7 (C-5), 71.9 (C-3), 70.5 (C-5), 69.5 (C-3), 65.7 (C-4/C-6), 65.5 (C-4/C-6), 65.4 (C-4/C-6), 65.3 (C-4/C-6), 62.3 (OCH_2), 62.2 (OCH_2), 51.7 (C-2), 51.4 (C-2), 30.31 ($=CH\underline{C}H_2$), 30.29 ($=CH\underline{C}H_2$), 28.50 ($OCH_2\underline{C}H_2$), 28.45 ($OCH_2\underline{C}H_2$), 21.3 ($C(O)\underline{C}H_3$), 21.18 ($C(O)\underline{C}H_3$), 21.12 ($C(O)\underline{C}H_3$), 21.11 ($C(O)\underline{C}H_3$), 21.09 ($C(O)\underline{C}H_3$), 21.04 ($C(O)\underline{C}H_3$), 21.01 ($C(O)\underline{C}H_3$) ppm.

DARinv in Solution

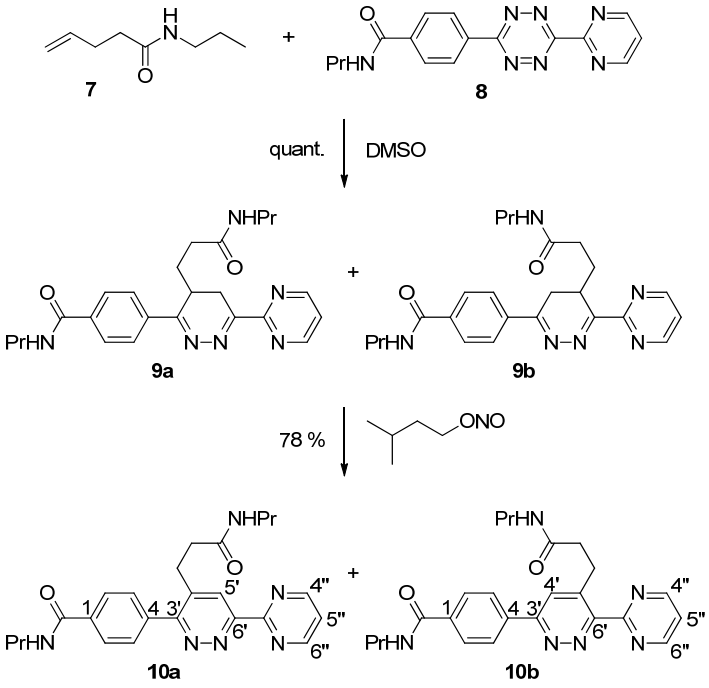

Figure S1. Model reaction of pentenamide **7** and tetrazine **8** and subsequent oxidation.

According to Figure S1 tetrazine **8**[3] (37 mg, 0.11 mmol) was dissolved in DMSO (2 mL) and a solution of pentenamide **7** (19 mg, 0.13 mmol) in DMSO (0.5 mL) was added. After completion of the reaction the solvent was evaporated in vacuum, and the residue was purified by silica gel chromatography (CH_2Cl_2/MeOH 10:1) to give dihydropyridazines **9a**/**b** as a mixture of tautomers (50 mg, quantitative). LC-MS analysis revealed that the products are easily oxidized to pyridazines **10a**/**b** (cf. Figure S2–S6). To complete this conversion the yellow solid was dissolved in acetic acid (2 mL), and isopentyl nitrite (17 µL) was added. After stirring at room temperature for 3 hours the solvent was removed under reduced pressure and the residue was purified by silica gel chromatography (CH_2Cl_2/MeOH 10:1) to afford a mixture of pyridazines **10a**/**b** in a ratio of 1:1.6 (37 mg, 78 %). LC-MS analysis of this mixture is shown in Figures S7–S9. A sample thereof was subjected to RP-HPLC using a gradient of acetonitrile in water with 0.1 % formic acid (30-80 % in 30 min) to separate the regioisomers. NMR spectroscopy revealed some minor impurities which could not be completely separated.

10a: ^{1}H NMR (600.1 MHz, $CDCl_3$): δ = 8.98 (d, J = 4.3 Hz, 2H; H-4‘‘ and H-6‘‘), 8.53 (s, 1H; H-5‘), 7.90 (d, J = 8.1 Hz, 2H; H-2 and H-6), 7.68 (d, J = 8.1 Hz, 2H; H-3 and H-5), 7.42 (t, J = 4.2 Hz, 1H; H-5‘‘), 6.30 (bs, 1H; Ar-C(O)NH), 5.46 (bs, 1H; $CH_2C(O)N\underline{H}$), 3.47 (q,

J = 6.7, 2H; Ar-C(O)NHC$\underline{H}_2$), 3.15 (m, 4H; C$\underline{H}_2$CH$_2$C(O)NHC$\underline{H}_2$), 2.41 (t, J = 7.8 Hz, 2H; C$\underline{H}_2$C(O)NH), 1.68 (sext., J = 7.3 Hz, 2H; Ar-C(O)NHCH$_2$C$\underline{H}_2$), 1.44 (sext., J = 7.3 Hz, 2H; CH$_2$C(O)NHCH$_2$C$\underline{H}_2$), 1.02 (t, J = 7.5 Hz, 3H; Ar-C(O)NHCH$_2$CH$_2$C$\underline{H}_3$), 0.84 (t, J = 7.2 Hz, 3H; CH$_2$C(O)NHCH$_2$CH$_2$C$\underline{H}_3$) ppm; **^{13}C NMR** (150.9 MHz, $CDCl_3$): δ = 170.3, 167.1, 162.1, 161.6 (quart. C), 157.9 (C-4'' and C-6''), 139.7, 139.4, 135.6 (quart. C), 129.5 (C-3 and C-5), 127.2 (C-2 and C-6), 126.9 (C-5'), 121.2 (C-5''), 41.9 (Ar-C(O)NH$\underline{C}$H$_2$), 41.4 (CH$_2$C(O)NH$\underline{C}$H$_2$), 35.3 ($\underline{C}$H$_2$C(O)NH), 27.7 ($\underline{C}$H$_2$CH$_2$C(O)NH), 23.0 (Ar-C(O)NHCH$_2\underline{C}$H$_2$), 22.8 (CH$_2$C(O)NHCH$_2\underline{C}$H$_2$), 11.5 (Ar-C(O)NHCH$_2$CH$_2\underline{C}$H$_3$), 11.3 (CH$_2$C(O)NHCH$_2$CH$_2\underline{C}$H$_3$) ppm.

10b: ^{1}H NMR (600.1 MHz, $CDCl_3$): δ = 8.98 (d, J = 4.9 Hz, 2H; H-4'' and H-6''), 8.21 (d, J = 8.0 Hz, 2H; H-3 and H-5), 7.92 (s, 1H; H-4'), 7.92 (d, J = 7.9 Hz, 2H; H-2 and H-6), 7.46 (t, J = 4.9 Hz, 1H; H-5''), 6.26 (bs, 1H; Ar-C(O)N$\underline{H}$), 5.72 (bs, 1H; CH$_2$C(O)N$\underline{H}$), 3.47 (q, J = 6.5 Hz, 2H; Ar-C(O)NHC$\underline{H}_2$), 3.26 (t, J = 7.4 Hz, 2H; C$\underline{H}_2$CH$_2$C(O)NH), 3.13 (q, J = 6.6 Hz, 2H; CH$_2$C(O)NHC$\underline{H}_2$), 2.59 (t, J = 7.4 Hz, 2H; C$\underline{H}_2$C(O)NH), 1.69 (sext., J = 7.1 Hz, 2H; Ar-C(O)NHCH$_2$C$\underline{H}_2$), 1.39 (sext., J = 7.4 Hz, 2H; CH$_2$C(O)NHCH$_2$C$\underline{H}_2$), 1.02 (t, J = 7.4 Hz, 3H; Ar-C(O)NHCH$_2$CH$_2$C$\underline{H}_3$), 0.79 (t, J = 7.2 Hz, 3H; CH$_2$C(O)NHCH$_2$CH$_2$C$\underline{H}_3$) ppm; **^{13}C NMR** (150.9 MHz, $CDCl_3$): δ = 171.0, 167.1, 164.1, 158.0, (quart. C), 157.6 (C-4'' and C-6''), 157.3, 140.7, 138.7, 136.4 (quart. C), 127.7 (C-2 and C-6 or C-3 and C-5), 127.7 (C-3 and C-5 or C-2 and C-6), 125.8 (C-4'), 120.8 (C-5''), 42.0 (Ar-C(O)NH$\underline{C}$H$_2$), 41.5 (CH$_2$C(O)NH$\underline{C}$H$_2$), 36.7 ($\underline{C}$H$_2$C(O)NH), 28.1 ($\underline{C}$H$_2$CH$_2$C(O)NH), 23.1 (Ar-C(O)NHCH$_2\underline{C}$H$_2$), 22.9 (CH$_2$C(O)NHCH$_2\underline{C}$H$_2$), 11.6 (Ar-C(O)NHCH$_2$CH$_2\underline{C}$H$_3$), 11.4 (CH$_2$C(O)NHCH$_2$CH$_2\underline{C}$H$_3$) ppm.

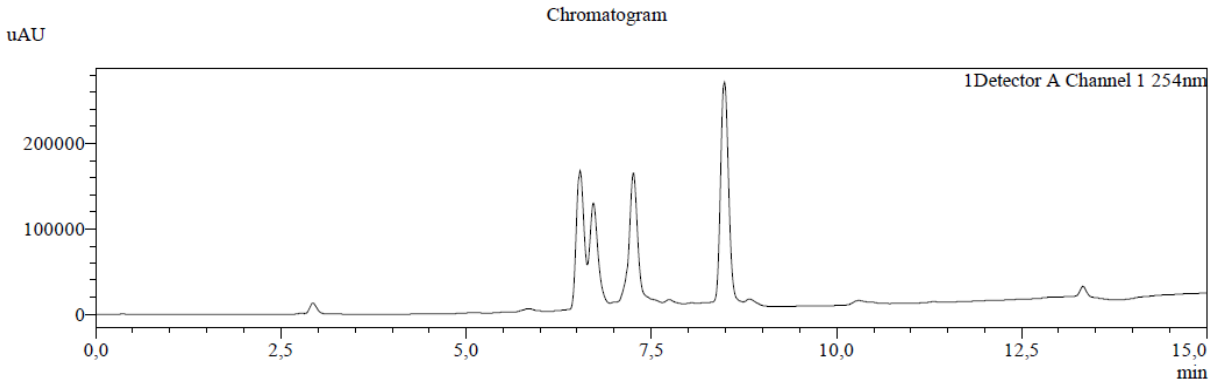

Figure S2. HPLC analysis of dihydropyridazines **9a**/**b**. The first two peaks correspond to *in situ*-formed oxidation products (**10a**/**b**). Conditions: Binary gradient of CH_3CN in H_2O with 0.1 % formic acid (20-90 % in 10 min).

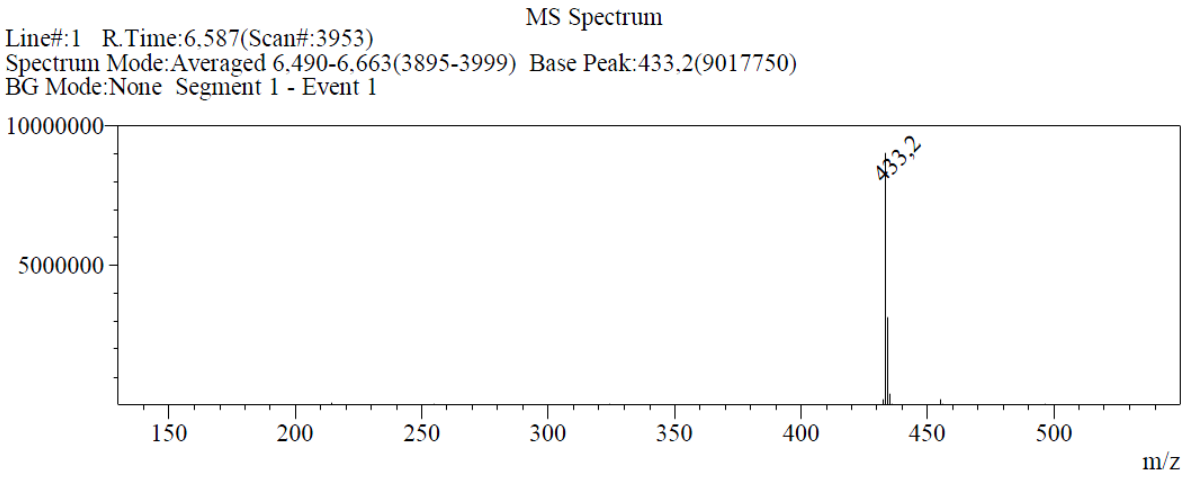

Figure S3. Mass spectrum of the first peak (**10a**) of the chromatogram shown in Figure S2.

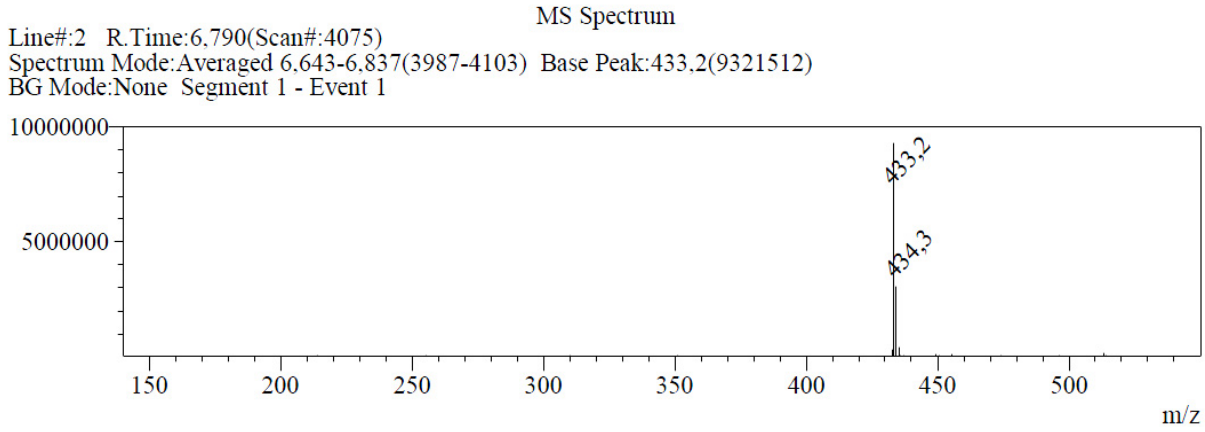

Figure S4. Mass spectrum of the second peak (**10b**) of the chromatogram shown in Figure S2.

