Comparison of *N*-acetyl-glucosamine to other monosaccharides reveals structural differences for the inhibition of α-synuclein aggregation

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*These authors contributed equally.

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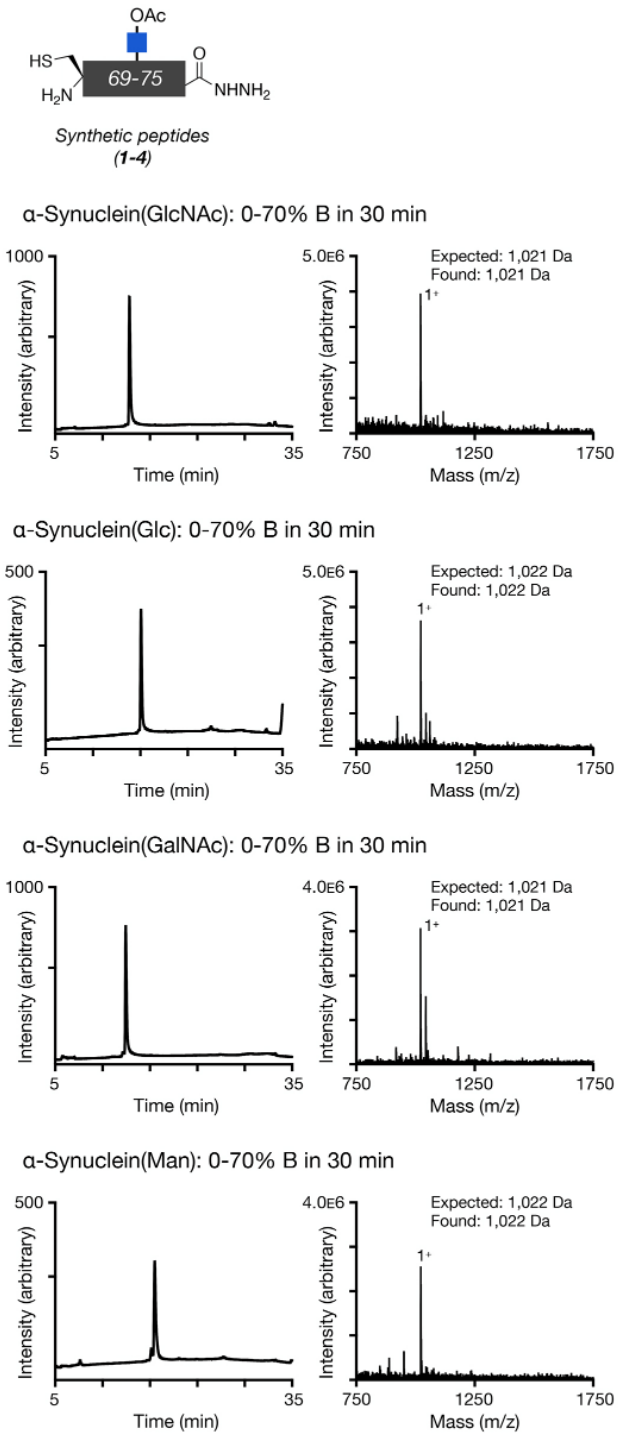


Figure S1. Characterization of glycosylated peptide building blocks. Analytical RP-HPLC traces and ESI-MS of the indicated synthetic glycopeptides. The different monosaccharides, exemplified by GlcNAc (blue square), are per-*O*-acetylated.

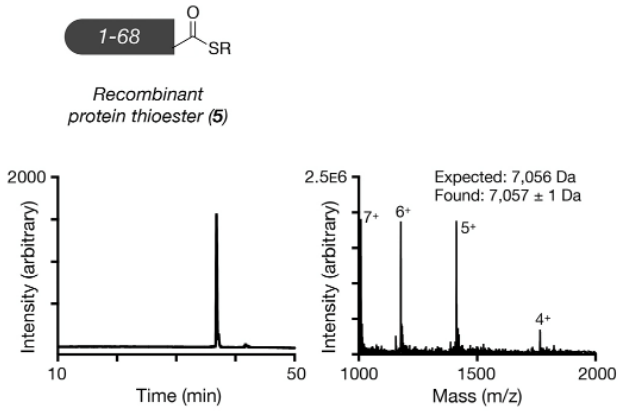


Figure S2. Characterization of N-terminal recombinant thioester. Analytical RP-HPLC traces and ESI-MS of the N-terminal thioester fragment.

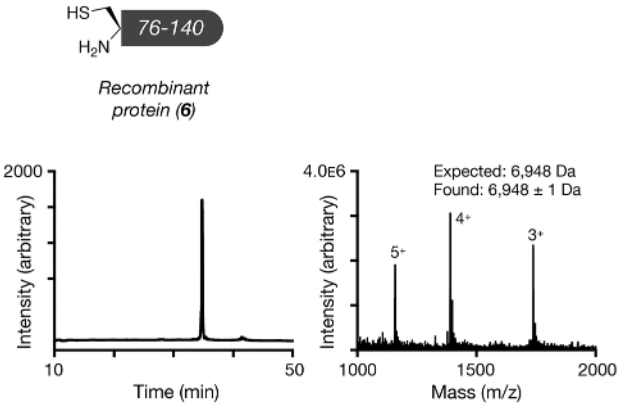


Figure S3. Characterization of C-terminal recombinant fragment. Analytical RP-HPLC traces and ESI-MS of the C-terminal fragment.

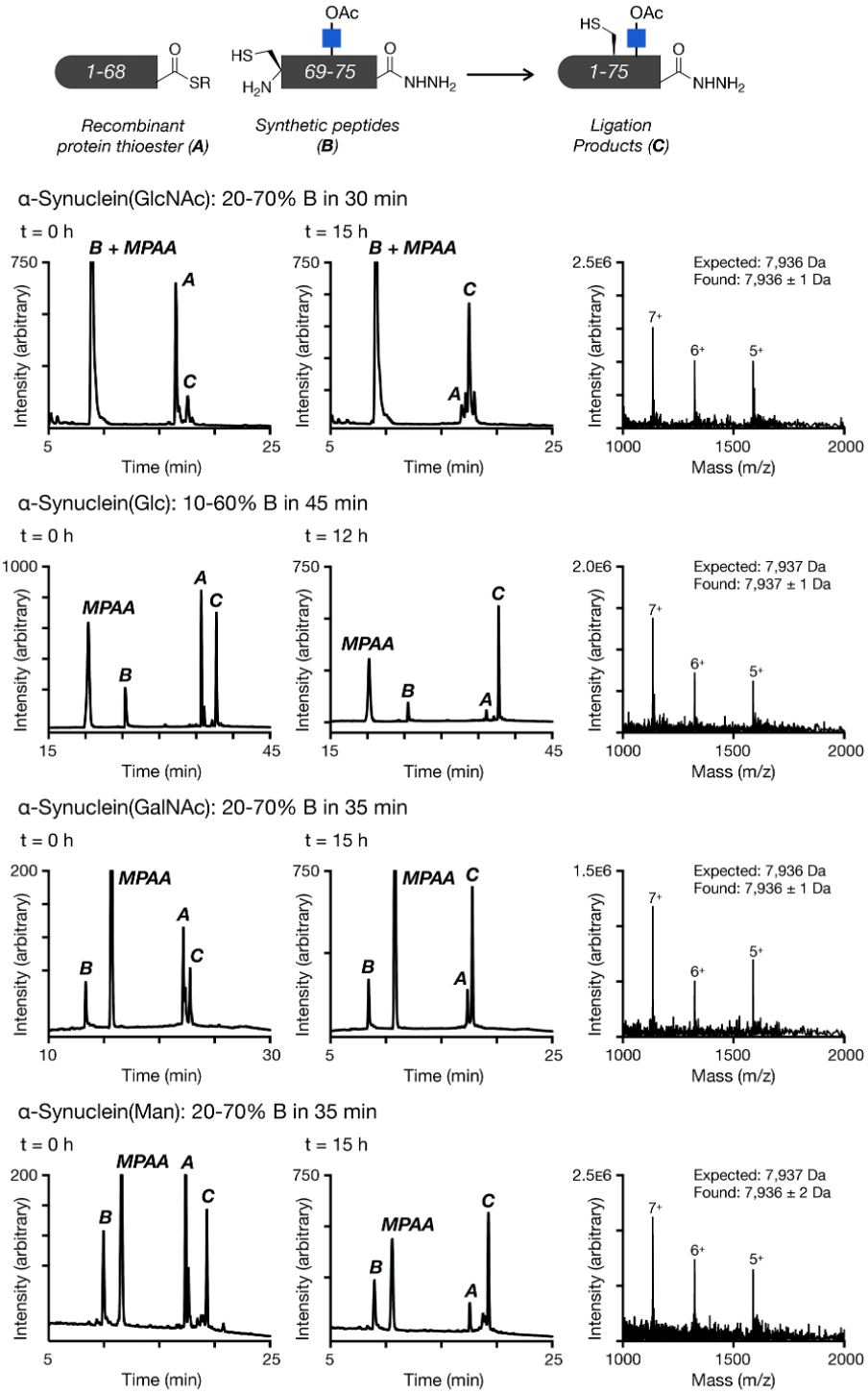


Figure S4. Ligation of synthetic glycopeptides with the N-terminal thioester. The indicated ligation reactions were monitored by RP-HPLC and the purified products were analyzed by ESI-MS. The different monosaccharides, exemplified by GlcNAc (blue square), are per-*O*-acetylated.

