

Supplementary Information

Synthesis and Protein Binding Studies of a Peptide Fragment of Clathrin Assembly Protein AP180 Bearing an *O*-Linked β -*N*- Acetylglucosaminyl-6-phosphate Modification

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Materials

Unless specified, commercial reagents were used without further purification. Amino acids, coupling reagents and resins were obtained from Novabiochem. Dichloromethane, toluene and methanol were distilled from calcium hydride. Tetrahydrofuran and diethyl ether were freshly distilled from sodium and benzophenone. DMF was obtained as peptide synthesis grade from Merck or Labscan. Analytical thin layer chromatography (TLC) was performed on commercially prepared silica plates (Merck Kieselgel 60 0.25 mm F254). Flash column chromatography was performed using 230-400 mesh Kieselgel 60 silica eluting with distilled solvents as described.

General Procedures

Melting points (mp) were recorded on a Stanford Research Systems Optimelt melting point apparatus and are uncorrected. ^1H NMR spectra were recorded using a Bruker DRX 400 spectrometer at a frequency of 400 MHz. ^{13}C NMR spectra were recorded on a Bruker DPX 400 spectrometer at a frequency of 100 MHz. The spectra are reported as parts per million (ppm) downfield shift using trimethylsilane as the internal reference. The data are reported as chemical shift (δ), multiplicity, relative integral, coupling constant (J Hz) and assignment where possible. IR spectra were measured on a Bruker ALPHA-E FTIR spectrometer fitted with a ZnSe ATR accessory as a thin film. Optical rotations were obtained using a Perkin Elmer model 341 polarimeter at 20 °C and $[\alpha]_{\text{D}}$ values are reported in $10^{-1} \text{ deg cm}^2 \text{ g}^{-1}$. Low resolution mass spectra were recorded on a Finnigan LCQ Deca ion trap mass spectrometer (ESI) operating in positive ion mode. High resolution ESI mass spectra were measured on a Bruker–Daltonics Apex Ultra 7.0T fourier transform mass spectrometer (FTICR).

Analytical reverse-phase HPLC was performed on a Waters System 2695 separations module with an Alliance series column heater at 30 °C and 2996 photodiode array detector. A Waters Sunfire 5 μm , 2.1 x 150 mm column (C-18) was used at a flow rate of 0.2 ml min^{-1} using a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) using linear gradients as described. Results were analysed with Waters Empower software. Preparative and semi-preparative reverse-phase HPLC were performed using a Waters 600 Multisolvant Delivery System and Waters 500 pump with 2996 photodiode array detector or Waters 490E Programmable wavelength detector operating at 230 nm. Peptide **1**, glycopeptide **2** and glycophosphopeptide **3** were purified on a Waters Sunfire 5 μm (C-18)

preparative column operating a flow rate of 7 ml min⁻¹ using a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B).

LC-MS was performed on a Thermo Separation Products: Spectra System consisting of P400 Pump and a UV6000LP Photodiode array detector on a Phenomenex Jupiter 5 μm, 2.1 x 150 mm column at a flow rate of 0.2 ml min⁻¹ coupled to a Thermoquest Finnigan LCQ Deca mass spectrometer (ESI) operating in positive mode. Separations involved a mobile phase of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) using a linear gradient of 0-25% B or 0-30%B over 30 min.

Synthetic Procedures

***N*-(Fluoren-9-ylmethoxycarbonyl)-*O*-[3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-L-threonine allyl ester (7)** To a solution of **5** (4.6 g, 7.35 mmol) and Fmoc-L-Thr-OAllyl (3.66 g, 9.59 mmol) in anhydrous CH₂Cl₂ (82 ml) was added activated 4Å molecular sieves (11 g) and the mixture stirred at rt for 1 h. The mixture was cooled to -78 °C before the addition of TMSOTf (143 μl, 0.74 mmol) and the reaction stirred at -78 °C for 90 min. The reaction was quenched by the addition of *N,N*-diisopropylethylamine (128 μl, 0.74 mmol) and allowed to warm to rt. The mixture was then filtered through Celite and the solvent removed under reduced pressure. The residue was purified by column chromatography on SiO₂ (eluent: 3:2 v/v hexane/EtOAc) to afford **7** as a white solid (5.59 g, 90%). ¹H NMR (400 MHz, CDCl₃) δ 7.76 (d, 2H, *J* = 8 Hz, ArH), 7.66 (d, 2H, *J* = 4 Hz, ArH), 7.40 (t, 2H, *J* = 8 Hz, ArH), 7.33 (t, 2H, *J* = 8 Hz, ArH), 5.94-5.86 (m, 1H, CH=CH₂), 5.75 (d, 1H, *J* = 8 Hz, NH), 5.36-5.22 (m, 4H, NH, CH=CH₂, H-3), 5.05 (t, 1H, *J* = 8 Hz, H-4), 4.76-4.63 (m, 5H, H-1, CH₂CCl₃, OCH₂CH of allyl), 4.49-4.39 (m, 3H, CH₂ of Fmoc, β-H), 4.32-4.25 (m, 3H, CH of Fmoc, α-H, H-6), 4.08 (dd, 1H, *J*_{6,5} = 4 Hz, *J*_{6,6'} = 12 Hz, H-6'), 3.66-3.64 (m, 1H, H-5), 3.61-3.54 (m, 1H, H-2), 2.07 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.04 (s, 3H, OAc), 1.24 (d, 3H, *J* = 8 Hz, γ-CH₃); ¹³C NMR (400 MHz, CDCl₃); 170.8, 170.6, 169.7, 169.4, 156.8, 154.1, 143.9, 143.8, 141.3, 131.7, 127.7, 127.1, 125.2, 124.8, 119.9, 118.5, 98.3, 95.3, 74.5, 73.9, 71.7, 71.5, 68.4, 67.4,

66.1, 61.8, 58.6, 56.3, 47.1, 20.7, 16.7, 14.2. These data are in agreement with those previously reported by Saha *et al.*¹

***N*-(Fluoren-9-ylmethoxycarbonyl)-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- β -D-glucopyranosyl)-L-threonine allyl ester (**8**)** To a solution of **7** (5.56 g, 6.69 mmol) in acetic anhydride (82 ml) cooled to 0 °C was added activated zinc dust (25.5 g) followed by glacial acetic acid (24.5 ml). The mixture was stirred at rt for 90 min before filtering through a plug of Celite. The solvent was removed under reduced pressure and the product purified by column chromatography on SiO₂ (eluent: 1:3 v/v hexane/EtOAc) to afford **8** as a white foam (4.36 g, 92 %). ¹H NMR (400 MHz, CDCl₃) δ 7.76 (d, 2H, *J* = 8 Hz, ArH), 7.63-7.60 (m, 2H, ArH), 7.36 (t, 2H, *J* = 8 Hz, ArH), 7.30-7.26 (t, 2H, *J* = 8 Hz, ArH), 5.96-5.84 (m, 2H, CH=CH₂, NH), 5.70 (d, 1H, *J* = 8 Hz, NH), 5.36-5.22 (m, 3H, CH=CH₂, H-3), 5.04 (t, 1H, *J* = 8 Hz, H-4), 4.72 (d, 1H, *J* = 8 Hz, H-1), 4.66-4.59 (m, 2H, OCH₂CH), 4.43-4.34 (m, 4H, CH₂ of Fmoc, α -H, β -H), 4.07 (d, 1H, *J* = 12 Hz, H-6), 3.75-3.64 (m, 2H, H-5, H-2), 2.06 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.90 (s, 3H, NHAc), 1.21 (d, 3H, *J* = 4 Hz, γ -CH₃); ¹³C NMR (400 MHz, CDCl₃) 170.0, 170.7, 169.9, 169.4, 156.8, 143.9, 143.7, 141.3, 131.7, 127.7, 127.1, 125.2, 119.9, 118.5, 98.4, 74.3, 71.9, 71.6, 68.4, 67.2, 66.1, 61.9, 58.6, 55.1, 47.1, 23.3, 20.7, 20.6, 20.5, 16.8. These data are in agreement with those previously reported by Saha *et al.*¹

***N*-(Fluoren-9-ylmethoxycarbonyl)-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- β -D-glucopyranosyl)-L-threonine (**4**)** To a solution of **8** (2.18 g, 3.07 mmol) in THF (44 ml) was added Pd(PPh₃)₄ (355 mg, 0.31 mmol) followed by *N*-methylaniline (3.3 ml, 30.7 mmol). The reaction was stirred at rt for 30 min before the solvent was removed under reduced pressure. The residue was purified by column chromatography on SiO₂ (eluent: 5:94:1 v/v/v MeOH/CH₂Cl₂/AcOH) to afford **4** as a pale yellow foam (2.03 g, 99%). ¹H NMR (300 MHz, CDCl₃) δ 7.75 (d, 2H, *J* = 8 Hz, ArH), 7.64 (d, 2H, *J* = 8 Hz, ArH), 7.41-7.28 (m, 4H, ArH), 6.03 (d, 1H, *J* = 8 Hz, NH), 5.87 (d, 1H, *J* = 12 Hz, NH), 5.29 (t, 1H, *J* = 12 Hz, H-3), 5.05 (t, 1H, *J* = 12 Hz, H-4), 4.79 (d, 1H, *J* = 12 Hz, H-1), 4.47-4.21 (m, 5H, CH₂ of Fmoc, α -H, β -H, H-6), 4.14 (dd, 1H, *J* = 4 Hz, 12 Hz, H-6'), 3.82-3.67 (m, 2H, H-5, H-2), 2.08 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.02 (s, 6H, 2 x OAc), 1.94 (s, 3H, NHAc), 1.23 (d, 3H, *J* = 4 Hz, γ -CH₃); ¹³C NMR (300 MHz, CDCl₃) 172.7, 171.9, 171.1, 171.0, 169.6, 157.0, 144.0, 141.4, 127.8,

127.2, 125.3, 120.1, 99.6, 72.2, 71.8, 68.7, 67.4, 61.9, 47.2, 23.3, 21.0, 20.8, 20.7, 17.7. These data are in agreement with those previously reported by Carvalho *et al.*²

Phenyl 2-deoxy-1-thio-2-(2,2,2-trichloroethoxycarbonyl-amino)-6-diphenylphosphoryl β -D-glucopyranoside (12). To a solution of **11** (3.98 g, 8.90 mmol) in anhydrous pyridine (195 ml) cooled to 0 °C was added diphenyl chlorophosphate (2.76 ml, 13.35 mmol). The mixture was allowed to warm to rt and stirred for 16 h. The solvent was removed under reduced pressure and the residue diluted in EtOAc (500 ml) and washed with water (500 ml) and brine (500 ml). The organic phase was dried over Na₂SO₄ and concentrated under reduced pressure. The product was purified by column chromatography on SiO₂ (eluent: 1:9 v/v MeOH:CH₂Cl₂) to afford **12** as a white foam (5.31 g, 88%). IR (CHCl₃) 3406, 1721, 1488, 1269, 1219, 1024, 960, 769, 689 cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 7.49-7.47 (m, 2H, 2 x ArH), 7.32-7.14 (m, 13H, 13 x ArH), 5.80 (d, 1H, *J* = 8 Hz, NH), 4.82 (d, 1H, *J* = 12 Hz, CHHCCl₃), 4.71 (d, 1H, *J* = 12 Hz, H-1), 4.64 (d, *J* = 12 Hz, CHHCCl₃), 4.52-4.39 (m, 2H, H-6,6'), 3.63 (t, 1H, *J* = 8 Hz, H-3), 3.45-3.31 (m, 3H, H-5, H-4, H-2); ¹³C NMR (400 MHz, CDCl₃) 154.8, 150.3, 132.6, 129.9, 128.9, 127.9, 125.8, 120.2, 95.5, 86.5, 78.1, 75.3, 74.7, 69.8, 67.9, 56.4. ESI-MS *m/z* 702.13 [M+Na]⁺, HRMS (ESI) *m/z* calcd for C₂₇H₂₇Cl₃NO₉PSNa (M+Na) 702.0107, found 702.0078.

Phenyl 3,4-di-O-acetyl-2-deoxy-1-thio-2-(2,2,2trichloroethoxy-carbonylamino)-6-diphenylphosphoryl β -D-glucopyranoside (13). To a solution of **12** (5.27 g, 7.76 mmol) in pyridine (71.6 ml) at 0 °C was added acetic anhydride (35.8 ml). The solution was stirred for 16 h at rt before the solvent was removed under reduced pressure. The residue was diluted with EtOAc (200 ml) and washed with water (200 ml) and brine (200 ml). The organic phase was dried over Na₂SO₄ and concentrated under reduced pressure. The product was purified by column chromatography on SiO₂ (eluent: 1:1 v/v hexane/EtOAc) to afford **13** as a white foam (5.28 g, 89%). IR (CHCl₃) 1750, 1489, 1223, 1026, 960, 754 cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 7.52 (d, 2H, *J* = 8 Hz, 2 x ArH), 7.50-7.17 (m, 13H, 13 x ArH), 5.33-5.25 (m, 2H, NH, H-3), 4.97 (t, 1H, *J* = 8 Hz, H-4), 4.86 (d, 1H, *J* = 8 Hz, H-1), 4.80 (d, 1H, *J* = 12 Hz, CHHCCl₃), 4.73 (d, 1H, *J* = 12 Hz, CHHCCl₃), 4.35-4.28 (m, 2H, H-6,6'), 3.75-3.74 (m, 1H, H-5), 3.65 (m, 1H, H-2), 2.00 (s, 3H, OAc), 1.98 (s, 3H, OAc); ¹³C NMR (CDCl₃, 400 MHz) 170.5, 169.5, 153.9, 150.4, 133.1, 131.7, 129.8, 129.3, 129.0, 128.4, 125.5, 120.1,

95.4, 86.5, 76.3, 74.5, 73.168.6, 67.1, 55.0, 20.6, 20.5; ESI-MS m/z 784.07 ($[M+Na]^+$), HRMS (ESI) m/z calcd for $C_{31}H_{31}Cl_3NO_{11}PSNa$ ($M+Na$) 784.0319, found 784.0304.

2-(2,2,2-trichloroethoxycarbonylamino)-3,4-di-*O*-acetyl-6-diphenylphosphoryl-2-deoxy- β -D-glucopyranoside (14). A solution of **13** (1.41 g, 1.81 mmol) in acetone (16.4 ml) was added to a solution of acetone/water (9:1 v/v, 125 ml). *N*-bromosuccinimide (1.65 g, 9.25 mmol) was added and the mixture stirred at rt for 10 min. The reaction was quenched by the addition of solid $NaHCO_3$ (4.23 g) and stirred for an additional 10 min. The solvent was removed under reduced pressure and the residue diluted in EtOAc (400 ml) and washed repeatedly with water (400 ml) until the aqueous phase was at pH 7. The organic phase was washed further with brine (400 ml) before concentrating under reduced pressure. The product was purified by column chromatography on SiO_2 (eluent: 3:2 v/v hexane/EtOAc) to afford **14** as a white foam (1.03 g, 83%). IR ($CHCl_3$) 1750, 1489, 1241, 1221, 1188, 965, 737 cm^{-1} ; 1H NMR (400 MHz, $CDCl_3$) δ 7.37-7.33 (m, 4H, 4 x ArH), 7.22-7.20 (m, 6H, 6 x ArH), 5.33 (d, 1H, $J = 8$ Hz, NH), 5.28 (t, 1H, $J = 12$ Hz, H-3), 5.07-5.01 (m, 2H, H-1, H-4), 4.80 (d, 1H, $J = 12$ Hz, $CHHCCl_3$), 4.69 (br s, 1H, OH), 4.62 (d, 1H, $J = 12$ Hz, $CHHCCl_3$), 4.33-4.20 (m, 3H, H-6,6', H-5), 3.88 (m, 1H, H-2). ^{13}C NMR (400 MHz, $CDCl_3$) 170.9, 169.4, 154.2, 150.4, 129.8, 125.6, 120.1, 95.4, 92.4, 91.4, 74.6, 71.0, 68.9, 68.4, 67.9, 67.4, 54.1, 20.7. ESI-MS m/z 692.20 ($[M+Na]^+$), HRMS (ESI) m/z calcd for $C_{25}H_{27}Cl_3NO_{12}PNa$ ($M+Na$) 692.0234, found 692.0216.

