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Supporting Information

Next-Generation Metabolic Glycosylation Reporters Enable Detection of Protein O–GlcNAcylation in Living Cells without *S*-Glyco Modification

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Supporting Information

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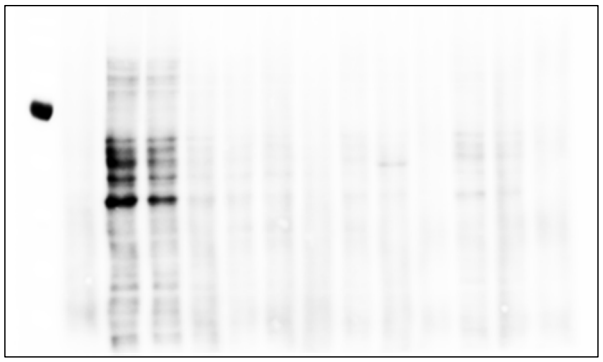

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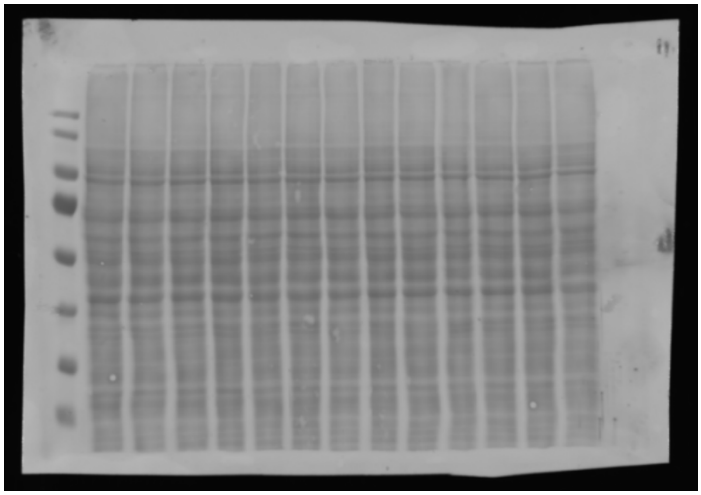

Ponceau S

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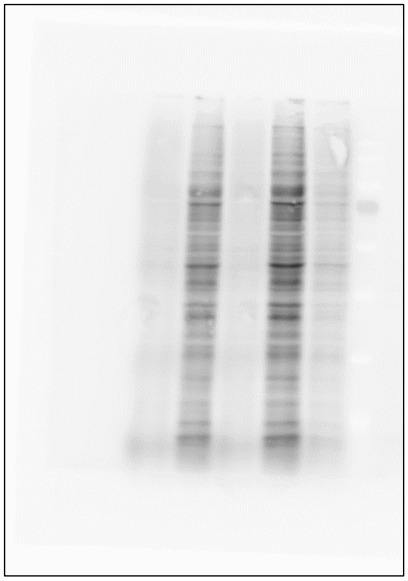

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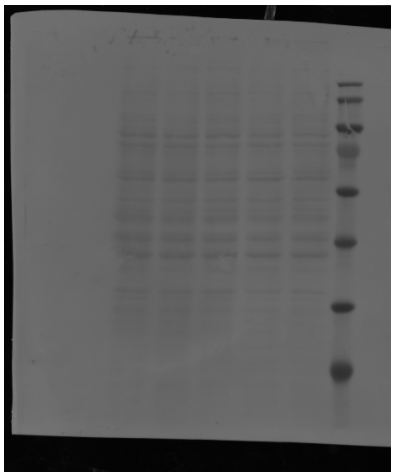

Ponceau S

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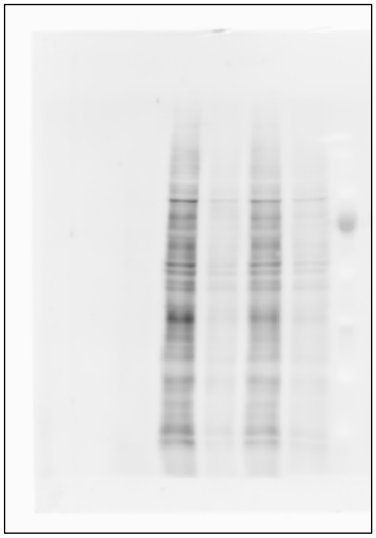

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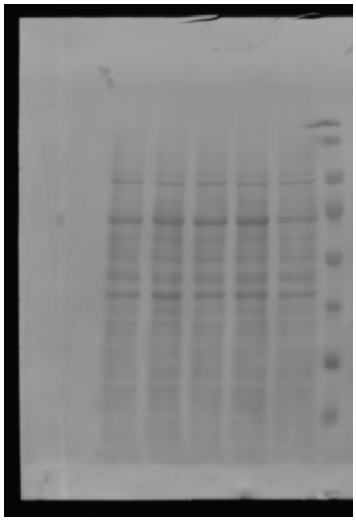

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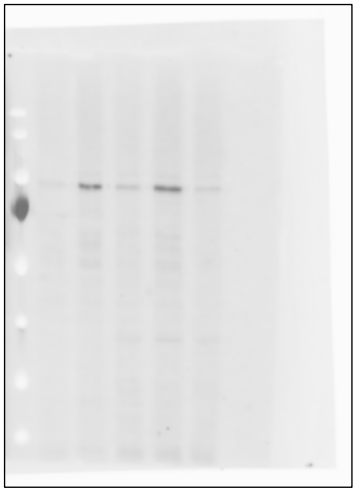

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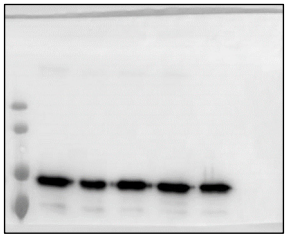

anti-GFP

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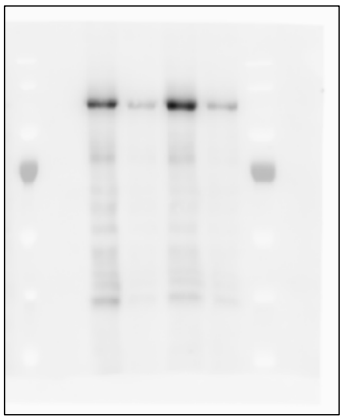

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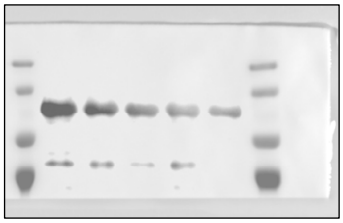

anti-GFP

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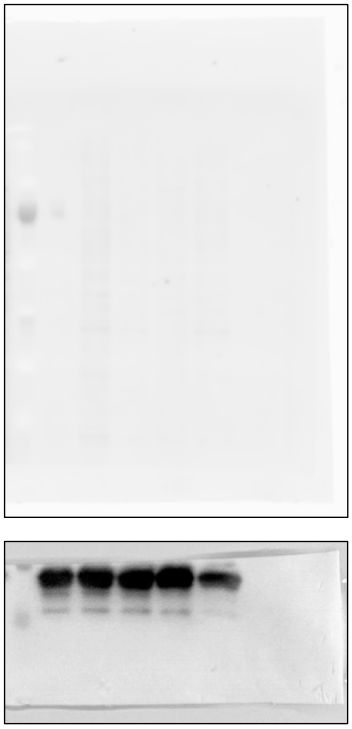

Cy3 fluorescence

anti-GFP

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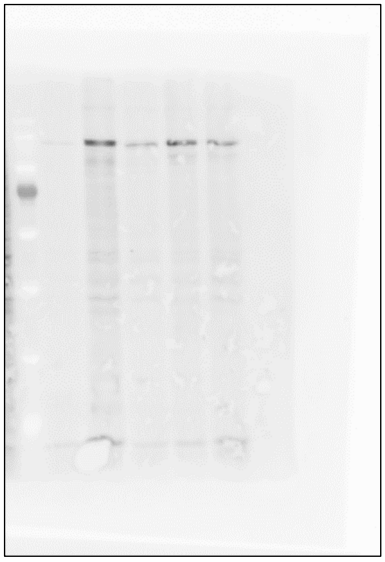
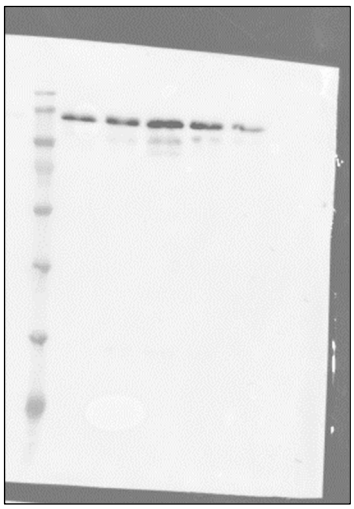


Cy3 fluorescence



anti-GFP

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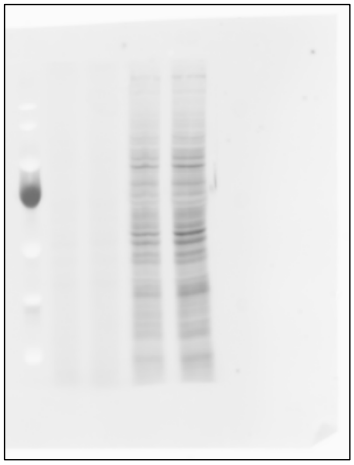
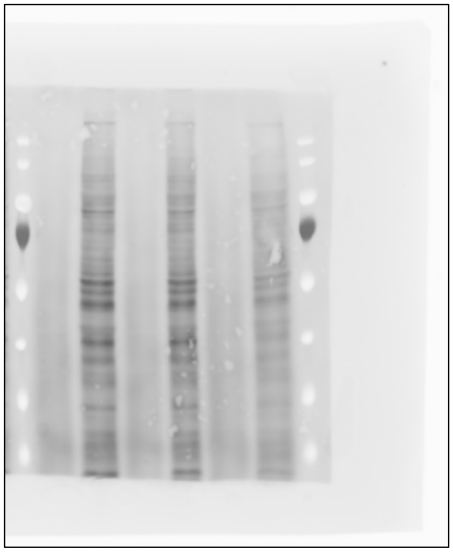


Cy3 fluorescence

Ponceau S

Uncropped blots for Figure 3A



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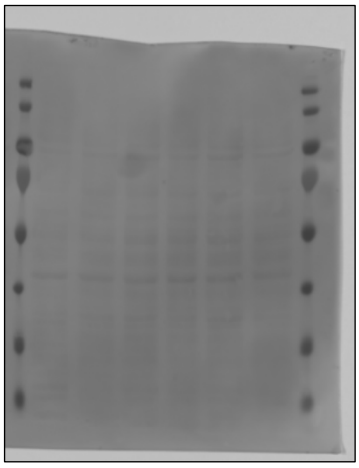


Ponceau S

Uncropped blots for Figure 3B

General Methods

All chemicals were obtained from commercial suppliers (Sigma-Aldrich, TCI, Merck, Roth) and used without further purification unless otherwise stated. Dichloromethane was dried over calcium hydride. All other dry solvents were purchased from Acros Organics. For chromatography, technical grade solvents were distilled prior to use. Preparative column chromatography was performed by flash column chromatography using silica gel 60 M from Macherey-Nagel or with an MPLC-Reveleris X2 system (Büchi). Reactions were monitored by TLC using aluminum sheets pre-coated with silica gel 60 F254 (Merck) with detection by UV light (λ = 254 nm). Additionally, acidic ethanolic *p*-anisaldehyde solution or basic potassium permanganate solution, followed by gentle heating, were used for visualization. NMR spectra were recorded at room temperature with an Avance III 400 or an Avance III 600 instrument from Bruker. Chemical shifts are reported in ppm relative to solvent signals ($CDCl_3$: δ_H = 7.26 ppm, δ_C = 77.16 ppm). Coupling constants (*J*) are given in Hz and multiplicity is abbreviated as follows: s = singlet, d = doublet, t = triplet, dd = doublet of doublet, m = multiplet. Signal assignments were carried out by two-dimensional $^1H,^1H$ and $^1H,^{13}C$ correlation spectroscopy (COSY, HSQC, and HMBC). Analytical RP-HPLC-MS was performed on an LCMS2020 prominence system (pumps LC-20AD, column oven CTO20AC, UV–vis detector SPD-20A, controller CBM20A, ESI detector, software LC-solution) from Shimadzu under the following conditions. Column: Kinetex Core-Shell C18 from Phenomenex, 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). Semi-preparative HPLC was performed on an LC20A Prominence system (high-pressure pumps LC-20AT, auto sampler SIL-20A, column oven CTO-20AC, diode array detector SPDM20A, controller CBM20A, software LC-solution) from Shimadzu under the following conditions. Column: Nucleodur 100-5 C18ec from Macherey Nagel (21.1 × 250 mm), 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). High-resolution mass spectra (HRMS) were recorded on a micrOTOF II instrument from Bruker in positive and negative mode with electrospray ionization (ESI) and time of flight (TOF) detection. Analysis of recorded mass spectra was performed using the software Xcalibur by Thermo Fischer Scientific.

Syntheses

Carbonate 2

p-Nitrophenyl chloroformate (3.68 g, 18.25 mmol) was dissolved in dry dichloromethane (50 mL). *Exo*-2-(hydroxymethyl)-5-norbornene (**31**) (1.03 g, 8.29 mmol) was added and the solution was cooled to 0 °C. Dry pyridine (1.35 mL, 16.59 mmol) was added slowly and the reaction mixture was kept at 0 °C before it was allowed to warm to room temperature. The

suspension was stirred at room temperature for 72 hours. The mixture was diluted with dichloromethane (100 mL) and washed with brine (3 x 100 mL). The organic layer was dried over magnesium sulfate and concentrated under reduced pressure. The crude product was purified by flash column chromatography (10% ethyl acetate in hexanes) and the product (2.26 g, 7.82 mmol, 94% yield) was obtained as a colorless oil. R_f = 0.63 (10% ethyl acetate in hexanes; UV or anisaldehyde staining). **^{1}H NMR** (500 MHz, $CDCl_3$): δ = 8.28 (d, *J* = 9.3 Hz, 2H), 7.40 (d, *J* = 9.3 Hz, 2H), 6.14–6.11 (m, 2H), 4.37 (dd, *J* = 10.6, 6.5 Hz, 1H), 4.19 (dd, *J* = 10.6, 9.3 Hz, 1H), 2.91–2.88 (m, 1H), 2.82–2.80 (m, 1H), 1.89–1.83 (m, 1H), 1.44–1.40 (m, 1H), 1.38–1.32 (m, 2H), 1.24–1.20 (m, 1H) ppm. **^{13}C NMR** (126 MHz, $CDCl_3$): δ = 155.8, 152.7,145.5, 137.3, 136.2, 125.5, 121.9, 73.6, 45.1, 43.7, 41.8, 38.1, 29.6 ppm.