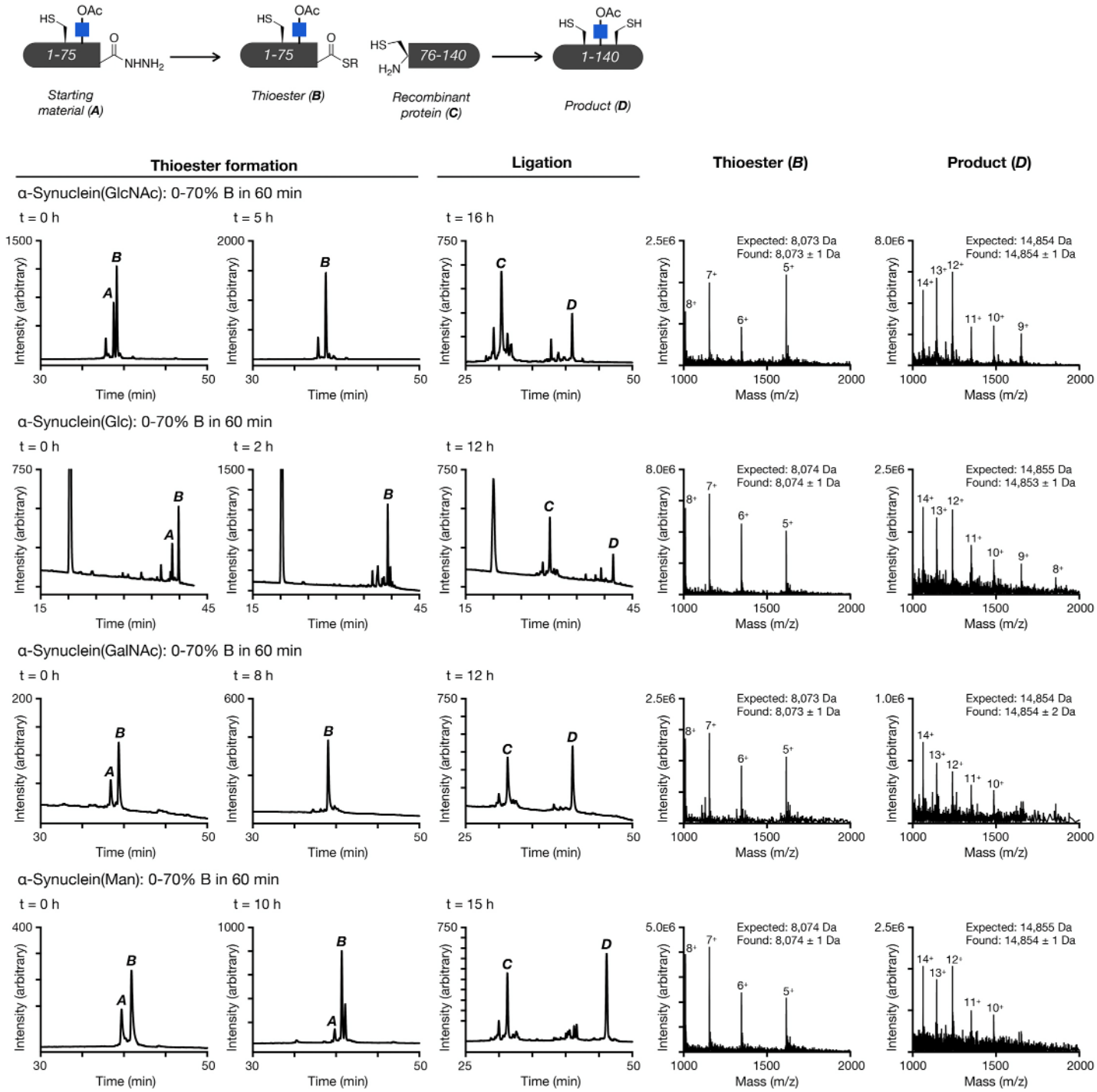


Figure S5. Activation of C-terminal hydrazide and ligation to the C-terminal fragment. The C-terminal hydrazides where converted to the corresponding thioesters and the subjected to one-pot ligation reactions with the C-terminal fragment. The reactions were monitored by RP-HPLC and the purified products were analyzed by ESI-MS. The different monosaccharides, exemplified by GlcNAc (blue square), are per-*O*-acetylated.

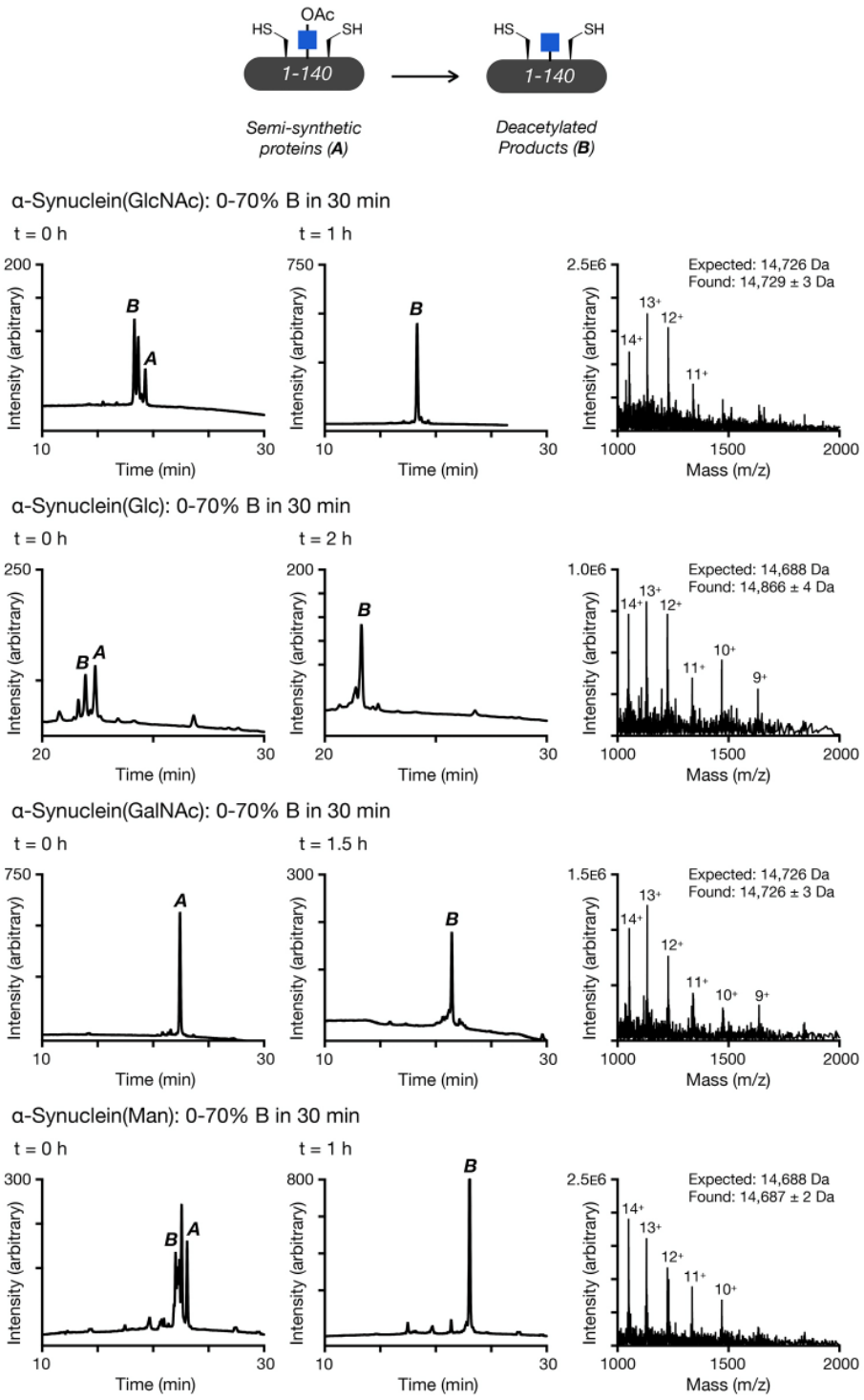


Figure S6. Deprotection of the monosaccharide *O*-acetates. The monosaccharide *O*-acetate protecting-groups, exemplified by GlcNAc (blue square), were removed by treatment with 5% hydrazine hydrate in H_2O. The reactions were monitored by RP-HPLC and the purified products were analyzed by ESI-MS.