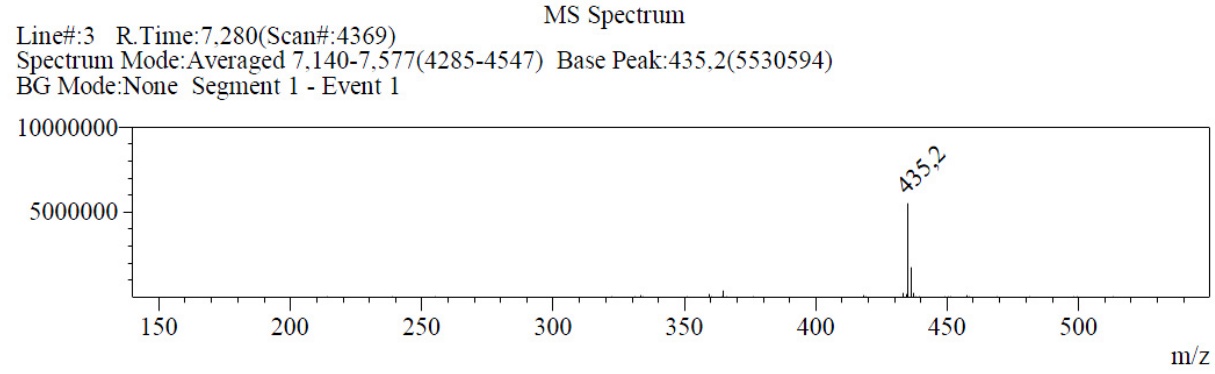

Figure S5. Mass spectrum of the third peak (**9a/b**) of the chromatogram shown in Figure S2.

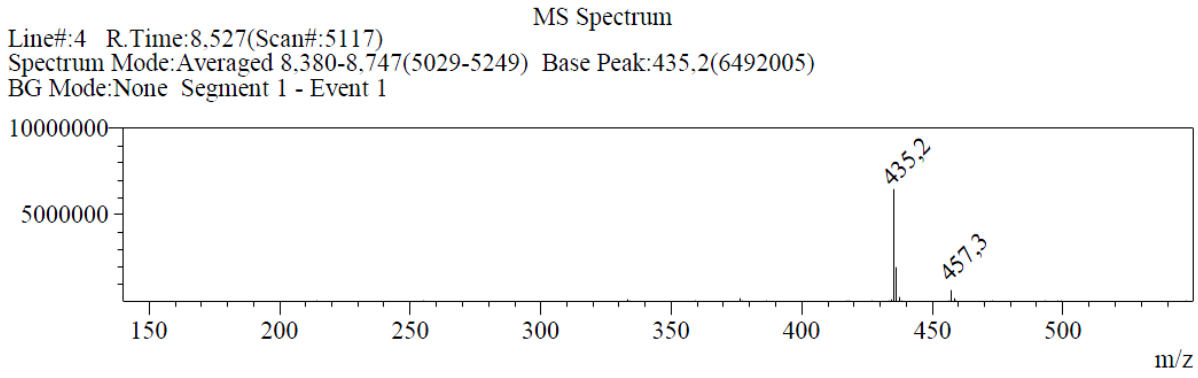

Figure S6. Mass spectrum of the fourth peak (**9a/b**) of the chromatogram shown in Figure S2.

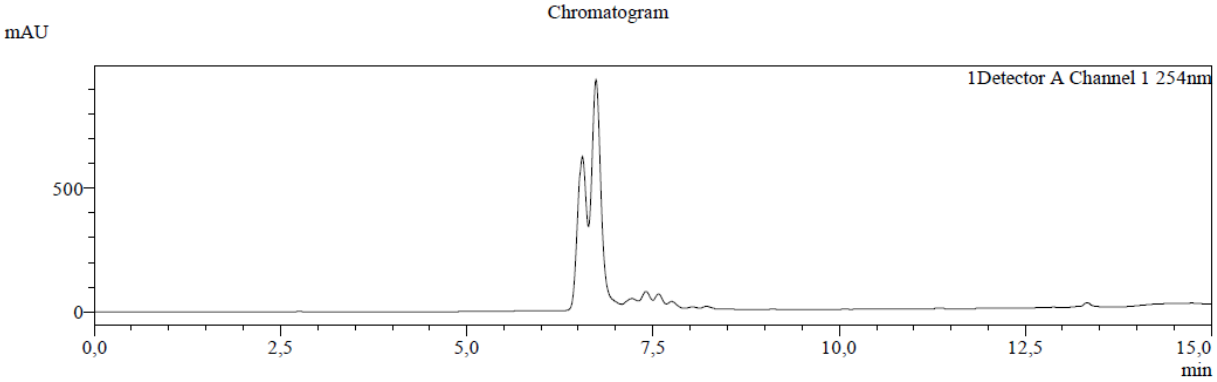

Figure S7. HPLC analysis of the mixture of isomeric pyridazines **10a**/**b** obtained after oxidation with isopentylnitrite (**10a**/**10b** 1:1.6). Conditions: Binary gradient of CH_3CN in H_2O with 0.1 % formic acid (20-90 % in 10 min).

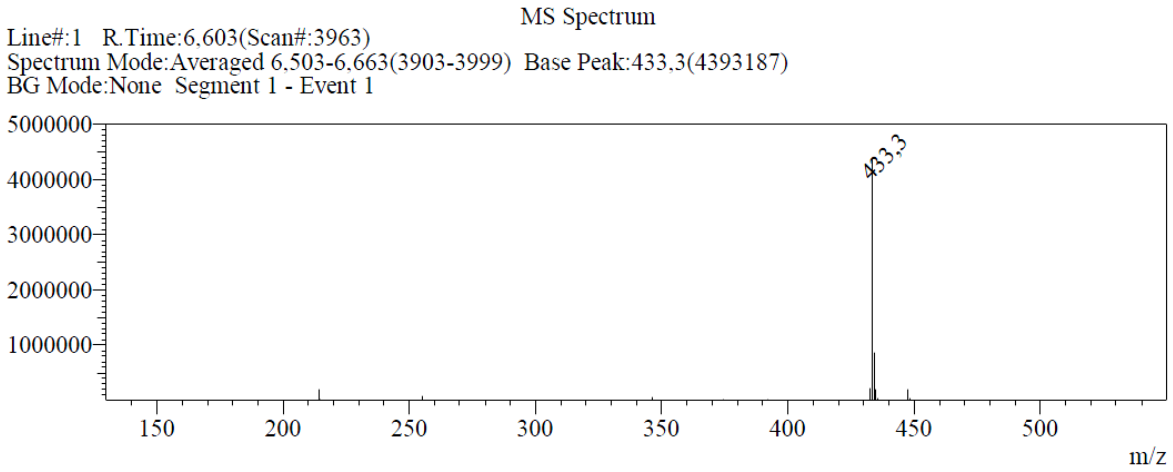

Figure S8. Mass spectrum of the first peak (**10a**) of the chromatogram shown in Figure S7.

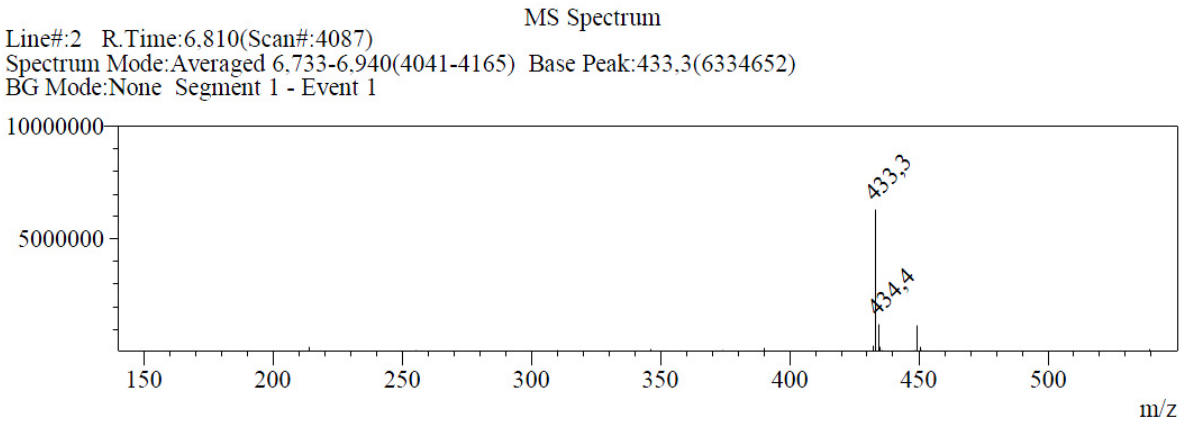

Figure S9. Mass spectrum of the second peak (**10b**) of the chromatogram shown in Figure S7.

N-(2-(2-(2-(2-Hydroxyethoxy)ethoxy)ethoxy)ethyl)-4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamide (11)

N-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC • HCl) (104 mg, 0.54 mmol) and *N*-hydroxysuccinimide (62.2 mg, 0.54 mmol) were added to a solution of 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid[3] (101 mg, 0.36 mmol) in DMSO (4 mL) and pyridine (0.2 mL). After stirring at 45 °C for 2.5 h, a solution of 11-amino-3,6,9-trioxa-undecan-1-ol[4] (70 mg, 0.36 mmol) in DMSO (1 mL) was added dropwise during 1 h at rt. After stirring for another 30 min, the solvents were removed in vacuum, the remainder was coevaporated with toluene, dissolved in CH_2Cl_2 (40 mL) and washed with water (2 x 30 mL). The aqueous solutions were extracted with CH_2Cl_2 (4 x 30 mL). The combined organic phases were washed with water (20 mL) and dried ($MgSO_4$). The solvent was removed in vacuum and the residue was purified by silica gel chromatography (CH_2Cl_2/MeOH 15:1) to afford **11** as a pink solid (91 mg, 55 %). **TLC**: $R_f = 0.31$ (CH_2Cl_2/MeOH 15:1); **^{1}H NMR** (400.1 MHz, $CDCl_3$): $\delta = 9.14$ (d, $J = 4.9$ Hz, 2H; H-4'' and H-6''), 8.79 (d, $J = 8.4$ Hz, 2H; H-2 and H-6 or H-3 and H-5), 8.13 (d, $J = 8.5$ Hz, 2H; H-3 and H-5 or H-2 and H-6), 8.05 (m, 1H, NH), 7.60 (t, $J = 4.9$ Hz, 1H; H-5''), 3.76-3.62 (m, 16H; CH_2) ppm; **^{13}C NMR** (100.6 MHz, $CDCl_3$): $\delta = 166.9$, 164.3, 163.3, 159.6 (quart. C), 158.6 (C-4'' and C-6''), 139.4, 133.7 (quart. C), 128.8 (C-2 and C-6 or C-3 and C-5), 128.6 (C-3 and C-5 or C-2 and C-6), 122.7 (C-5''), 72.6, 70.7, 70.6, 70.2, 69.9 (CH_2), 61.5 (CH_2OH), 40.2 (CH_2NH) ppm; **CHN analysis** (in %): found C 55.15, H 5.51, N 21.44 (calcd. C 55.38, H 5.53, N 21.53).

_N_-(15-Oxo-19-((4_S_)-2-oxohexahydro-1_H_-thieno[3,4-_d_]imidazol-4-yl)-4,7,10-trioxa-14-azanonadecyl)-4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamide (12)

A solution of *N*-(13-amino-4,7,10-trioxatridecanyl)biotinamide[5] (390 mg, 0.88 mmol) in DMF (1 mL) was added to a solution of 2,5-dioxopyrrolidin-1-yl 6-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoate[3] (300 mg, 0.8 mmol) in a mixture of DMF (15 mL) and pyridine (1.5 mL). After stirring the solution for 30 h at room temperature, the solvents were evaporated. The residue was dissolved in CH_2Cl_2 (40 mL) and washed with water (40 mL). The aqueous phase was extracted with CH_2Cl_2 (3 x 40 mL). The combined organic extracts were washed with water (40 mL) and dried ($MgSO_4$). The solvents were removed under reduced pressure and the residue was purified by silica gel chromatography (CH_2Cl_2/MeOH 8:1 to 7:1) to afford **12** as a red crystalline solid (98 mg, 17 %). **TLC**: $R_f = 0.1$ (CH_2Cl_2/MeOH 10:1); **^{1}H NMR** (400.1 MHz, $CDCl_3$): $\delta = 9.11$ (d, $J = 4.9$ Hz, 2H; H-4‘‘ and H-6‘‘), 8.74 (d, $J = 8.5$ Hz, 2H; H-2 and H-6 or H-3 and H-5), 8.09 (d, $J = 8.5$ Hz, 2H; H-3 and H-5 or H-2 and H-6), 8.25 (t, $J = 5.3$ Hz, 1H; NH), 7.59 (t, $J = 4.9$ Hz, 1H; H-5‘‘), 6.7 (t, $J = 5.5$ Hz, 1H; NH), 6.46 (bs, 1H; NH), 4.47 (dd, $J = 7.9$, 4.7 Hz, 1H; C<u>H</u>NH), 4.26 (dd, $J = 7.8$, 4.6 Hz, 1H; C<u>H</u>NH), 3.72-3.47 (m, 16H; CH_2CH_2O and CH_2), 3.26 (q, $J = 6.2$ Hz, 2H; CH_2), 3.08 (td, $J = 7.3$, 4.5 Hz, 1H; CHS), 2.84 (dd, $J = 12.8$, 4.8 Hz, 1H; CH_{exo}), 2.7 (d, $J = 12.7$ Hz, 1H; CH_{endo}), 2.14 (t, $J = 7.5$ Hz, 2H, CH_2), 1.92 (quin., $J = 5.8$ Hz, 2H; CH_2), 1.81-1.55 (m, 6H; CH_2), 1.36 (quin., $J = 7.3$ Hz, 2H; CHC<u>H$_2$</u>) ppm; **^{13}C NMR** (100.6 MHz, $CDCl_3$)): $\delta = 173.3$, 166.3, 164.2, 164.0, 163.2, 159.4 (quart. C), 158.5 (C-4‘‘ and C-6‘‘), 139.1, 133.6 (quart. C), 128.9 (C-2 and C-6 or C-3 and C-5), 128.3 (C-3 and C-5 or C-2 and C-6), 122.8 (C-5‘‘), 70.7, 70.52, 70.46, 70.3, 70.0, 69.9 (OCH_2), 61.9 (CHNH), 60.3 (CHNH), 55.7 (CHS), 40.6 (CH_2S), 39.1, 37.7, 36.1, 29.1, 28.9, 28.3, 28.2, 25.8 (CH_2) ppm; **ESI-TOF-MS**: found 709.3217 $[M + H]^+$, 731.3037 $[M + Na]^+$, 747.2788 $[M + K]^+$ (calcd. 709.3239 $[M + H]^+$, 731.3058 $[M + Na]^+$, 747.2798 $[M + K]^+$).