Ac_4GlcNNorboc

Glucosamine hydrochloride (1.36 g, 6.33 mmol) was suspended in dry DMF (12 mL) and *N*,*N*-diisopropylethylamine (4.30 mL, 25.30 mmol) and stirred at room temperature for 2 hours. Carbonate **2** (1.83 g, 6.33 mmol) was added and the reaction mixture was stirred at room temperature for 15 hours. The yellow solution was concentrated under reduced pressure and coevaporated with *n*-heptane (2 x 10 mL). The residue was dissolved in pyridine (10 mL) and cooled to 0 °C. Acetic anhydride (10 mL) was added and the mixture was allowed to warm to room temperature. The reaction was stirred at room temperature for 15 hours before it was concentrated under reduced pressure and coevaporated with toluene (2 x 10 mL). The residue was dissolved dichloromethane (50 mL) and washed with potassium bisulfate solution (10% in water, 50 mL), saturated aqueous sodium bicarbonate solution (50 mL) and brine (50 mL). The organic layer was dried over magnesium sulfate and concentrated under reduced pressure. The crude product was purified by flash column chromatography (25 to 50 % ethyl acetate in hexanes) and the product (2.65 g, 5.55 mmol, 84% yield over two steps) was obtained as a white solid. R_f = 0.51 (50% ethyl acetate in hexanes; anisaldehyde staining). α-Anomer: **^{1}H NMR** (400 MHz, $CDCl_3$): δ = 6.19 (d, *J* = 2.7 Hz, 1H), 6.08–9.05 (m, 2H), 5.26–5.14 (m, 2H), 4.78 (d, *J* = 9.5 Hz, 1H), 4.30–3.88 (m, 6H), 2.85–2.80 (m, 1H), 2.67–2.61 (m, 1H), 2.18 (s, 3H), 2.08 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.69–1.65 (m, 1H), 1.36–1.27 (m, 2H), 1.25–1.19 (m, 1H), 1.15–1.08 (m, 1H) ppm. **^{13}C NMR** (101 MHz, $CDCl_3$): δ = 170.79, 170.76, 168.8, 156.0, 137.1, 136.2, 91.0, 70.8, 69.8, 67.8, 61.7, 52.9, 45.1, 43.6, 41.7, 31.3, 29.5, 21.04, 20.81, 20.80, 20.68 ppm. β-Anomer: **^{1}H NMR** (400 MHz, $CDCl_3$): δ = 6.08–9.05 (m, 2H, H-a), 5.69 (d, *J* = 8.8 Hz, 1H, H-1), 5.26–5.14 (m, 1H, H-4), 5.13–5.08 (t, *J* = 9.7 Hz, 1H, H-3), 4.78 (d, *J* = 9.5 Hz, 1H, NH), 4.30–3.88 (m, 6H, H-2, H-5, H-6, H-f), 3.80 (ddd, *J* = 9.7, 4.5, 2.2 Hz, 1H, H-5),2.85–2.80 (m, 1H, H-b), 2.67–2.61 (m, 1H, H-b), 2.11 (s, 3H, CH_3), 2.08 (s, 3H, CH_3), 2.03 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 1.69–1.65 (m, 1H, H-e), 1.36–1.27 (m, 2H, H-c), 1.25–1.19 (m, 1H, H-d), 1.15–1.08 (m, 1H, H-d) ppm. **^{13}C NMR** (101 MHz, $CDCl_3$): δ = 171.4

(OC(O)), 169.5 (OC(O)), 169.3 (2x OC(O)), 156.1 (OC(O)N), 137.1 (C-a), 136.2 (C-a), 92.7 (C-1), 73.0 (C-5), 72.5 (C-4), 69.8 (C-f), 68.1 (C-3), 61.8 (C-6), 55.0 (C-2), 45.1 (C-c), 43.6 (C-b), 41.7 (C-b), 31.3 (C-e), 29.5 (C-d), 21.00 (CH_3), 20.84 (CH_3), 20.73 (CH_3), 20.70 (CH_3) ppm. **HRMS** (ESI, *m/z*): calcd. for $C_{23}H_{31}NO_{11}$ $[M+Na]^+$: 520.1789, found: 520.1777.

Ac_3GlcNNorboc (3)

Ethylendiamine (129 µL, 1.93 mmol) was dissolved in THF (10 mL) and cooled to 0 °C. Acetic acid (129 µL, 2.25 mmol) was added to the solution followed by Ac_4GlcNNorboc (800 mg, 1.61 mmol). The reaction mixture was allowed to slowly warm to room temperature and stirred for 15 hours. The suspension was diluted with ethyl acetate (100 mL) and water (100 mL). Layers were separated and the organic layer was washed with brine (50 mL), dried over magnesium sulfate and concentrated under reduced pressure. The crude product was purified by flash column chromatography (50% ethyl acetate in hexanes) and the product (719 mg, 1.58 mmol, 98% yield) was obtained as a white solid. R_f = 0.38 (50% ethyl acetate in hexanes; anisaldehyde staining). **^{1}H NMR** (400 MHz, $CDCl_3$): δ = 6.07 (s, 2H), 5.32–5.24 (m, 2H, H-1), 5.14–5.06 (m, 2H), 4.25–3.85 (m, 6H), 3.69 (s, 1H), 2.84–2.80 (m, 1H), 2.68–2.64 (m, 1H), 2.09 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.70–1.63 (m, 1H), 1.35–1.19 (m, 3H), 1.15–1.08 (m, 1H) ppm. **^{13}C NMR** (101 MHz, $CDCl_3$): δ = 171.3, 171.1, 169.6, 156.4, 137.1, 136.3, 92.1, 71.1, 69.5, 68.5, 67.8, 62.2, 54.0, 45.0, 43.6, 41.7, 38.4, 29.5, 20.91, 20.85, 20.8 ppm. **HRMS** (ESI, *m/z*): calcd. for $C_{21}H_{29}NO_{10}$ $[M+Na]^+$: 478.1684, found: 478.1672.

Di-(*S*-acetyl-thioethyl)-*p*-nitrophenyl-phosphate (7)

p-Nitrophenyl dichlorophosphate (**4**) (3.00 g, 11.72 mmol) was dissolved in dry THF (45 mL) with molecular sieve (4 Å). The solution was cooled to −78 °C and *S*-acetyl-2-thioethanol (**5**) (3.10 g, 25.78 mmol) dissolved in dry THF (15 mL) and triethylamine (3.74 mL, 26.96 mmol) was added dropwise. The reaction mixture was stirred at −78 °C for 1 hour and, subsequently, allowed to warm to room temperature. The mixture was filtered over celite and the crude product was purified by flash column chromatography (10 to 50% ethyl acetate in hexanes) to obtain the product (2.55 g, 6.02 mmol, 51% yield) as a colorless oil. R_f = 0.53 (50% ethyl acetate in hexanes; UV or anisaldehyde staining). **^{1}H NMR** (400 MHz, $CDCl_3$): δ = 8.21 (d, J = 9.1 Hz, 2H), 7.36 (d, J = 9.1 Hz, 2H), 4.25–4.19 (m, 4H), 3.17–3.13 (m, 4H), 2.31 (s, 6H) ppm. **^{13}C NMR** (101 MHz, $CDCl_3$): δ = 194.5, 155.1, 144.9, 125.7, 120.8, 120.7, 67.13, 67.07, 30.6, 29.1, 29.0 ppm. **^{31}P NMR** (162 MHz, $CDCl_3$): δ = −7.99 ppm. **HRMS** (ESI, *m/z*): calcd. for $C_{14}H_{18}NO_8PS_2$ $[M+Na]^+$: 446.0104, found: 446.0085.

***p*-Nitrophenyl-di-(*S*-pivaloyl-thioethyl)-phosphate (8)**

p-Nitrophenyl dichlorophosphate (**4**) (3.00 g, 11.72 mmol) was dissolved in dry THF (45 mL) with molecular sieve (4 Å). The solution was cooled to −78 °C and *S*-pivaloyl-2-thioethanol (**6**) (4.18 g, 25.78 mmol) dissolved in dry THF (15 mL) and triethylamine (3.74 mL, 26.96 mmol) was added dropwise. The reaction mixture was stirred at −78 °C for 1 hour and, subsequently, allowed to warm to room temperature. The mixture was filtered over celite and the crude product was purified by flash column chromatography (10 to 40% ethyl acetate in hexanes) to obtain the product (1.48 g, 2.91 mmol, 26% yield) as a colorless oil. R_f = 0.64 (35% ethyl acetate in hexanes; UV or anisaldehyde staining). **^{1}H NMR** (500 MHz, $CDCl_3$): δ = 8.23 (d, *J* = 9.0 Hz, 2H), 7.39 (d, *J* = 9.2 Hz, 2H), 4.25–4.19 (m, 4H), 3.16–3.12 (m, 4H), 1.21 (s, 18H) ppm. **^{13}C NMR** (101 MHz, $CDCl_3$): δ = 205.6, 155.3, 145.0, 125.8, 120.9, 120.8, 67.4, 67.3, 46.7, 28.53, 28.47, 27.4 ppm. **^{31}P NMR** (202 MHz, $CDCl_3$): δ = −7.31 ppm. **HRMS** (ESI, *m/z*): calcd. for $C_{20}H_{30}NO_8PS_2$ $[M+Na]^+$: 530.1043, found: 530.1028.

Ac_3GlcNNorboc-1-P(SATE)$_2$

Sugar **3** (150 mg, 0.329 mmol) was dissolved in dry DMF (2.5 mL) and stirred with molecular sieve (4 Å) at room temperature for 45 minutes. The solution was cooled to 0 °C and *t*BuMgCl (1 M in 2-MeTHF, 0.36 mL, 0.362 mmol) was added the reaction mixture was kept at 0 °C for 30 minutes. Phosphate **7** (278 mg, 0.659 mmol) dissolved in dry THF (2.5 mL) was added and the reaction mixture was allowed to warm to room temperature. After stirring for 2 hours, the turbid solution was poured into cold saturated aqueous sodium bicarbonate solution (40 mL). The aqueous mixture was extracted with dichloromethane (3 x 20 mL). All organic layers were combined, washed with brine (50 mL), dried over magnesium sulfate and concentrated under reduced pressure. The crude product was purified by flash column chromatography (0 to 50% ethyl acetate in hexanes) and the product (105 mg, 0.142 mmol, 42% yield) was obtained as a colorless oil. R_f = 0.38 (50% ethyl acetate in hexanes; anisaldehyde staining). **^{1}H NMR** (500 MHz, $CDCl_3$): δ = 6.07 (bs, 2H), 5.75 (dd, *J* = 5.4, 3.3 Hz, 1H), 5.25 (t, *J* = 10.1 Hz, 1H), 5.20–5.15 (m, 2H, H-3), 4.28 (dd, *J* = 12.5, 3.9 Hz, 1H), 4.22–4.07 (m, 8H), 4.03–3.89 (m, 1H), 3.21–3.16 (m, 4H), 2.82 (bs, 1H), 2.67–2.63 (m, 1H), 2.37–2.36 (m, 6H), 2.10 (s, 3H), 2.04 (s, 6H), 1.70–1.64 (m, 1H), 1.34–1.22 (m, 3H), 1.16–1.10 (m, 1H) ppm. **^{13}C NMR** (126 MHz, $CDCl_3$): δ = 194.8, 194.7, 171.1, 170.7, 169.4, 156.2, 137.2, 136.3, 96.8, 70.2, 69.9, 69.8, 67.7, 66.8, 66.6, 61.6, 53.9, 45.1, 43.6, 41.7, 38.4, 30.7, 29.6, 29.3, 29.2, 20.84, 20.80, 20.7 ppm. **^{31}P NMR** (202 MHz, $CDCl_3$): δ = –2.53 ppm. **HRMS** (ESI, *m/z*): calcd. for $C_{29}H_{42}NO_{15}PS_2$ $[M+Na]^+$: 762.1626, found: 762.1619.

Ac_3GlcNNorboc-1-P(SPTE)$_2$

Sugar **3** (67 mg, 0.147 mmol) was dissolved in dry DMF (1 mL) and stirred with molecular sieve (4 Å) at room temperature for 45 minutes. The solution was cooled to 0 °C and *t*BuMgCl (1 M in 2-MeTHF, 0.16 mL, 0.162 mmol) was added the reaction mixture was kept at 0 °C for 30 minutes. Phosphate **8** (149 mg, 0.294 mmol) dissolved in dry THF (1.5 mL) was added and the reaction mixture was allowed to warm to room temperature. After stirring for 2 hours, the turbid solution was poured into cold saturated aqueous sodium bicarbonate solution (30 mL). The aqueous mixture was extracted with dichloromethane (3 x 15 mL). All organic layers were combined, washed with brine (30 mL), dried over magnesium sulfate and concentrated under reduced pressure. The crude product was purified by flash column chromatography (0 to 40% ethyl acetate in hexanes) and the product (63 mg, 0.076 mmol, 52% yield) was obtained as a colorless oil. R_f = 0.43 (30% ethyl acetate in hexanes; anisaldehyde staining). **^{1}H NMR** (500 MHz, $CDCl_3$): δ = 6.04 (bs, 2H), 5.74 (dd, *J* = 5.2, 3.4 Hz, 1H), 5.27–5.22 (m, 2H, H-4), 5.16 (t, *J* = 9.9 Hz, 1H),4.28 (dd, *J* = 12.5, 3.8 Hz, 1H),4.20 (dt, *J* = 10.3, 3.4 Hz, 1H), 4.14 (dt, *J* = 8.8, 6.8 Hz, 4H), 4.13–3.88 (m, 4H), 3.13 (dt, *J* = 8.8, 6.8 Hz, 4H), 2.80 (bs, 1H), 2.66–2.61 (m, 1H), 2.08 (s, 3H), 2.01 (s, 6H), 1.68–1.62 (m, 1H), 1.35–1.25 (m, 3H), 1.22 (s, 18H), 1.13–1-08 (m, 1H) ppm. **^{13}C NMR** (126 MHz, $CDCl_3$): δ = 205.8, 205.7, 170.9, 170.7, 169.4, 156.2, 137.1, 136.25, 136.22, 96.7, 77.4, 77.4, 77.2, 76.9, 70.2, 69.8, 69.7, 67.7, 66.7, 66.6, 61.6, 53.8, 46.7, 45.1, 45.0, 43.6, 41.7, 38.3, 29.5, 28.6, 28.5, 27.4, 20.8, 20.73, 20.66 ppm. **HRMS** (ESI, *m/z*): calcd. for $C_{35}H_{54}NO_{15}PS_2$ $[M+Na]^+$: 846.2565, found: 846.2541.