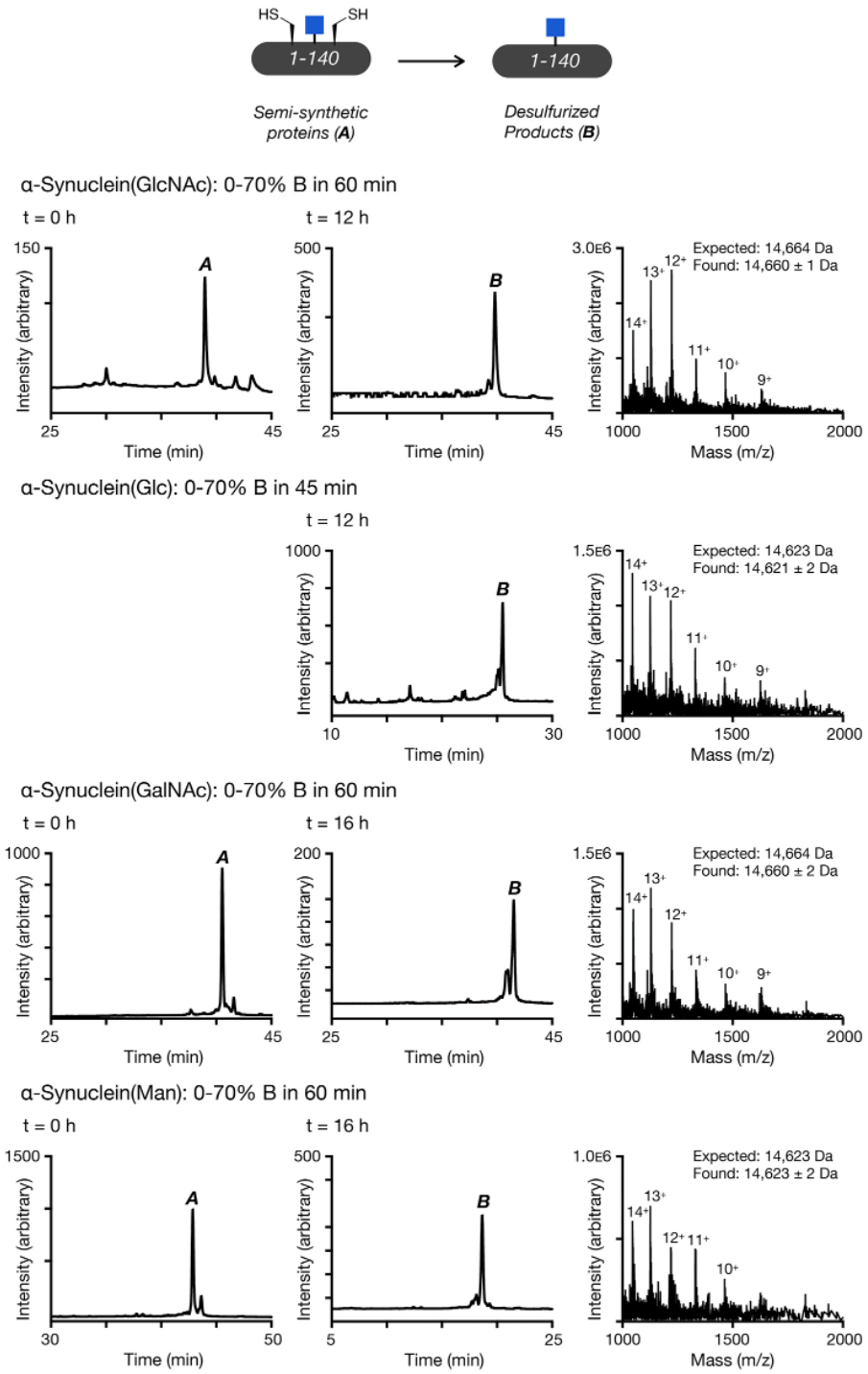


Figure S7. Desulfurization of semi-synthetic proteins. The indicated synuclein proteins, exemplified by GlcNAc (blue square), were subjected to radical desulfurization conditions. The reactions were monitored by RP-HPLC and the purified products were analyzed by ESI-MS.

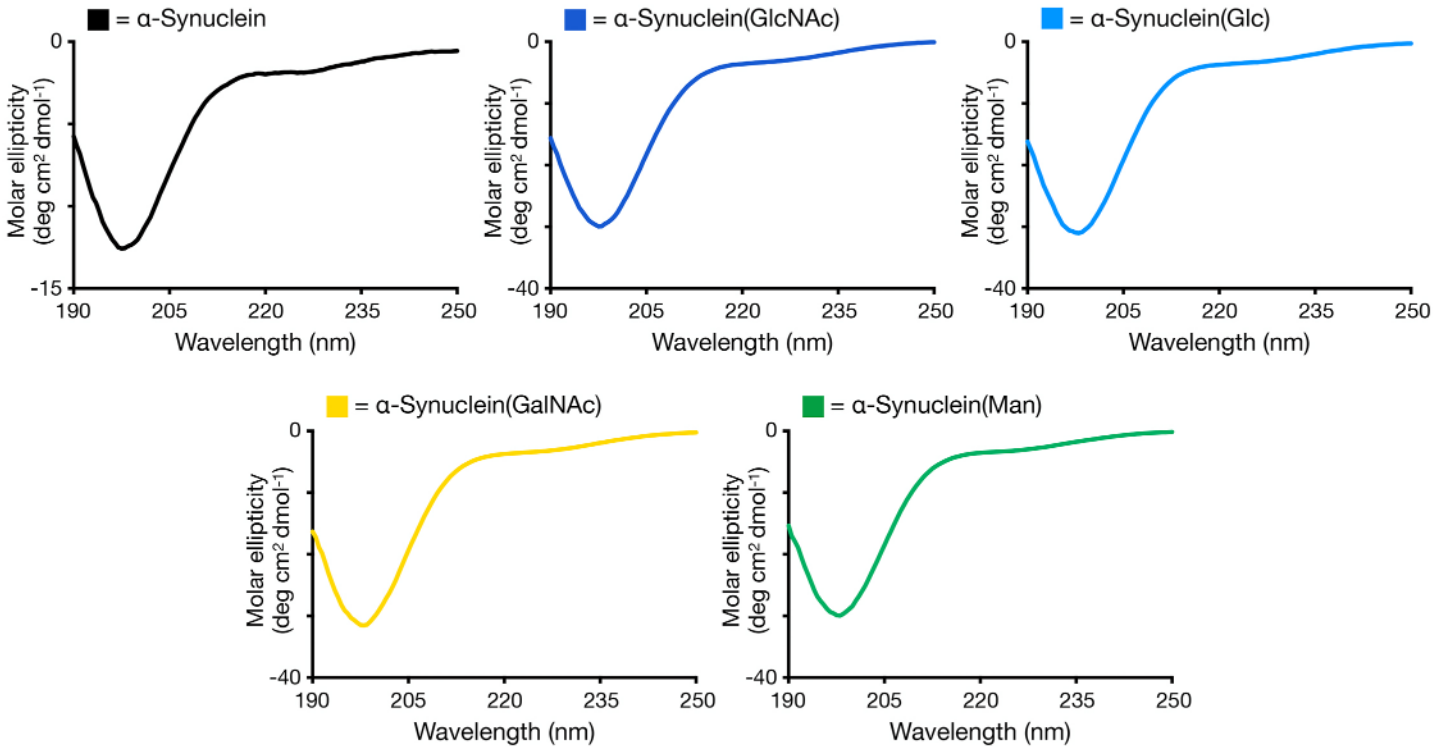


Figure S8. No monosaccharides alter α-synuclein secondary structure. Circular dichroism (CD) spectra were collected for freshly dissolved samples of the indicated proteins.

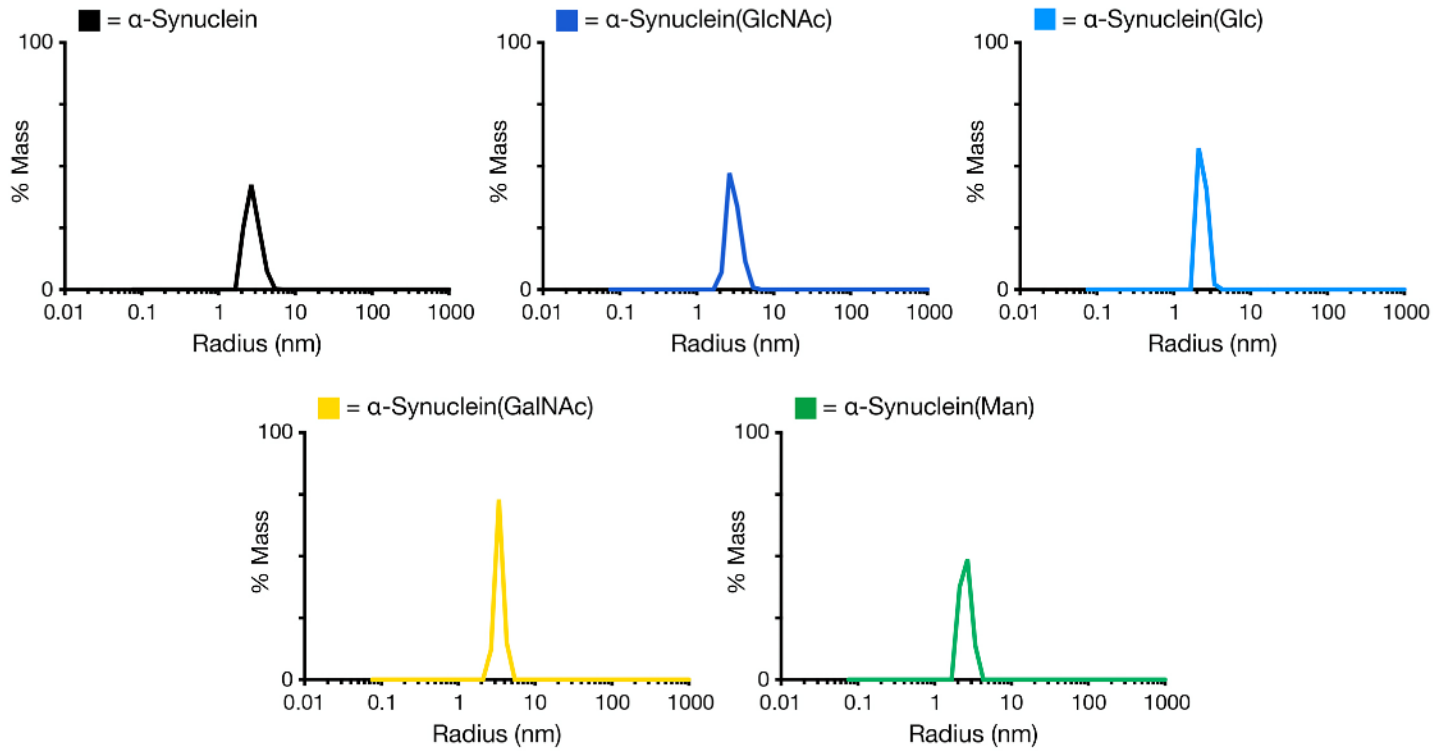


Figure S9. All of the glycoproteins are monomeric in solution. The indicated proteins were analyzed using dynamic light scattering (DLS). All preparations showed a single peak with a Stoke's Radius less than the 10 nm, consistent with monomeric protein.