Kinetic Measurements

Kinetic measurements were carried out under second order conditions. Peracetylated compounds were deacetylated under standard Zemplén conditions[6]. Stock solutions of **11** (6 mM or 10 mM) and deacetylated dienophiles **1**, **3** or **5** (6 mM or 10 mM) in acetate buffer (pH 4.8) were mixed in a quartz cuvette. To monitor the reaction over time the absorption at 522 nm was measured (Figures S10 and S13). The absorption at 522 nm corresponds to the concentration of tetrazine **11**. Product formation was confirmed by HPLC and ESI-MS analysis (cf. Figures S11 and S12). The second-order rate constant was determined by plotting 1/[tetrazine] *versus* time, followed by analysis by linear regression. All measurements were carried out at least in duplicates. Stability of tetrazine **11** was verified by measuring the absorption at 522 nm of a solution of **11** in acetate buffer (pH 4.8) (Figure S14).

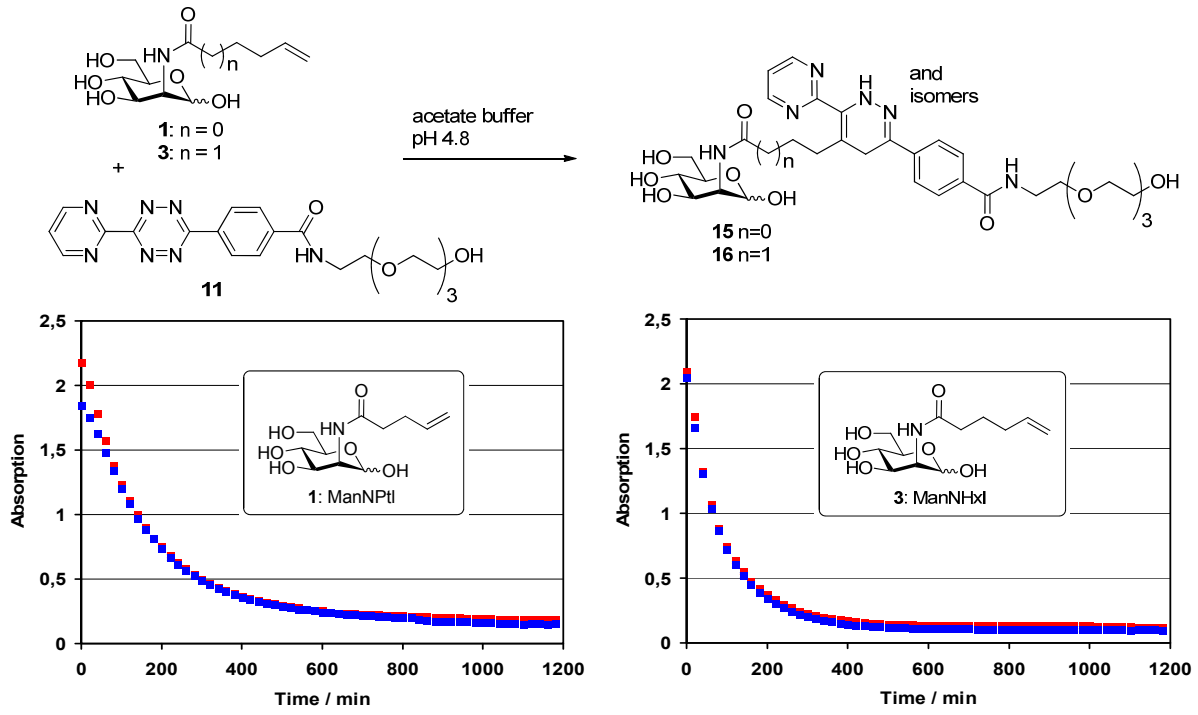

Figure S10. Monitoring of the reaction of tetrazine **11** (5 mM) with ManNPtl **1** (5 mM) (left) or ManNHxl **3** (5 mM) (right) in acetate buffer by measuring the absorption of **11** at 522 nm in duplicate.

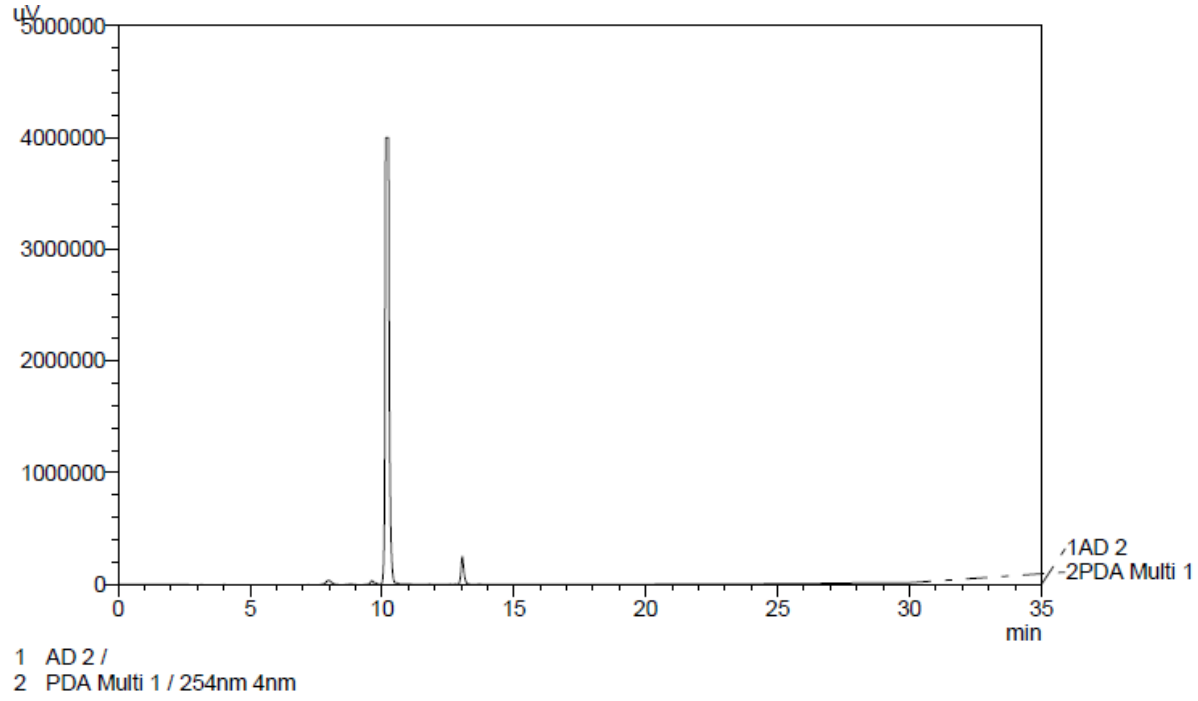

Figure S11. HPLC analysis of tetrazine **11** using a gradient of CH_3CN in H_2O with 0.1 % formic acid (20-90 % in 20 min).

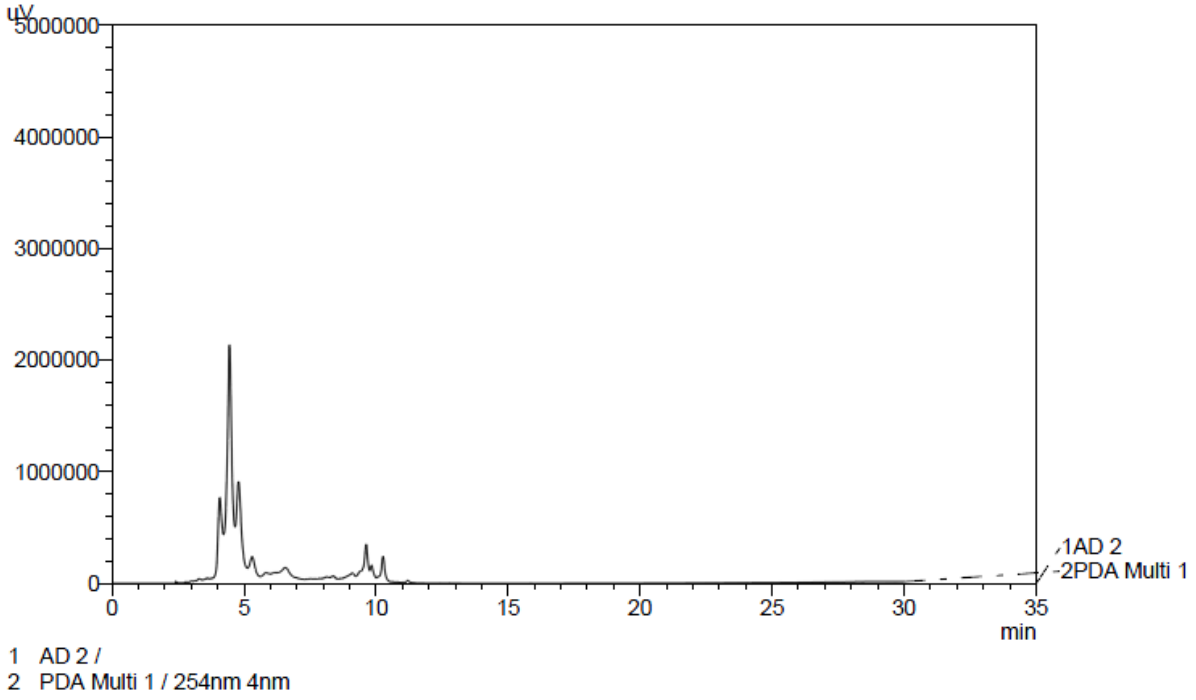

Figure S12. HPLC analysis of crude reaction mixture after reaction of tetrazine **11** with ManNHxl **3.** A gradient of CH_3CN in H_2O with 0.1 % formic acid (20-90 % in 20 min) was used. The major peaks were further analyzed by ESI-MS and showed the expected mass of the oxidized forms of dihydropyridazines **16**. **ESI-MS**: found 701.2 $[M + H]^+$, 723.1 $[M + Na]^+$, 739.0 $[M + K]^+$, calcd: 701.3 $[M + H]^+$,723.3 $[M + Na]^+$, 739.3 $[M + K]^+$

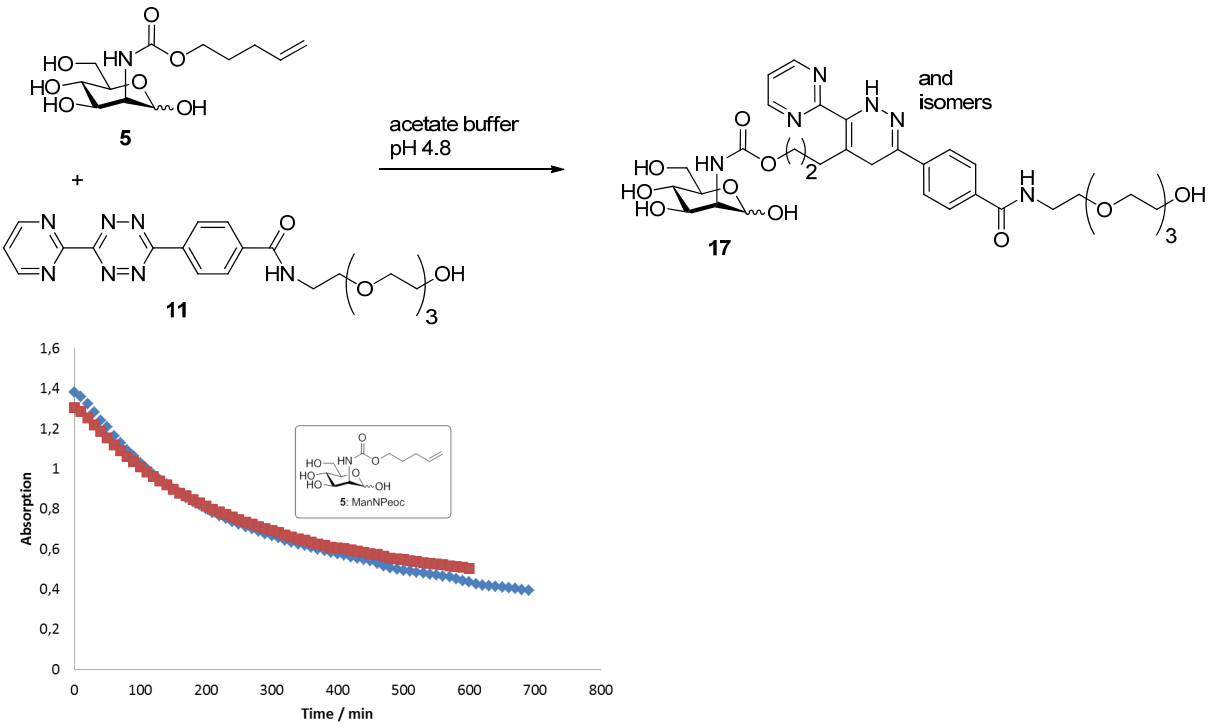

Figure S13. Reaction of tetrazine **11** (3 mM) with ManNPeoc **5** (3 mM) in acetate buffer monitored by measuring the absorption of **11** at 522 nm in duplicate.