Ac_3GlcNNorboc-1-P(OPh)N-Ala-O*i*Pr

Sugar **3** (83 mg, 0.182 mmol) was dissolved in dry THF (1.5 mL) and stirred with molecular sieve (4 Å) at room temperature for 30 minutes. The solution was cooled to −78 °C and *n*BuLi (2.5 M in hexanes, 0.07 mL, 0.182 mmol) was added. Afterwards, phosphoramidate **9** (124 mg, 0.273 mmol) dissolved in dry THF (1.5 mL) was added immediately. The reaction mixture was stirred at −78 °C for 1 hour. Without warming, it was poured into cold saturated aqueous sodium bicarbonate solution (30 mL). The aqueous mixture was extracted with dichloromethane (3 x 15 mL). All organic layers were combined, washed with brine (40 mL), dried over magnesium sulfate and concentrated under reduced pressure. The crude product was purified by flash column chromatography (0 to 5% acetone in dichloromethane) and the product (44 mg, 0.061 mmol, 34% yield) was obtained as a colorless gum. R_f = 0.51 (30% ethyl acetate in hexanes; anisaldehyde staining). **^{1}H NMR** (400 MHz, $CDCl_3$): δ = 7.37–7.29 (m, 2H), 7.25–7.21 (m, 2H), 7.20–7.15 (m, 1H), 6.07–6.04 (m, 2H), 5.87 (dd, *J* = 6.3, 3.2 Hz, 1H), 5.44 (d, *J* = 9.7 Hz, 1H), 5.18–4.98 (m, 3H), 4.28–3.84 (m, 7H), 3.62 (t, *J* = 10.2 Hz, 1H), 2.80 (bs, 1H), 2.69–2.63 (m, 1H), 2.01 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.70–1.63 (m, 1H), 1.39 (d, *J* = 7.0 Hz, 3H), 1.33–1.27 (m, 2H), 1.27 (d, *J* = 6.0 Hz, 3H), 1.27–1.22 (m, 1H), 1.24

(d, J = 6.0 Hz, 3H), 1.15–1.09 (m, 1H) ppm. **^{13}C NMR** (101 MHz, $CDCl_3$): δ = 173.3, 170.9, 170.8, 169.3, 156.3, 150.5, 137.1, 136.3, 130.0, 125.4, 120.3, 120.2, 96.4, 70.6, 69.9, 69.6, 67.8, 61.5, 53.8, 50.6, 45.1, 43.6, 41.7, 38.3, 29.5, 21.8, 21.8, 21.1, 20.8, 20.7, 20.7 ppm. **^{31}P NMR** (162 MHz, $CDCl_3$): δ = 0.72 ppm. **HRMS** (ESI, *m/z*): calcd. for $C_{33}H_{45}N_2O_{14}P$ $[M+Na]^+$: 747.2501, found: 747.2498.

***p*NP-GlcNNorboc**

*p*NP-Ac_4GlcNH_2 (**10**) (138 mg, 0.325 mmol) was solved in dry DMF (0.65 mL). An *endo/exo*-mixture of carbonate **2** (34 mg, 0.649 mmol) and triethylamine (0.06 mL, 0.422 mmol) were added. The resulting mixture was stirred at room temperature for 48 hours. The solvent was removed under reduced pressure and the crude product was purified by flash column chromatography (MPLC-Reveleris system, 0 to 10% ethyl acetate in hexanes in 20 minutes). The acetylated product (118 mg, 0.204 mmol) was obtained as a white solid. The acetyl groups were removed by dissolving the acetylated product in dry methanol (2.4 mL) and addition of sodium methoxide (0.5 M in methanol, 0.08 mL, 41 mmol) in methanol (2.4 mL). The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure and the product was purified by flash column chromatography (MPLC-Reveleris system, 0 to 100% ethyl acetate in hexanes in 15 minutes). The product was obtained as a white solid (34 mg, 0.075 mmol, 23% yield over 2 steps). $\boldsymbol{R_f}$ = 0.17 (ethyl acetate; anisaldehyde staining). **^{1}H NMR** (500 MHz, CD_3OD) δ = 8.20 (dt, J = 9.1, 1.4 Hz, 2H), 7.24–7.14 (m, 2H), 6.15−5.93 (m, 4H), 5.18 (d, J = 8.3 Hz, 1H), 4.20–4.06 (m, 1H), 4.02 (dd, J = 10.7, 9.0 Hz, 1H), 3.93 (dd, J = 12.2, 2.3 Hz, 1H), 3.86 (dd, J = 10.5, 6.5 Hz, 1H), 3.72 (dd, J = 12.1, 5.8 Hz, 1H), 3.66 (tt, J = 9.5, 4.8 Hz, 1H), 3.57 (t, J = 9.9 Hz, 1H), 3.51 (ddd, J = 9.8, 5.7, 2.3 Hz, 1H), 3.47–3.43 (m, 1H), 3.42−2.68 (m, 4H), 2.38 (s, 1H), 1.81 (d, J = 12.7 Hz, 1H), 1.68 (s, 1H), 1.45–1.06 (m, 6H), 0.54 (d, J = 12.1 Hz, 1H) ppm. **^{13}C NMR** (126 MHz, CD_3OD) δ = 163.8, 159.0, 144.6, 138.5, 138.0, 137.3, 133.2, 128.1, 119.4, 99.7, 79.4, 75.6, 71.4, 70.4, 69.4, 62.5, 57.8, 50.2, 45.8, 45.1, 44.8, 43.4, 42.8, 39.8, 39.4, 30.3, 29.7 ppm. **HRMS** (ESI, *m/z*): calcd. for $C_{21}H_{26}N_2O_9$ $[M+Na]^+$: 473.1531, found: 473.1523.

Cell Culture

HeLa cells were grown in Dulbecco's modified Eagle medium supplemented with 10 V-% tetracycline-free fetal bovine serum, 0.8 V-% Hygromycin B (stock concentration = 50 mg·mL^{-1}) and 1 V-% penicillin-streptomycin (stock concentration = 10000 U·mL^{-1} penicillin and 10 mg·mL^{-1} streptomycin) at 37 °C under a CO_2 atmosphere of 5 %. HEK 293T cells were grown in Dulbecco's modified Eagle medium supplemented with 10 V-% fetal bovine serum and 1 V-% penicillin-streptomycin (stock concentration = 10000 U·mL^{-1} penicillin and 10 mg·mL^{-1} streptomycin) at 37 °C under a CO_2 atmosphere of 5 %.

Plasmids

The eGFP (pEGFP-C1-loxP) plasmid was purchased from Clontech. The pEGFP-N1-Foxo1 plasmid was a gift from Domenico Accili (Addgene plasmid # 17551).[1] Human p53 DNA was amplified by polymerase chain reaction (PCR) from p53 plasmid (kindly provided by Prof. Martin Scheffner, University of Konstanz).[2] These primers were used: 5'-TAAGCAGTCGACATGGAGGAGCCGCAGTCA-3' (forward primer) and 5'-TGCTTAGGATCCAAGTCTGAGTCAGGCCCTTCTGT-3' (reverse primer). The coding sequence was inserted into pEGFP-N1 (Clontech) via BamHI/SalI restriction sites to yield pEGFP-N1-p53.

Transient Transfection and Sugar Treatment of HEK 293T Cells

HEK 293T cells were transiently transfected by calcium phosphate co-precipitation using a total amount of 4 µg plasmid DNA per 10 cm culture dish. The day prior transfection, one to two million cells were seeded per 10 cm cell culture dish. 500 µL MQ water were mixed with plasmid DNA, 500 µL 2x HEPES buffered saline (280 mM NaCl, 50 mM HEPES, 1.48 mM Na_2HPO_4 x 7 H_2O) and 50 µL 2.5 M $CaCl_2$ solution while vortexing. The transfection mixture as incubated at room temperature for 15 minutes. Cells were treated with 1 µL chloroquine (25 mM) and 100 µL transfection mixture. After incubation at 37°C for 6-8 hours the cells were washed once with DPBS and treated with fresh medium. After 36- 48 hours the gene expression of the transfected plasmids is optimal. 20 hours before cells should be harvested 100 µM or 200 µM sugar were added to the cell culture medium.

Cell Assay with HeLa Cells

6 Mio HeLa eGFP or HeLa Kif18A-eGFP cells were seeded per 10 cm dish and treated with tetracycline (0.01 µg·mL^{-1} for HeLa eGFP and 2 µg·mL^{-1} for Kif18A-eGFP) and thymidine

(2 mM). After 24 h, cells were washed tree times with DPBS. Then, the cells were incubated for two hours at 37 °C and 5 % CO_2 with the respectively amount of tetracycline. After this, media was changed for a thymidine release and the cells were treated with tetracycline as before, nocodazole (0.15 $\mu g \cdot mL^{-1}$) and additionally the corresponding sugar was added (100 µM or 200 µM). 18 h later all cells should be in mitosis and can be harvested.

Mitotic Shake-Off and Lysis of HeLa Cells

Cell culture dished were shaken gentle to detach mitotic cells from the substratum. Cells were centrifuged (5 min, 600 g) and washed with PBS (7 mL). Then, new medium without any additives was added and cells were incubated for 20 min at 37 °C to get them into metaphase. Cells were lysed by resuspending the cell pellet in 400 µL lysis buffer (0.5 V-% Triton X-100, 25 mM Tris pH 7.4, 300 mM NaCl, 5 mM EDTA, 20 mM β-glycerophosphate, 20 mM NaF, 0.3 mM Na_3VO_4, 10 $U \cdot mL^{-1}$ DNase I, 1x protease inhibitor cocktail) and incubating this mixture for 30 min on ice. Afterwards, the mixture was centrifuged at 18500 g for 30 min at 4 °C. The supernatant contained the desired whole cell lysate, which was transferred into a new 1.5 mL reaction tube. These probes can be used directly or stored at -80 °C.

Lysis of HEK 293T Cells

HEK 293T cells were directly harvested and lysed without any incubation time. Lysis buffer and further procedure as described for HeLa cells.

Immunoprecipitation

GFP-Trap®_A (Chromotek) beads (10 µL) were prepared using ice cold dilution buffer (500 µL, 10 mM Tris pH 7.4, 150 mM NaCl in Milli Q water) and centrifugation for 2 min at 3000 g. The supernatant was aspirated down to approximately 50 µL. This washing step was repeated one more time. Then, the cell lysate (350 µL) and dilution buffer (650 µL) were added. The Triton X-100 concentration must be reduced below 0,2 V-% in order to secure proper functioning of the GFP-Trap beads. The mixture was incubated over night at 4 °C with overhead rotation. After that the beads were washed three times using dilution buffer (500 µL), leaving 30 µL dilution buffer in the mixture after the last washing step.

IEDDA Reaction

For the IEDDA reaction, tetrazine-Cy3 (Tz-Cy3, Jena Bioscience, 10 µM) was added to the IP probes and to the whole cell lysate probes. The probes were incubated for 90 min at 25 °C.

Denaturation

After adding 4x SDS loading buffer (100 mM Tris pH 6.8, 30 V-% glycerol, 0.21 M SDS, 2.84 M β-mercaptoethanol, 0.29 mM bromophenol blue) to the samples to a final concentration of 1x SDS buffer, the samples were denatured for 10 min at 98 °C. These samples were directly loaded on SDS-page gel or stored at -20 °C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-Page) and Western Blotting

For the gel electrophoresis, a 10 % SDS-page gel was used. The samples (10 µL) were loaded on the gel and run at 30 mA. The proteins were transferred to a nitrocellulose membrane at 120 V for 1 h (Western blotting). After that, the fluorescence could be read out directly using *Amersham Imager 600* fluorescence imager. The Cy3-tetrazine signal was detected with an excitation of 520 nm. Then, the membrane was blocked with 5 V-% milk in PBS-T for 1 h at r.t. The primary antibody (monoclonal mouse anti-GFP, JI-8, *Clonetech* or monoclonal mouse anti-O-GlcNAc IgG1; RL2, *Thermo Fisher*) was diluted in 2 w-% BSA, 0.05 w-% NaAz in PBS-T (1:3000) and incubated overnight at 4 °C. After washing the membrane three times for 10 min, the secondary antibody (anti-mouse) (1:3000 in 5 V-% milk in PBS-T) was added for 1 h at r.t. The secondary antibody was washed out three times for 10 min. The chemiluminescence was read out by adding ECL solution (*BioRad*) to the membrane and developing a picture at the *ChemiDoc Touch Imaging System* by *BioRad*. As loading control, a Ponceau S solution (0,1 % Ponceau S in 5 % acetic acid) was added to the membrane for 5 min, removed, and the membrane rinsed twice with distilled water.

OGA Cleavage Assay

Reactions were initiated by addition of 5.3 nM OGA (R&D systems, #Q89ZI2) to PBS (pH 7.0-7.2) containing 0.01% BSA, 8% DMSO, and varying concentrations of *p*NP-GlcNNorboc or *p*NP-GlcNAc. The substrate concentrations measured were: 10 mM, 5 mM, 2,5 mM, 1,25 mM, 0,63 mM, and 0,31 mM. Absorbance of the *p*-nitrophenol leaving group was detected at 405 nm by using FLUOstar Optima plate reader. The reactions were monitored until complete conversion of the substrate. *p*-nitrophenol was measured under the same conditions to generate standard regression. Michaelis-Menten parameters were calculated by using Origin 2020 version 9.7.0.188.

FLIM-FRET in Living Cells

HEK 293T cells were seeded in with fibronectin coated ibiTreat µ-Slides (ibidi) (50000 cells/cm²) and were transfected with eGFP-fusion proteins as described above. After 24 h cells were treated with $Ac_3GlcNNorboc\text{-}1\text{-}P(SATE)_2$ or DMSO for 20h. IEDDA reaction was performed by adding 25 µM TAMRA-tetrazine (Jena Bioscience, CLK-017-05) for 1 h to the cell culture medium. Afterwards, cells were washed with DPBS and phenol red-free DMEM containing 10 µM HEPES was added.