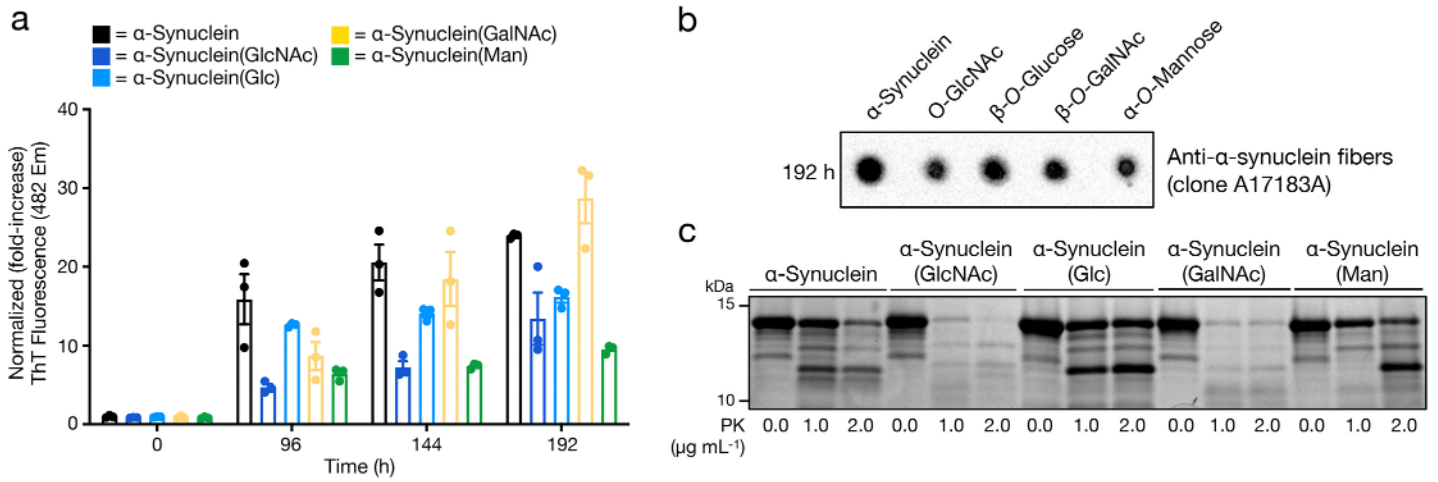


Figure S10. Different monosaccharides have distinct effects on α-synuclein aggregation. a) Analysis of amyloid formation using thioflavin T (ThT) fluorescence (λ_{ex} = 450 nm, λ_{em} = 482 nm). The indicated α-synuclein proteins (50 μM) were subjected to aggregation conditions and analyzed by fluorescence at the indicated timepoints. b) The same reactions (192 h timepoint) were analyzed by dot-blotting using an α-synuclein amyloid-selective antibody (A17183A). c) The aggregation reaction mixtures (192 h timepoint) were subjected to the indicted concentrations of proteinase K (PK) before analysis by SDS-PAGE and staining with Coomassie blue. The persistence of bands correlates with the amount and gross structure of amyloids formed.

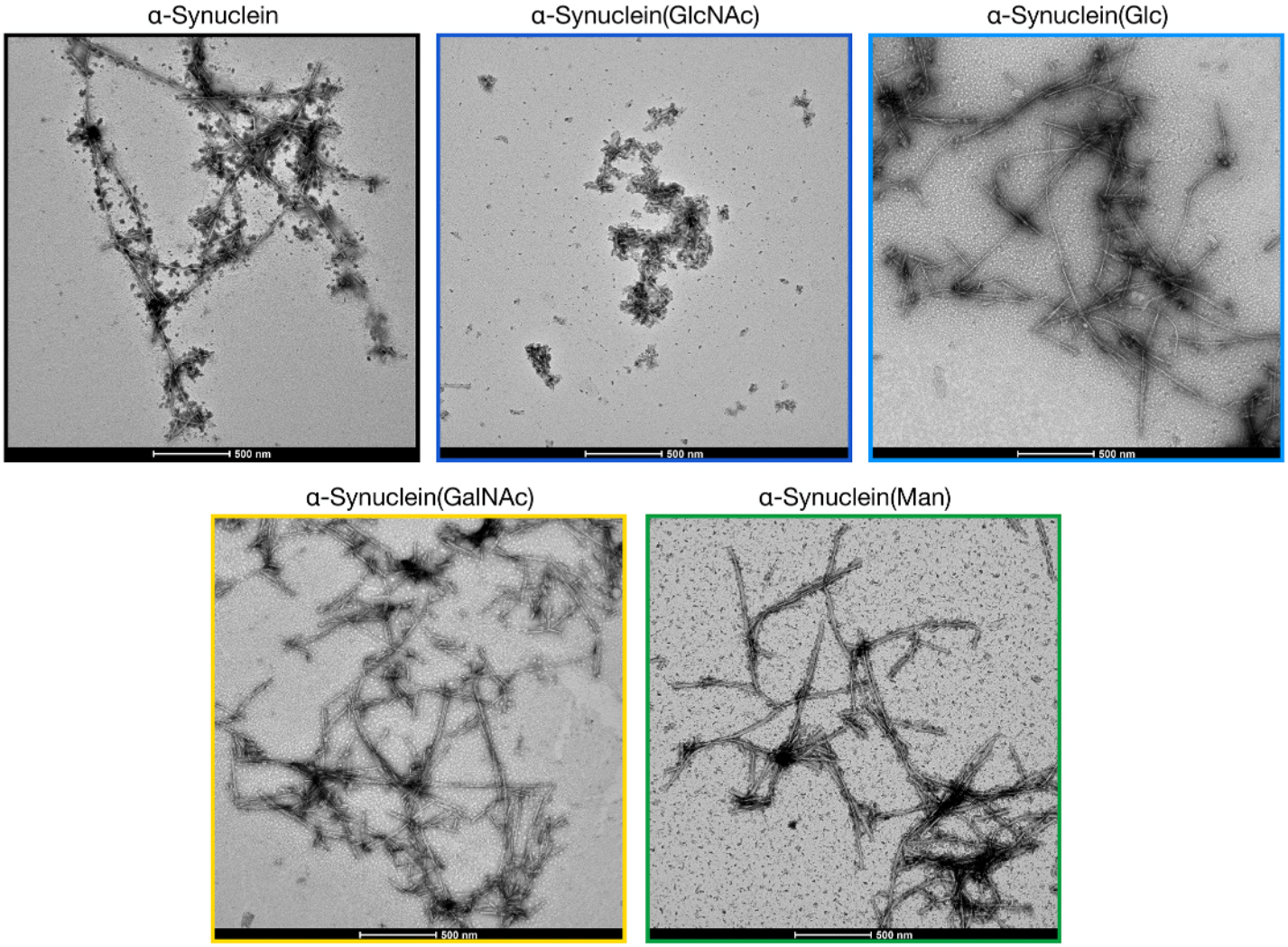


Figure S11. Large format TEM images. Representative TEM images from Figure 4.

Experimental Methods:

General:

Unless otherwise mentioned, all solvents and reagents were purchased from Sigma-Aldrich, EMD, Fluka, Novagen, VWR etc. and used without any further purification. Luria Bertani-Miller and Terrific broth medium were procured from EMD and solutions were prepared, autoclaved, stored and used following protocols from the manufacturer. Aqueous solutions were prepared using deionized, distilled and filtered water (18 MΩ at 25 °C). E. coli BL21 (DE3) chemically competent cells were obtained from VWR. Precasted 12% Bis-Tris gels, MES running buffer and Coomassie Brilliant Blue were purchased from Bio-Rad. N-Fmoc and side chain protected amino acids, HBTU, as well as resin beads for peptide synthesis were obtained from NovaBiochem. Fmoc-Thr(ß-O-Ac_4Glc)-OH was purchased from Polt laboratory, University of Arizona. Stock solutions of ampicillin sodium salt and kanamycin sulfate were prepared at concentration of 100 mg mL^{-1} and 50 mg mL^{-1} respectively and stored at -20 °C. All silica gel column chromatography was performed using 60 Å silica gel (EMD) and all thin-layer chromatography (TLC) was performed using 60 Å, F254 silica gel plates (EMD) with detection by ultraviolet light and staining with either ceric ammonium molybdate (CAM) or ninhydrin. ^{1}H and ^{13}C NMR spectra were collected in either $CDCl_3$ or CD_3OD as a solvent (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C on a Varian 600 MHz spectrometer. All chemical shifts are reported in the standard notation of parts per million (ppm) using the peaks of proton and carbon signals of residual solvents for calibration ($CDCl_3$: ^{1}H - 7.26 ppm and ^{13}C - 77.16 ppm and CD_3OD: 1H - 4.78, 3.31 ppm and ^{13}C - 49.15 ppm) and coupling constants (J) given in Hertz. The abbreviations used for the proton spectra multiplicities are: s, singlet; b, broad; d, doublet; t, triplet; q, quartet; m, multiplet. A Biotage Isolera Spektra FLASH system (solvent A, 0.1% TFA in water; solvent B, 0.1 % TFA in acetonitrile) or an Agilent 1200 Series HPLC (solvent A: 0.1 % TFA in water; solvent B: 0.1 % TFA and 90 % acetonitrile in water) system was used for reverse-phase high performance liquid chromatography (RP-HPLC). Mass spectra was recorded either on an API-150EX (Applied Biosystems/MDS SCIEX) or on an Agilent HPLC/Q TOF MS/MS Spectrometer.

