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One-pot Total Chemical Synthesis of Human α-Synuclein

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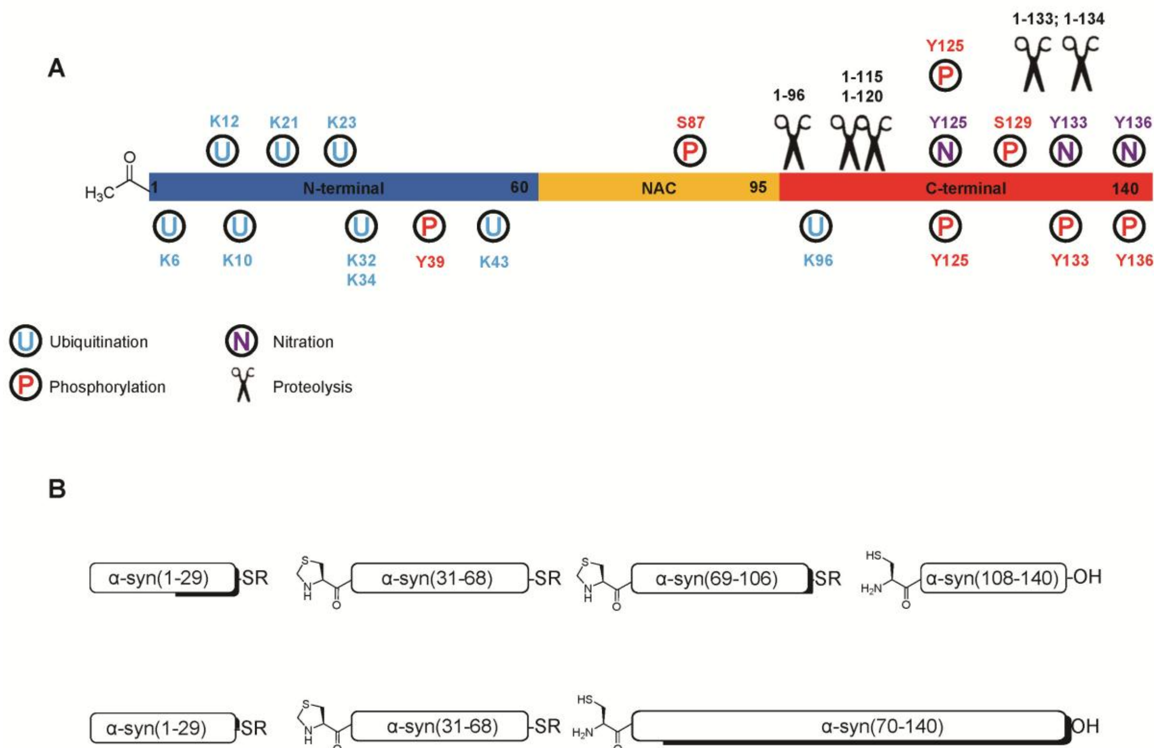

Fig. S1: A: Post-translational modifications of α-syn. PTMs identified in Lewy Bodies are shown above the protein's domains; and PTMs identified from *in-vitro* studies are shown below. **B:** Fragment design for the initial, four-fragment ligation strategy (top) and the optimized, three-fragment scheme (bottom).

Peptide	Strategy #	Sequence
α-syn(69-140) A69C	2	**C**VVTGVTAVAQ**KT**VEGAGSIAAATGFVKKDQLGKNEEGAPQEGILEDMPVDP DNEAYEMPSEEGYQDYEPEA-**OH**
Thz-α-syn(31-68)SR	1 and 2	**Thz**-GKTKEGVLYVGS**KT**KEGVVHGVATVAE**KT**KEQVTNVGG-**SR**
α-syn(1-29)SR	1 and 2	MDVFMKGLSKAKEGVVAAAEKTKQGVAEA-**SR**
Thz-α-syn(70-106)SR	1	**Thz**-VVTGVTAVAQ**KT**VEGAGSIAAATGFVKKDQLGKNEEG-**SR**
α-syn(107-140) A107C	1	**C**PQEGILEDMPVDPDNEAYEMPSEEGYQDYEPEA-**OH**

Table S1: Sequences used for the total synthesis of a-synuclein and the structure of (R) thiazolidine-4-carboxylic acid (Thz) which was used as a protecting group for N-terminal Cys. The sites in which structure-disrupting Lys-Thr pseudoproline dipeptides were introduced during the synthesis are shown in bold. The phosphorylation site at S87 is shown in red.