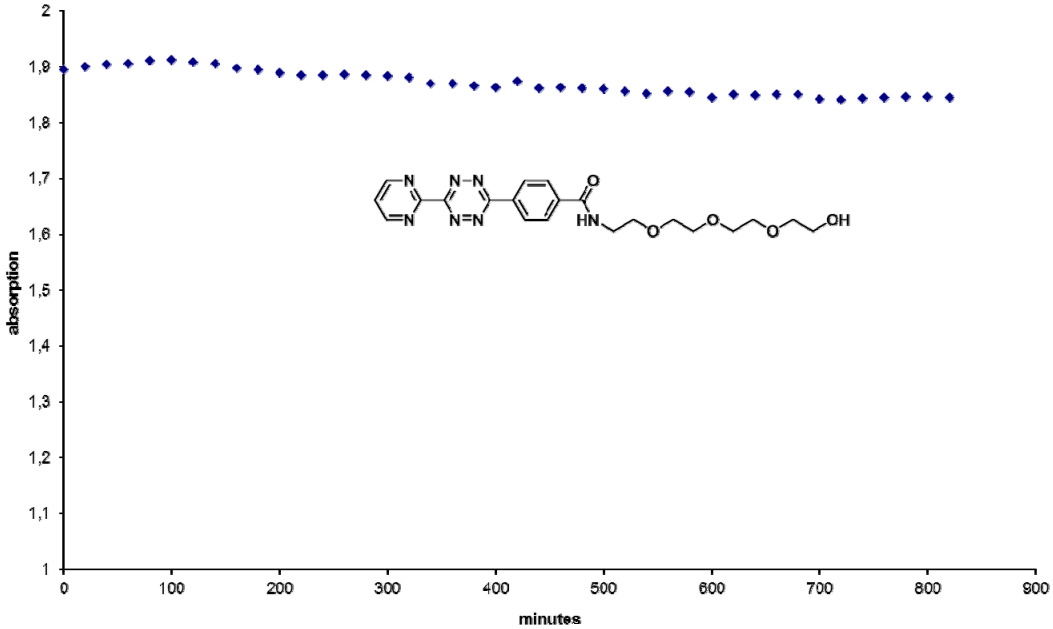

Figure S14. Stability of tetrazine **11** in acetate buffer (pH 4.8) at a concentration of 5 mM. Absorption at 522 nm was measured over time and no significant decrease could be observed.

Cell Growth Conditions

HeLa S3 and HEK293 T cells were grown in Dulbecco's Modified Essential Medium 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

Cells (5000 cells/cm^2) were grown in 8 well ibiTreat µ-Slides (ibidi). 12 h after seeding the cells were incubated for three days with the specified functionalized mannosamine derivatives. No sugar was added as negative control. For live-cell experiments, cells were washed three times with PBS and then treated with tetrazine biotin **12** (1 mM) for 6 hours at 37 °C. After three washes with PBS, cells were incubated with AlexaFluor®647-labeled streptavidin (6.6 µg/mL) for 20 minutes at room temperature in the dark. The cells were washed three times with PBS and medium was added. For experiments with fixed cells, cells were incubated for 8 min at room temperature with aqueous paraformaldehyde (4 %) before incubation with biotinylated tetrazine **12**.

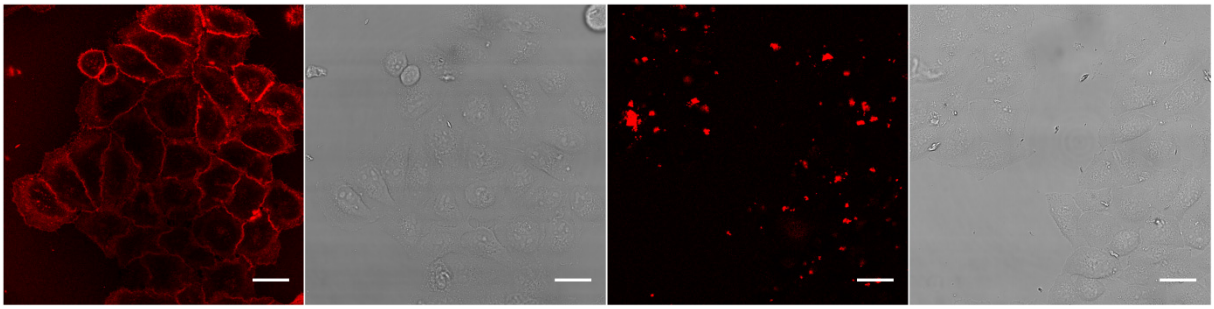

Figure S15. HeLa S3 cells were grown for 2 days with 100 µM Ac_4ManNPtl **2** (left) or without sugar (right). Living cells were labeled with 1 mM tetrazine biotin **12** at 37 °C for 6 h followed by incubation with AlexaFluor®647-streptavidin for 20 min at rt. (Scale bar 30 µm)

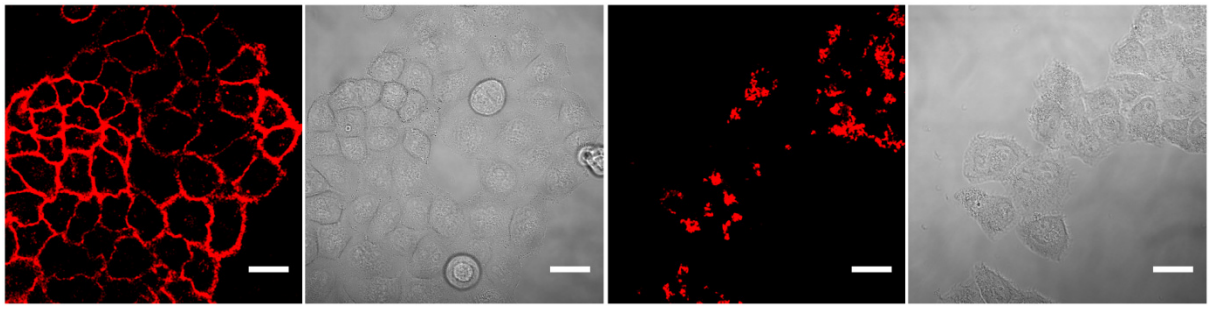

Figure S16. HeLa S3 cells were grown for 2 days with 100 µM Ac_4ManNHxl **4** (left) or without sugar (right). Living cells were labeled with 1 mM tetrazine biotin **12** at 37 °C for 6 h followed by incubation with AlexaFluor®647-streptavidin for 20 min at rt. (Scale bar 30 µm)

For dual labeling experiments cells (5000 cells/cm^2) were grown in 8 well ibiTreat µ-Slides (ibidi). 12 h after seeding the cells were incubated for three days with either Ac_4ManNPtl **2** (final concentration 100 µM) or Ac_4GalNAz **14** (final concentration 25 µM) or both or without sugar as negative control. Cells were washed three times with PBS and then incubated with 1 mM tetrazine biotin **12** for 6 hours at 37 °C. After three washes with PBS, cells were incubated with AlexaFluor®647-labeled streptavidin (6.6 µg/ mL) for 20 minutes at rt in the dark. The cells were washed three times with PBS before incubation with AlexaFluor®488-DIBO **13** (final concentration 4 µM) for 30 minutes at rt. After three washes, medium was added. A Zeiss LSM 510 Meta equipped with a 40 x 1.3 NA Plan-Neofluar oil DIC immersion objective was employed for imaging. Analysis of the obtained data was carried out using Image J software version 1.45 S.

Cytotoxicity Assay

Cells were seeded in 96-well plates (4000 HeLa S3 cells/well) and allowed to grow for 24 h. Then they were treated with different concentrations of the compound to be tested. Solutions of compounds were prepared by dissolving the respective compound in PBS and diluting with medium to give final concentrations. Cells were incubated for 72 h, the media were discarded and a solution of AlamarBlue® (1:10 in DMEM, 100 µL/well) was added and the cells were incubated for 1.5 hours. After excitation at 530 nm, fluorescence at 590 nm was measured using a Synergy 2 HT Fluorescence Microplate Reader (*BioTek*). Cell viability is expressed in percent with respective to a control containing only pure medium. All experiments were repeated for a minimum of three times with each experiment done in four replicates. The resulting curves were fitted using Sigma plot 8.0 and EC_{50} values for 50 % cell viability were determined (Table S1).

Table S1. Results of the cytotoxicity assay.

Compound	*EC_{50} [µM]*
Ac_4ManNPtl **2**	253 ± 28
Ac_4ManNHxl **4**	252 ± 21
Ac_4ManNPeoc **6**	245 ± 13

NMR spectra

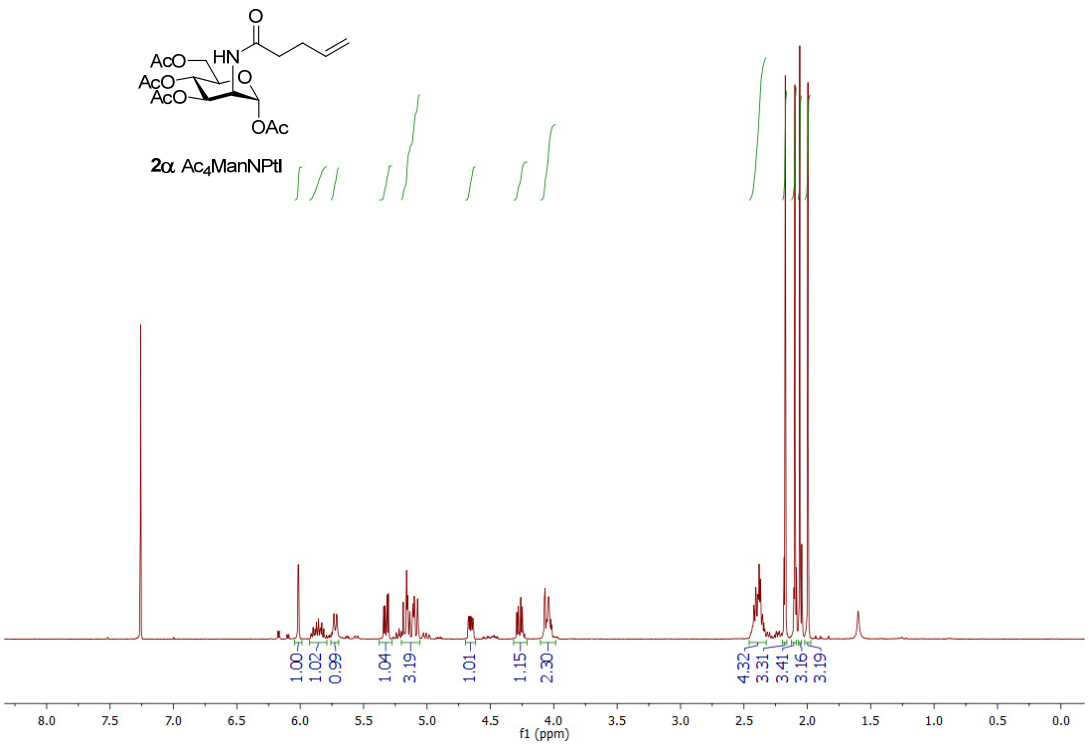

Figure S17. ^{1}H NMR spectrum ($CDCl_3$, 400.1 MHz) of the α isomer of compound **2**.

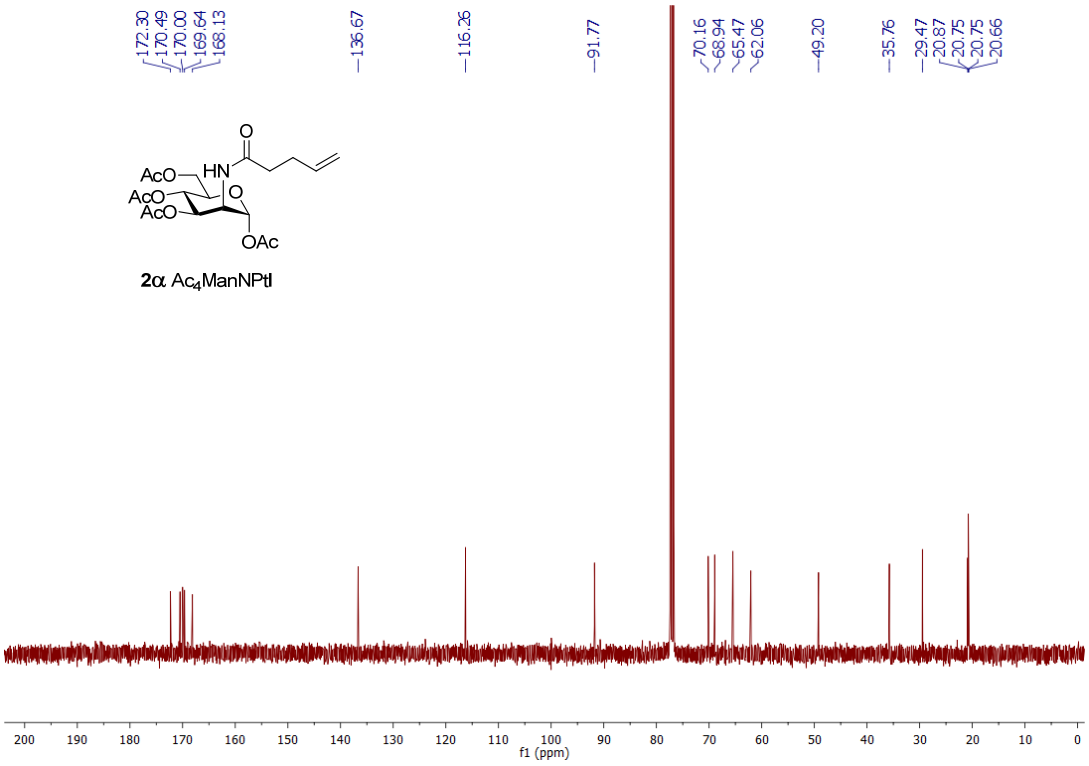

Figure S18. ^{13}C NMR spectrum (CDCl3, 100.6 MHz) of the α isomer of compound **2**.