Unmodified α-synuclein expression and purification:

E. coli BL21 (DE3) cells were transformed with α-synuclein-coding pRK172 construct[1] via a 30 s heat shock at 42 °C. The cells were grown on an LB agar ampicillin plate (100 μg mL^{-1}) at 37 °C for 12 h and then stored at 4 °C. A started culture was used to inoculate 300 mL of TB culture (100 μg mL^{-1} ampicillin) which was shaken at 250 rpm at 37 °C till an OD600nm of around 0.6-0.7. To induce α-synuclein expression, IPTG (0.5 M) was added to the cultures with shaking at same conditions for 16 h. Bacteria were harvested by centrifugation at 6,000 × g for 15 min at 4 °C. Cell pellets were re-suspended in lysis buffer (100 mM Tris, 500 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 8) after performing freeze thaw cycles thrice using liquid nitrogen and a 37 °C incubator respectively. The cells were then boiled at 80 °C for 10 mins while agitating the resuspension every minute. The lysed cells were then kept at room temperature for 30 mins. PMSF solution in isopropanol (100 mM stock) was added to the cell lysates to give a 2 mM final concentration, and cells were incubated on ice for another 30 min. After that, the cell lysate was centrifuged (6000 × g, 4 °C, 20 min) and the supernatant was collected and acidified to pH 3.5 using 1M HCl. It was again incubated on ice for 30 mins before another centrifugation (6000 × g, 4 °C, 20 min). After centrifugation, the supernatant was dialyzed into a 2 L degassed, 1% acetic acid solution using SnakeSkin dialysis tubing (3.5K MWCO). After 12 h, the dialyzed protein solution was centrifuged and the clear solution was purified by a C4 semi-preparative column (Higgins Analytical) using an Agilent 1200 Series HPLC system (20-70 % solvent B in solvent A gradient for 40 mins, 2.5 ml/min flow rate) and then characterized by ESI-MS. Pure protein was lyophilized and stored at -20 °C. The amount of pure protein was calculated by Pierce BCA assay (Thermo Scientific).

Expression of α-synuclein N-terminal (1-68) thioester:

E. coli BL21 (DE3) cells were transformed with a modified pTXB1 plasmid encoding α-synuclein(1-68) fused to the Ava-DnaE N137A intein[2] by heat shock at 42 °C for 30 s, plated on a LB agar plate containing ampicillin (100 μg mL^{-1}) and incubated overnight at 37 °C. N-terminal (1-68) fragment was expressed in E. coli cells as wild type α-synuclein protein. After centrifuging (6,000 x g, 15 min, 4 °C), the cell pellet was resuspended in 40

mL (per 2L of TB culture) of cold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, 2 mM PMSF, 2 mM TCEP.HCl, pH 7.4) and subjected to tip sonication (45% amplitude, 30 sec pulse ON, 30 sec rest, 8 min, on ice). The cell lysate was centrifuged again (6,000 x g, 45 min, 4 °C) to remove cell debris and supernatant was incubated with Co-NTA Agarose resin beads, previously equilibrated with the wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 2 mM TCEP.HCl, pH 7.4), for 1 h with continuous agitation at 4 °C. The resin beads were thoroughly washed with wash buffer before treating with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 2 mM TCEP.HCl, pH 7.4). The eluted protein was dialyzed into a 0.5X DPBS (2 L) solution using SnakeSkin dialysis tubing (3.5K MWCO) overnight at 4 °C followed by addition of sodium mercaptoethane sulfonate (MESNa) (250 mM) to induced intein cleavage and thioester generation. The thiolysis reaction was carried out for 12 h at room temperature. After completion, the reaction mixture was purified from the intein fusion by C4 column using a Biotage Isolera Spektra FLASH system and stored as lyophilized powder at -20 °C. The purity of α-synuclein N-terminal (1-68) thioester fragment was confirmed by HPLC and ESI-MS.

Expression of α-synuclein C-terminal fragment (76-140):
E. coli BL21 (DE3) cells were transformed with the specific pET42b construct1 for α-synuclein(A76C-140) via a 30 s heat shock at 42 °C and plated on a kanamycin (50 μg mL^{-1}) containing LB agar plate. Protein was expressed and purified following protocol as described above for full length, unmodified α-synuclein.

Solid phase synthesis of 69-75 peptides:
Peptides were synthesized manually by using solid phase peptide synthesis strategy on 2-Cl-(Trt)-Cl resin (loading 0.68 mmol/g resin) on a 0.1 mmol scale. These resin beads were activated to 2-Cl-(Trt)-$NHNH_2$ with hydrazine hydrate solution following a previously reported procedure. [3] Before coupling, commercially available N-Fmoc and side chain protected amino acids (5 eq) were activated for 5 min with HBTU (5 eq) and DIEA (10 eq) and then coupled to the resin for 1 hour. After coupling, the terminal Fmoc group was deprotected with 20% v/v piperidine in DMF for 7 min twice. For the first coupling, N-Fmoc protected amino acid was coupled twice for 45 mins. After that, the resin beads were capped with a capping solution (156 μl Ac_2O, 40 μl pyridine in 2 mL DMF) for 30 mins. Deprotection of Fmoc group was then carried out. For the incorporation of the glycosylated threonine amino acids, pentafluorophenyl (PFP) activated O-modified Fmoc-threonines were synthesized and purified as described in the synthesis section. Two equivalents of this amino acid were incubated with the peptide resin overnight, followed by standard coupling cycles for the remaining amino acids. After the final Fmoc-deprotection, peptides were cleaved from resin by treatment with 3 mL of cleavage cocktail (TFA:H_2O:Triisopropylsilane; 95:2.5:2.5) for 4 h at room temperature with constant rocking. The crude peptide was filtered and diluted in cold diethyl ether (35 mL) and precipitated overnight at -80 °C. The resulting suspension was then centrifuged (6,000 x g, 30 min, 4 °C). The pellet was dried under nitrogen flow to remove traces of diethylether and then resuspended in 20% acetonitrile in water solution before HPLC purification using a C18 semi-preparative column. Purified peptides were then characterized by HPLC and ESI-MS.

α-Synuclein semi-synthesis:
A common ligation procedure was followed to make all four glycosylated proteins. N-terminal (1-68) thioester fragment (2 mM, 1 eq) and an individual peptide (4 mM, 2 eq) were dissolved in ligation buffer (3 M guanidine-HCl, 300 mM phosphate, pH 7.0). To this solution were added TCEP (300 mM stock in ligation buffer) and 4-mercaptophenylacetic acid "MPAA" (250 mM stock in ligation buffer) to a final concentration of 30 mM each. The mixture was rocked for 12 h at room temperature after adjusting the pH of the reaction to 7.0 with 3M NaOH solution. After 12 h, ligation product (α-synuclein(1-75)-$NHNH_2$ fragment) was purified using a C18 semi-prep column (Higgins Analytical) and lyophilized.

The α-synuclein(1-75)-$NHNH_2$ fragment was first activated using the Dawson Knorr-Pyrazole method. [4]. Specifically, the fragment was dissolved in activation buffer (6 M guanidine-HCl, 200 mM MPAA, pH 3.5) and acetylacetone (1:20 v/v) in activation buffer at pH 3.5 and stirred at room temperature until the MPAA activation is complete (~2 h). The thioester formation was monitored by RP-HPLC (0–70 % of solvent B over 60 min) and activation was confirmed by ESI-MS. After completion, the α-synuclein(A76C-140) fragment was added to the reaction mixture and equal volume of ligation buffer (6 M guanidine-HCl, 200 NaH_2PO_4, pH 8.3) was added followed by TCEP.HCl (50 mM final concentration). The pH of the resulting solution was adjusted to 7.0 with

addition of 3M NaOH solution. The reaction mixture was stirred for 12 h at room temperature. This product was purified using a C4 semi-prep column (Higgins Analytical) and lyophilized. Deacetylation of the monosaccharide on the lyophilized full length protein was done in presence of 5 % hydrazine monohydrate aqueous solution at room temperature for 1 h and then quenched with 5% acetic acid solution. The de-acetylated full length α-synuclein was then purified using a C4 semi-prep column (Higgins Analytical) and lyophilized. Desulfurization on the full length protein was carried out in degassed buffer (6 M guanidine-HCl, 300 mM phosphate, 300 mM TCEP, 2.5% v/v ethanethiol, 10% v/v tertbutylthiol, pH 7.0) in the presence of a radical initiator VA-061 (200 mM in MeOH, 2 mM final concentration) under inert atmosphere. The reaction was stirred at 37 °C for 16 h. The product was purified and characterized by C4 analytical column (Higgins Analytical) and ESI-MS respectively.

Circular dichroism (CD):
All circular dichroism (CD) spectra were taken at room temperature with a Jasco-J-815 spectrometer. In 10 mM phosphate buffer, pH-7.4, sample aliquots were diluted to 7.5 μM final concentration, with in a 1 mm path length quartz cuvette at 25 °C. The far UV spectra (195 nm-250 nm) were obtained by averaging three scans with a 50 nm min^{-1} scanning speed, 1 nm bandwidth, 0.1 nm step size, and data integral speed of 4. For all samples, buffer reading was subtracted and the data were converted into mean residue ellipticity.

Dynamic light scattering (DLS):
Dynamic light scattering data were obtained with Wyatt Technologies Dynastar. All samples were at t = 0 h of aggregation reaction and 50 μM concentration. An average of 10 scans at 25 °C was obtained for all samples and with laser power adjusted to intensity of 2.6E6 counts s^{-1}. To calculate radii, Raleigh sphere approximation was used.

α-Synuclein aggregation:
Purified unmodified α-synuclein and the glycosylated derivatives were individually dissolved in aggregation buffer (10 mM phosphate, 0.05% NaN_3, pH 7.4, filtered) and sonicated for 10 mins. The solutions were then centrifuged at 20,000 x g for 20 min at 4 °C to remove any pre-formed seeds. The supernatant was transferred into a fresh tube and BCA assay was performed to exactly determine the concentration of proteins in solution. The volume of solution was adjusted with aggregation buffer to get a final concentration of 50 μM of each protein which was then partitioned into triplicates with a final volume of 150 μl in each tube. These triplicates solutions were then left at 37 °C incubator with constant agitation (1,000 x g) in a Thermomixer F1.5 (Eppendorf) for the indicated lengths of time, and aliquots were collected at different time points and stored at -80 °C for analysis by Thioflavin T.