1-(2,2,2-trichloroetanimidate)-2-(2,2,2-trichloroethoxy-carbonylamino)-3,4-di-*O*-acetyl-6-diphenylphosphoryl-2-deoxy- α -D-glucopyranoside (15). To a solution of **14** (880 mg, 1.31 mmol) in anhydrous CH_2Cl_2 (5.5 ml) and Cl_3CCN (1.3 ml, 13.1 mmol) was added DBU (20 μ l, 0.13 mmol). The reaction was stirred at rt for 16 h before the solvent was removed under reduced pressure. The product was purified using a SiO_2 plug (eluent: 3:2 v/v hexane/EtOAc) to afford **15** as a white foam (862 mg, 81%). IR ($CHCl_3$) 1752, 1538, 1220, 1027, 964, 772 cm^{-1} ; 1H NMR (400 MHz, $CDCl_3$) δ 8.77 (s, 1H, NH), 7.36-7.26 (m, 4H, 4 x ArH), 7.21-7.18 (m, 6H, 6 x ArH), 6.40 (d, 1H, $J = 4$ Hz, H-1), 5.33 (t, 1H, $J = 12$ Hz, H-3), 5.21-5.17 (m, 2H, H-4, NH), 4.72 (s, 2H, CH_2CCl_3), 4.36-4.28 (m, 2H, H-6,6'), 4.20-4.17 (m, 2H, H-5, H-2), 2.05 (s, 3H, OAc), 2.03 (s, 3H, OAc). ^{13}C NMR (400 MHz, $CDCl_3$) 171.1, 169.1, 160.2, 54.1, 150.4, 129.8, 125.1120.1, 100.4, 95.0, 90.4, 74.6, 70.8, 70.2, 67.5, 66.4,

54.4, 20.6, 20.5. ESI-MS m/z 837.07 $[M+Na]^+$, HRMS (ESI) m/z calcd for $C_{27}H_{27}Cl_6N_2O_{12}PNa$ (M+Na) 836.9301, found 836.9293.

***N*-(Fluoren-9-ylmethoxycarbonyl)-*O*-(2-acetamido-3,4-di-*O*-acetyl-2-[2,2,2-trichloroethoxycarbonylamino]-6-diphenyl-phosphoryl-2-deoxy- β -D-glucopyranosyl)-L-threonine allyl ester (**16**).** To a solution of **15** (826 mg, 1.01 mmol) and Fmoc-Thr-OAllyl (524 mg, 1.37 mmol) in anhydrous CH_2Cl_2 (20 ml) was added activated 4Å molecular sieves (2.2 g) and the mixture stirred at rt for 1 h. The mixture was cooled to 0 °C before the addition of TMSOTf (20 μ l, 0.11 mmol) and the reaction stirred at 0 °C for 2 h. The reaction was quenched by the addition of *N,N*-diisopropylethylamine (18 μ l, 0.11 mmol) and allowed to warm to rt. The mixture was filtered through a plug of Celite and the solvent removed under reduced pressure. The residue was purified through a silica plug (3:2 v/v hexane/EtOAc) to afford **16** as a white foam (958 mg, 88%). IR ($CHCl_3$) 1748, 1489, 1218, 1187, 1025, 954, 737 cm^{-1} ; 1H NMR (400 MHz, $CDCl_3$) δ : 7.75 (d, 2H, J = 4 Hz, 2 x ArH), 7.63 (d, 2H, J = 8 Hz, 2 x ArH), 7.39-7.16 (m, 14H, 14 x ArH), 5.88 (m, 1H, $CH=CH_2$), 5.32 (s, 1H, NH), 5.27 (t, 1H, J = 8 Hz, H-3), 5.19 (m, 3H, NH, $CH=CH_2$), 4.96 (t, 1H, J = 12 Hz, H-4), 4.74 (s, 2H, CH_2CCl_3), 4.70-4.57 (m, 3H, H-1, OCH_2CH of allyl), 4.45-4.21 (m, 7H, α -H, β -H, CH of Fmoc, CH_2 of Fmoc, H-6,6'), 3.72-3.71 (m, 1H, H-5), 3.54-3.47 (m, 1H, H-2), 2.02 (s, 3H, OAc), 1.98 (s, 3H, OAc), 1.18 (d, 3H, J = 8 Hz, γ - CH_3); ^{13}C NMR (400 MHz, $CDCl_3$) 170.6, 169.6, 169.3, 156.8, 154.0, 150.5, 150.4, 144.0, 143.9, 141.3, 131.8, 129.8, 127.7, 127.1, 125.5, 125.2, 120.1, 120.0, 119.9, 118.5, 98.1, 95.3, 74.4, 73.7, 72.4, 71.5, 68.9, 67.4, 67.1, 67.0, 66.1, 58.7, 56.4, 47.2, 20.5, 16.4. ESI-MS m/z 1033.19 $[M+H]^+$, HRMS m/z calcd for $C_{47}H_{49}Cl_3N_2O_{16}P$ (M+H) 1033.1885, found 1033.1890.

***N*-(Fluoren-9-ylmethoxycarbonyl)-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-6-diphenylphosphoryl-2-deoxy- β -D-glucopyranosyl)-L-threonine allyl ester (**17**).** To a solution of **16** (1.18 g, 1.14 mmol) in acetic anhydride (13.9 ml) cooled to 0 °C was added activated zinc dust (4.40 g) followed by glacial acetic acid (0.16 ml). The mixture was stirred at rt for 90 min before filtering through a plug of Celite. The solvent was removed under reduced pressure and the product purified by column chromatography on SiO_2 (eluent: 1:3 v/v hexane/EtOAc) to afford **17** as a white foam (831 mg, 81%). IR ($CHCl_3$) 1748, 1489, 1218, 1187, 1025, 954, 737 cm^{-1} ; 1H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, 2H, J = 8 Hz, 2 x ArH), 7.63-7.60 (m, 2H, 2 x ArH), 7.38-7.16 (m, 14H, 14 x ArH), 5.88-5.80 (m, 1H,

$\text{CH}=\text{CH}_2$), 5.28 (s, 1H, NH), 5.22 (t, 1H, $J = 8$ Hz, H-3), 5.16-5.13 (m, 2H, $\text{CH}=\text{CH}_2$), 4.94 (t, 1H, $J = 8$ Hz, H-4), 4.66-4.52 (m, 3H, H-1, OCH_2CH), 4.39-4.24 (m, 6H, H-6,6', α -H, β -H, CH_2 of Fmoc), 4.20 (t, 1H, $J = 8$ Hz, CH of Fmoc), 3.77-3.75 (m, 2H, H-5, H-2), 1.99 (s, 3H, OAc), 1.96 (s, 3H, OAc), 1.91 (s, 3H, NHAc), 1.16 (d, 3H, γ - CH_3); ESI-MS m/z 923.60 $[\text{M}+\text{Na}]^+$, HRMS (ESI) m/z calcd for $\text{C}_{46}\text{H}_{49}\text{N}_2\text{O}_{15}\text{PNa}$ 923.2768, found 923.2756.

***N*-(9H-fluoren-9-ylmethoxycarbonyl)-*O*-(2-acetamido-3,4-di-*O*-acetyl-6-**

diphenylphosphoryl-2-deoxy- β -D-glucopyranosyl)-L-threonine (9). To a solution of **17** (825 mg, 0.92 mmol) in THF (16.4 ml) was added $\text{Pd}(\text{PPh}_3)_4$ (106 mg, 0.09 mmol) followed by *N*-methylaniline (1 ml, 9.16 mmol). The reaction was stirred at rt for 30 min before the solvent was removed under reduced pressure. The residue was purified by column chromatography on SiO_2 (eluent: 5:94:1 v/v/v MeOH/ CH_2Cl_2 /AcOH) to afford **9** as a pale yellow foam (709 mg, 90%). $[\alpha]_D +14.1$ (c 1.0, CHCl_3); IR (CHCl_3) 3296, 3067, 2979, 2250, 1748, 1722, 1515, 1214, 1037, 958, 728; ^1H NMR (400 MHz, CDCl_3) δ 7.75 (d, 2H, $J = 8$ Hz, 2 x ArH), 7.64 (t, 2H, $J = 8$ Hz, 2 x ArH), 7.39-7.19 (m, 14H, 14 x ArH), 5.83 (d, 1H, $J = 8$ Hz, NHAc), 5.73 (d, 1H, $J = 8$ Hz, NHFmoc), 5.28 (t, 1H, $J = 8$ Hz, H-3), 4.95 (t, 1H, $J = 8$ Hz, H-4), 4.72 (d, 1H, $J = 8$ Hz, H-1), 4.43-4.22 (m, 5H, CH_2 of Fmoc, CH of Fmoc, α -H, β -H), 3.62-3.54 (m, 2H, H-5, H-2), 2.03 (s, 3H, OAc), 1.98 (s, 3H, OAc), 1.92 (s, 3H, NHAc), 1.18 (d, 3H, $J = 8$ Hz, γ - CH_3); ^{13}C NMR (400 MHz, CDCl_3) 171.5, 170.8, 170.4, 169.1, 156.9, 150.2, 150.1, 144.0, 143.8, 141.2, 132.0, 129.9, 128.6, 127.6, 127.1, 125.9, 125.9, 125.7, 125.3, 125.2, 120.4, 120.0, 119.9, 100.1, 72.0, 71.8, 68.1, 67.2, 66.6, 66.5, 58.4, 54.7, 47.1, 23.2, 20.6, 17.9; ESI-MS m/z 883.33 $[\text{M}+\text{Na}]^+$, HRMS (ESI) m/z calcd for $\text{C}_{43}\text{H}_{45}\text{N}_2\text{O}_{15}\text{PNa}$ (M+Na) 883.2455, found 883.2445.

General procedure for the solid-phase peptide synthesis (SPPS) of peptide 1 (20 μmol scale), glycopeptide 2 and glycoposphopeptide 3:

Resin loading: Rink amide resin (Novabiochem) (540 mg, 0.4 mmol) was swollen and washed with DMF (5×3 ml), DCM (5×3 ml) and DMF (5×3 ml). Fmoc deprotection was performed by suspending the resin in a solution of piperidine/DMF (1:9 v/v, 4 ml) for 3 min and the procedure repeated before the resin was washed with DMF (5×3 ml), DCM (5×3 ml) and DMF (5×3 ml). A solution of Fmoc-Asn(Trt)-OH (955 mg, 1.6 mmol, 4.0 equiv.),

benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) (832 mg, 1.6 mmol) and *N*-methylmorpholine (NMM) (352 μ l, 3.2 mmol) in DMF (3 ml) was added and the resin shaken at rt for 1 h. The resin was filtered and washed with DMF (5×3 ml), DCM (5×3 ml) and DMF (5×3 ml).

Fmoc Deprotection: Pre-loaded Rink Amide resin was suspended in a solution of piperidine/DMF (1:9 v/v, 5 ml) and shaken for 3 min. The procedure was repeated. The resin was subsequently washed with DMF (5×3 ml), DCM (5×3 ml), and DMF (5×3 ml).

Coupling (25 μ mol scale): A solution of a Fmoc-protected amino acid (100 μ mol, 4.0 equiv.), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) (52 mg, 100 μ mol) and *N*-methylmorpholine (NMM) (22 μ l, 200 μ mol) in DMF (3 ml) was added and the resin shaken at rt for 1 h. The resin was filtered and washed with DMF (5×3 ml), DCM (5×3 ml) and DMF (5×3 ml).

For **2** and **3**: *Coupling of Glycosylamino Acids 4 and 9*: A solution of **4** or **9** (30 μ mol, 1.2 equiv.), 2-(1H-7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU) (11 mg, 30 μ mol, 1.2 equiv.) and NMM (6.6 μ L, 60 μ mol, 2.4 equiv.) in DMF (0.7 ml) was added to the resin and shaken. After 18 h the resin was washed with DMF (5×3 ml), DCM (5×3 ml), and DMF (5×3 ml).

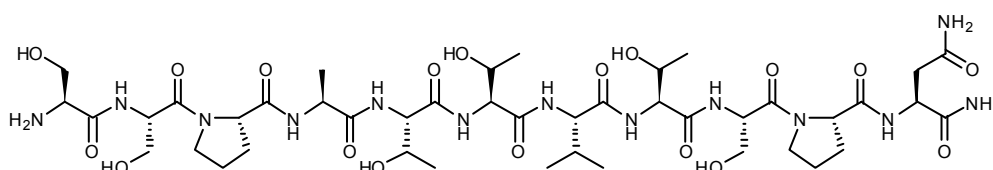
Capping: Acetic anhydride/pyridine (1:9 v/v, 2 ml) was added to the resin and shaken. After 3 min the resin was washed DMF (5×3 ml), DCM (5×3 ml), and DMF (5×3 ml).

The above steps were repeated in an iterative fashion to assemble the desired peptide/glycopeptide on the resin.

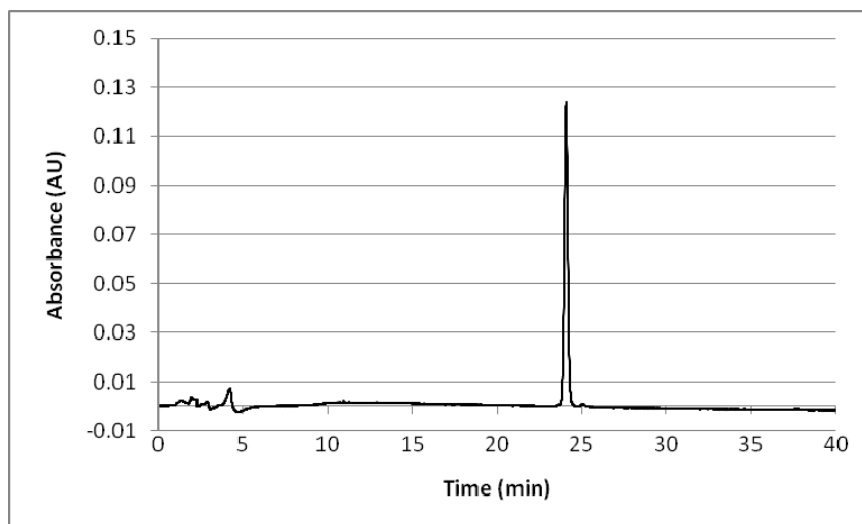
Resin cleavage: The resin was washed thoroughly with DCM (20×3 ml) and treated with a solution of TFA/TIS/water (90:5:5 v/v/v, 2 ml) and shaken for 2 h at rt. The resin was filtered and the filtrate was evaporated to dryness.

Synthesis and Analytical Data for AP180 peptide (**1**) and glycopeptides (**2** and **3**).

H₂N-Ser-Ser-Pro-Ala-Thr-Thr-Val-Thr-Ser-Pro-Asn-Ser-NH₂ (**1**)

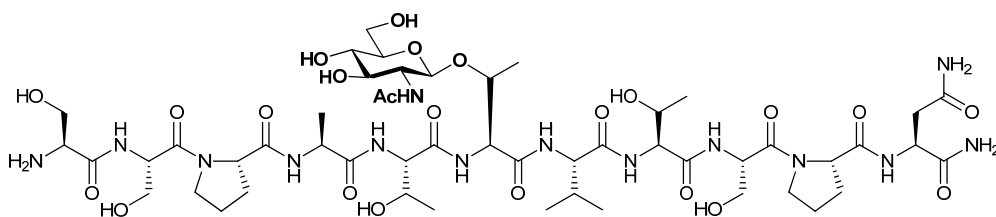


Peptide **1** was prepared according to the Fmoc-strategy SPPS method outlined in the general procedures. Purification by preparative reverse phase HPLC (0 to 15% B over 60 min) afforded **1** as a white solid following lyophilisation (12.2 mg, 46% yield based on the original 25 μ mol resin loading).