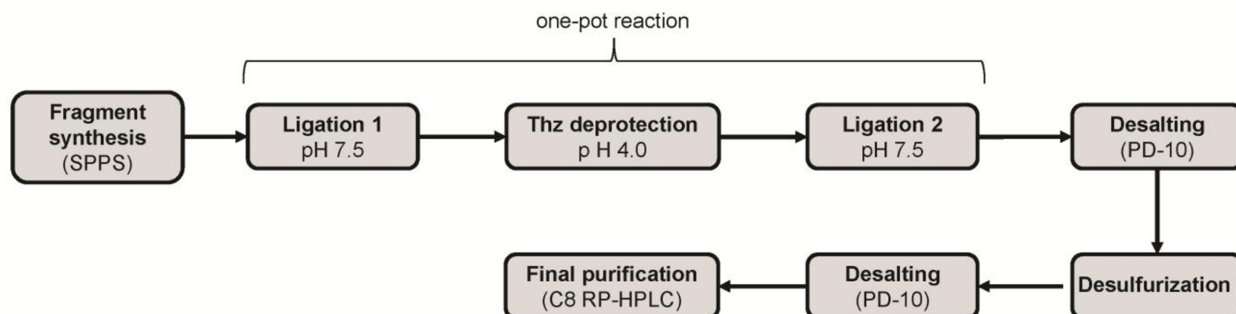

Fig. S2: Workflow diagram describing the synthesis and purification steps for the final (three-fragment) synthesis scheme.