Thioflavin T fluorescence:
The progression of α-synuclein aggregation was quantified by Thioflavin T fluorescence. Samples from the aggregation assay reaction mixture were diluted in a 96-well plate to a concentration of 1.25 μM with reaction buffer (10 mM PBS, pH 7.4 and 0.05% NaN_3) containing 20 μM Thioflavin T (dissolved from a 2000X stock prepared in DMSO). Fluorescence was measured using a Synergy H4 hybrid reader (BioTek). The plate was shaken for 3 min, and data was collected (λ_{ex} = 450 nm, 9 nm band path, λ_{em} = 482 nm, 9 nm band path). Triplicate measurements were performed for all aggregation reaction conditions.

Dot blotting:
Ten nanograms (10 ng) of protein from aggregation reactions were spotted onto nitrocellulose membrane and air dried for 30 min. The proteins were fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. Membrane was washed 3 times in PBS for 5 minutes each before blocking for 1 hour at room temperature with OneBlock Western-CL buffer (Genessee Scientific). Primary antibody (A17183A, BioLegend) was added to the blocking buffer at 1:5000 dilution and the membrane was incubated for 16 hours at 4 °C. The membrane was washed three times in TBST for 10 minutes each, after which it was incubated with anti-rat-HRP secondary antibody (1:10,000 in blocking buffer) for one hour at room temperature. The membrane was washed again with TBST three times for 10 minutes each before developing in Western blotting substrate (Bio-Rad Clarity Western ECL).

Transmission electron microscopy:
At the end of the aggregation process, each protein solution was diluted to 15 μM by adding the aggregation reaction buffer, and the diluted solution (10 μL) was incubated with a Formvar coated copper grid (150 mesh, Electron Microscopy Science) for 5 min. Subsequently, the grid was incubated with 1% uranyl acetate three times for 2 min. Each time, excess liquid was removed with filter paper. The grid was dried for 24 h. Grids were visualized with a JEOL JEM-2100F transmission electron microscope operated at 200 kV and 600,000× magnification and an Orius Pre-GIF CCD.

Proteinase K digestion:
Ten micrograms of aggregation protein sample were incubated with Proteinase K (Sigma Aldrich P2308) at the indicated concentrations for 30 min at 37 °C. Reactions were quenched by the addition of sample loading buffer (2% final SDS concentration) and boiling samples at 95 °C for 10 min. Digestion products were separated by SDS- PAGE using precast 12% Bis-Tris gels with MES running buffer. Bands were visualized with Coommassie Brilliant Blue.

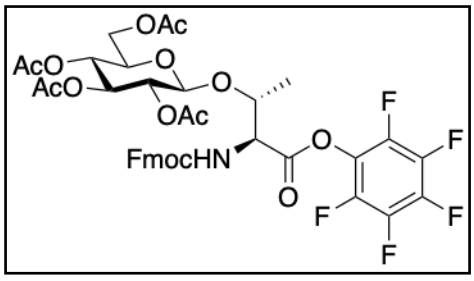
Visualization of soluble protein and aggregate pellet:
At the end of aggregation assay, 10 uL of aggregation reaction sample was centrifuged at 20 000g for 1 h at 25 °C. The supernatant was lyophilized to dryness. The lyophilized sample was solubilized in 15 uL of fresh 8 M urea, 20 mM HEPES buffer (pH 8.0) and 5 uL of 4 x SDS loading buffer (with BME) was added. The sample was boiled for 10 min at 95°C, loaded on 4–20% Criterion precast gel (BioRad) and separated by SDS-PAGE at 195 V. The gel was stained with Coomassie brilliant blue for 30 min and destained with acetic acid/water/methanol solution (1:4:5) overnight.

β-O-Ac$_3$GlcNAc-threonine pentafluorophenyl ester was synthesized as previously described. [5]

General glycosylation reaction for the preparation of glycosyl-threonines: [5]
A solution of the per-O-acetylated donor sugar (3 equiv.), $InBr_3$ (0.6 equiv., 20 mol % with respect to donor), and the Fmoc-threonine acceptor (1 equiv.) in 1,2-dichloroethane (200 mM concentration of the acceptor) was refluxed for 16 h at 85 °C under nitrogen atmosphere. The reaction was monitored by TLC (7:2:1: EtOAc: MeOH: H2O). The reaction was allowed to cool to room temperature and concentrated under vacuum. The crude was then resuspended in dichloromethane (DCM), loaded onto a flash column chromatography and purified with a mixture of 5% MeOH in DCM (0.1% AcOH). The amounts of individual reagents can be found in the specific methods below.

General preparation of the pentafluorophenyl amino-acid esters:
Pentafluorophenyl trifluoroacetate (3 equiv.) was added to a stirring solution of the free acid (1 equiv.), anhydrous pyridine (4 equiv.) in anhydrous dimethylformamide (100 mM concentration of the amino acid) under N_2. The reaction was allowed to stir at room temperature overnight. The reaction progress was monitored by TLC (4:6 EtOAc: Hexane). After the reaction went to completion, the mixture was concentrated in vacuum and the residue was then suspended in DCM and purified by flash chromatography (Hexane/ Acetone, 7/3). The amounts of individual reagents can be found in the specific methods below.

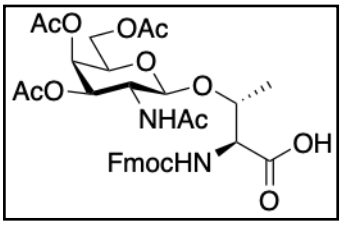
***O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-*N*-(9-fluorenylmethyloxycarbonyl)-L-threonine pentafluorophenyl ester (β-*O*-Ac$_4$Glc-threonine):**



Following the general pentafluorophenyl ester synthesis using commercially available β-O-Ac$_4$Glc-threonine (250 mg), β-*O*-Ac$_4$Glc-threonine pentafluorophenyl ester was obtained as a white or light brown foam (310 mg, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.67 – 7.59 (m, 2H), 7.40 (t, *J* = 7.5 Hz, 2H), 7.31 (t, *J* = 7.5 Hz, 2H), 5.72 (d, *J* = 8.9 Hz, 1H), 5.23 (t, *J* = 9.6 Hz, 1H), 5.08 (t, *J* = 9.7 Hz, 1H), 4.97 (dd, *J* = 9.8, 7.9 Hz, 1H), 4.72 (dd, *J* = 8.9, 2.8 Hz, 1H), 4.59 – 4.54 (m, 2H), 4.50 – 4.39 (m, 2H), 4.26 (t, *J* = 7.1 Hz, 1H), 4.20 (dd, *J* = 12.3, 4.8 Hz, 1H), 4.05 (dd, *J* = 12.3, 2.5 Hz, 1H), 3.69 (ddd, *J* = 9.9, 4.8, 2.5 Hz, 1H), 2.05

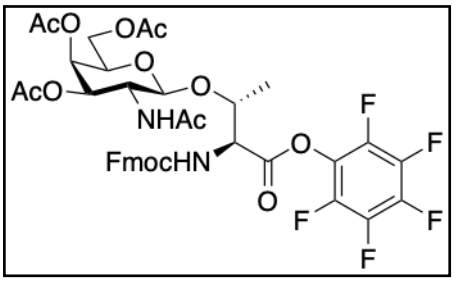
(s, 3H), 2.03 (s, 3H), 2.03 (s, 3H), 2.00 (s, 3H), 1.31 (d, J = 6.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.56, 170.23, 169.32, 169.28, 166.30, 166.28, 156.45, 143.78, 143.52, 141.31, 127.77, 127.75, 127.09, 127.08, 125.10, 119.99, 119.98, 98.30, 73.87, 72.40, 71.87, 71.29, 68.23, 67.44, 61.67, 58.48, 47.11, 20.63, 20.60, 20.56, 20.42, 16.74.

_O_-(2-Acetamido-3,4,6-tri-_O_-acetyl-2-deoxy-β-D-galactopyranosyl)-_N_-(9-fluorenylmethyloxycarbonyl)-L-threonine (β-_O_-Ac_3GalNAc-threonine):



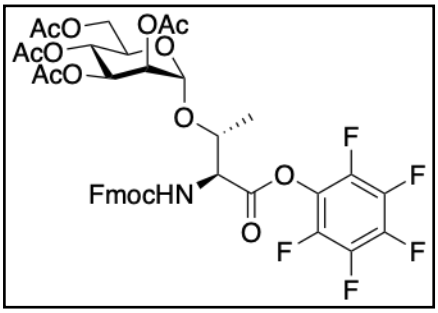
Following the general glycosylation reaction: β-Ac_4GalNAc (3.14 g, 8.07 mmol), Fmoc-Thr-OH (800 mg, 2.69 mmol), and $InBr_3$ (572 mg, 1.61 mmol). Following purification, β-_O_-Ac_3GalNAc threonine was obtained as a white or light brown foam (550 mg, 61% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 7.77 (d, _J_ = 7.5 Hz, 1H), 7.68 (t, _J_ = 7.2 Hz, 2H), 7.37 (t, _J_ = 7.5 Hz, 2H), 7.30 (t, _J_ = 6.8 Hz, 2H), 5.33 (d, _J_ = 3.3 Hz, 1H), 5.08 (dd, _J_ = 11.3, 3.4 Hz, 2H), 4.59 (d, _J_ = 8.5 Hz, 1H), 4.45 – 4.39 (m, 1H), 4.35 (dd, _J_ = 7.1, 4.2 Hz, 2H), 4.25 – 4.21 (m, 2H), 4.18 – 4.02 (m, 3H), 3.97 (t, _J_ = 6.8 Hz, 1H), 2.09 (s, 3H), 2.00 (s, 3H), 1.95 (s, 3H), 1.94 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 173.95, 173.33, 172.13, 172.04, 171.69, 158.98, 145.29, 145.05, 142.51, 128.78, 128.20, 128.19, 126.34, 126.30, 120.93, 101.57, 76.72, 71.79, 71.57, 68.19, 67.81, 62.19, 59.93, 51.62, 48.33, 22.95, 20.59, 20.55, 17.99.