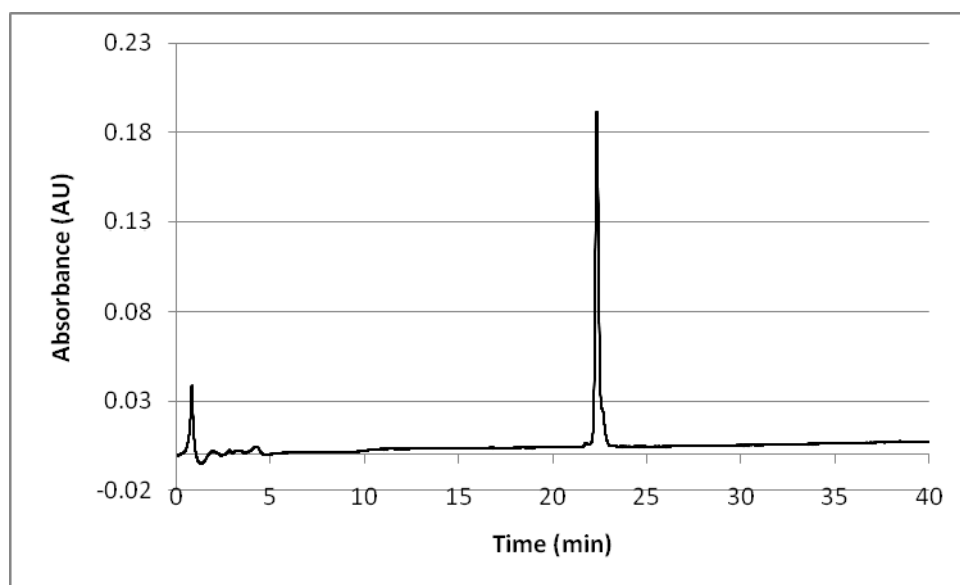
Analytical HPLC: R_t 24.1 min (0-15% B over 60 min, λ = 230 nm); HRMS (ESI) m/z calcd for $C_{43}H_{74}N_{13}O_{18}$ ($M+H$) 1060.5272, found 1060.5273 [$M+H$] $^+$, 672.2904 [$M+2H$] $^{2+}$.

H₂N-Ser-Ser-Pro-Ala-Thr-Thr(OGlcNAc)-Val-Thr-Ser-Pro-Asn-Ser-NH₂ (2)



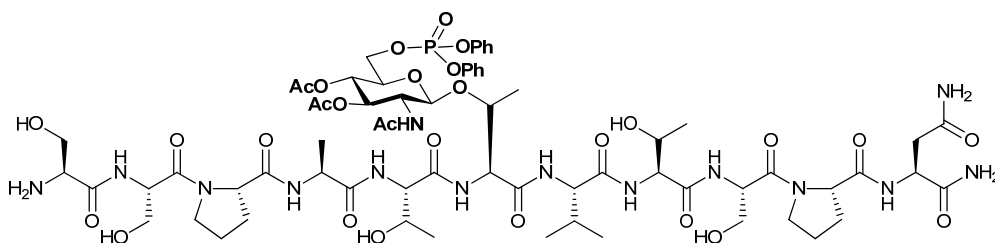
Glycopeptide **2** was prepared according to the Fmoc-strategy SPPS method outlined in the general procedures. After assembly of the resin bound glycopeptide, the resin was suspended

in a solution of hydrazine hydrate in methanol (1:6, 4 ml) and shaken for 6 h. The resin was washed with MeOH ($\times 10$) and DCM ($\times 10$) before acidic cleavage from the resin as outlined in the general procedures. Purification by preparative reverse phase HPLC (0 to 25% B over 60 min) afforded **2** as a white solid following lyophilisation (12.6 mg, 40% yield based on the original 25 μ mol resin loading).



Analytical HPLC: R_t 22.4 min (0-25% B over 60 min, $\lambda = 230$ nm); HRMS (ESI) m/z calcd for $C_{51}H_{87}N_{14}O_{23}$ ($M+H$) 1262.5990, found 1263.61 [$M+H$] $^+$, 632.31 [$M+2H$] $^{2+}$.

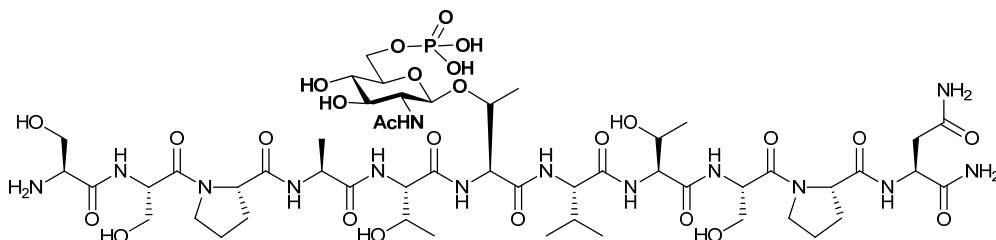
H₂N-Ser-Ser-Pro-Ala-Thr-Thr[β -GlcNAc-6-PO(OPh)₂]-Val-Thr-Ser-Pro-Asn-Ser-NH₂
(**18**)



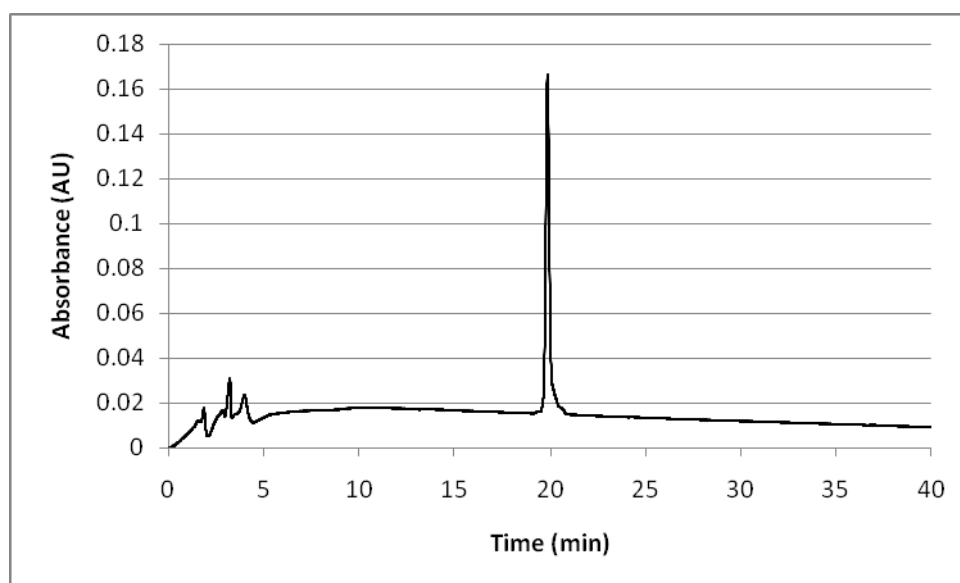
Glycopeptide **18** was prepared on 50 μ mol Rink amide resin according to the Fmoc-strategy SPPS method outlined in the general procedures. Purification by preparative reverse

phase HPLC (2 to 45% B over 45 min) afforded glycopeptide **18** as a white solid after lyophilisation (30.65 mg, 73% yield based on the original 25 μ mol resin loading)

Glycophosphopeptide 3: H₂N-Ser-Ser-Pro-Ala-Thr-Thr[β -GlcNAc-6-PO(OH)₂]-Val-Thr-Ser-Pro-Asn-Ser-NH₂



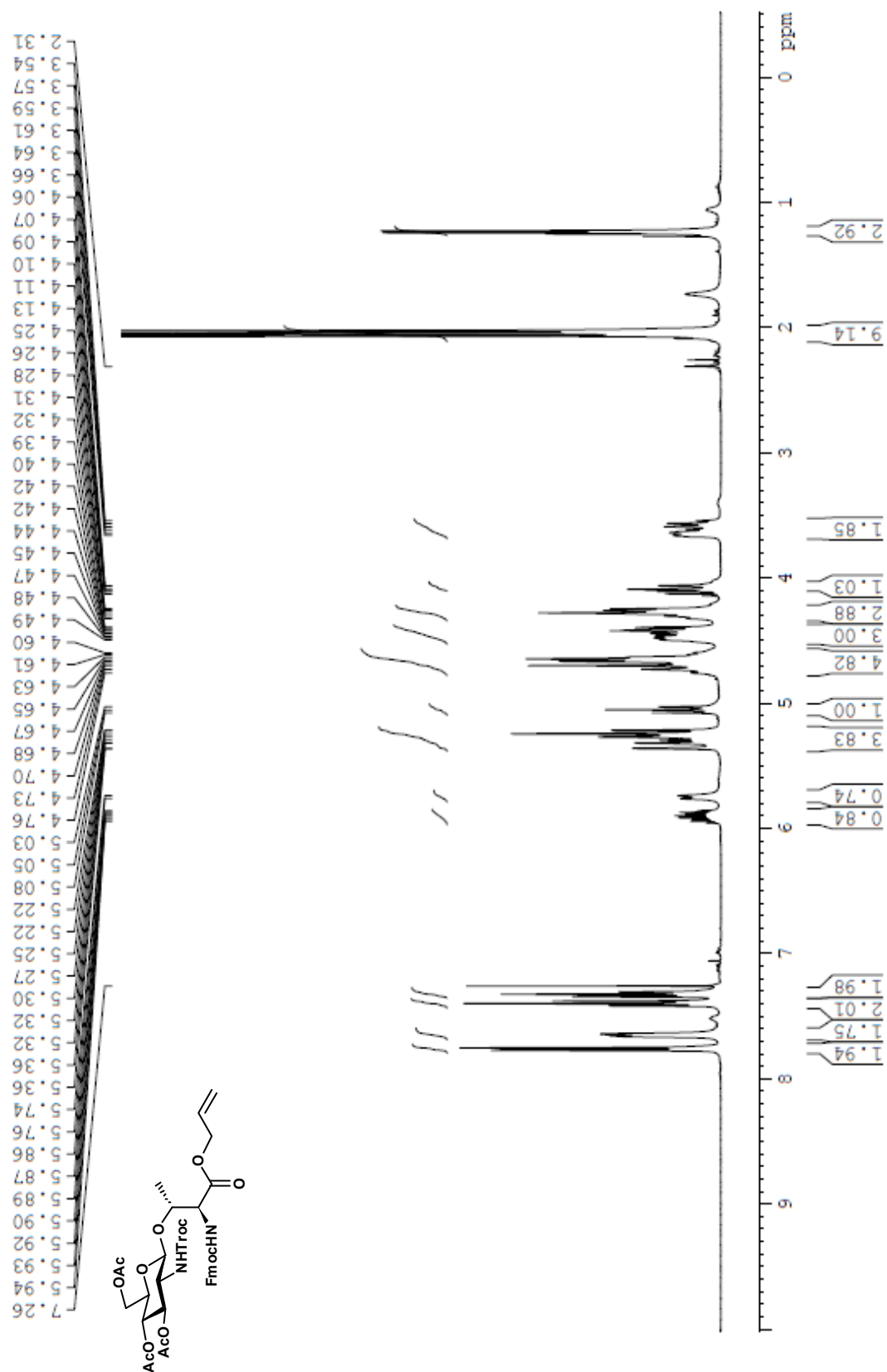
Glycopeptide **18** (10.4 mg, 6.3 μ mol) was dissolved in glacial acetic acid (0.3 ml) and PtO₂ (8.6 mg, 13.9 μ mol) was added. The suspension was placed under a hydrogen atmosphere and the reaction stirred at rt for 16 h. The suspension was filtered, washed with acetic acid followed by a 50 mM aqueous ammonium bicarbonate solution (10 ml). The filtrate was lyophilised and the crude material dissolved in methanol (0.4 ml). A solution of sodium methoxide in methanol (0.5 M) was added dropwise until a pH of 9 was reached. The reaction was stirred at rt for 2.5 h before neutralising with Amberlite 120H⁺ resin. The resin was filtered and washed with 0.1% TFA in water (3 ml). Purification by preparative reverse phase HPLC (0 to 25% B over 40 min) afforded **3** as a white solid following lyophilisation (4.2 mg, 50% yield over 2 steps).

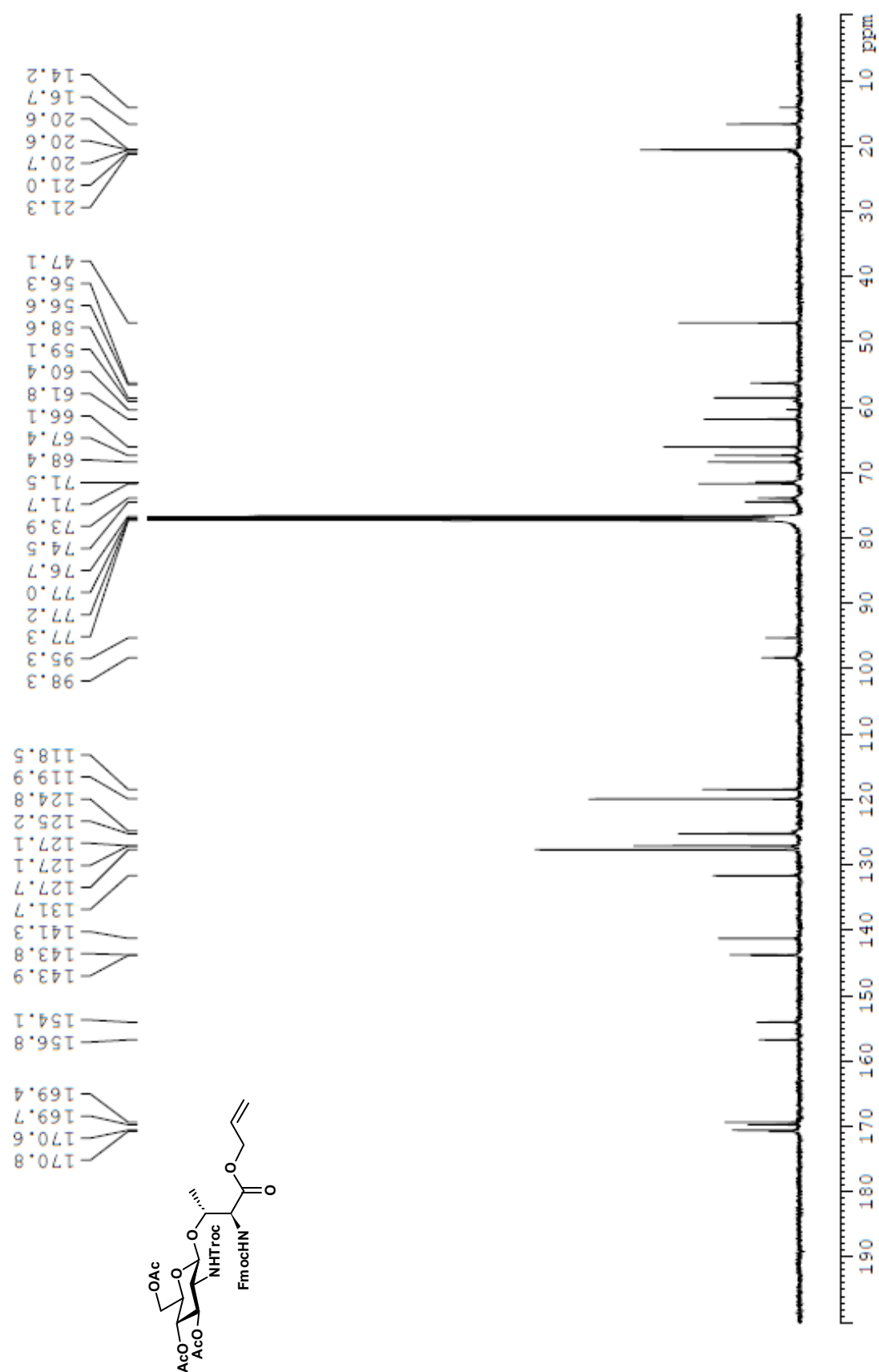


Analytical HPLC: R_t 19.9 min (0-25% B over 40 min, $\lambda = 230$ nm); HRMS (ESI) m/z calcd for $C_{51}H_{88}N_{14}O_{26}P$ (M+H) 1343.5732, found 1342.5729 [M+H]⁺, 672.2904 [M+2H]²⁺.