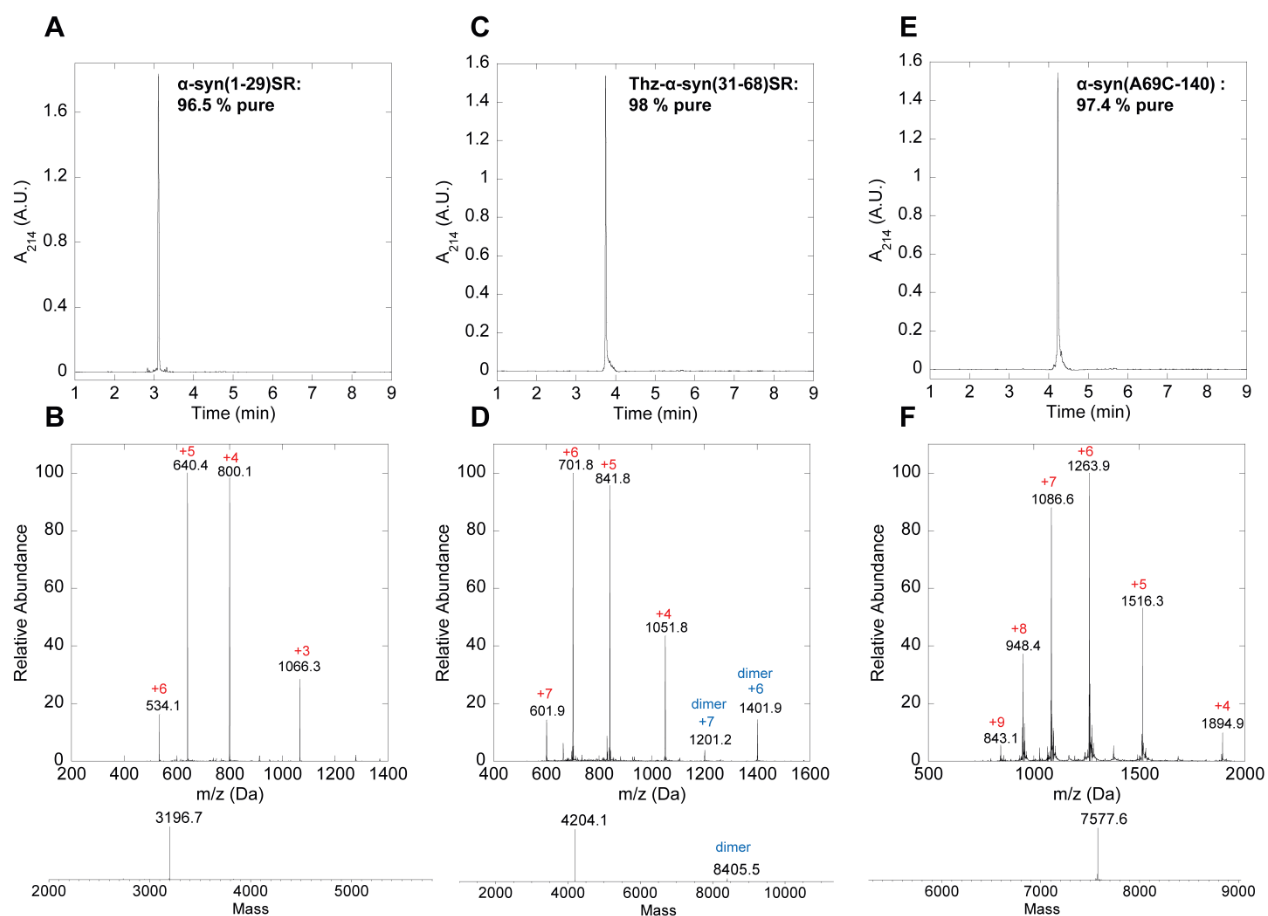

Fig. S3: Purity analyses of the peptides used for WT α-syn(1-140) total synthesis. **A-B**: analytical RP-UHPLC (**A**) and ESI-MS (**B**) of purified α-syn(1-29)SR. **C-D**: analytical RP-UHPLC (**C**) and ESI-MS (**D** of purified Thz-α-syn(31-68)SR. **E-F**: analytical RP-UHPLC (**E**) and ESI-MS (**F**) of purified α-syn(A69C-140). Panels below ESI-MS spectra correspond to the mass calculated by deconvolution of the ESI-MS spectra (performed with MagTran).