Fluorescence lifetime measurements were performed on a home built confocal microscopy set up at 37° C. A 100x objective (PLAN APO, NA 1.4, oil immersion, Leica) was used for all measurements. eGFP was excited at 470 nm using a pulsed diode laser with repetition rate of 20 MHz. Fluorescence was filtered by a 488 nm longpass and 525/50 nm emission filter. TAMRA was excited at 561 nm using a CW diode pumped laser (Cobolt Jive, Hübner Photonics) and emission was collected with a 561 nm longpass filter. The photons were counted by a time correlated single photon counting module (MultiHarp 150P, PicoQuant) and a single photon counting detector (PMA Hybrid, PicoQuant). Fluorescence images were acquired by fast scanning (10 µs pixel dwell time, 5 frames) with 512 x 512 pixels using ScanImage (MBF Bioscience) and SymPhoTime 64 software (PicoQuant). To get average lifetime information of the cellular samples, regions of interest were chosen in the image and the corresponding TCSPC histograms (>300 kcounts) were fitted with a double exponential reconvolution fitting model. For FRET analysis, the amplitude weighted average lifetime was used and the FRET efficiency (E) was calculated as

$$\mathrm{E} = 1 - {}^{\tau_{DA}}/_{\tau_D} \times 100\%$$

where τ_{DA} is the fluorescence lifetime of donor in the presence of acceptor and τ_D that of donor in the absence of acceptor. The pixel colors in the image corresponds to the lifetimes and brightness corresponds to the fluorescence intensities.[3]

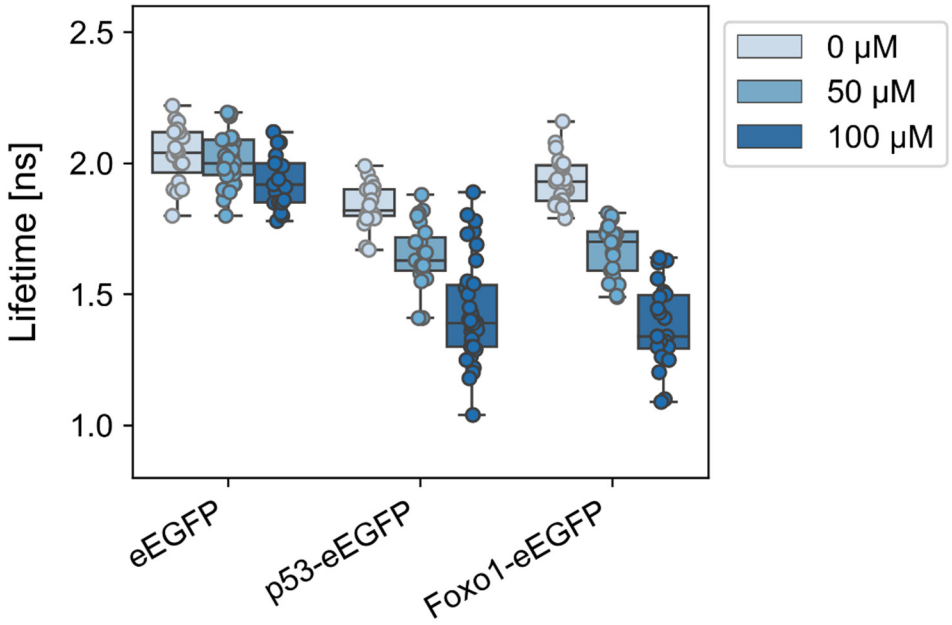
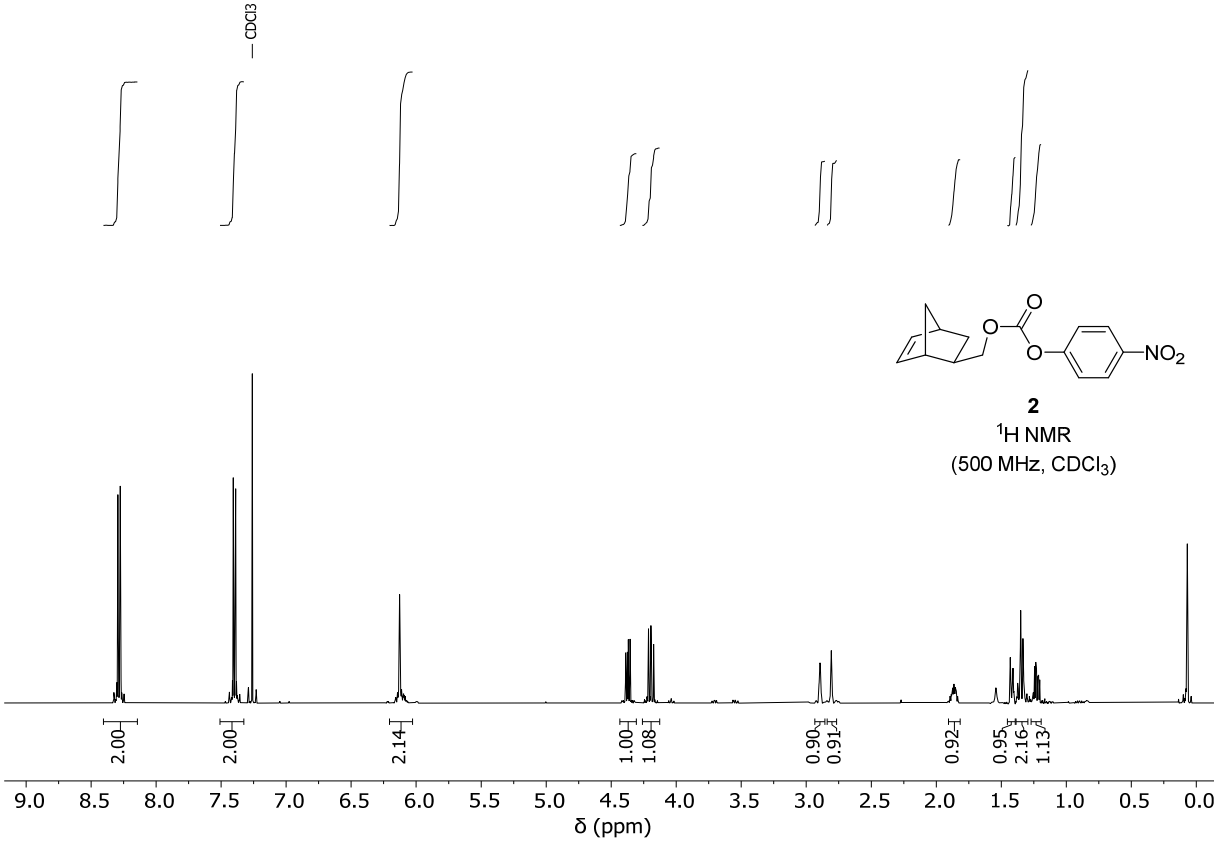


Figure S1. Fluorescence lifetimes obtained from HEK 293T cells expressing eGFP, p53-eGFP, or Foxo1-eGFP after addition of 0 µM, 50 µM, or 100 µM of Ac_3GlcNNorboc-1-P(SATE)$_2$. Each data point was obtained from an individual cell. At least five cells from three or more independent experiments were imaged for each protein under the different sugar treatment conditions.

References

[1] D. Frescas, L. Valenti, D. Accili, *J. Biol. Chem.* **2005**, *280*, 20589-20595.
[2] A. Hengstermann, L. K. Linares, A. Ciechanover, N. J. Whitaker, M. Scheffner, *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 1218-1223.
[3] *Principles of Fluorescence Spectroscopy*, 3rd ed. (Ed.: J. R. Lakowicz), Springer, New York, **2006**.

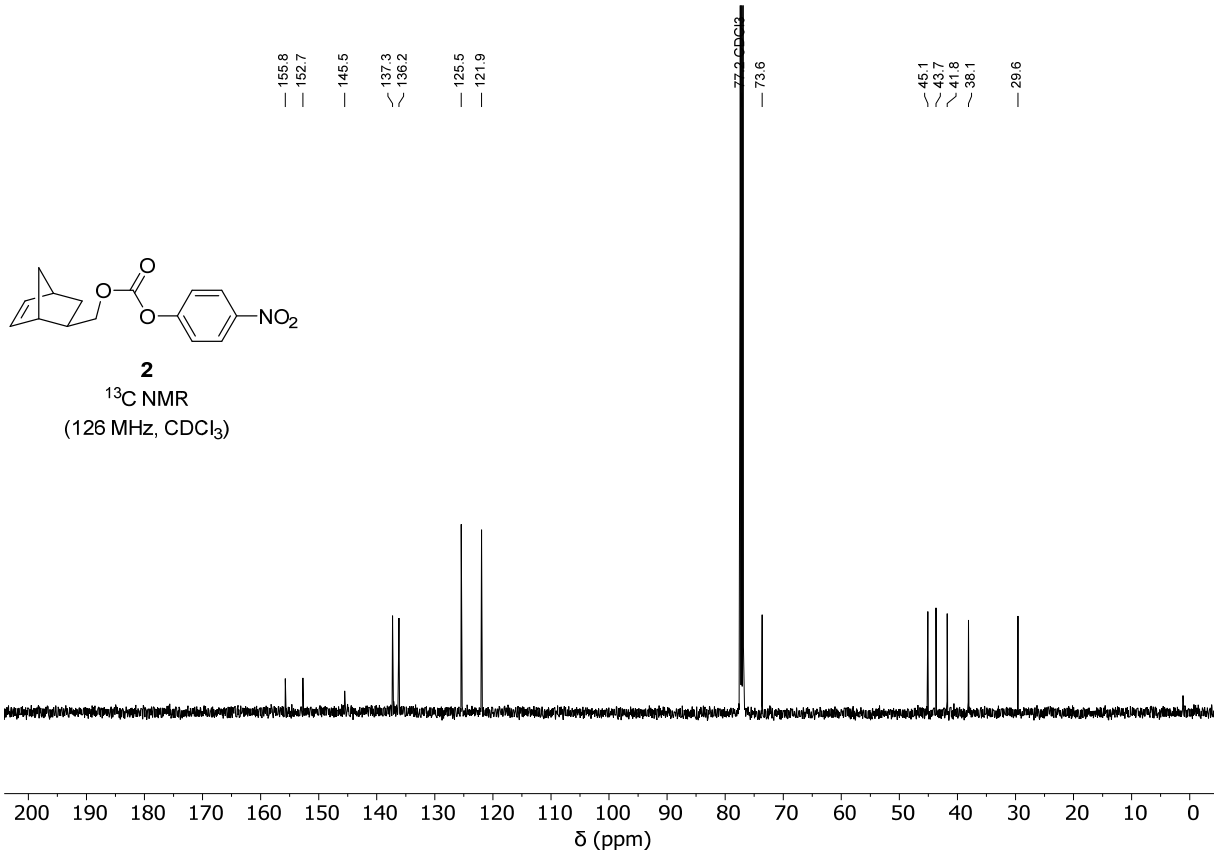
NMR Spectra



2

^{1}H NMR

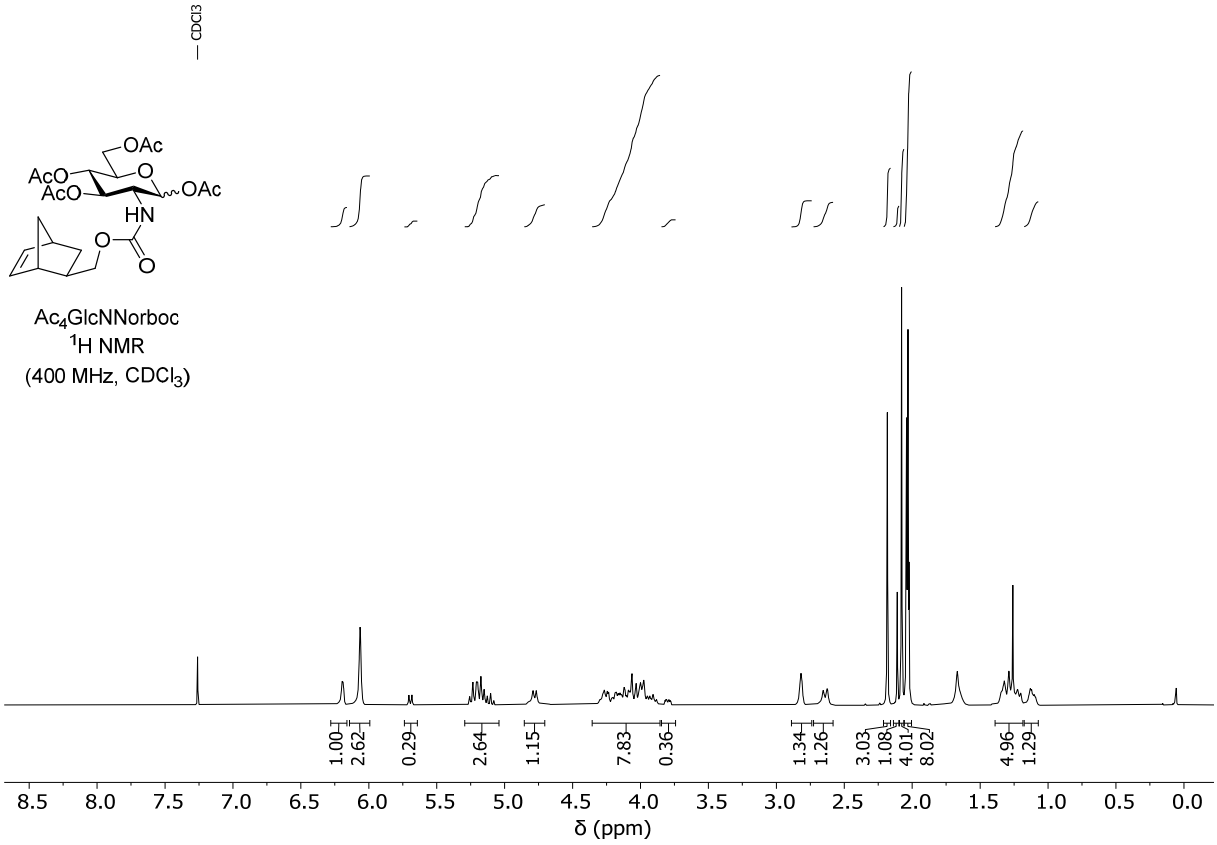
(500 MHz, $CDCl_3$)

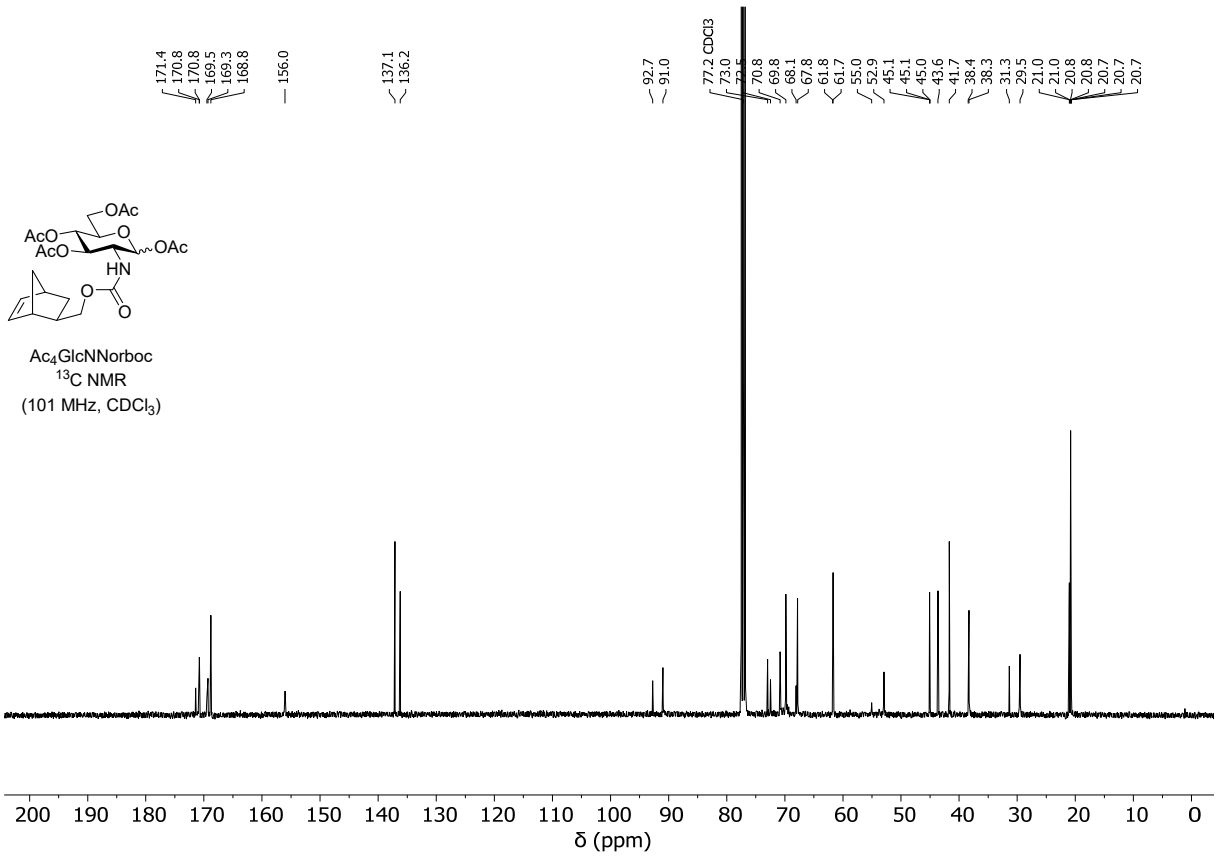


2

^{13}C NMR

(126 MHz, $CDCl_3$)