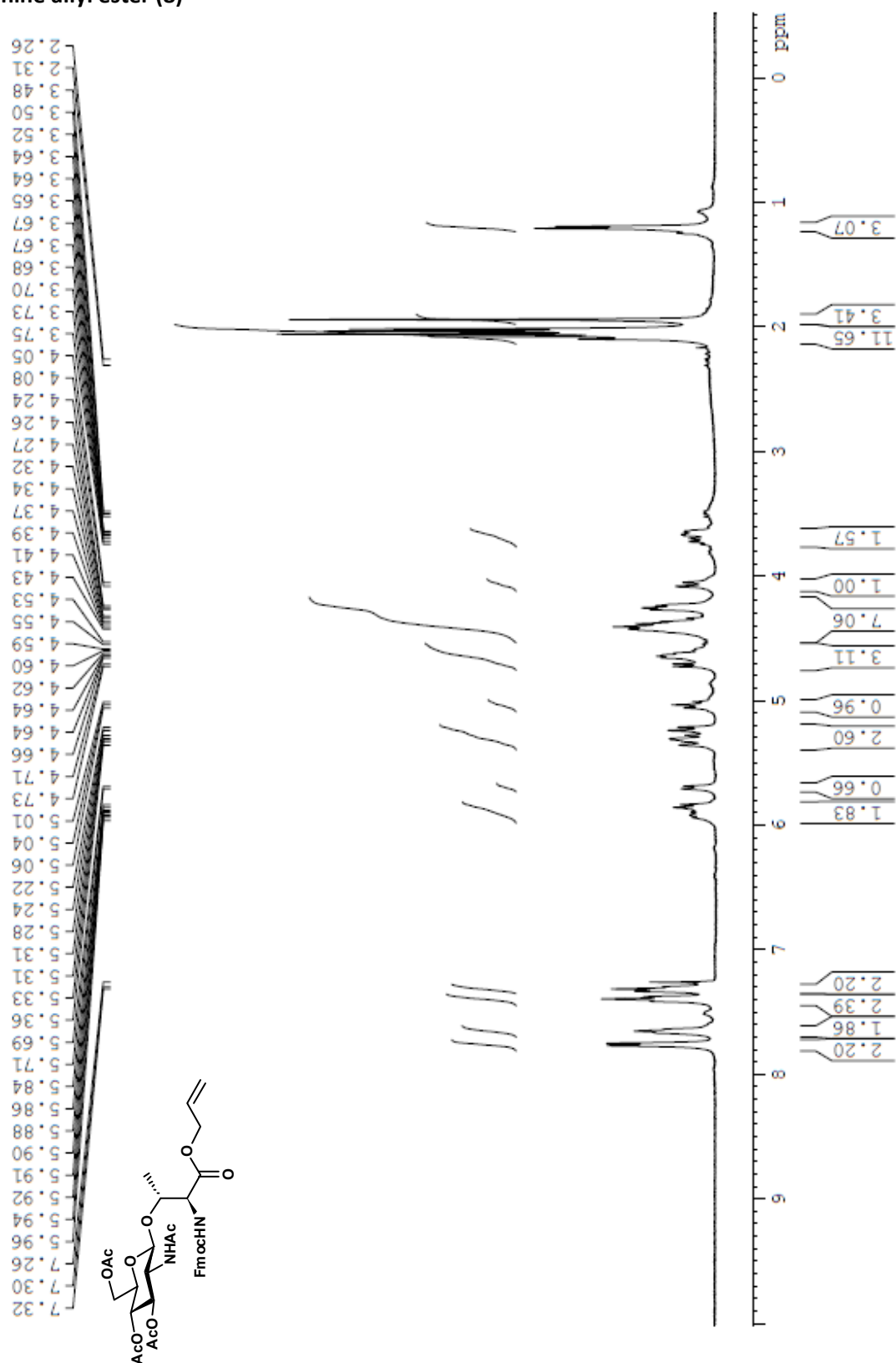
¹H and ¹³C NMR spectra for novel compounds

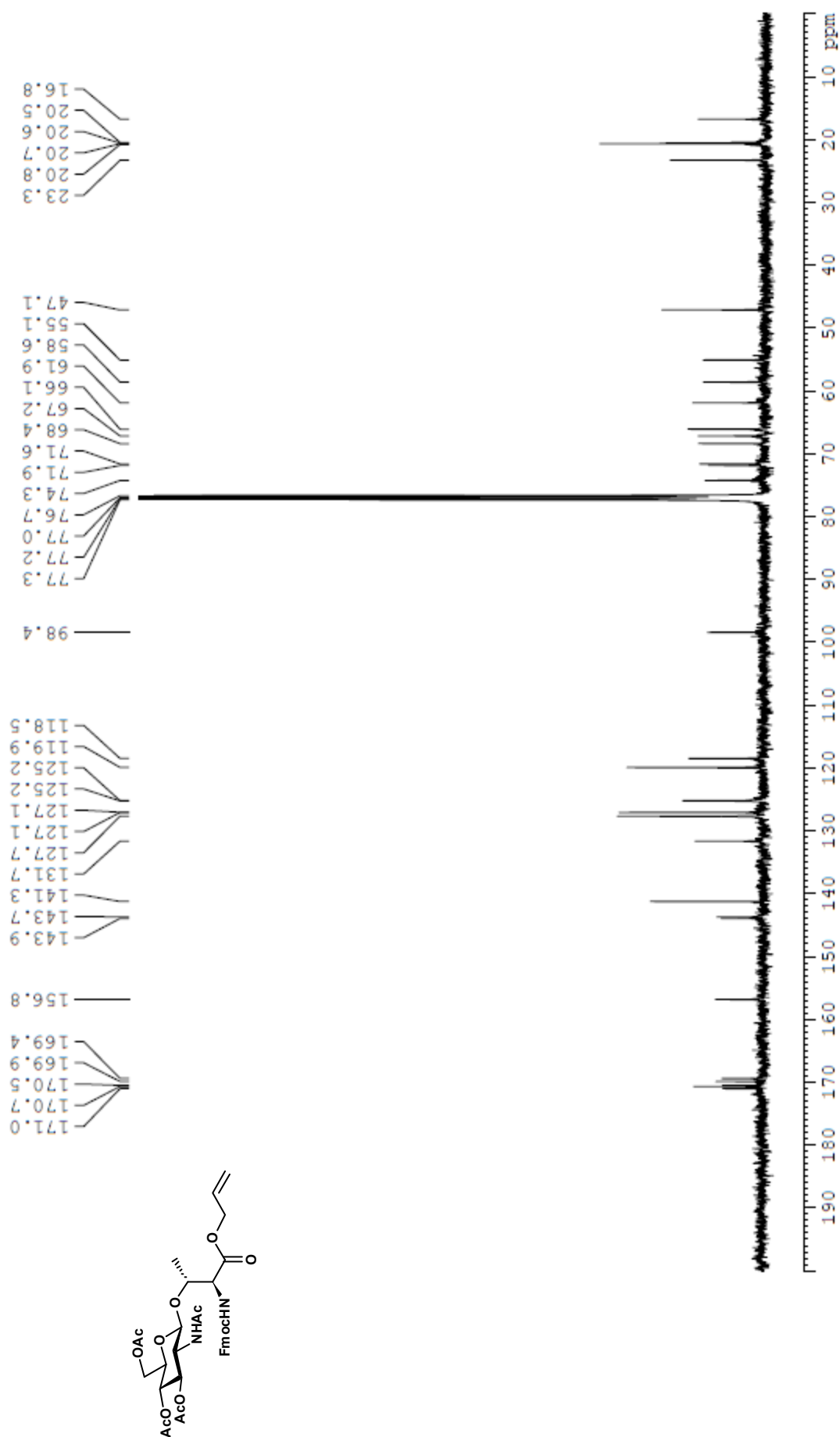
N-(Fluoren-9-ylmethoxycarbonyl)-*O*-[3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-*D*-glucopyranosyl]-*L*-threonine allyl ester (7)



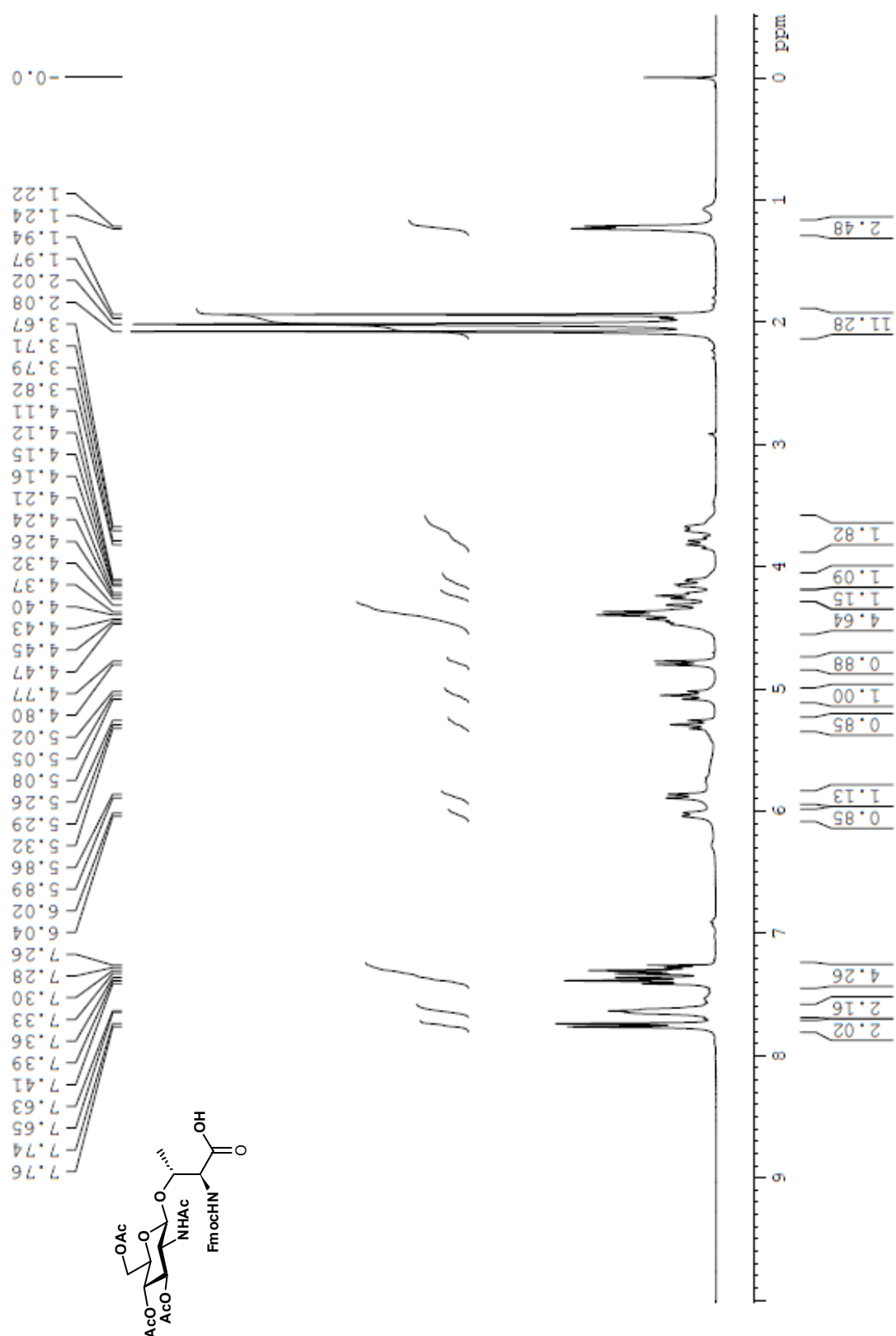


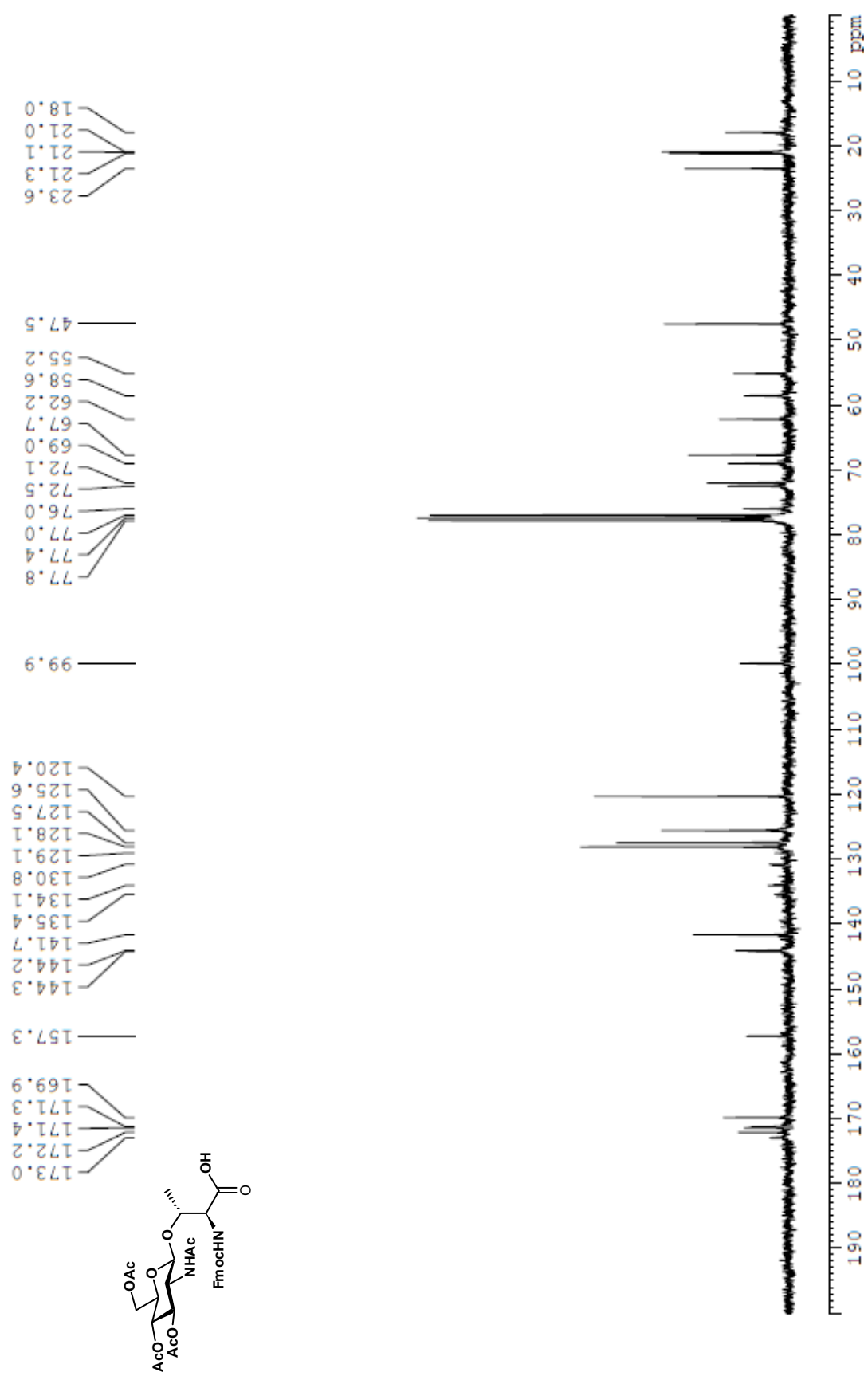
***N*-(Fluoren-9-ylmethoxycarbonyl)-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- β -D-glucopyranosyl)-
L-threonine allyl ester (8)**