_O_-(2-Acetamido-3,4,6-tri-_O_-acetyl-2-deoxy-β-D-galactopyranosyl)-_N_-(9-fluorenylmethyloxycarbonyl)-L-threonine pentafluorophenyl ester (β-_O_-Ac_3GalNAc threonine pentafluorophenyl ester):



Following the general pentafluorophenyl ester synthesis using β-_O_-Ac_3GalNAc Threonine (330 mg, 8.07 mmol), β-_O_-Ac_3GalNAc threonine pentafluorophenyl ester was obtained as a white or light brown foam after purification (365 mg, 87% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, _J_ = 7.5 Hz, 2H), 7.66 – 7.59 (m, 2H), 7.34 (t, _J_ = 7.4 Hz, 2H), 7.26 (t, _J_ = 7.2 Hz, 2H), 6.07 (dd, _J_ = 8.8, 3.9 Hz, 2H), 5.30 (d, _J_ = 3.5 Hz, 1H), 5.20 (dd, J = 11.4, 3.4 Hz, 1H), 4.70 – 4.63 (m, 2H), 4.56 – 4.51 (m, 1H), 4.47 – 4.44 (m, 3H), 4.22 (t, _J_ = 7.2 Hz, 1H), 4.02 – 3.92 (m, 3H), 3.85 (t, _J_ = 6.6 Hz, 1H), 2.10 (s, 3H), 1.96 (s, 3H), 1.95 (s, 3H), 1.92 (s, 3H), 1.25 (d, _J_ = 6.3 Hz, 3H).^{13}C NMR (100 MHz, $CDCl_3$) δ 207.16, 170.71, 170.55, 170.31, 170.26, 166.51, 156.65, 143.84, 143.56, 141.24, 127.70, 127.67, 127.06, 127.02, 125.19, 125.13, 119.91, 98.53, 73.18, 70.46, 69.65, 67.30, 66.41, 61.15, 58.84, 51.41, 47.08, 30.85, 23.37, 20.61, 20.47, 20.42, 16.36.

_O_-(2,3,4,6-tetra-_O_-acetyl-α-D-mannopyranosyl)-_N_-(9-fluorenylmethyloxycarbonyl)-L-threonine pentafluorophenyl ester (α-_O_-Ac_4Man-threonine pentafluorophenyl ester):



Following the general glycosylation reaction: _O_-Ac_5Man (3.90 g, 10.09 mmol), Fmoc-Thr-OH (1.00 g, 3.36 mmol), and $InBr_3$ (715 mg, 2.02 mmol); α-_O_-Ac_4Man-threonine was first purified by column chromatography using 5% MeOH in DCM (0.1% AcOH). The product was obtained as a mixture of compounds and used for the next step without further purification. Following the general pentafluorophenyl ester synthesis using α-_O_-Ac_4Man-threonine (600 mg), α-_O_-Ac_4Man-threonine pentafluorophenyl ester was obtained as a white or light brown foam (650 mg, 24% yield over two steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, _J_ = 6.6 Hz, 2H), 7.69 – 7.64 (m, 1H), 7.46 – 7.22 (m, 5H), 5.72 (d, _J_ = 8.3 Hz, 1H), 5.34 – 5.21 (m, 2H), 5.15 (s, 1H), 5.00 (s, 1H), 4.83 (d, J = 9.5 Hz, 1H), 4.62 – 4.42 (m, 3H), 4.34 – 4.22 (m, 2H), 4.19 – 4.02 (m, 2H), 2.14 (s, 3H), 2.09 (s, 3H), 2.08 – 2.06 (m, 3H), 1.99 (s, 3H), 1.48 – 1.39 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.50, 169.84, 169.66, 169.62, 166.47, 156.41, 143.68, 143.56, 141.31, 127.79, 127.13, 125.13, 120.03, 120.00, 99.16, 69.34, 69.20, 68.66, 67.73, 66.26, 62.56, 58.53, 47.10, 29.68, 20.59, 18.07.

NMR Characterization:

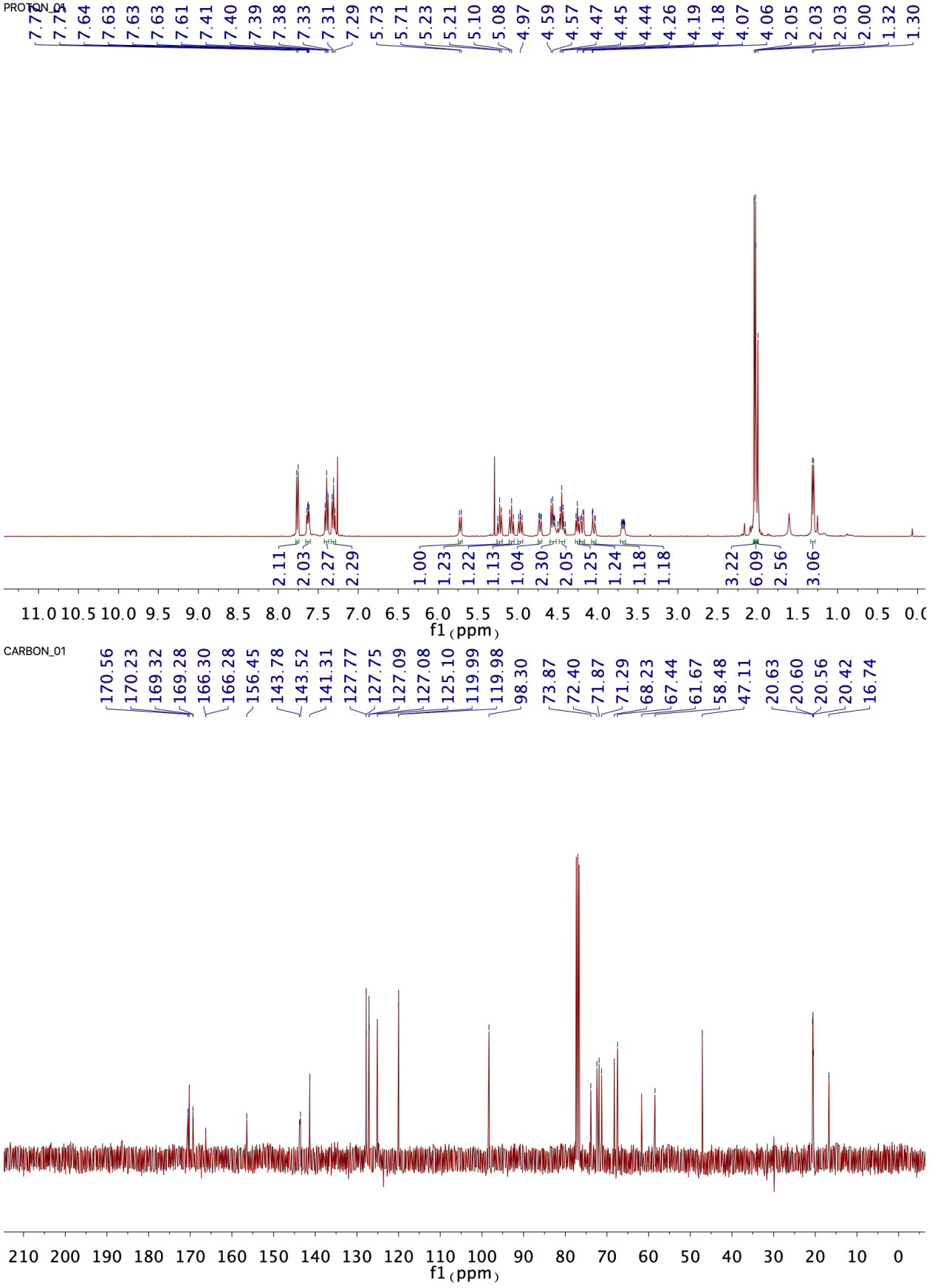


Figure S12: a) ^{1}H NMR and b)^{13}C NMR spectra of β-*O*-Ac$_4$Glc-threonine pentafluorophenyl ester.