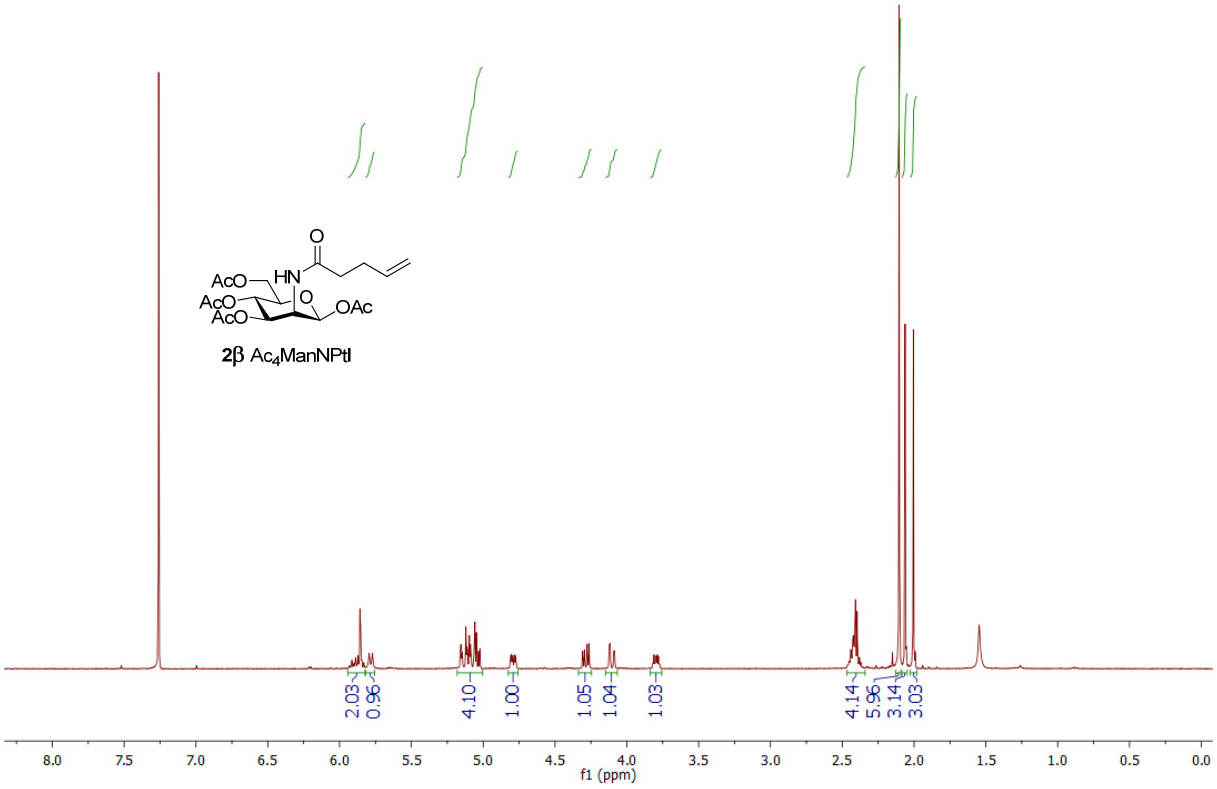

Figure S19. ^{1}H NMR spectrum ($CDCl_3$, 400.1 MHz) of the β isomer of compound **2**.

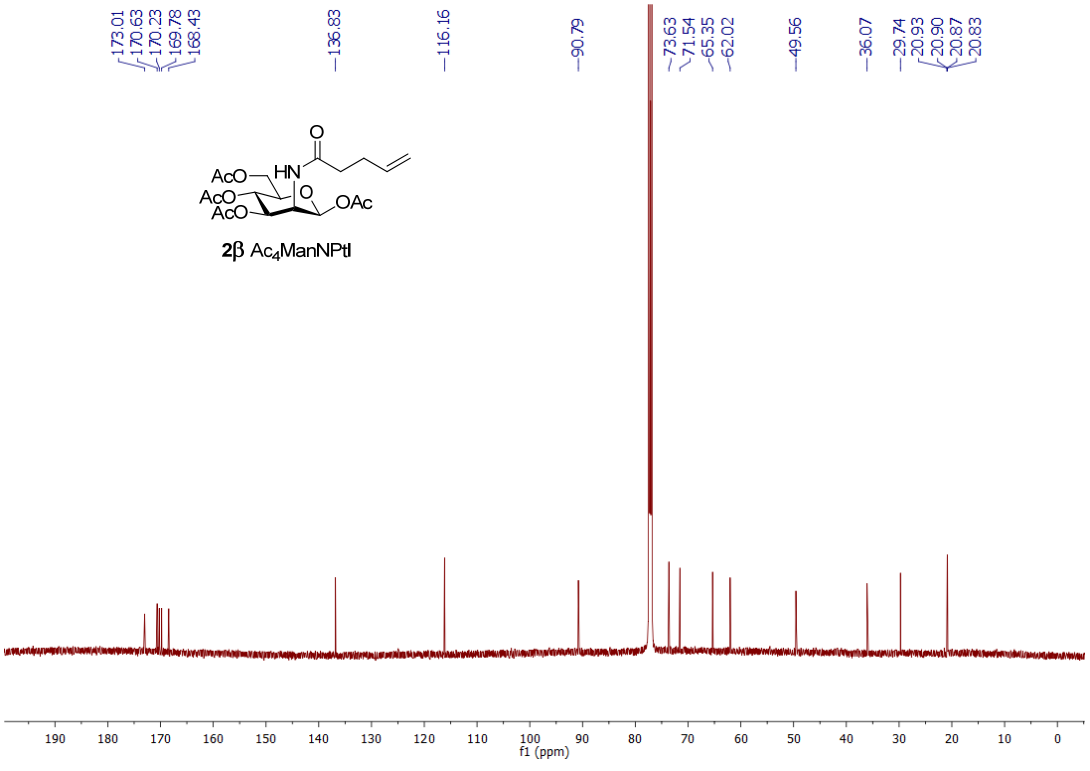

Figure S20. ^{13}C NMR spectrum ($CDCl_3$, 150.9 MHz) of the β isomer of compound **2**.

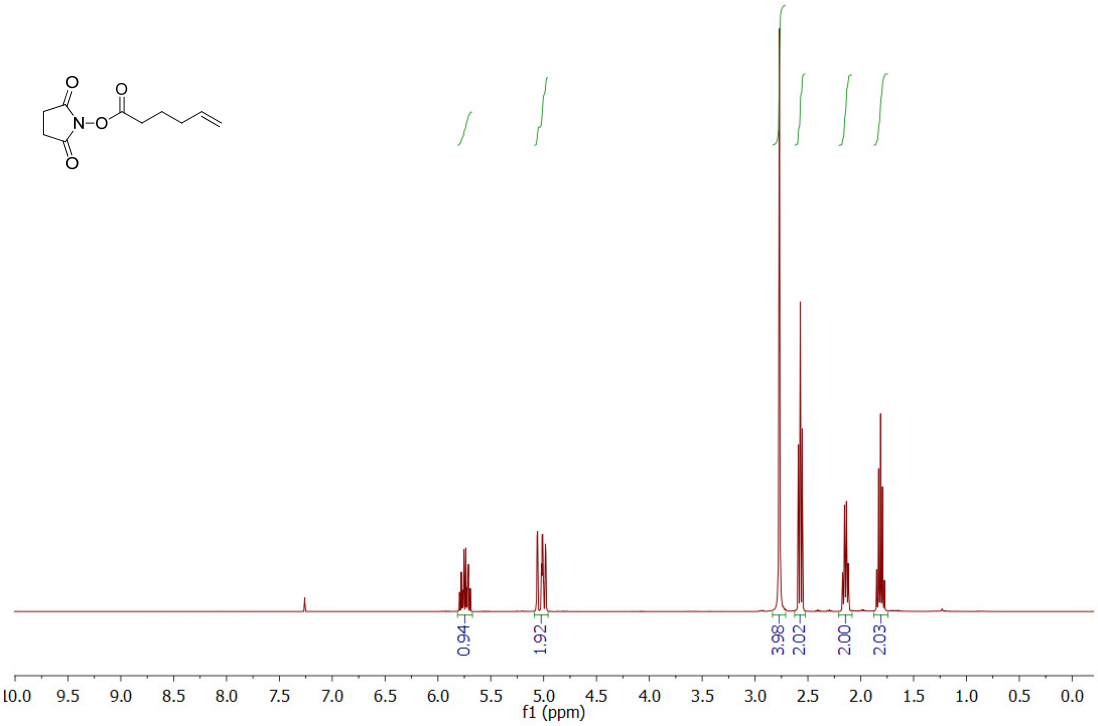

Figure S21. ^{1}H NMR spectrum ($CDCl_3$, 400.1 MHz) of succinimidyl hex-5-enoate.

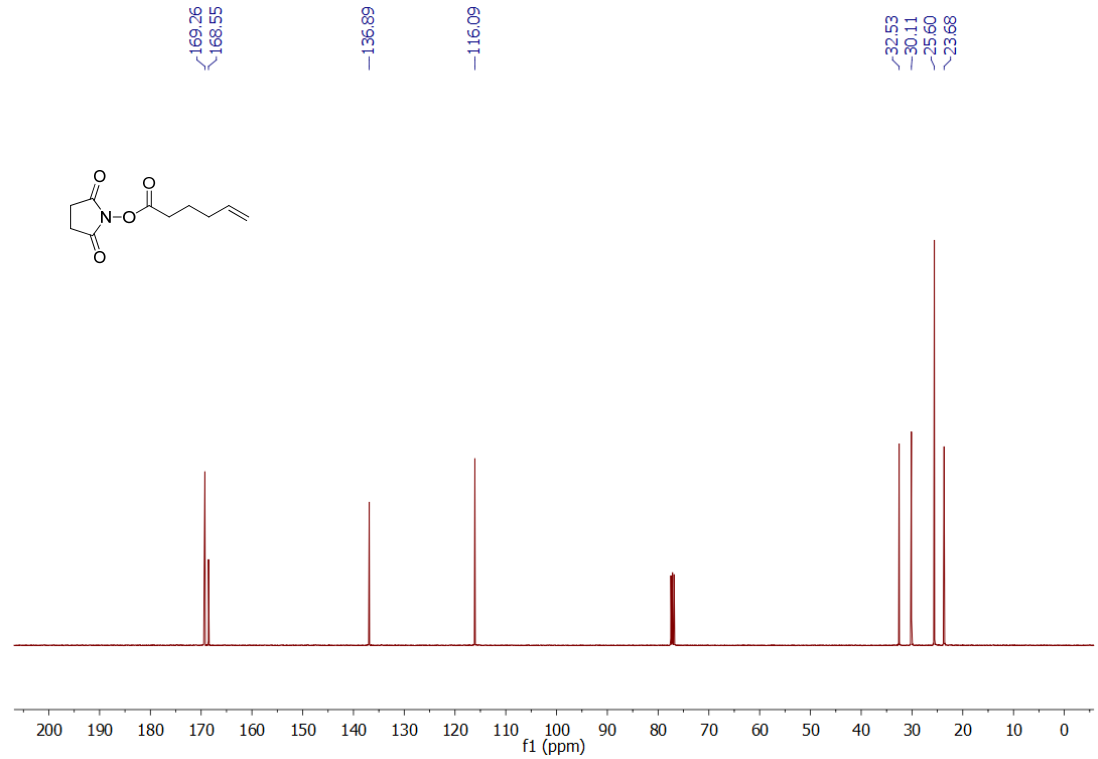

Figure S22. ^{13}C NMR spectrum ($CDCl_3$, 100.6 MHz) of succinimidyl hex-5-enoate.

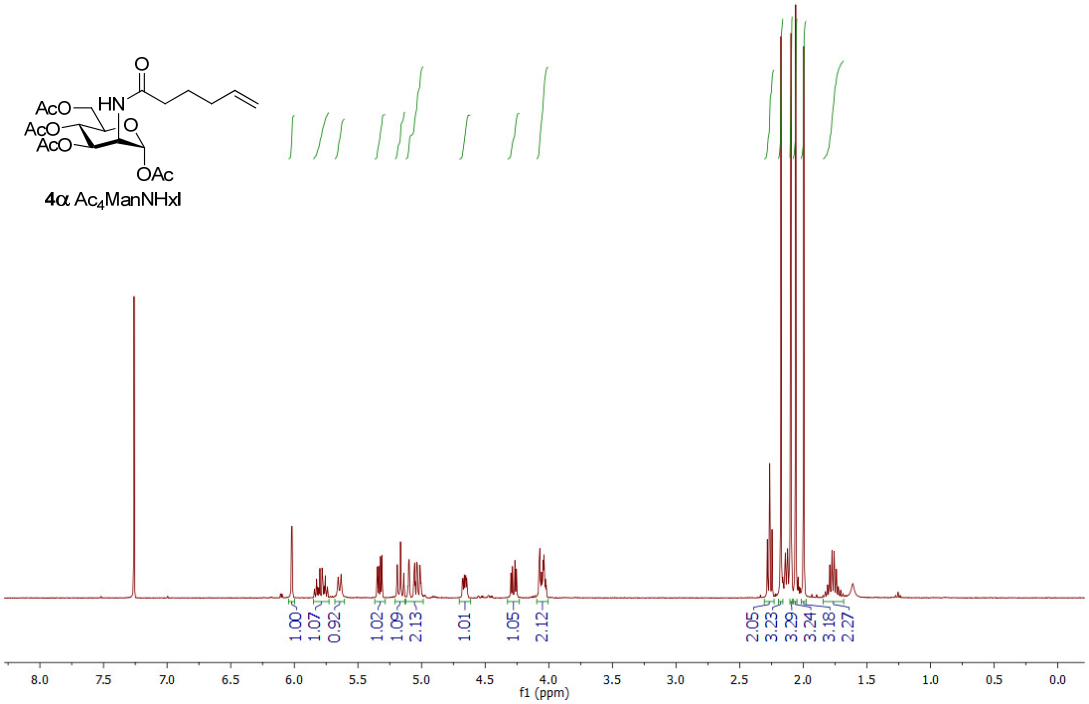

Figure S23. ^{1}H NMR spectrum ($CDCl_3$, 400.1 MHz) of the α isomer of compound **4**.