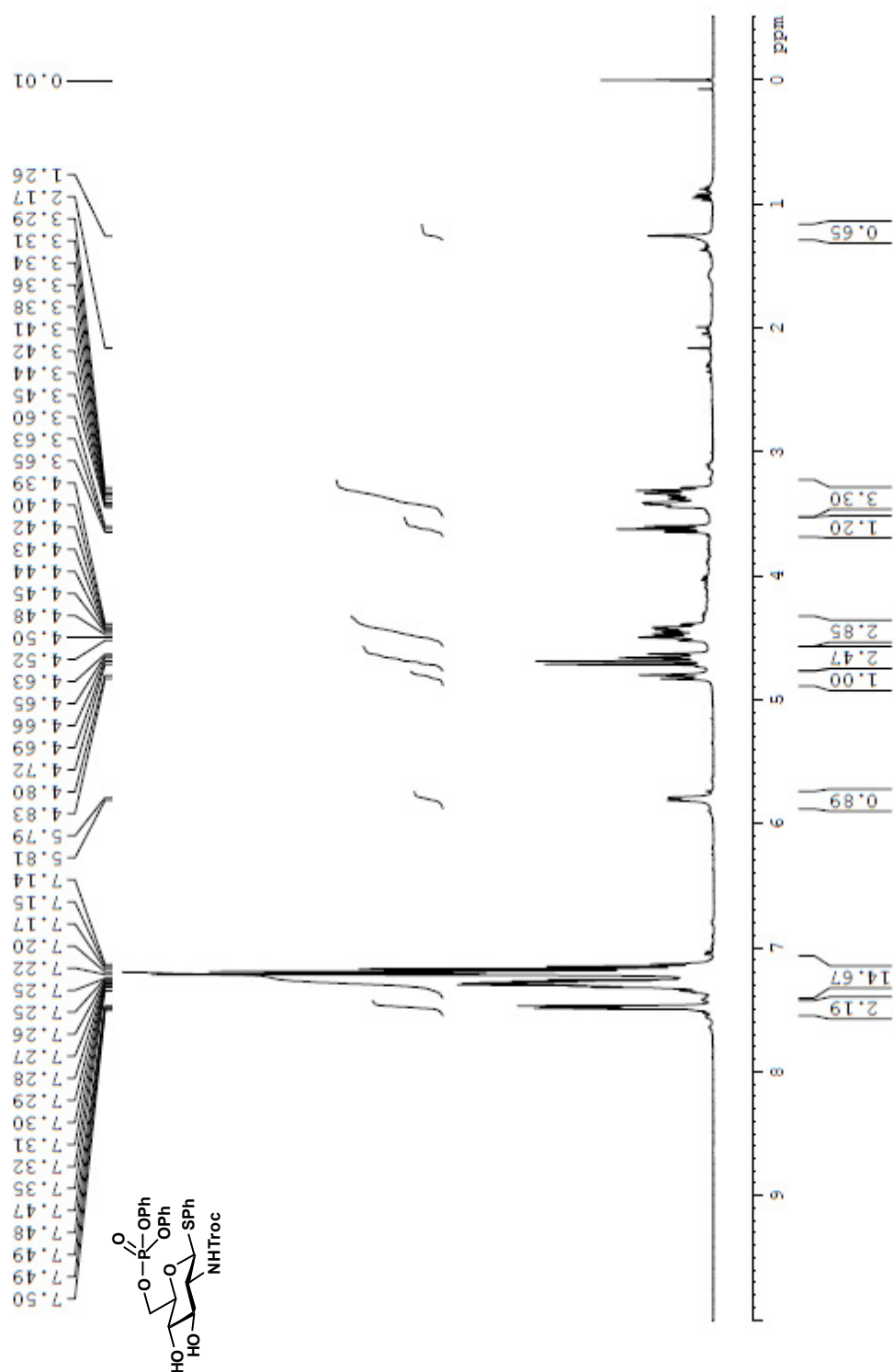


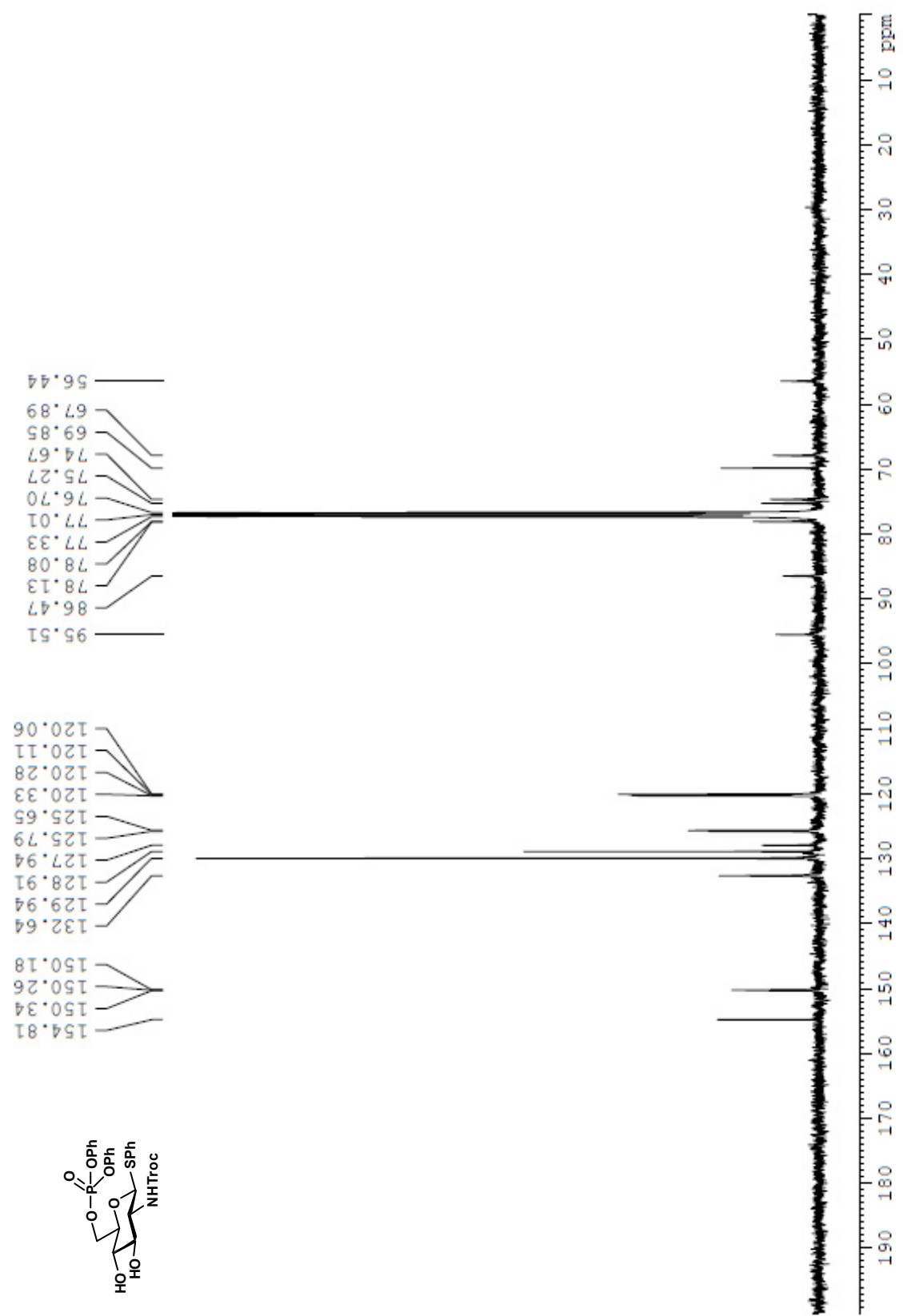
***N*-(Fluoren-9-ylmethoxycarbonyl)-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- β -D-glucopyranosyl)-
L-threonine (**4**)**



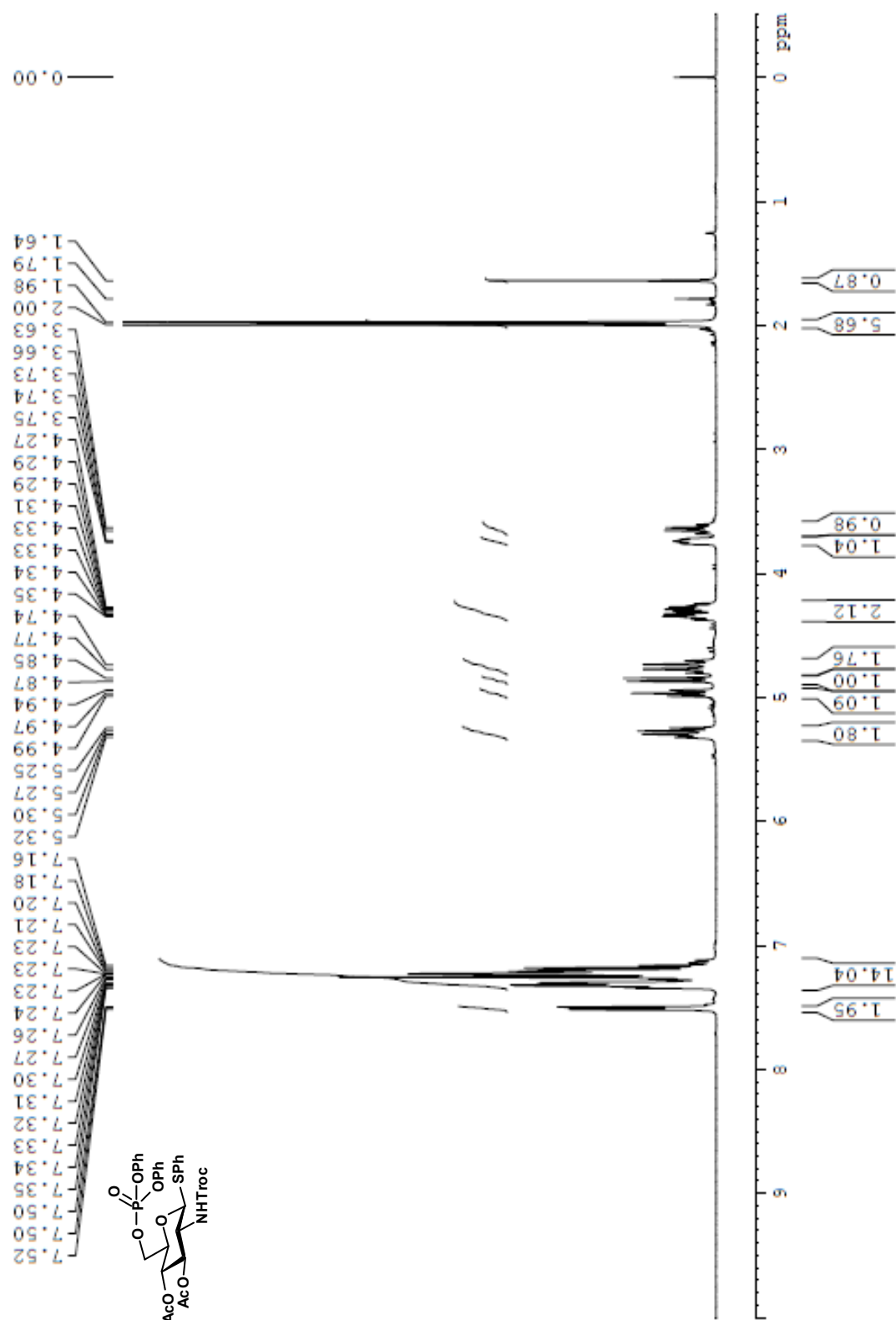


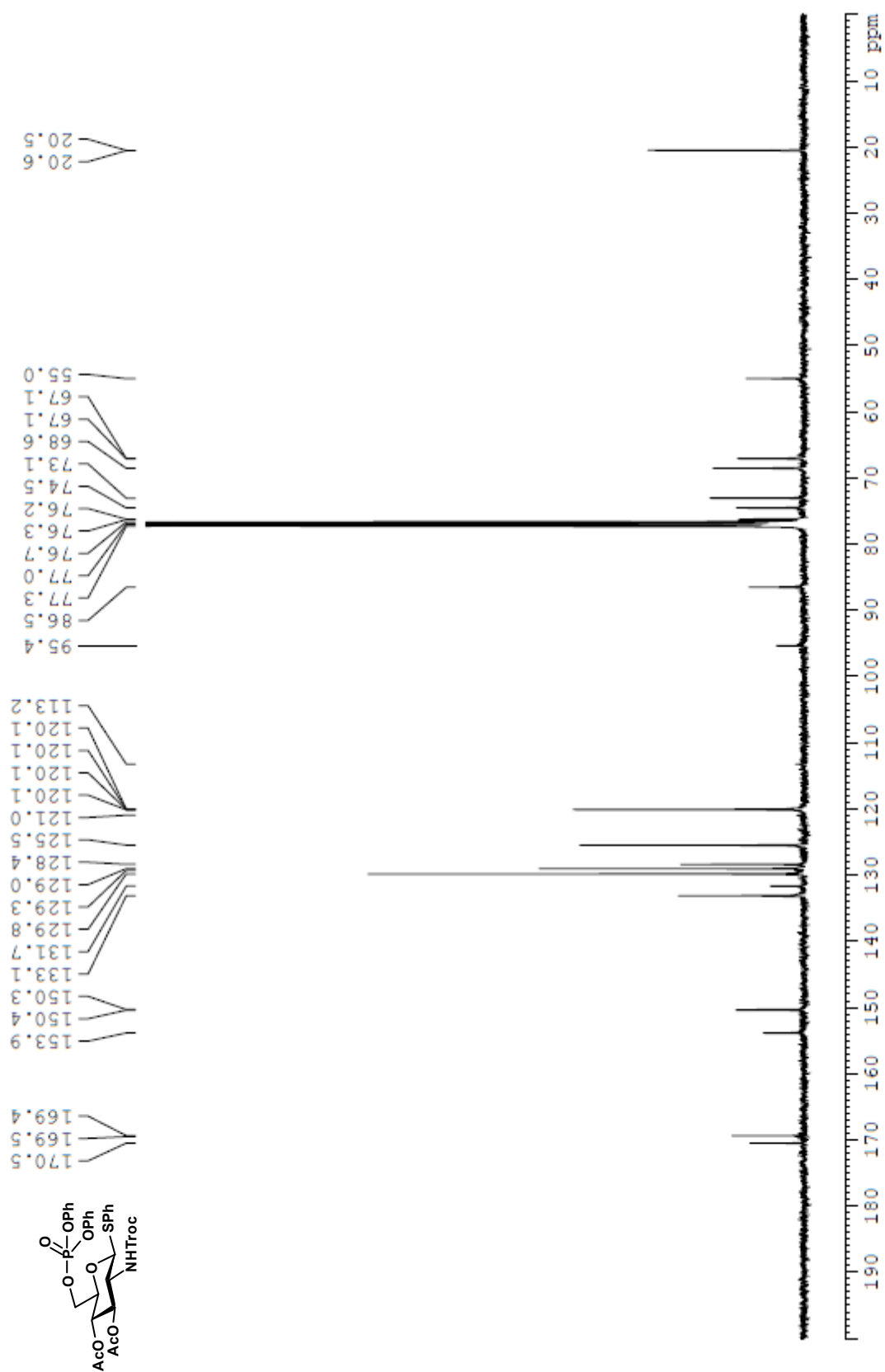
Phenyl 2-deoxy-1-thio-2-(2,2,2-trichloroethoxycarbonylamino)-6-diphenylphosphoryl β-D-glucopyranoside (12)