CDCl3
OAc
AcO
AcO
OAc
HN
O
O
Ac4GlcNNorboc
1H NMR
(400 MHz, CDCl3)
1.00
2.62
0.29
2.64
1.15
7.83
0.36
1.34
1.26
3.03
1.08
4.01
8.02
4.96
1.29
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
δ (ppm)


171.4
170.8
170.8
169.5
169.3
168.8
156.0
137.1
136.2
92.7
91.0
77.2 CDCl3
73.0
70.8
69.8
68.1
67.8
61.8
61.7
55.0
52.9
45.1
45.1
45.0
43.6
41.7
38.4
38.3
31.3
29.5
21.0
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20.8
20.8
20.7
20.7
20.7
OAc
AcO
AcO
OAc
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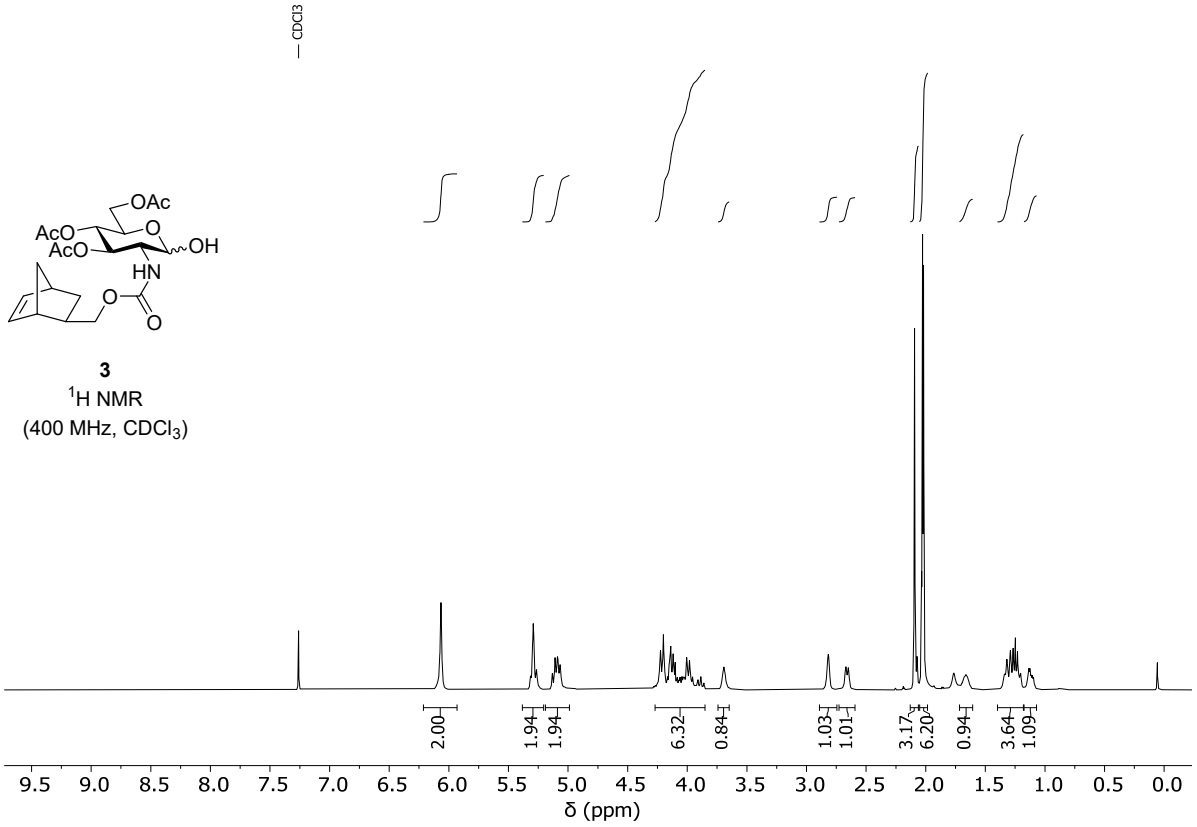
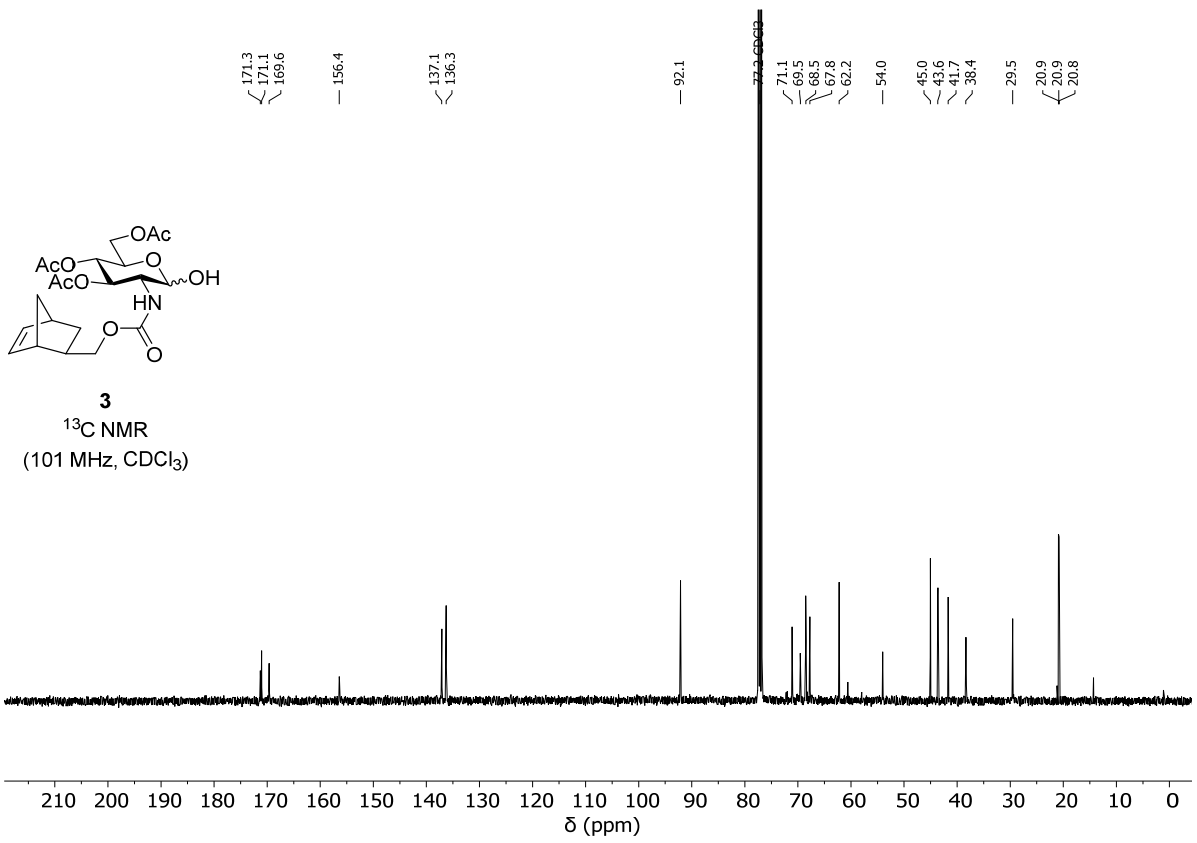
13C NMR
(101 MHz, CDCl3)
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
δ (ppm)



3

^{1}H NMR

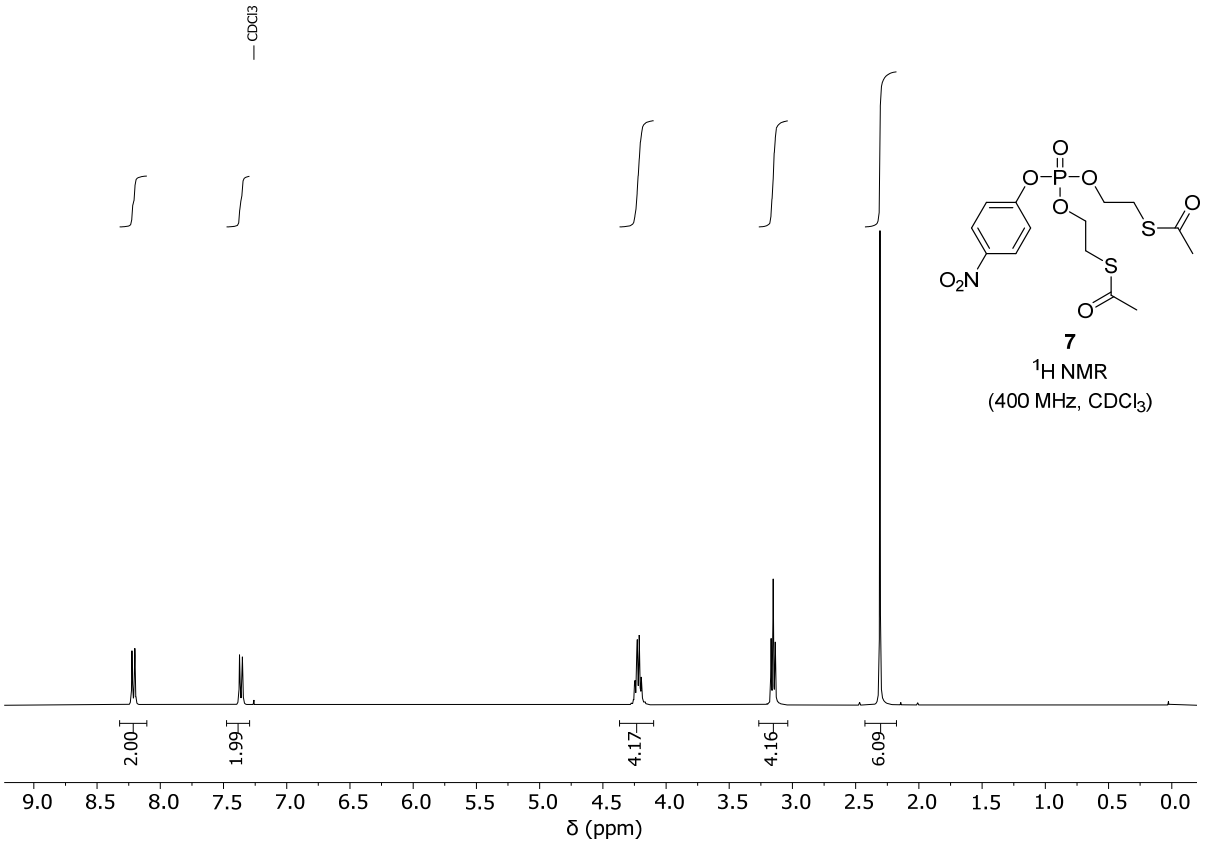
(400 MHz, $CDCl_3$)



3

^{13}C NMR

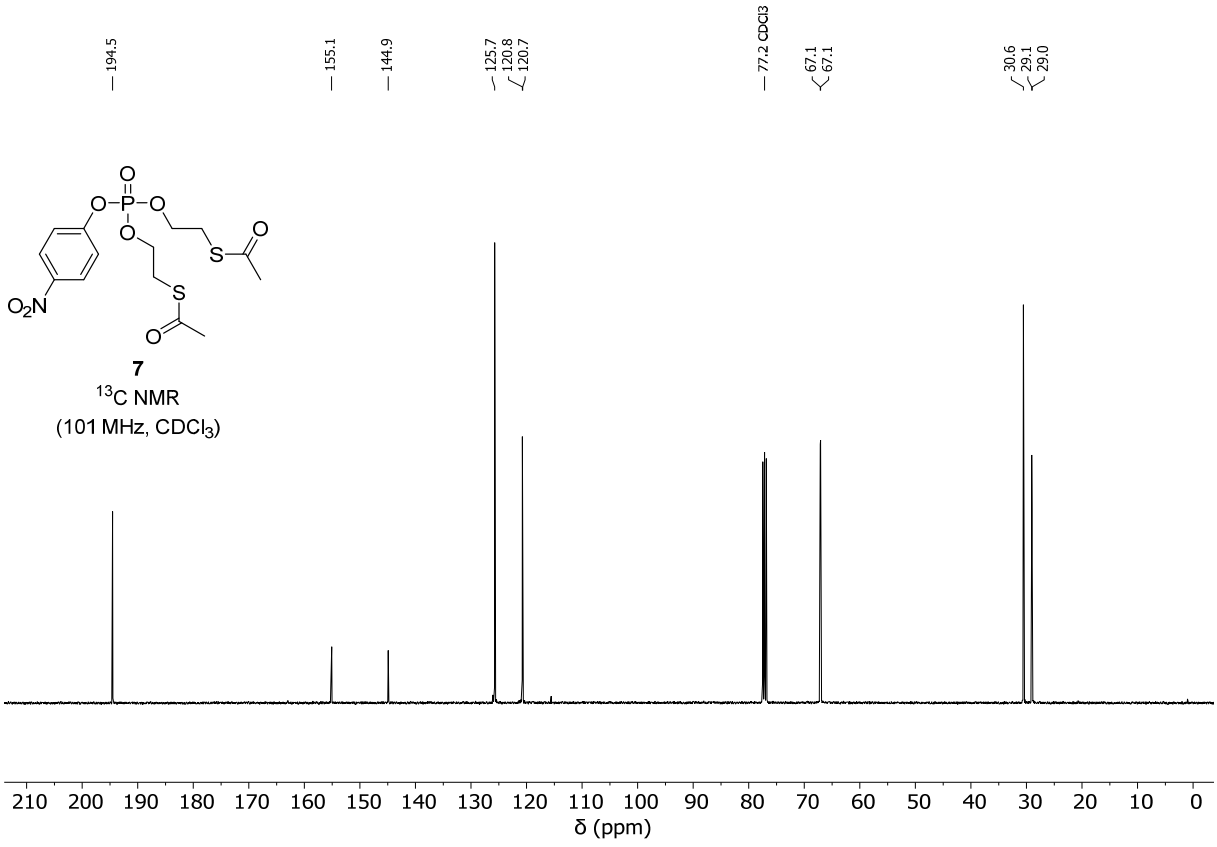
(101 MHz, $CDCl_3$)