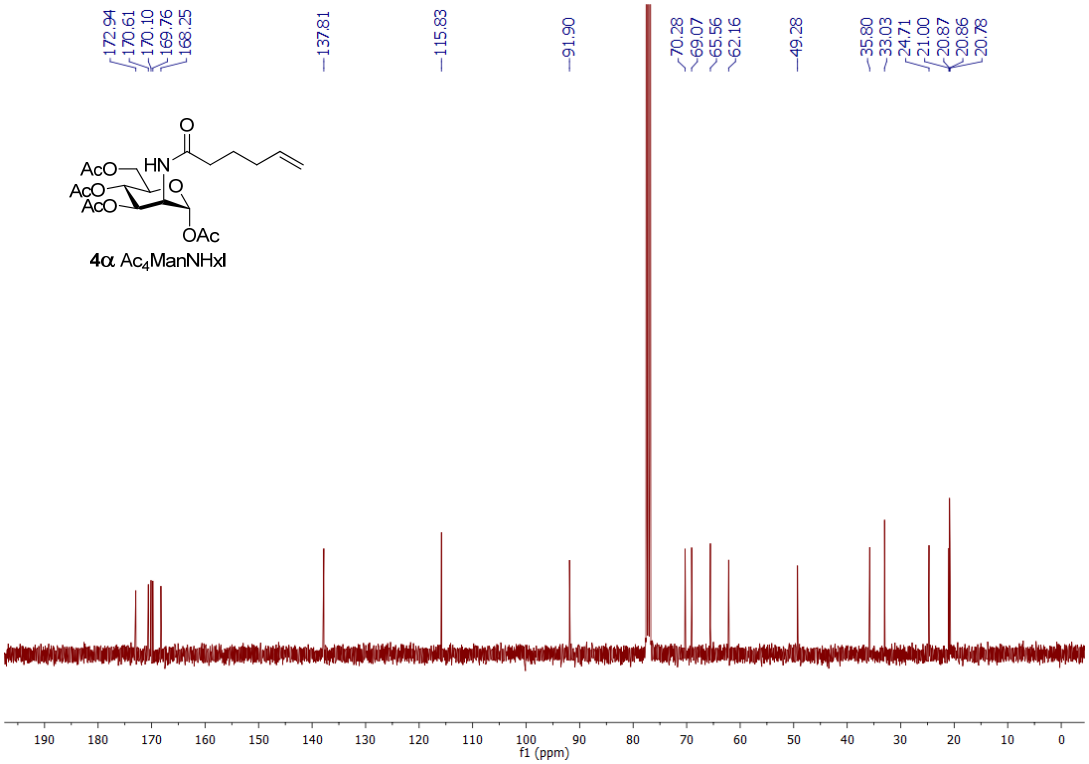

Figure S24. ^{13}C NMR spectrum ($CDCl_3$, 100.6 MHz) of the α isomer of compound **4**.

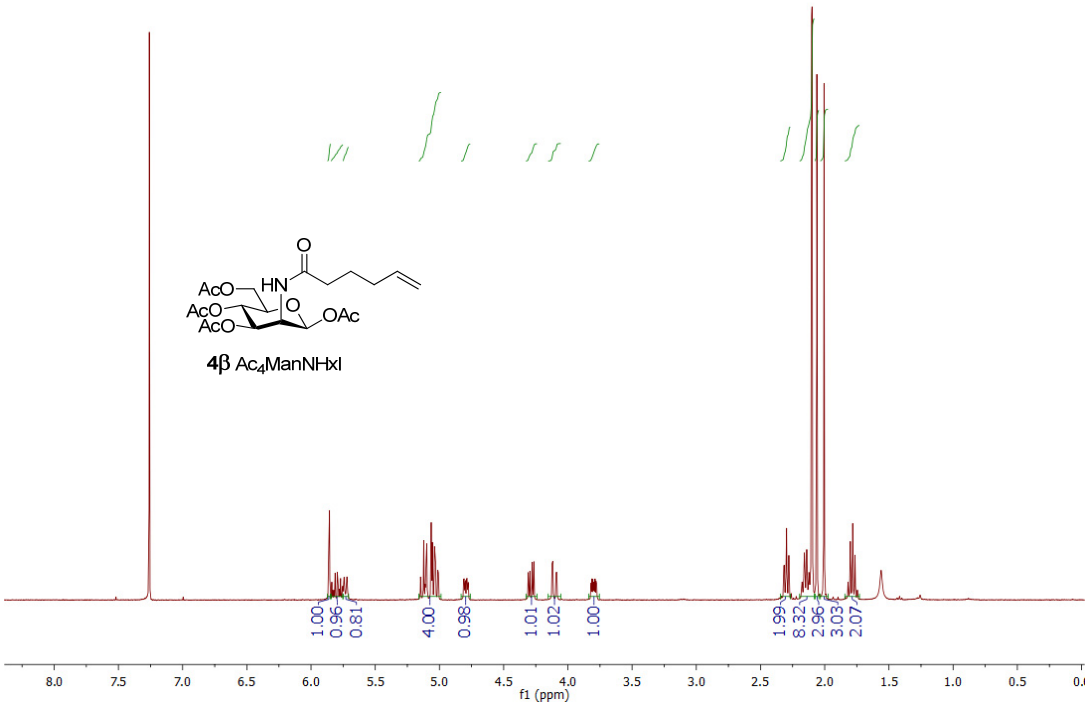

Figure S25. ^{1}H NMR spectrum ($CDCl_3$, 400.1 MHz) of the β isomer of compound **4**.

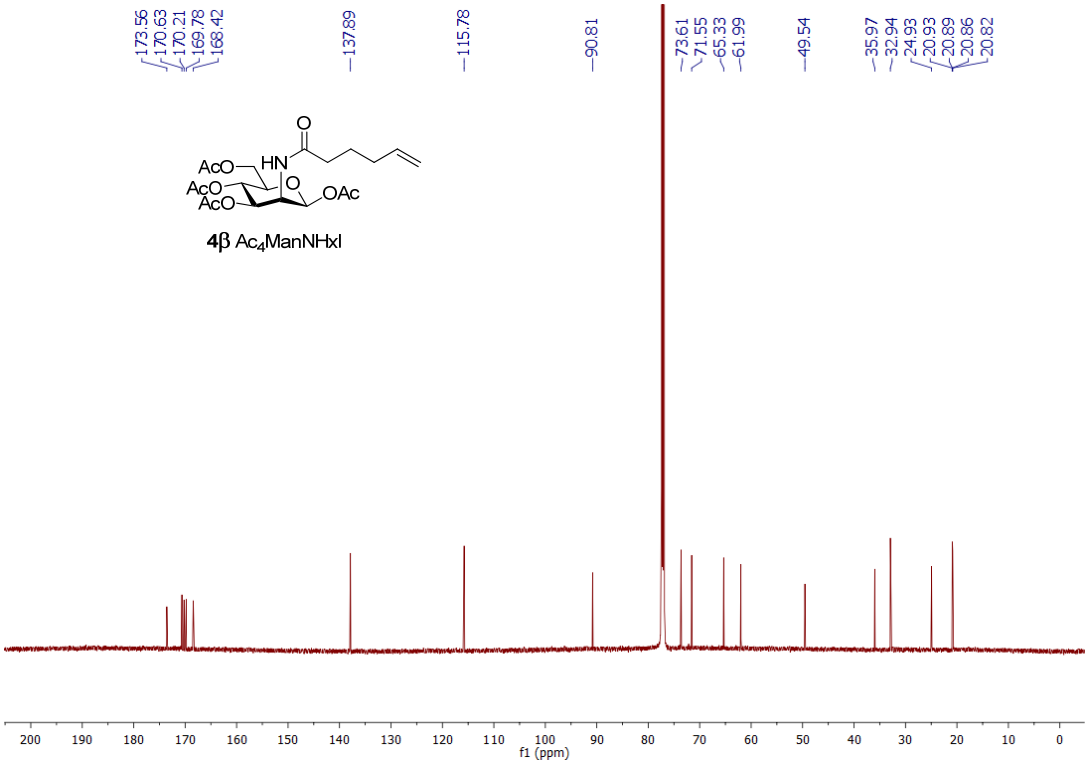

Figure S26. ^{13}C NMR spectrum ($CDCl_3$, 150.9 MHz) of the β isomer of compound **4**.

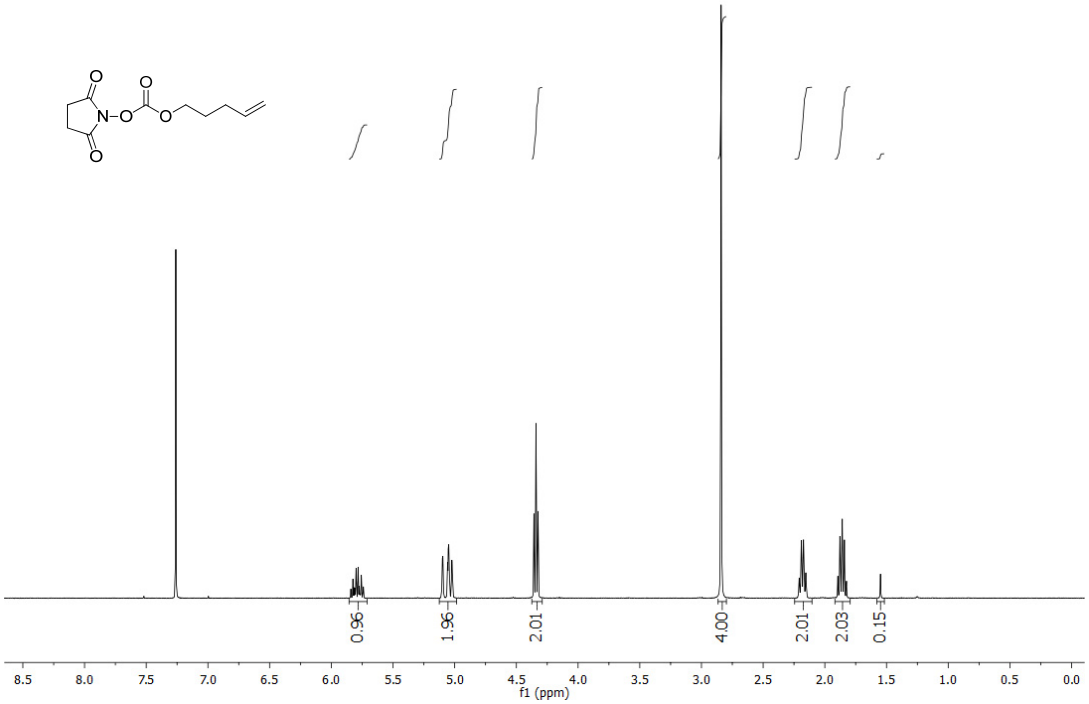

Figure S27. ^{1}H NMR spectrum ($CDCl_3$, 400.1 MHz) of succinimidyl pent-4-en-1-yl carbonate.

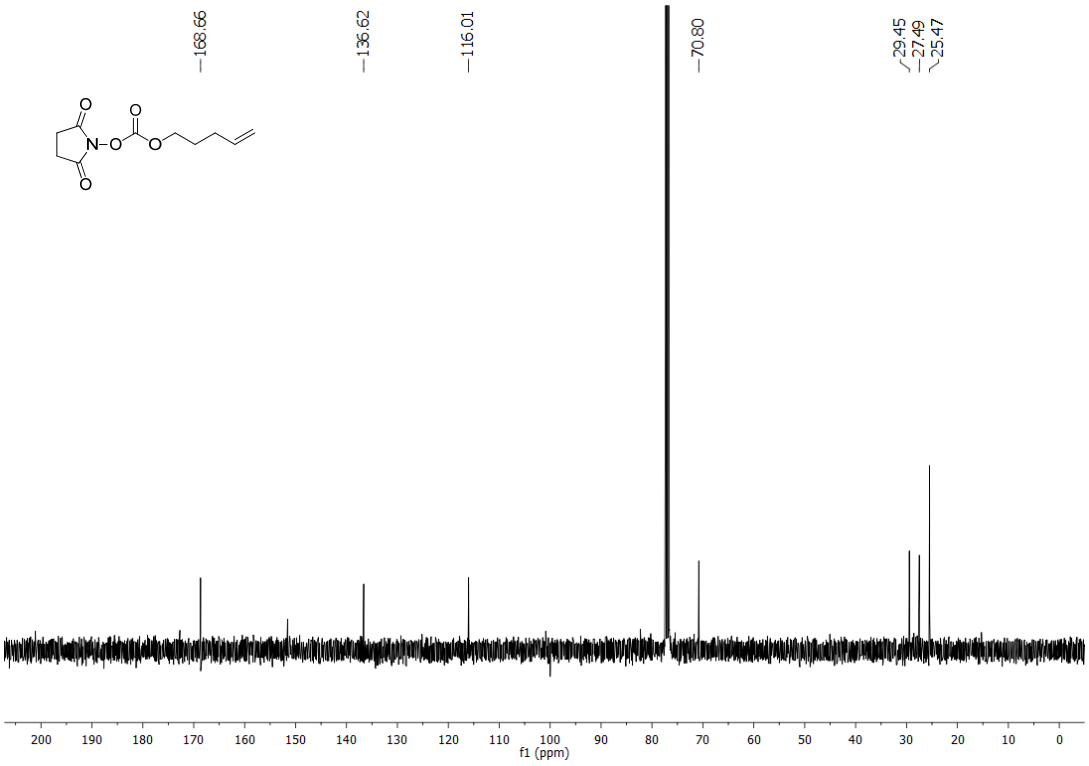

Figure S28. ^{13}C NMR spectrum ($CDCl_3$, 100.6 MHz) of succinimidyl pent-4-en-1-yl carbonate.