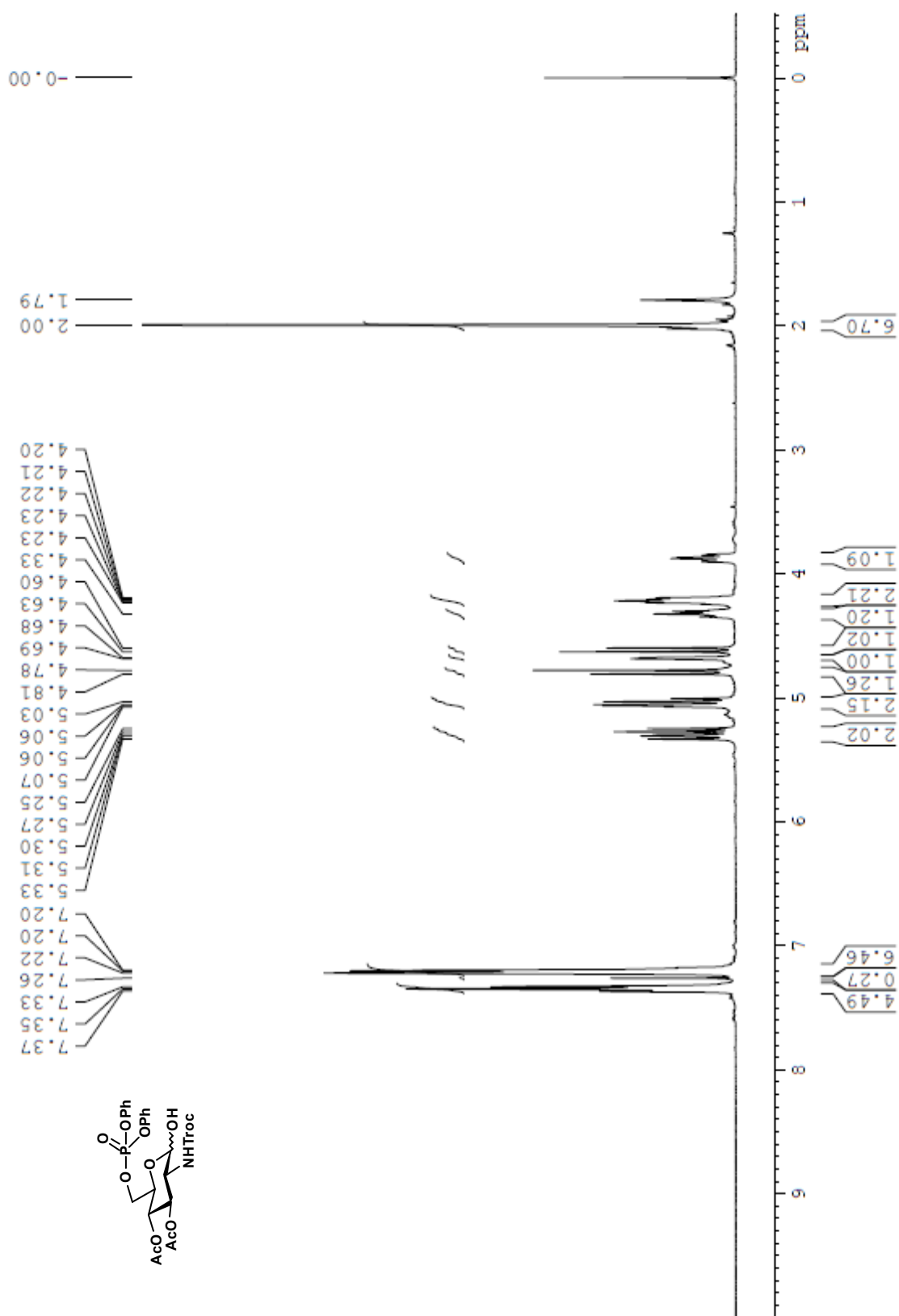


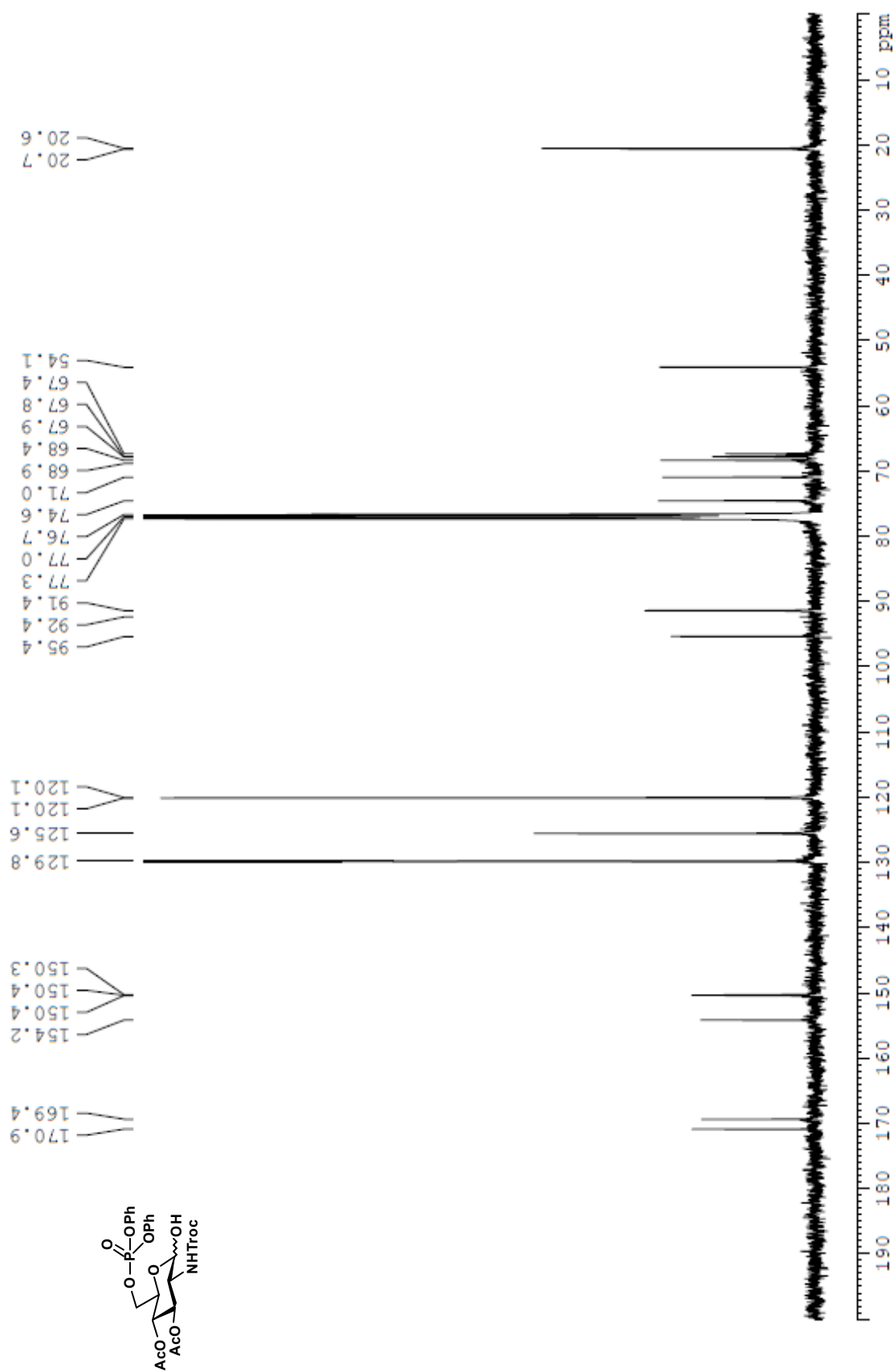
Phenyl 3,4-di-*O*-acetyl-2-deoxy-1-thio-2-(2,2,2-trichloroethoxycarbonylamino)-6-diphenylphosphoryl β-D-glucopyranoside (13)



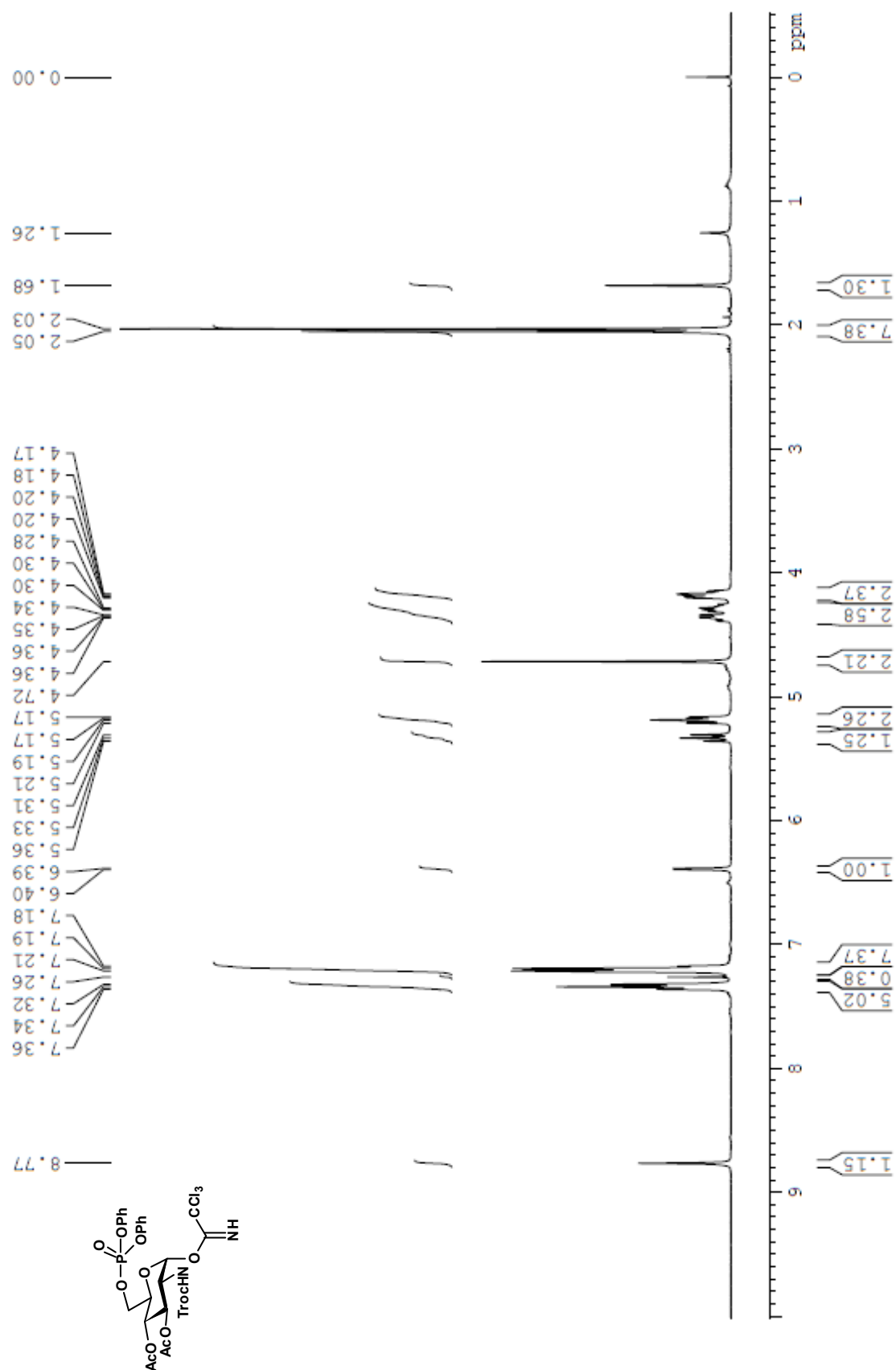


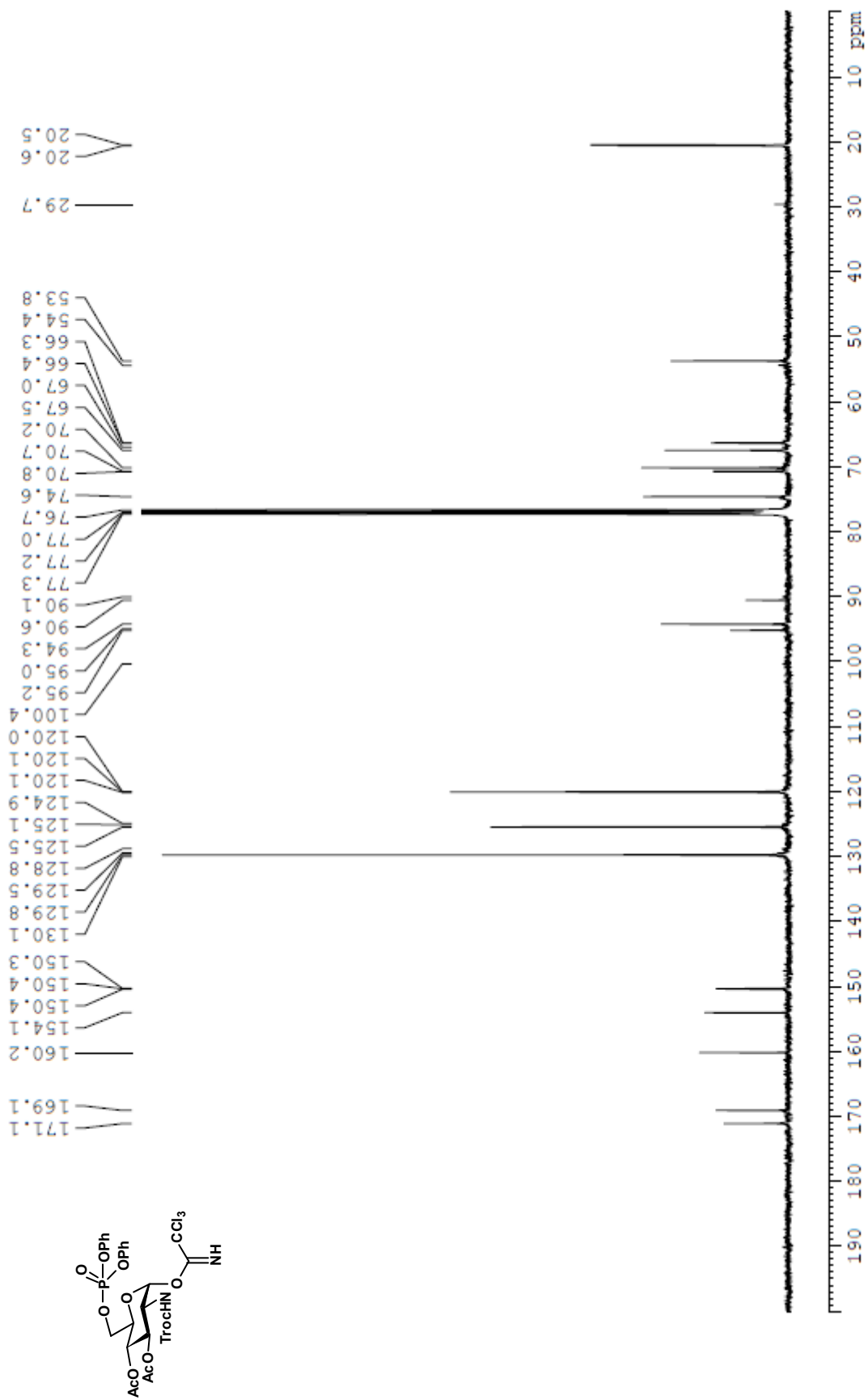
2-(2,2,2-trichloroethoxycarbonylamino)-3,4-di-*O*-acetyl-6-diphenylphosphoryl-2-deoxy- β -D-glucopyranoside (14)