7

^{1}H NMR

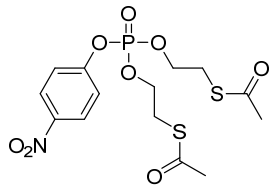
(400 MHz, $CDCl_3$)



7

^{13}C NMR

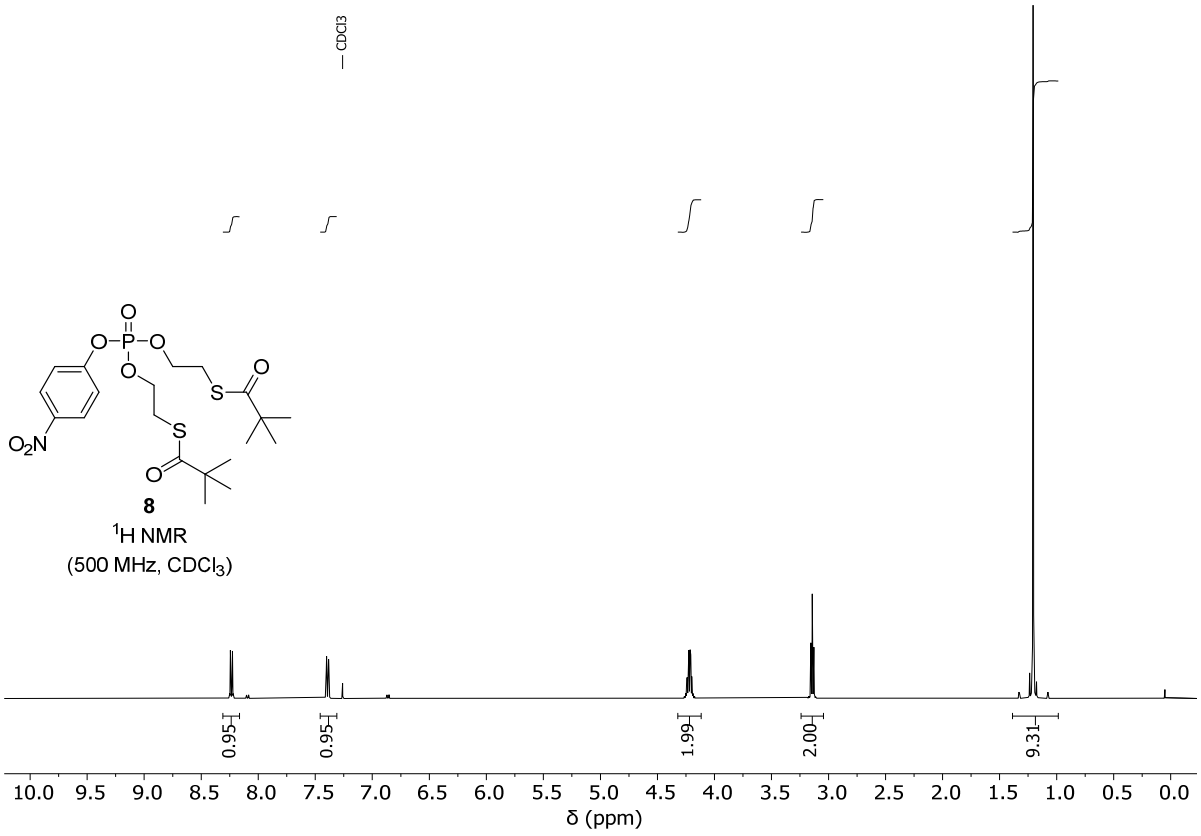
(101 MHz, $CDCl_3$)



7

^{31}P NMR

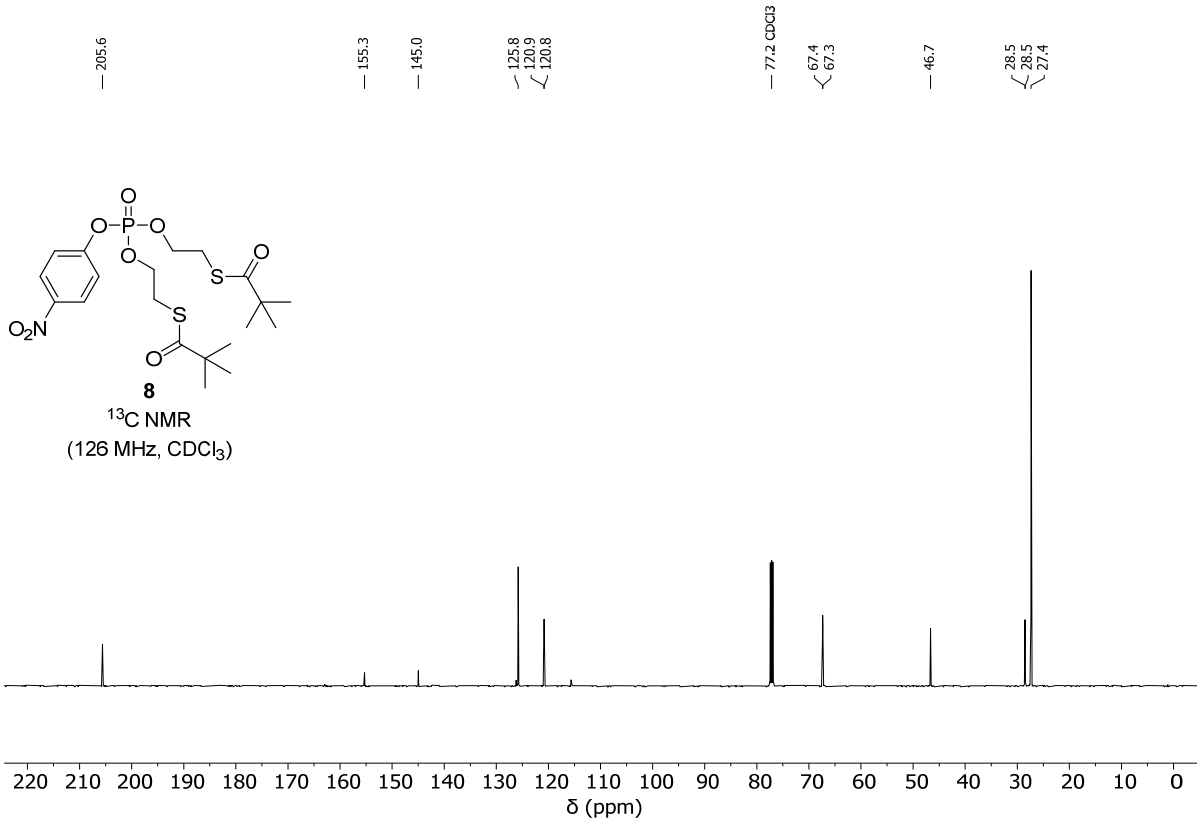
(162 MHz, $CDCl_3$)



8

^{1}H NMR

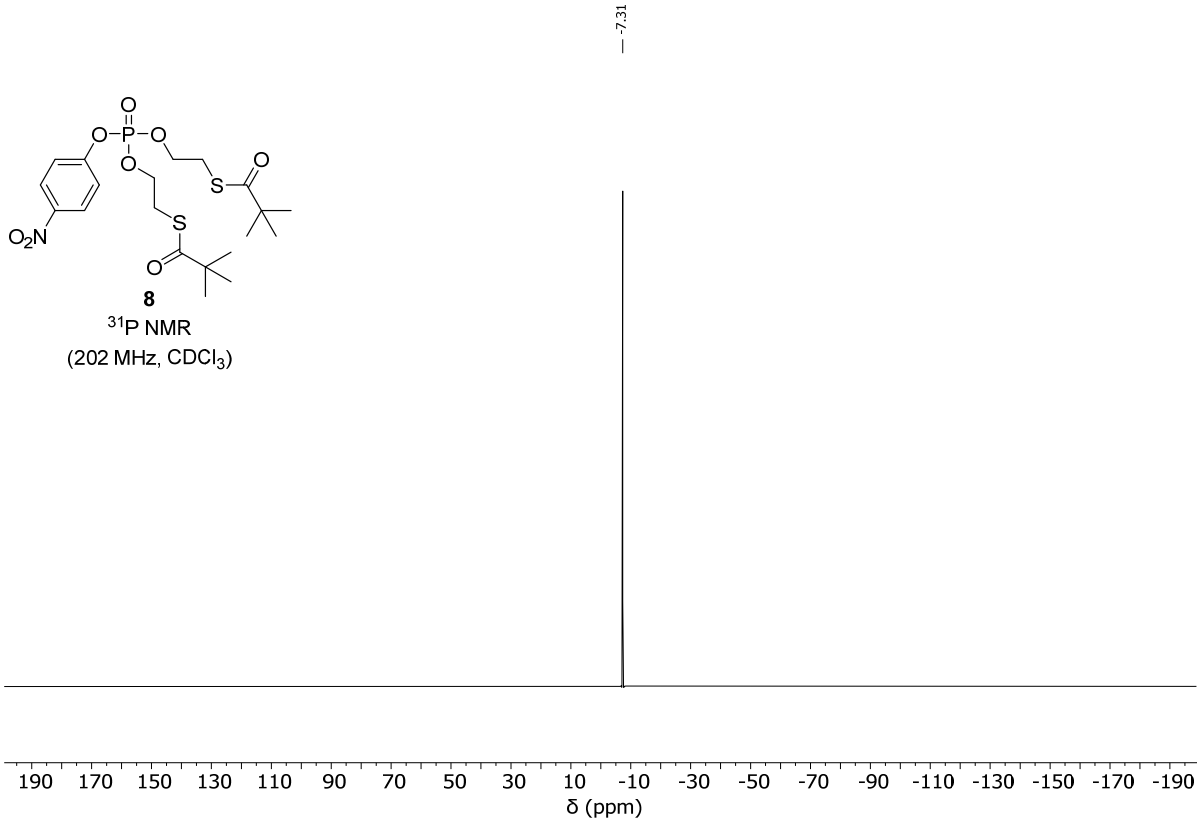
(500 MHz, $CDCl_3$)



8

^{13}C NMR

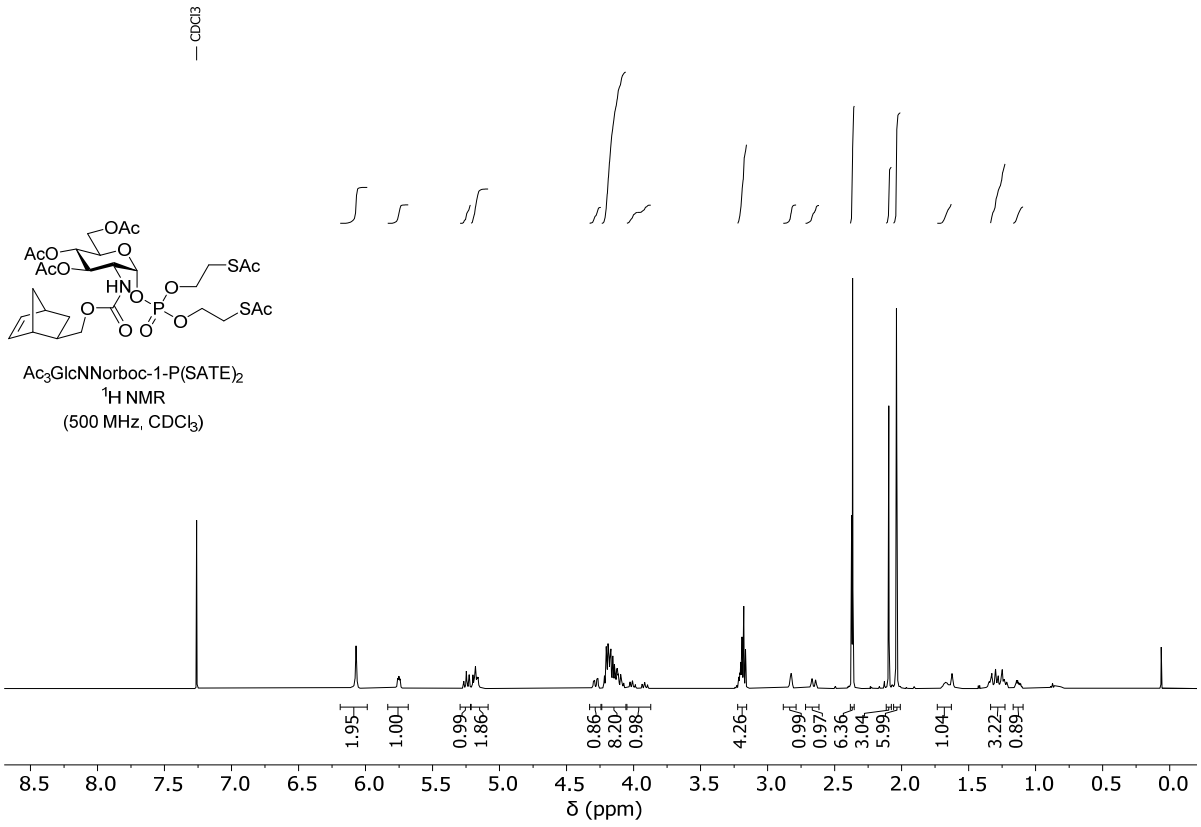
(126 MHz, $CDCl_3$)