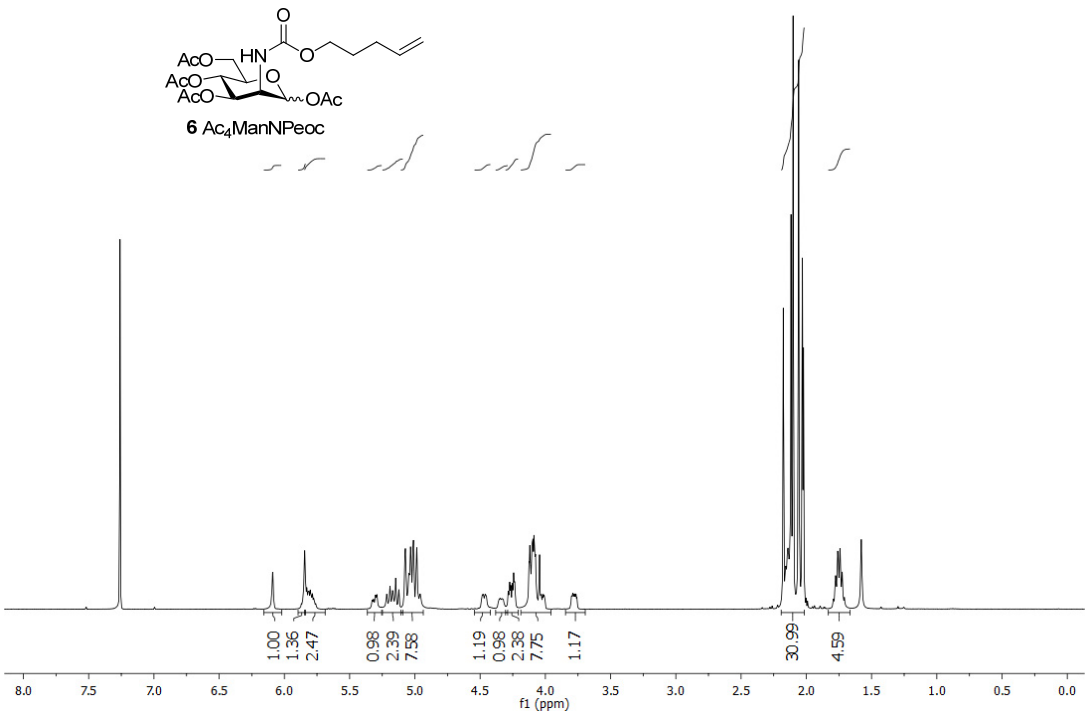

Figure S29. ^{1}H NMR spectrum (400.1 MHz, $CDCl_3$) of compound **6**.

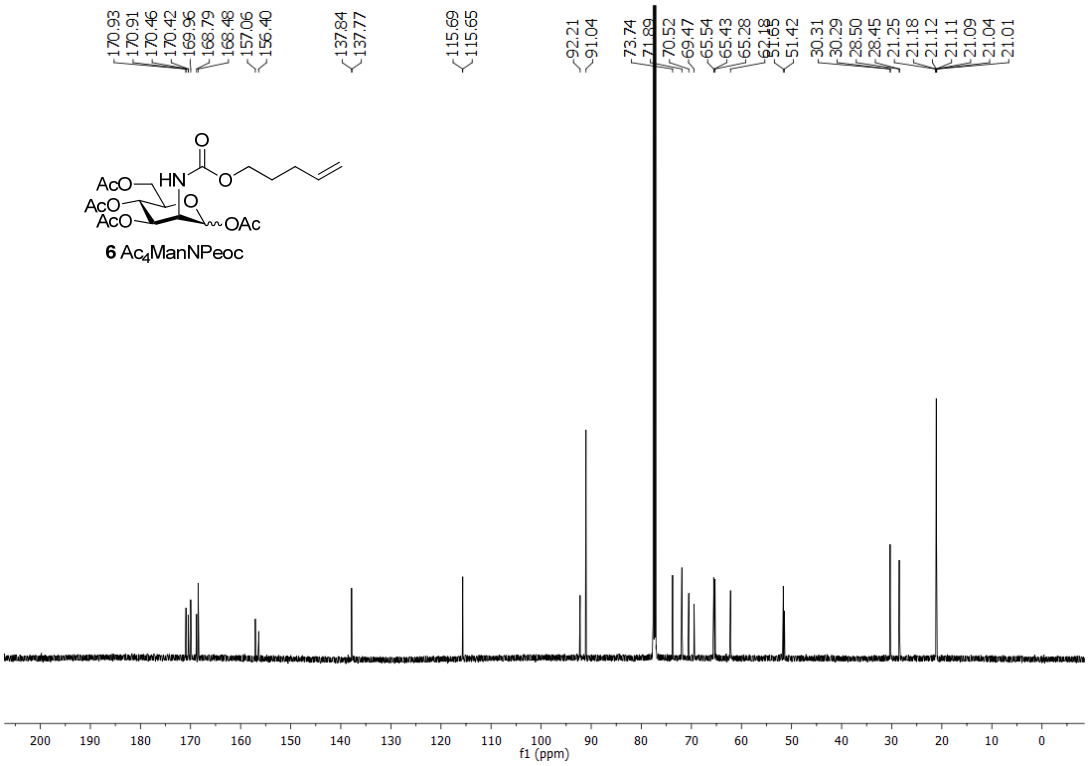

Figure S30. ^{13}C NMR spectrum ($CDCl_3$, 100.6 MHz) of compound **6**.

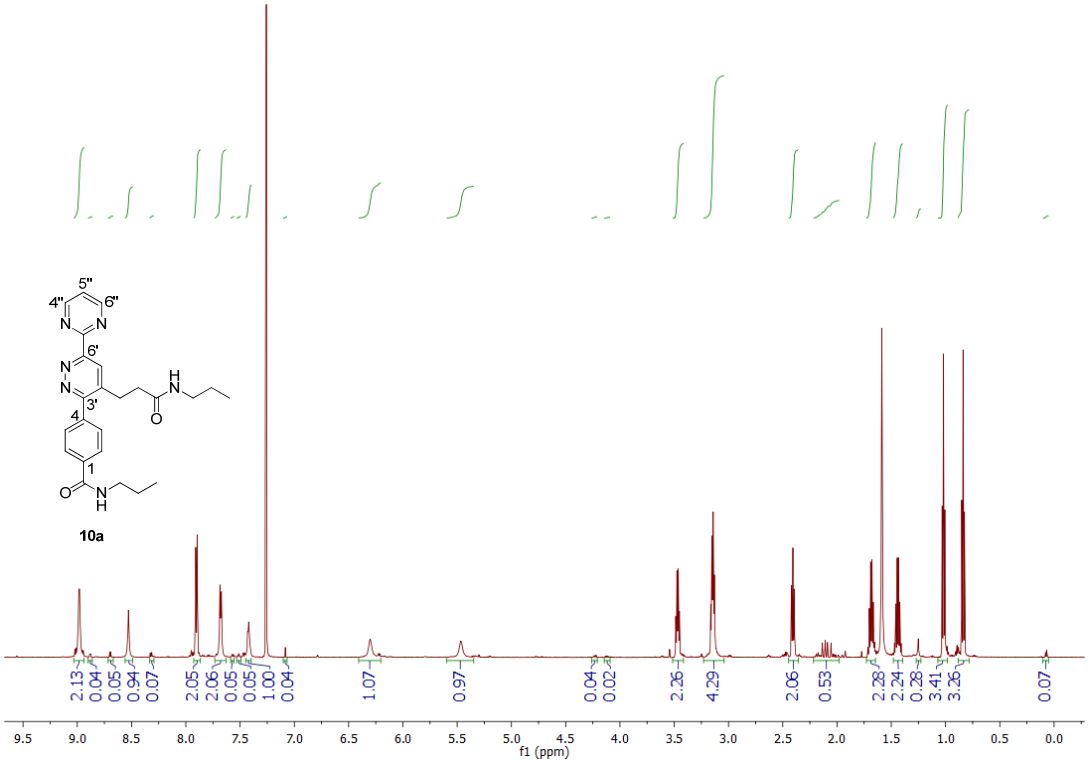

Figure S31. ^{1}H NMR spectrum (400.1 MHz, $CDCl_3$) of compound **10a**.

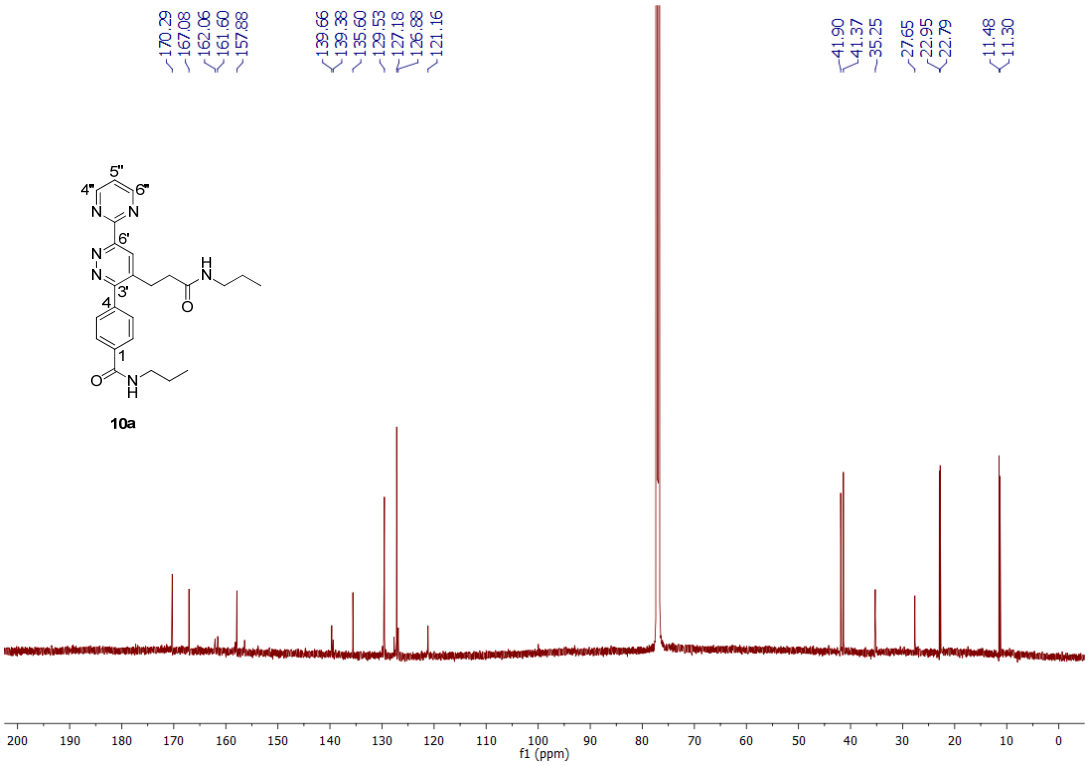

Figure S32. ^{13}C NMR spectrum (100.6 MHz, $CDCl_3$) of compound **10a**.

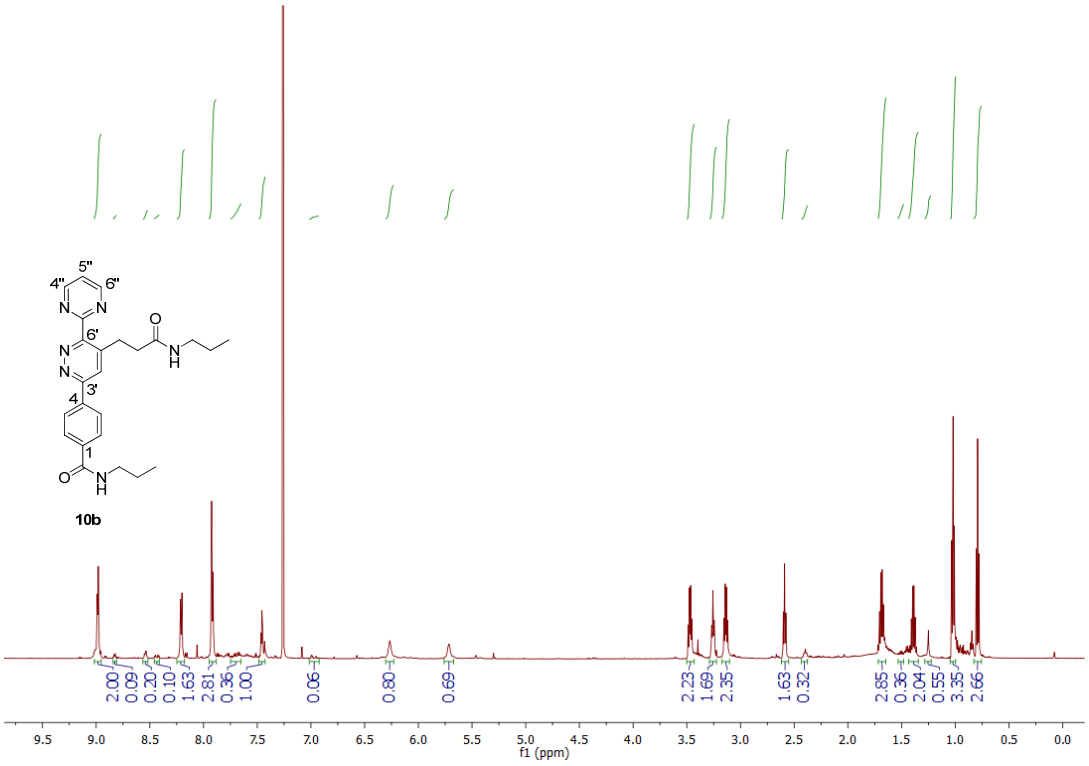

Figure S33. ^{1}H NMR spectrum (400.1 MHz, $CDCl_3$) of compound **10b**.