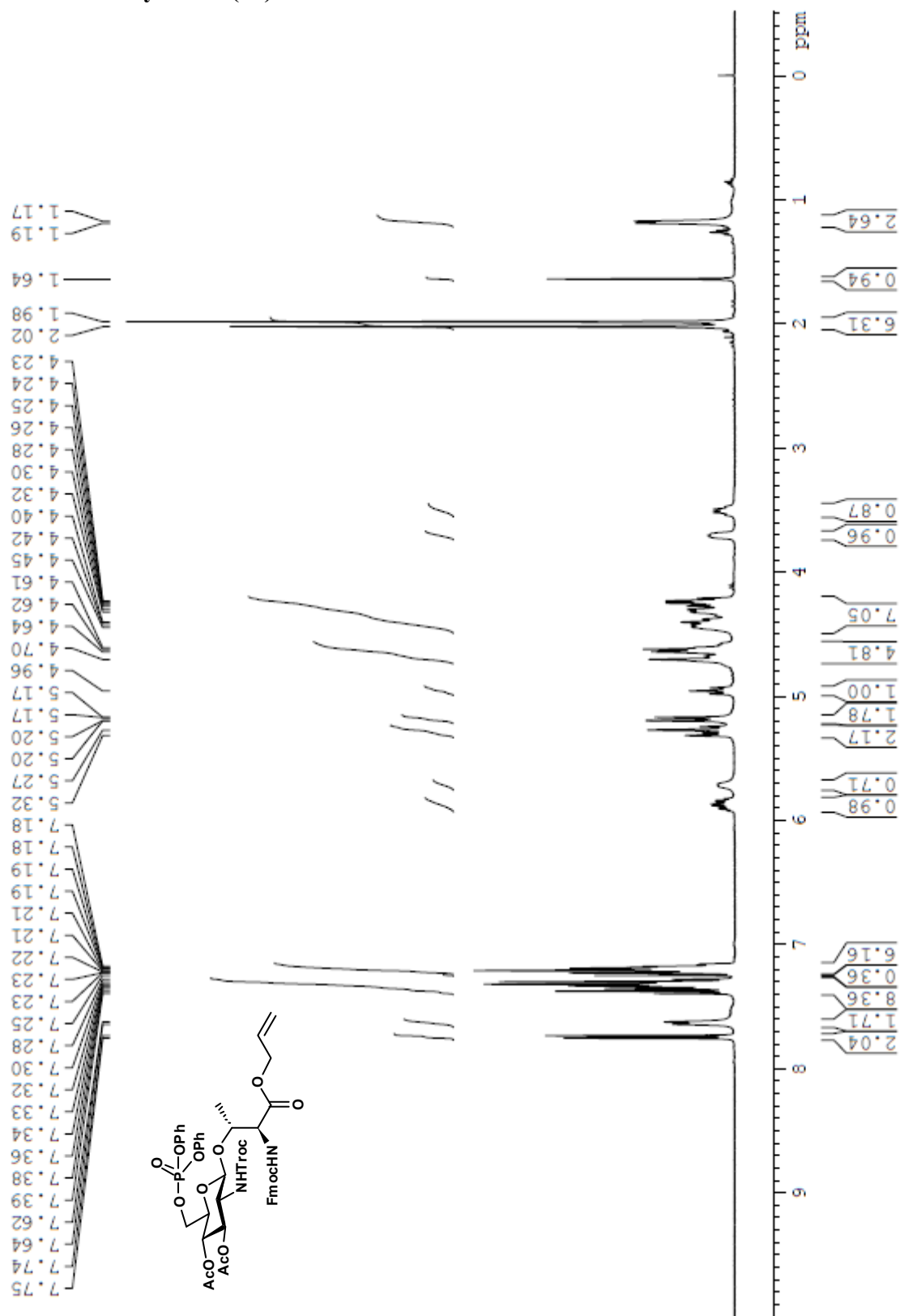


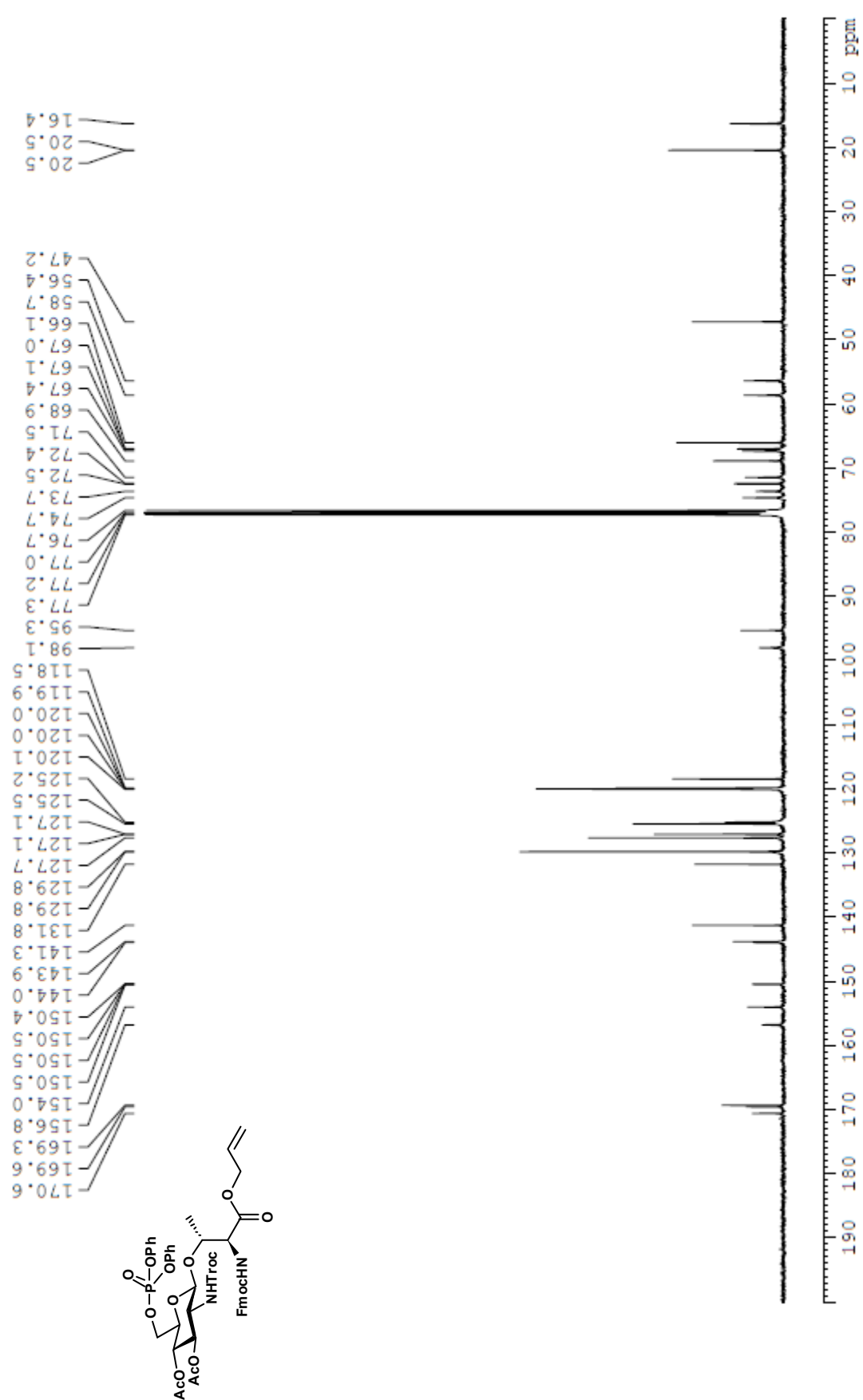
1-(2,2,2-trichloroetanimidate)-2-(2,2,2-trichloroethoxycarbonylamino)-3,4-di-*O*-acetyl-6-diphenylphosphoryl-2-deoxy- α -D-glucopyranoside (15)



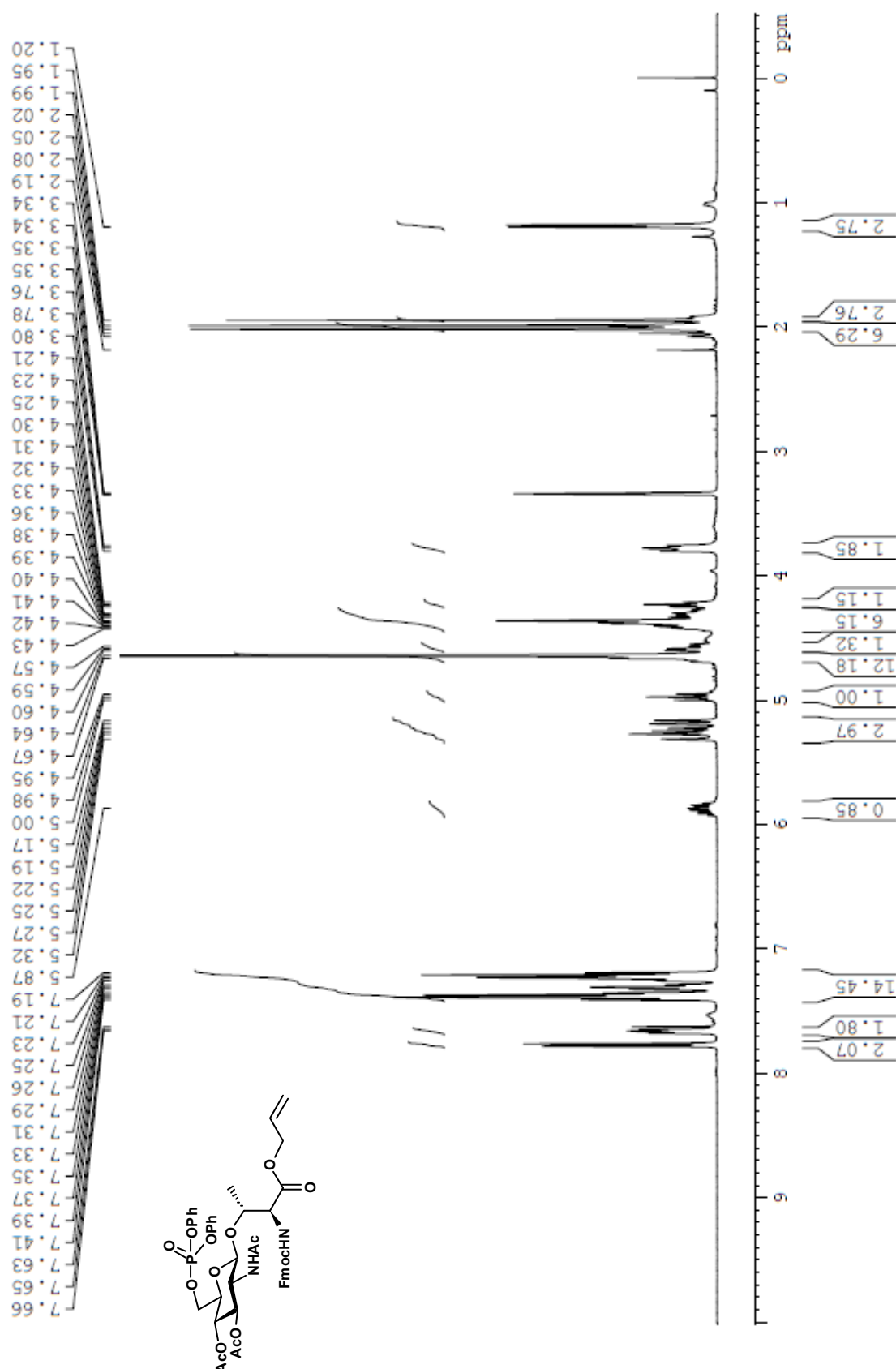


***N*-(Fluoren-9-ylmethoxycarbonyl)-*O*-(2-acetamido-3,4-di-*O*-acetyl-2-[2,2,2-trichloroethoxycarbonylamino]-6-diphenylphosphoryl-2-deoxy- β -D-glucopyranosyl)-L-threonine allyl ester (16)**