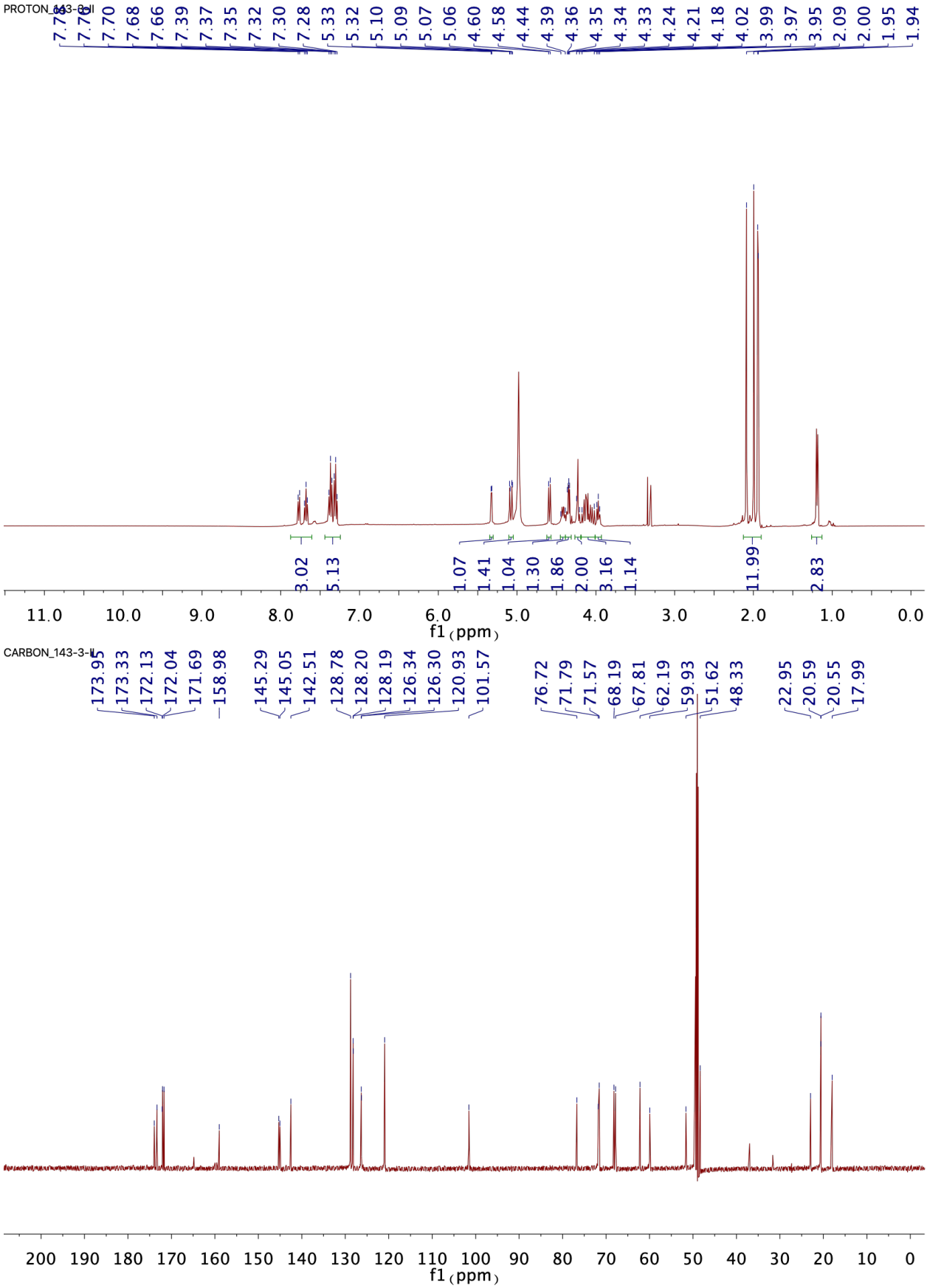


Figure S13: a) ^{1}H NMR and b)^{13}C NMR spectra of β-*O*-Ac_3GalNAc-threonine.

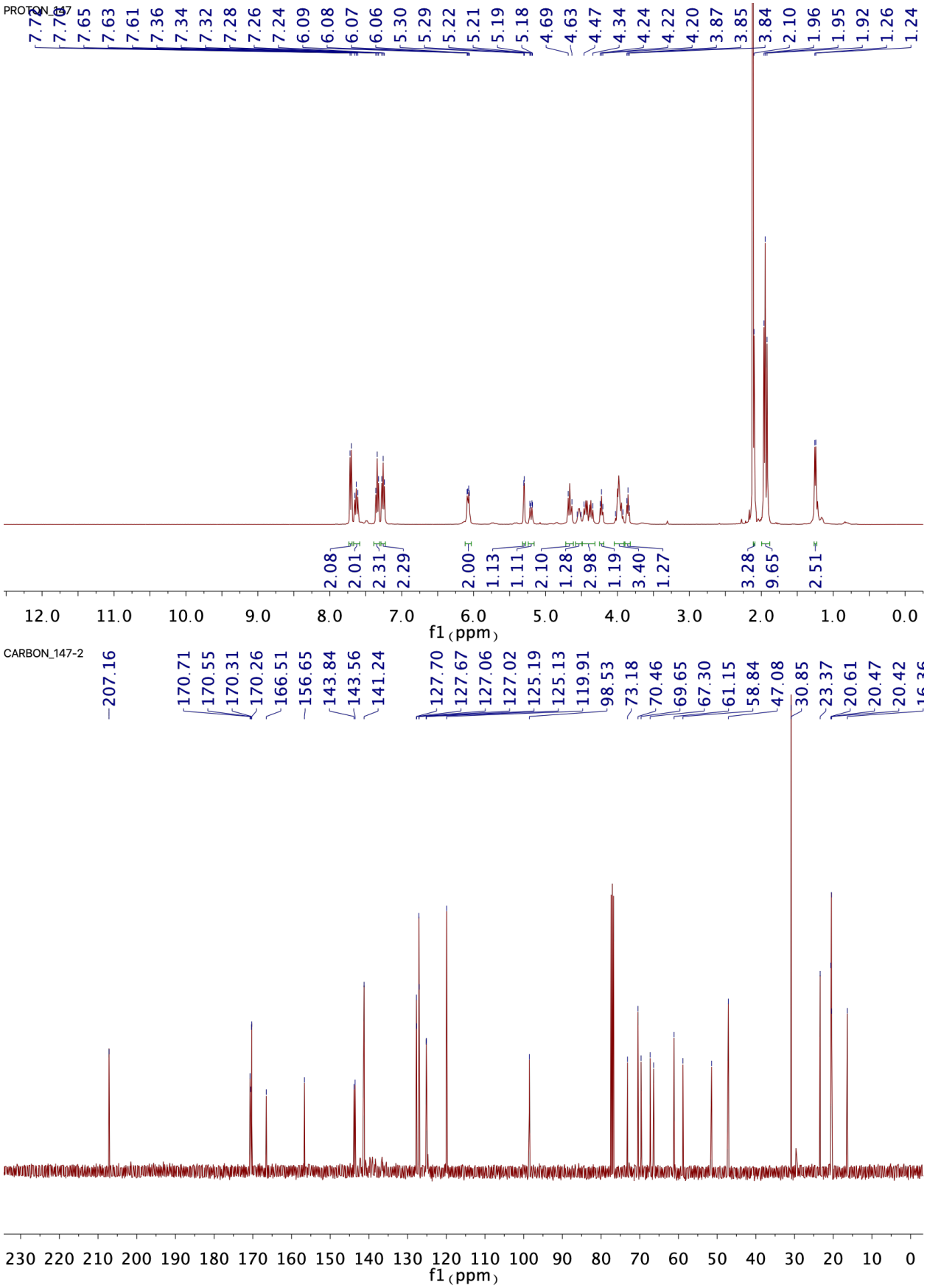


Figure S14: a) ^{1}H NMR and b)^{13}C NMR spectra of β-*O*-Ac_3GalNAc-threonine pentafluorophenyl ester.

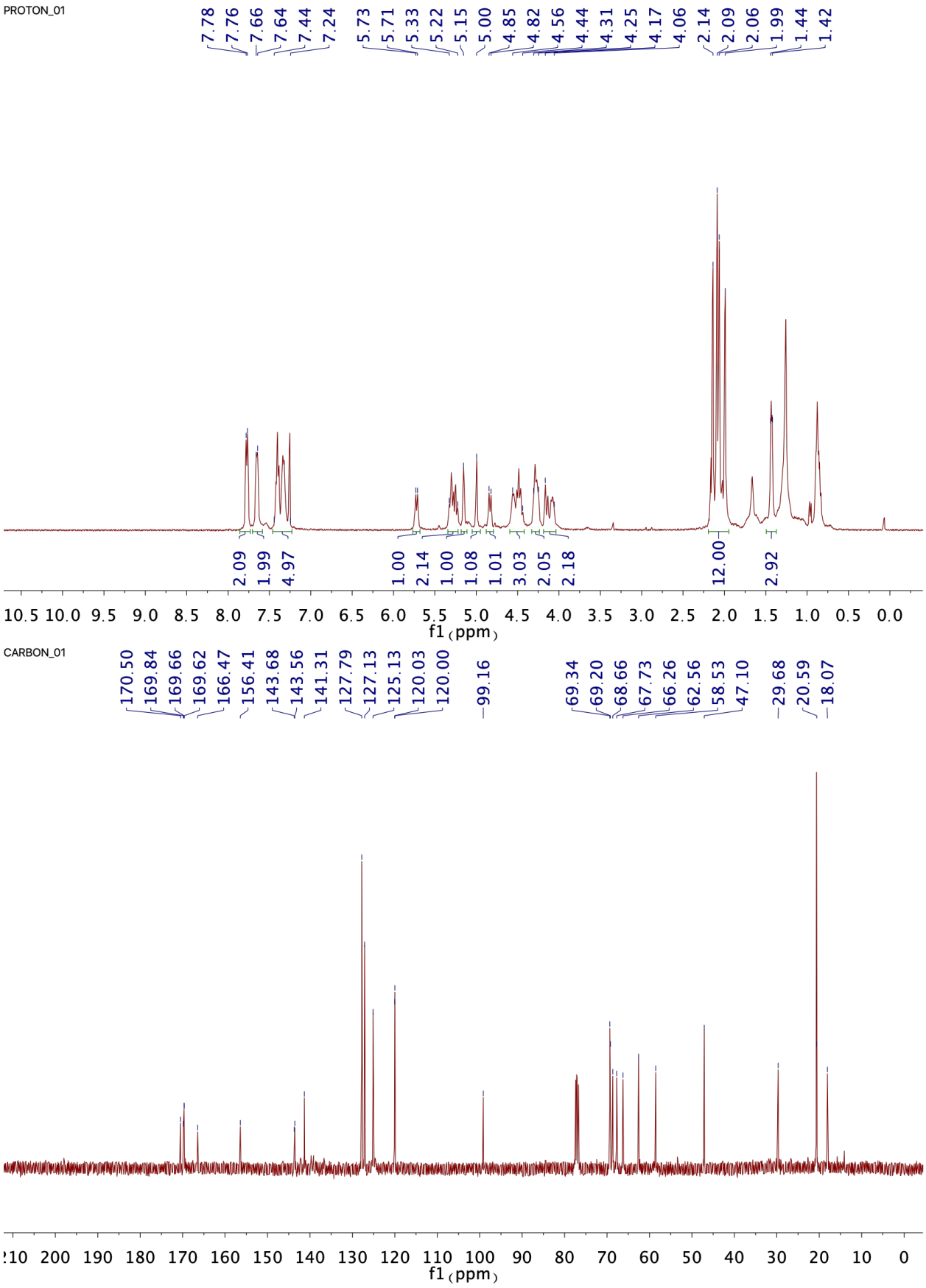


Figure S15: a) ^{1}H NMR and b)^{13}C NMR spectra of α-*O*-Ac$_4$Man-threonine pentafluorophenyl ester.

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