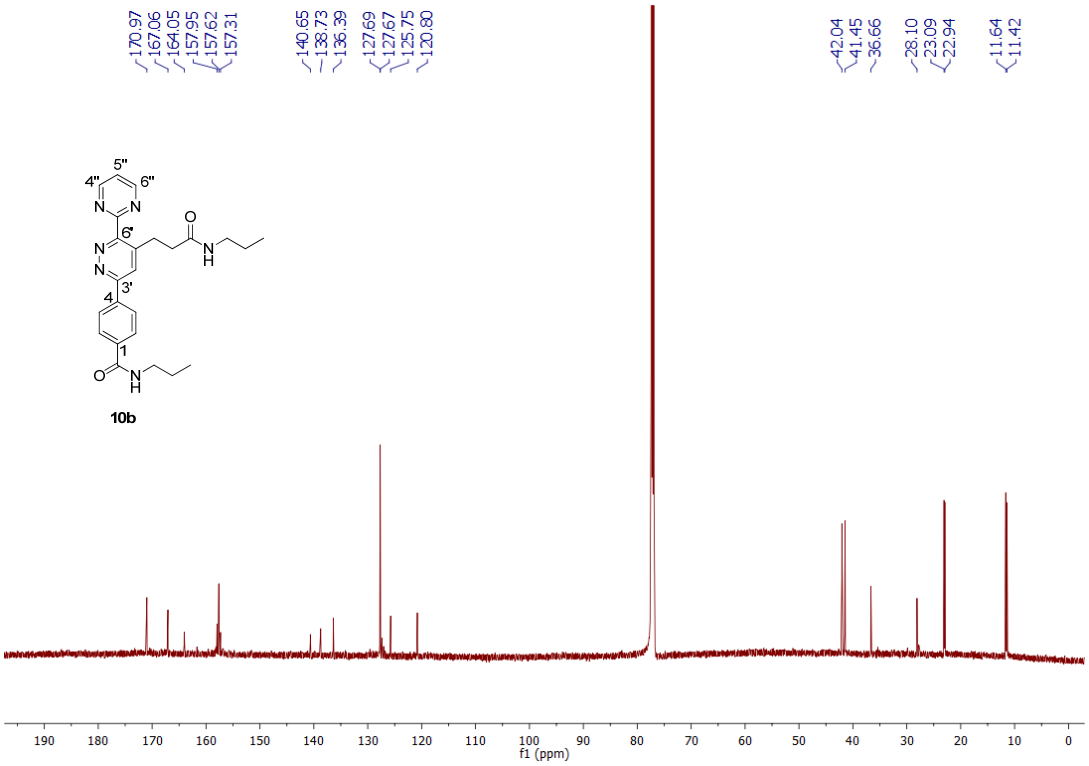

Figure S34. ^{13}C NMR spectrum (100.6 MHz, $CDCl_3$) of compound **10b**.

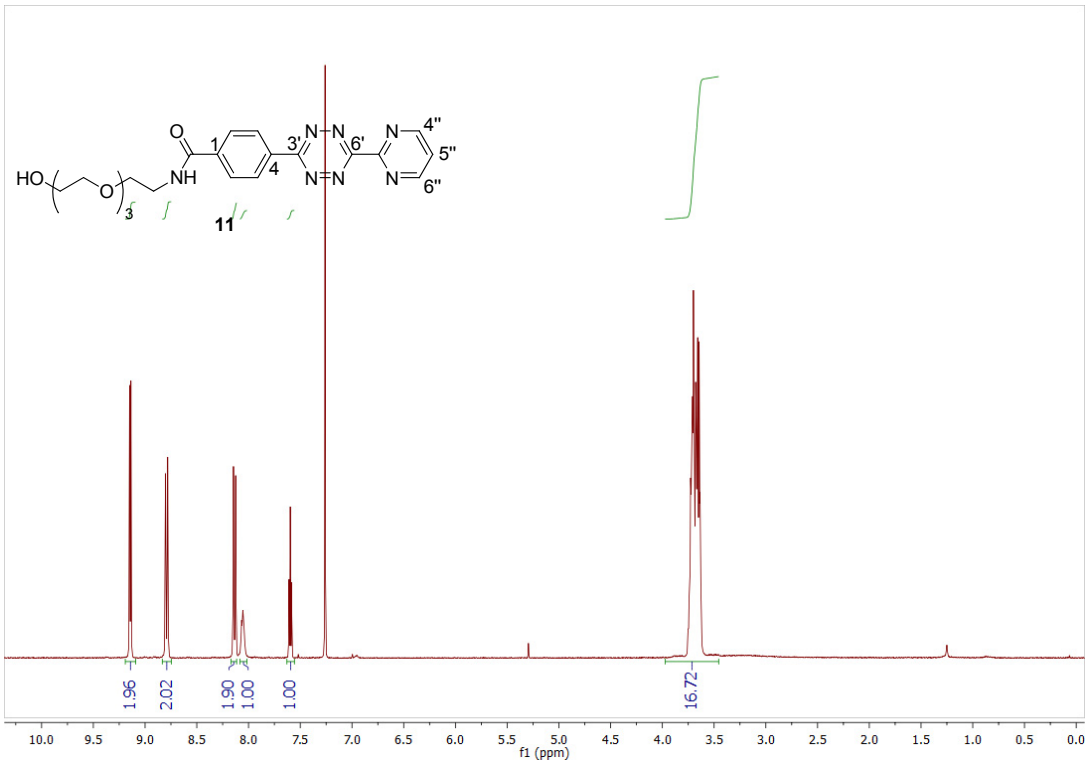

Figure S35. ^{1}H NMR spectrum ($CDCl_3$, 400.1 MHz) of compound **11**.

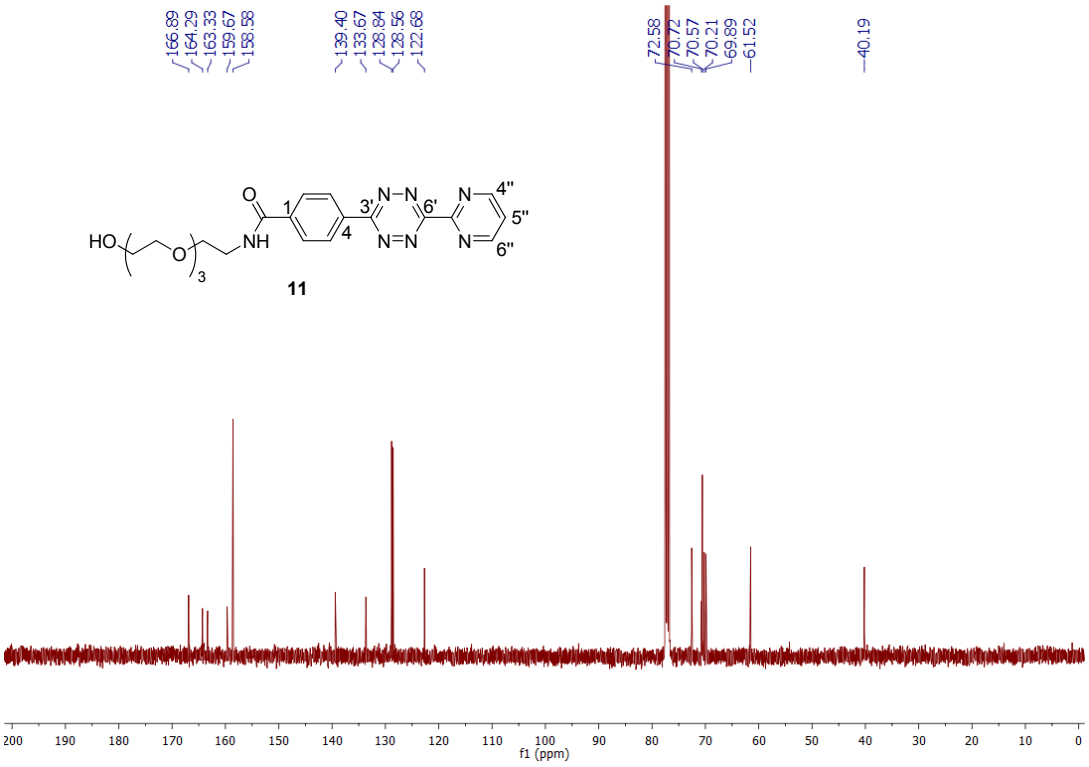

Figure S36. ^{13}C NMR spectrum ($CDCl_3$, 100.6 MHz) of compound **11**.

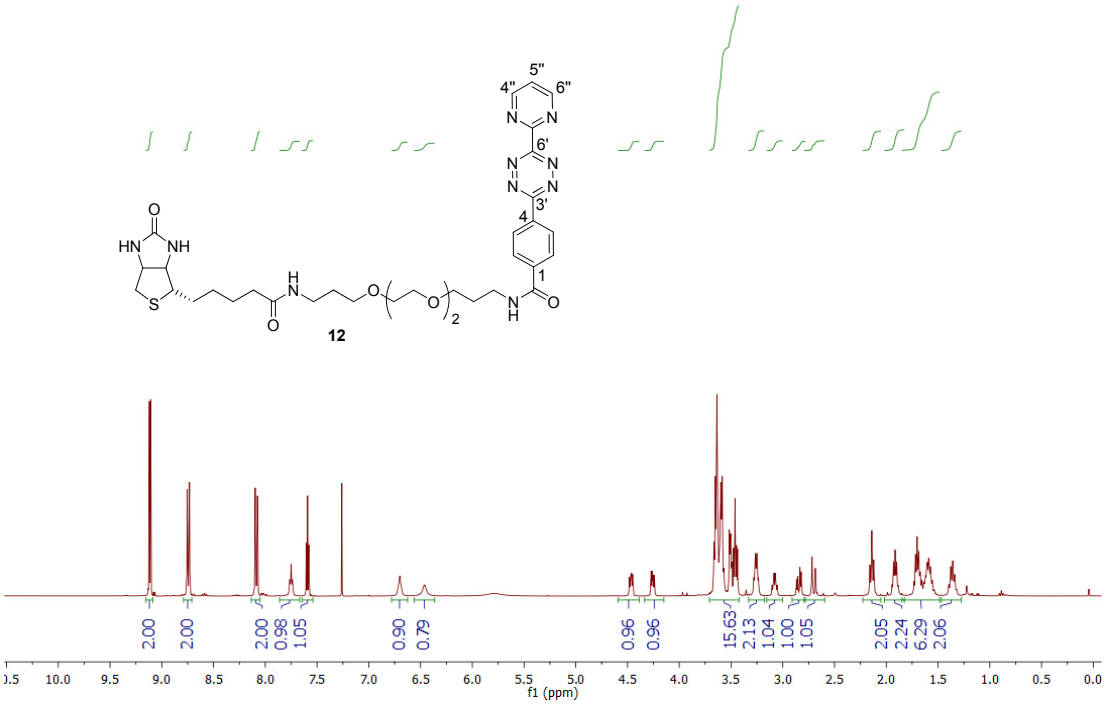

Figure S37. ^{1}H NMR spectrum ($CDCl_3$, 400.1 MHz) of compound **12**.

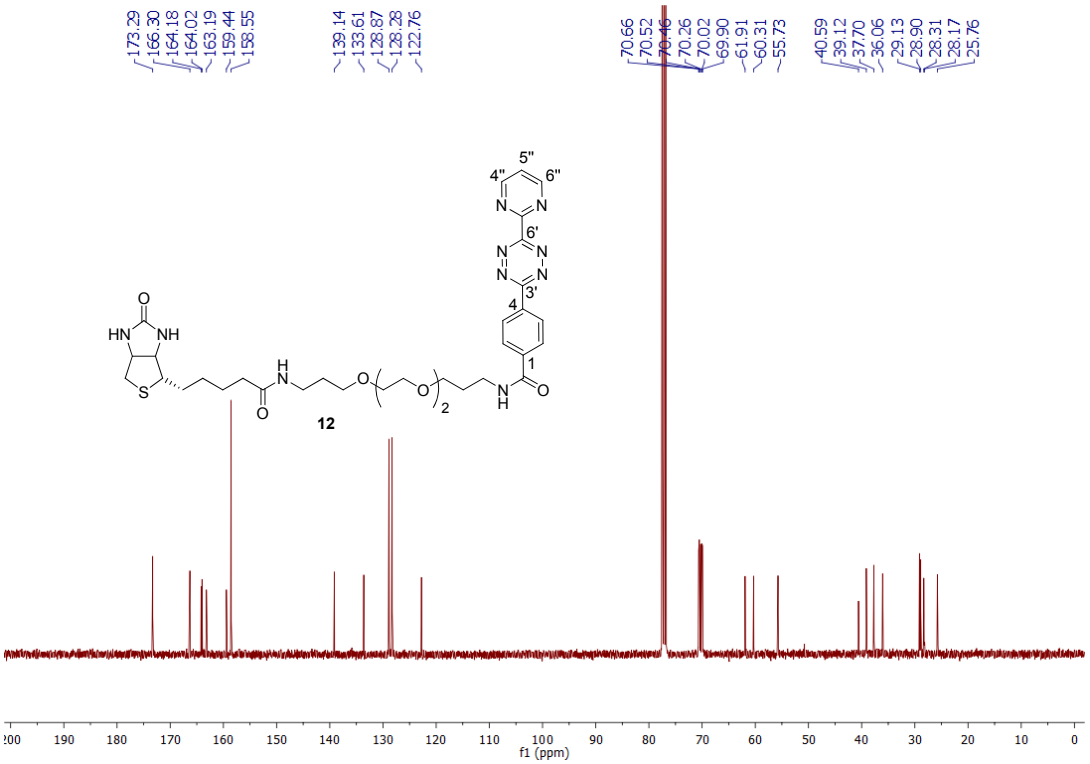

Figure S38. ^{13}C NMR spectrum ($CDCl_3$, 100.6 MHz) of compound **12**.

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