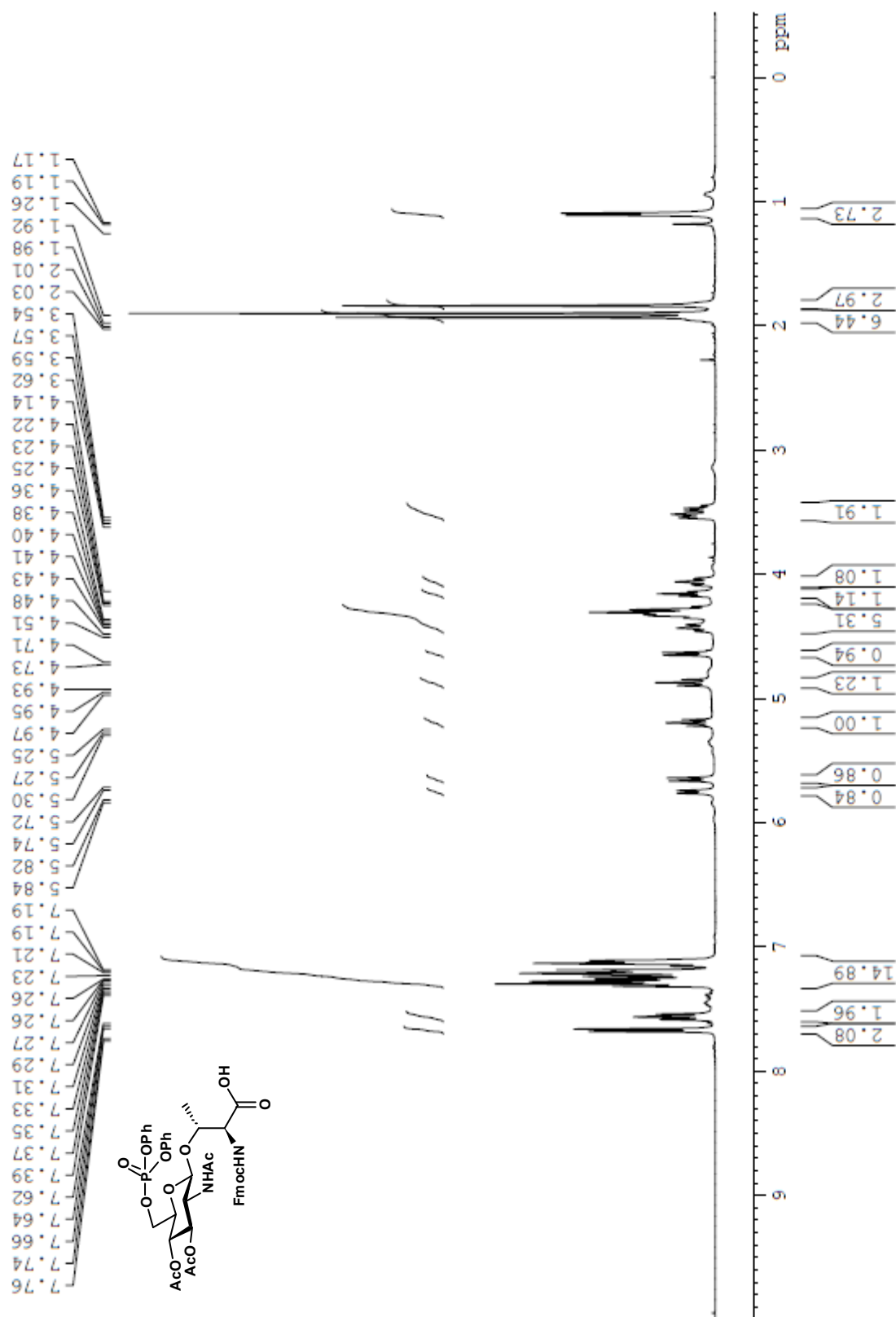


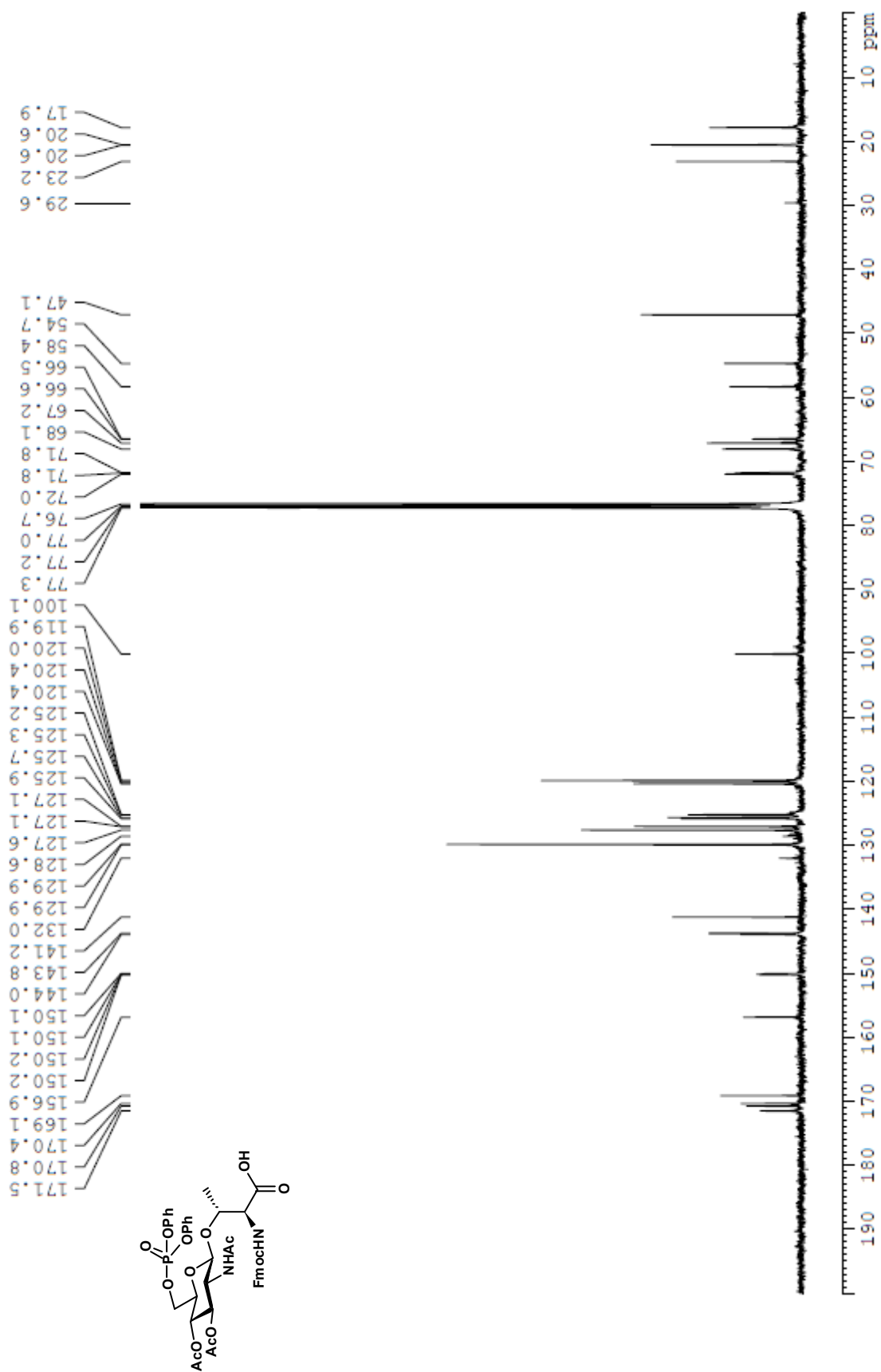


***N*-(Fluoren-9-ylmethoxycarbonyl)-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-6-diphenylphosphoryl-2-deoxy- β -D-glucopyranosyl)-L-threonine allyl ester (17)**



***N*-(9H-fluoren-9-ylmethoxycarbonyl)-*O*-(2-acetamido-3,4-di-*O*-acetyl-6-diphenylphosphoryl-2-deoxy- β -D-glucopyranosyl)-L-threonine (9)**





Peptide Immobilisation, Binding Experiment and Mass Spectrometry

NHS-activated sepharose 4 Fast Flow (GE Healthcare) (200 µl slurry) was washed with ice-cold 1 M HCl (4×400 µl). Peptide **1** (1 mg) was dissolved in Coupling Solution (400 µl, 0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3) before addition to the sepharose. The suspension was incubated on a rotating wheel for 16 h at 4 °C before washing with Washing Solution (400 µl, 0.1 M AcOH, 0.5 M NaCl, pH 4.0). Treatment with 0.1 M Tris-HCl at rt for 2 h was then used to block any residual reactive sites on the beads. The peptide-bound beads were washed alternately with Coupling Solution and Washing Solution (4×400 µl) before suspending in 20% ethanol for storage at 4 °C. Peptides **2** and **3** were immobilised using the same procedure and the blank beads were prepared using the same conditions excluding the peptide in the Coupling Solution.

Crude (P2) synaptosomes were prepared from three rat brains and rested for 30 minutes at 37 °C in low calcium Krebs-like buffer (118.5 mM NaCl, 4.7 mM KCl, 1.18 mM MgCl₂, 0.1 mM K₂HPO₄, 20 mM HEPES, 10 mM glucose, with 0.1 mM calcium, pH 7.4) as described previously.³ The synaptosomes were pelleted, the supernatant was suctioned off and then lysed in 25 mM Tris, pH 7.4, containing 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 2 mM EDTA, 50 mM NaF, 20 µg/ml leupeptin, 1 mM PMSF and EDTA-free protease inhibitor cocktail (Roche). Proteins in the lysate binding the beads alone or the various peptides (**1**, **2** and **3**) were purified by incubation with the beads for 1 h at 4 °C on a rotating wheel. The beads were washed extensively and covered with 40 µl of 0.5 M triethylammonium bicarbonate and 0.05% SDS. The proteins were reduced with 2.5 mM TCEP at 85 °C for 5 min and alkylated with 5 mM S-methyl methanethiosulfonate at 23 °C for 10 min. Trypsin was added (1:100 trypsin to protein by mass) and digestion was carried out for 18 h at 37 °C. The tryptic peptides from each sample were desalted using C18 material packed into a pipette tip as described previously.⁴ Fifteen percent of the sample was analysed by LC-MS/MS using a Dionex Ultimate 3000 HPLC and a Thermo Fisher Scientific Velos Orbitrap mass spectrometer. The sample was loaded onto a 300 µm inside diameter 5 mm long trap column packed with Acclaim PepMap100 C18, 5 µm, 100Å material (Dionex) and eluted through a 75 µm inside diameter 16 cm long C18 column (ReproSil-Pur 120 C18-AQ, 3 µm beads, Dr Maisch, Germany). The main part of the gradient was from 0% eluent B (90% acetonitrile, 0.1% formic acid and 9.9% water) to 35% eluent B in 60 minutes, to 60% eluent B in 4

minutes and to 100% eluent B in 1 minute and held at 100% eluent B for 4 minutes. The outlet of the column was connected to a 10 μm i.d. uncoated SilicaTip (New Objective, USA) to electrospray the eluent into the mass spectrometer with 2.2 kV applied. Other parameters were as follows: capillary temperature was 275 $^{\circ}\text{C}$, the S-Lens radiofrequency level was 69%, peptide detection was between m/z 350-2000 at 60,000 resolution in the orbitrap, the top 20 peptides above 10,000 counts were selected for fragmentation, fragment ion detection was at 7,500 resolution in the orbitrap, lock mass was used on background signal m/z 445.120024, predict ion injection time was enabled, dynamic exclusion was enabled, exclusion duration was 40 seconds.

The data was imported to Proteome Discover 1.2 (Thermo Fisher Scientific) and searched using Mascot 2.3 (Matrix Science, UK) against the SwissProtKB-Uniprot August 2011 database (Rattus 7645 sequences) with the following parameters: variable modifications were deamidation (NQ), oxidation (M) and methylthio (C) was a fixed modification, the precursor ion mass tolerance was 7 ppm and fragment ion tolerance was 0.05 Da, enzyme specificity for tryptic digests was selected to trypsin with up to 3 missed cleavages. A Mascot Ion Score of 30 and Mascot Expect (probability) of 0.05 were the minimum requirements for confident identification. The peptide matches were correlated by retention time and the area under the peak was calculated by Proteome Discover 1.2. A quantitative value for each protein was determined by summing the chromatographic peak areas for all the detected peptides to obtain a total area for the protein. Only peptides unique to each protein and only those peptides that were detected more than twice in any sample were used in this calculation. Protein identifications were discarded if less than three peptides were detected. The single exception to these criteria was for Vamp1 and Vamp2, as discussed in the results. An average for each condition (Beads alone, and beads with peptides **1**, **2** and **3**) was obtained from three binding experiments using synaptome lysate from different rat brains.

Comparative mass spectral analysis of native rat brain AP180 and synthetic glycoposphopeptide 3.

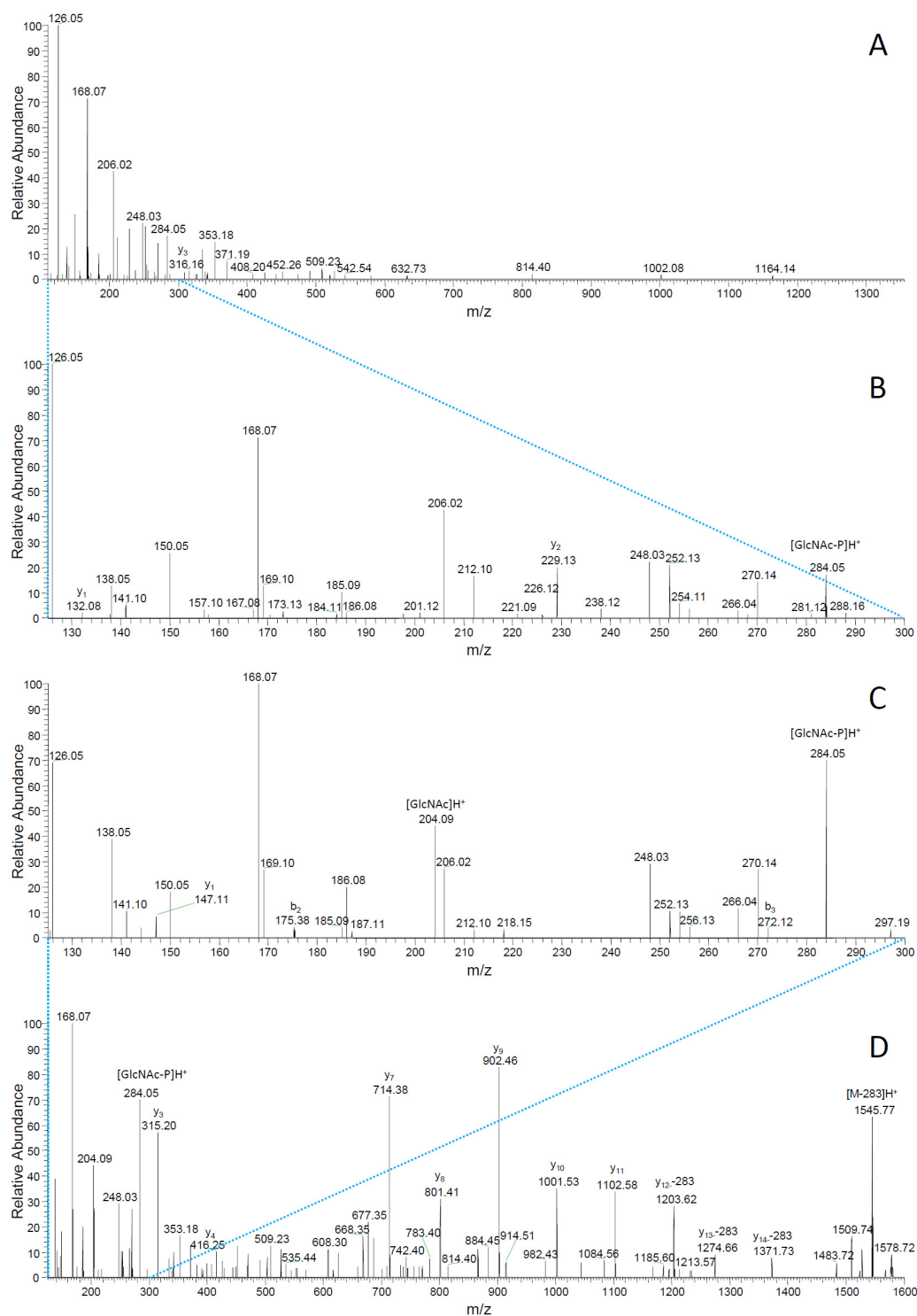


Fig. 1. The secondary MS/MS fragmentation of [GlcNAc-P] H^+ from native and synthetic O-GlcNAc-P modified peptides.

The MS/MS spectra of peptide **3** (O-GlcNAc-P modified AP180 305-315) from the fragmentation of the doubly charged precursor at m/z 672.29 is shown in **A** and the MS/MS spectra of rat brain O-GlcNAc-P modified AP180 305-320 from fragmentation of the precursor at m/z 914.91 is shown in **D**. Some y - and b -type ions are present for these peptides. Peptide **3** is expected to provide poor fragment ions since it lacks a charged Arg/Lys at the C-terminus typical of tryptic peptides. Only y_1 to y_3 peptide fragment ions were detected for peptide **3**. The spectra were dominated by low mass ions typical of glycosylated peptides. **B** and **C** showed magnified parts of spectra **A** and **D**, respectively. The [GlcNAc-P] H^+ ion at m/z 284.05 was previously reported by Graham *et al.*, using native AP180, to fragment further to produce a distinctive fragmentation pattern (e.g. as in **C** and **D**).⁵ We now observe a very similar pattern of fragmentation pattern for a synthetic O-GlcNAc-6-P modified synthetic peptide (**A** and **B**). Identical molecules should give the same fragmentation pattern in a mass spectrometer under the same conditions. In the LTQ Orbitrap Velos mass spectrometer, these peptides were not fragmented with the same energy and [GlcNAc-P] H^+ was not produced from the same peptide. However, the fragmentation products of [GlcNAc-P] H^+ (e.g. m/z 138.05, 168.07, 206.02 and 248.03) are very similar in **B** and **C**. This is supportive, but not definitive proof that native O-GlcNAc-P is phosphorylated at the 6-position. Note that O-GlcNAc (m/z 204.09) is also present in **C** and **D** because of the presence of O-GlcNAc AP180 305-320 where S313 is phosphorylated to give it the same molecular mass. Native AP180 was obtained as described previously.⁵

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- 4 L. S. Chan, G. Hansra, P. J. Robinson, M. E. Graham, *J. Proteome Res.* 2010, **9**, 4028.
- 5 M. E. Graham, M. Thaysen-Andersen, N. Bache, G. E. Craft, M. R. Larsen, N. H. Packer, P. J. Robinson, *J. Proteome Res.* 2011, **10**, 2725.