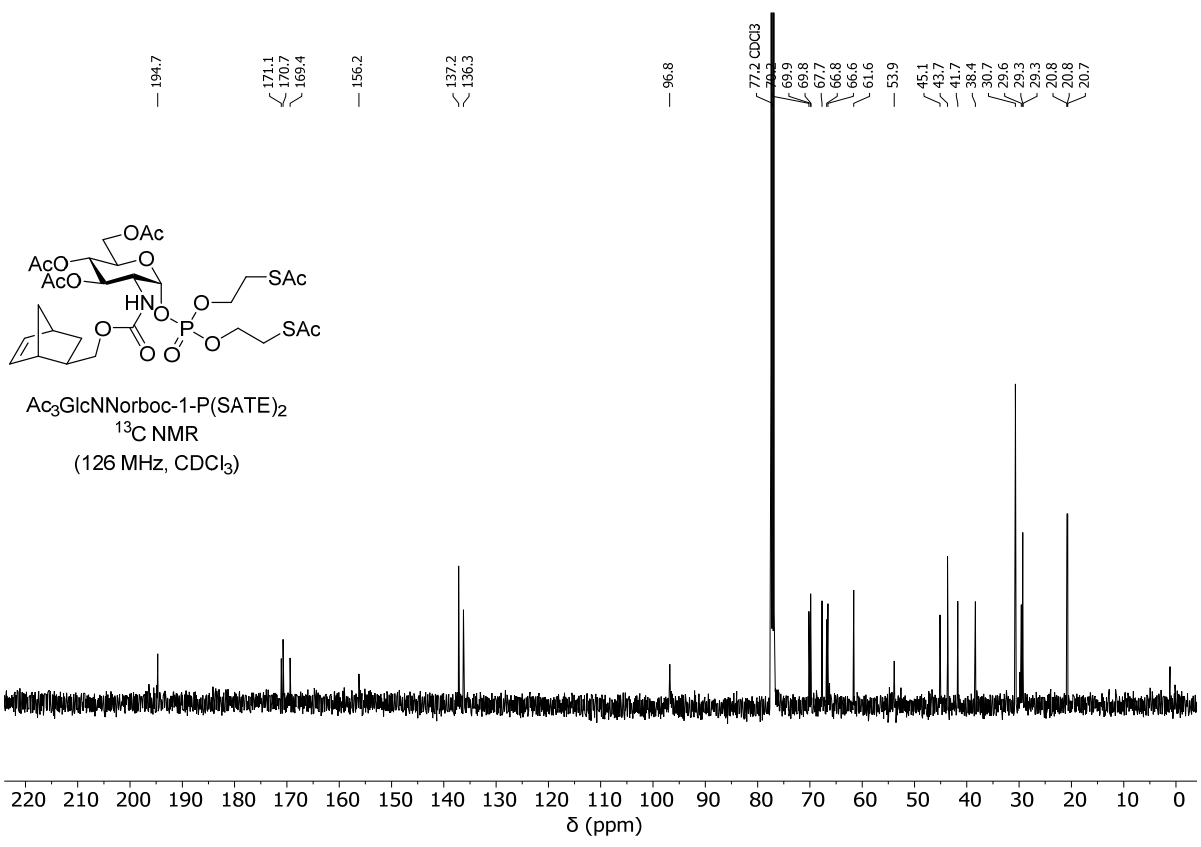
8

^{31}P NMR

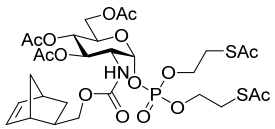
(202 MHz, $CDCl_3$)



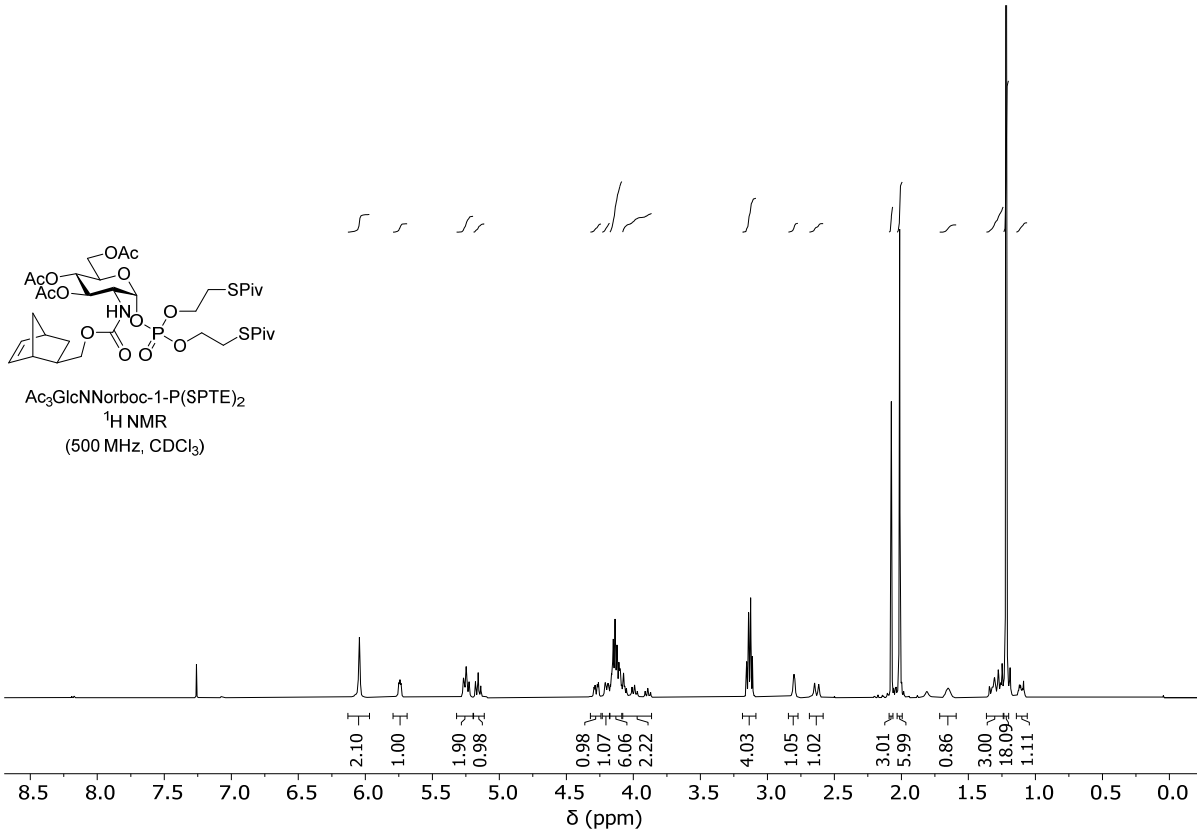
$Ac_3GlcNNorboc\text{-}1\text{-}P(SATE)_2$
^1H NMR
(500 MHz, $CDCl_3$)



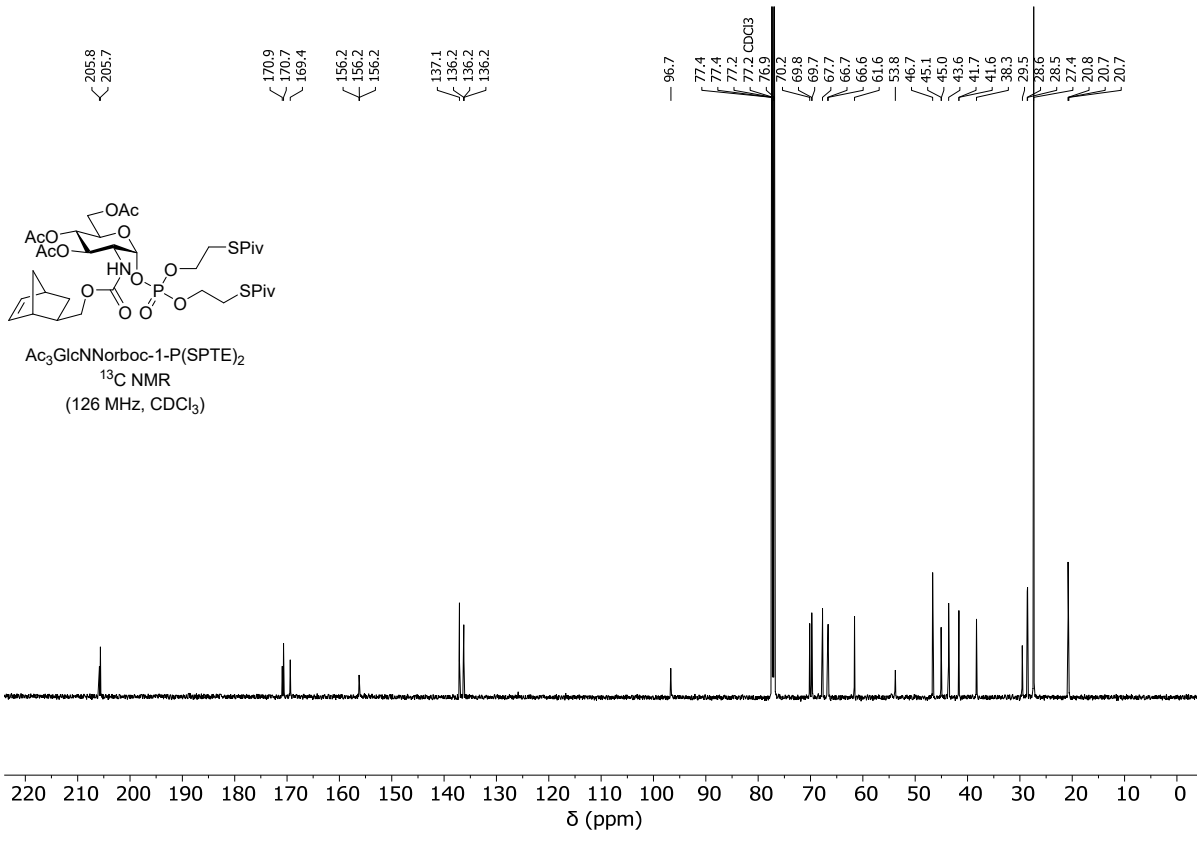
$Ac_3GlcNNorboc\text{-}1\text{-}P(SATE)_2$
^{13}C NMR
(126 MHz, $CDCl_3$)



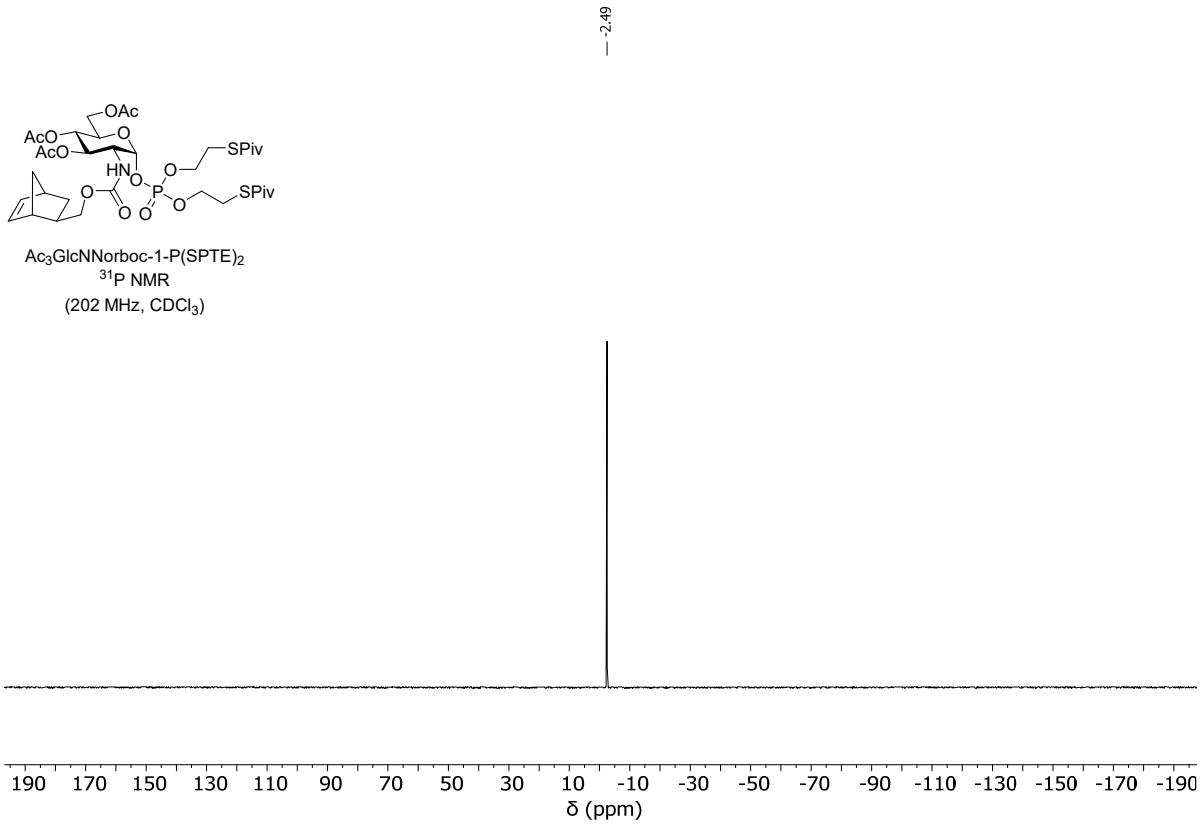
$Ac_3GlcNNorboc\text{-}1\text{-}P(SATE)_2$
^{31}P NMR
(202 MHz, $CDCl_3$)



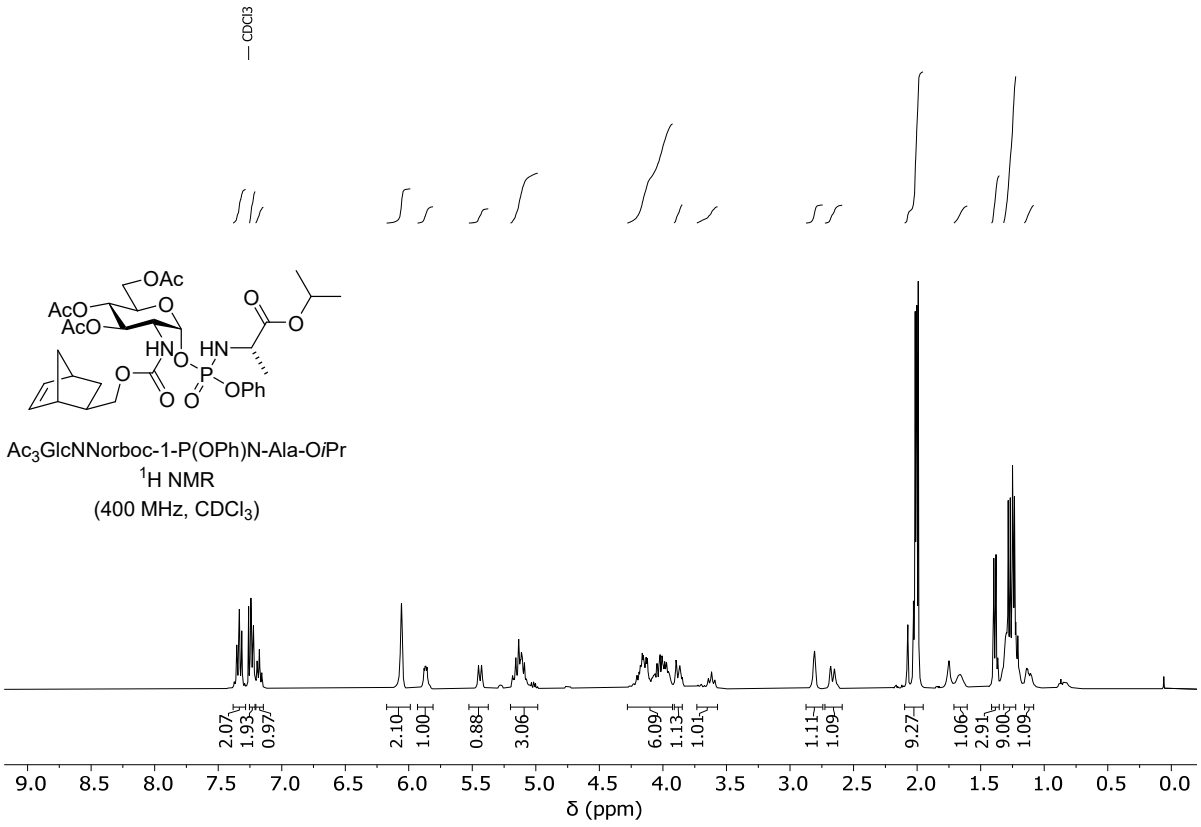
$Ac_3GlcNNorboc\text{-}1\text{-}P(SPTE)_2$
^1H NMR
(500 MHz, $CDCl_3$)



$Ac_3GlcNNorboc\text{-}1\text{-}P(SPTE)_2$
^{13}C NMR
(126 MHz, $CDCl_3$)



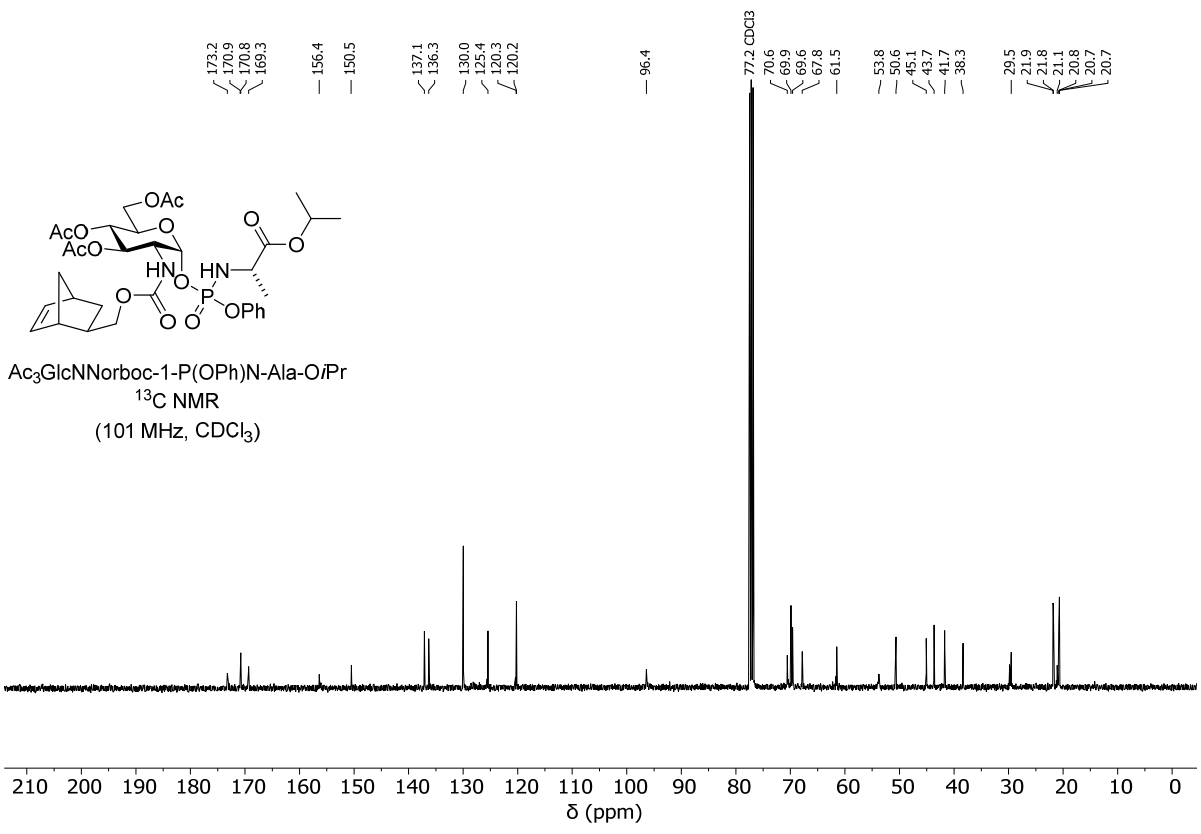
$Ac_3GlcNNorboc\text{-}1\text{-}P(SPTE)_2$
^{31}P NMR
(202 MHz, $CDCl_3$)



$Ac_3GlcNNorboc$-1-P(OPh)N-Ala-O*i*Pr

^1H NMR

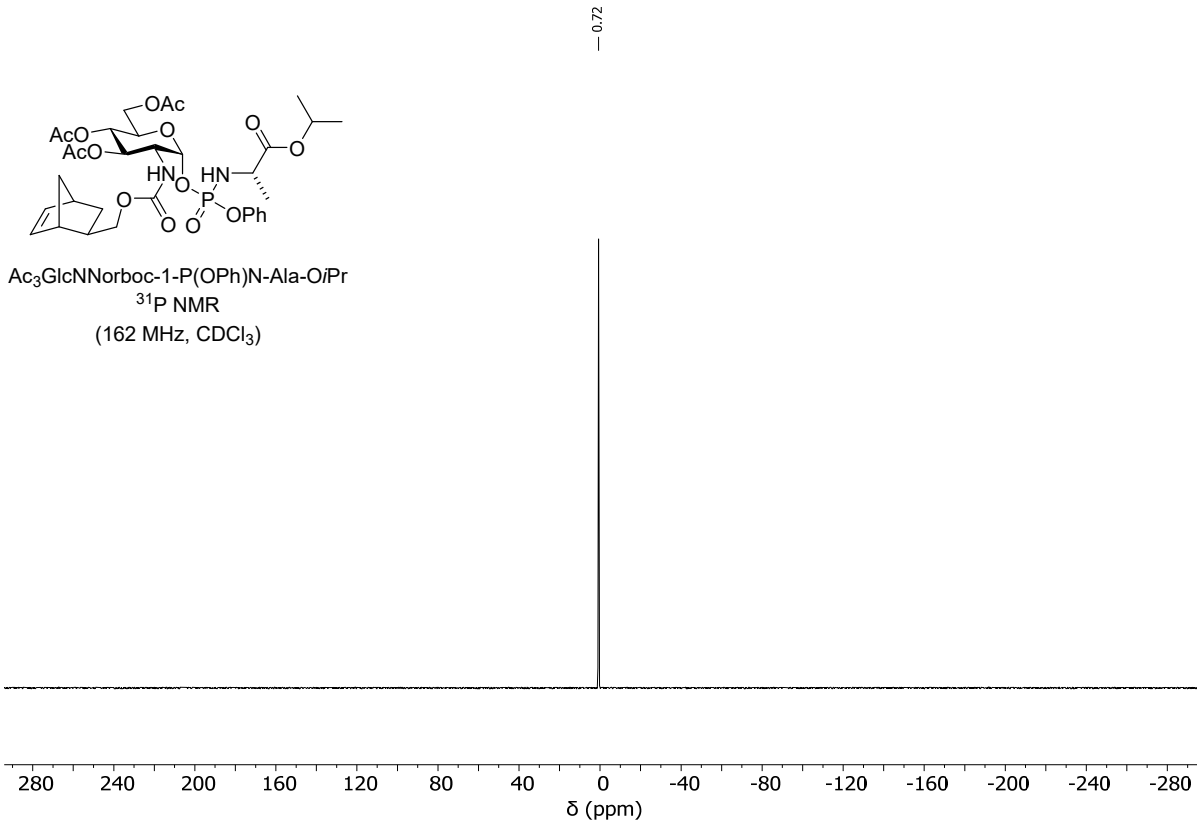
(400 MHz, $CDCl_3$)



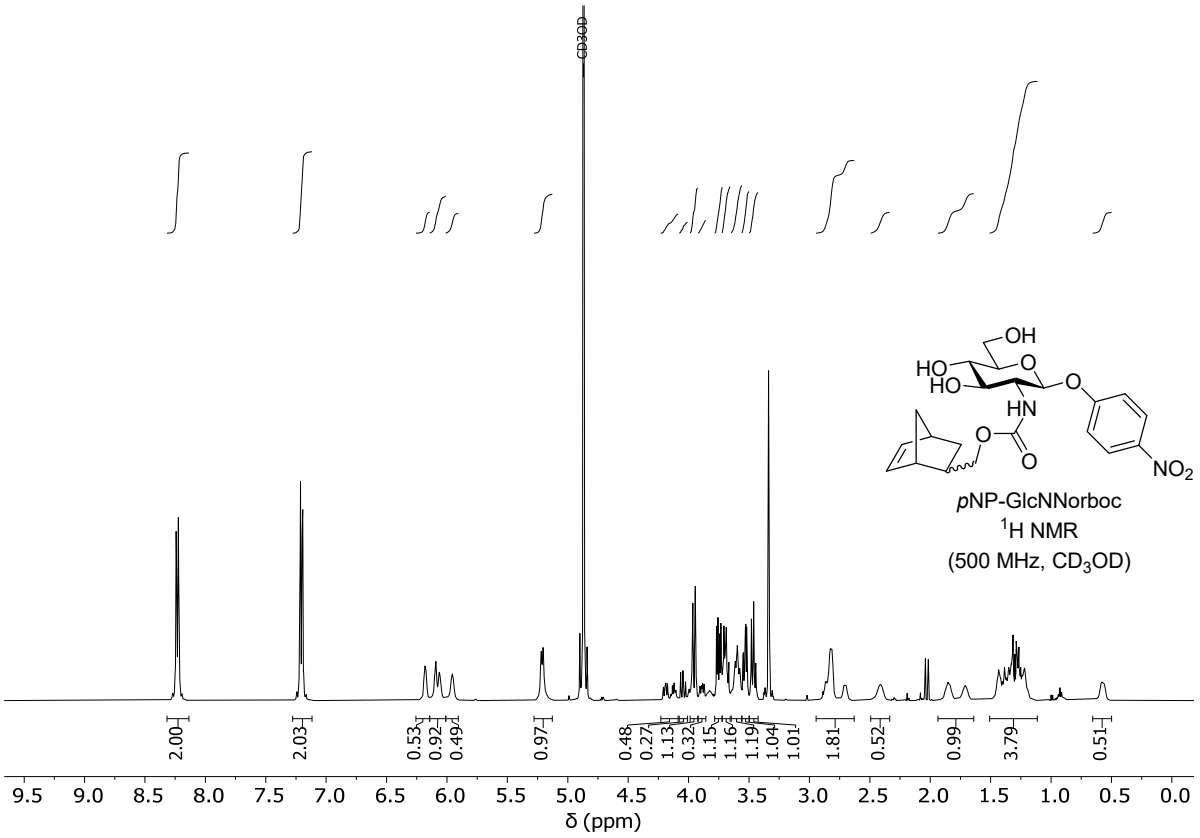
$Ac_3GlcNNorboc$-1-P(OPh)N-Ala-O*i*Pr

^{13}C NMR

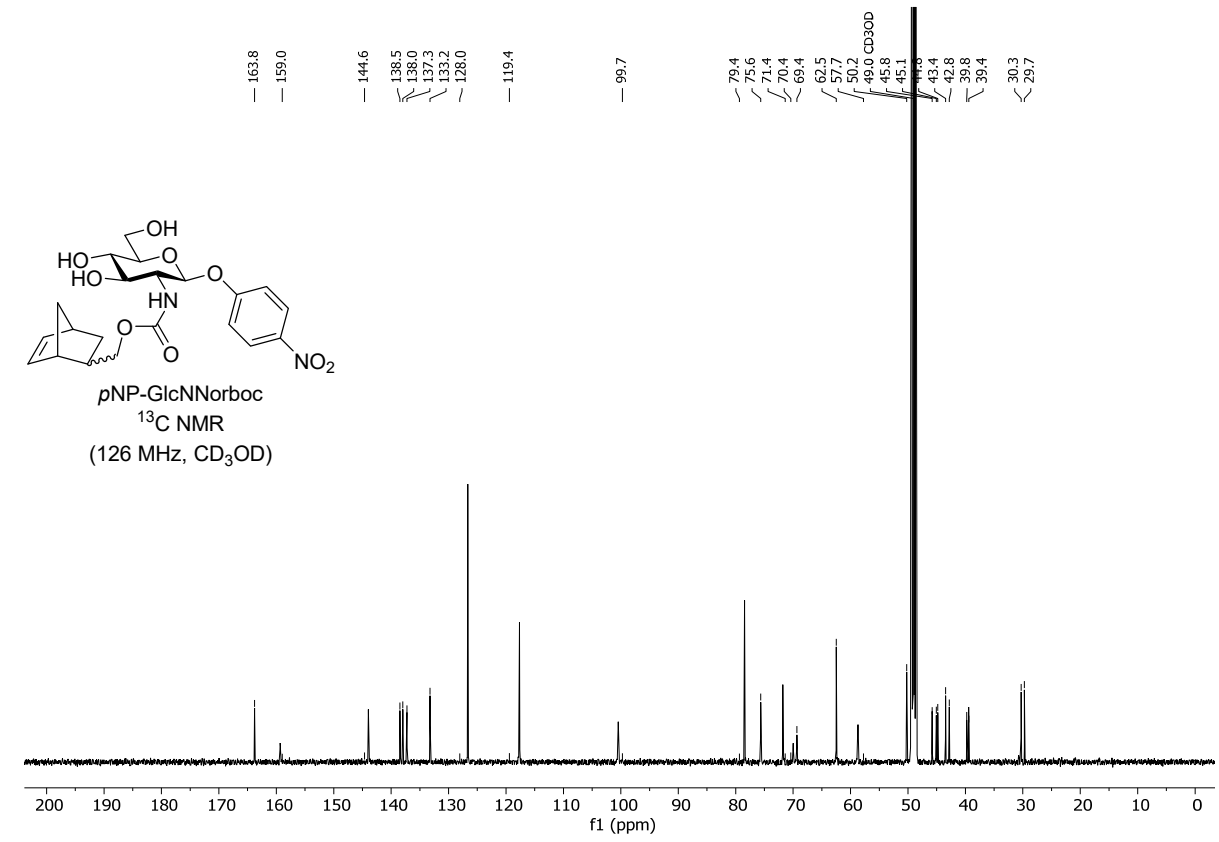
(101 MHz, $CDCl_3$)



Ac_3GlcNNorboc-1-P(OPh)N-Ala-O*i*Pr
^{31}P NMR
(162 MHz, $CDCl_3$)



*p*NP-GlcNNorboc
^{1}H NMR
(500 MHz, CD_3OD)



*p*NP-GlcNNorboc
^{13}C NMR
(126 MHz, CD_3OD)