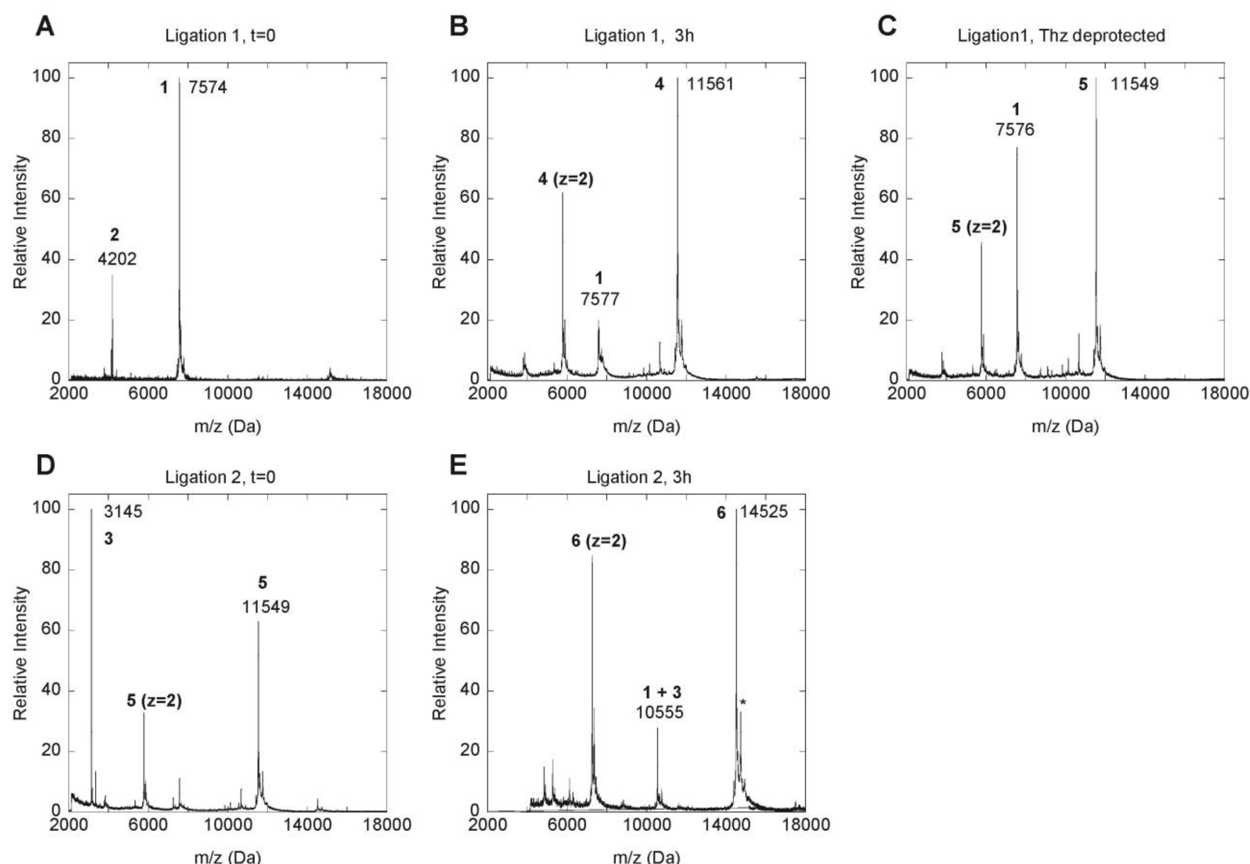

Fig. S4: MALDI-TOF MS analysis of the wt α-syn one-pot synthesis Samples were diluted 100X in 95:5 water:acetonitrile (each with 0.1% TFA) before being mixed 1:1 with sinapinic acid and spotted on the MALDI plate . **A-B**: Spectra taken at t=0 (**A**) and t=3h (**B**) of the first ligation, between α-syn(A69C-140) and Thz-α-syn(31-140)SR. **C**: Analysis after 1 h of reaction with methoxyamine at pH 4.0, showing the -12 Da mass difference indicative of conversion of the N-terminal Thz in the intermediate 30-140 fragment to a ligation-competent N-terminal cysteine. **D-E**: Spectra taken at t=0 (**D**) and t=3h (**E**) of the first ligation, between α-syn(30-140) A30C A69C and α-syn(1-29)SR.

Experimental section

Chemicals

9-Fluorenylmethoxycarbonyl (Fmoc)-amino acids, t-butyloxycarbonyl (Boc)-amino acids, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), H-Ala-2-ClTrt resin, H-Gly-2-ClTrt resin, and Fmoc-Ala-Wang resin. The side-chain functionalities of the Fmoc-amino acids were protected as follows: Asp(OtBu), Glu(OtBu), Lys(Boc), Thr(tBu), Ser(tBu), Tyr(tBu), Gln(Trt), and Asn(Trt). All unlisted amino acids were not protected. The Boc-amino acids included Boc-Met-OH and Boc-Cys(Trt)-OH. Fmoc-Lys(Boc)-Thr($\psi^{Me,Me}$pro)-OH was also obtained from Novabiochem. N-Boc-(R)-thiazolidine-4-carboxylic acid (Boc-Thz-OH), Dimethylamino)-*N*,*N*-dimethyl(3*H*-[1,2,3]triazolo[4,5-*b*]pyridin-3-yloxy)methaniminium hexafluorophosphate (HATU) and 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Anaspec. Guanidine hydrochloride (GdnHCl), N,N-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), trifluoroethanol (TFE), triisopropylsilane (TIPS), benzyl mercaptan, sodium 2-mercaptoethanesulfonate (MesNa), 1,2-ethanedithiol (EDT), β-mercaptoethanol (BME), thioanisole, dimethylsulfide, ammonium iodide, methoxyamine hydrochloride, and tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) were all purchased from Sigma-Aldrich. Piperidine and diethyl ether were purchased from Acros. Thiophenol and 2-methyl-2-propanethiol were purchased from Fluka. 2-2'-Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) was purchased from Wako. Chitin beads were purchased from Neb and Tev protease was obtained from Promega. Solvents N,N-dimethylformamide (DMF), dichloromethane, and HPLC grade acetonitrile were purchased from VWR.

Solid-Phase peptide synthesis (SPPS) and peptide purification

Peptides were synthesized on an automated CS 336X peptide synthesizer from CS Bio using standard Fmoc protocols including the in situ neutralization protocol described by Alewood [1] For peptides with a C-terminal acid functionality, the Wang resin was used in which the first Ala residue was pre-loaded. For peptides with a C-terminal thioester, the 2ClTrt resin was used in which the first amino acid was pre-loaded. Syntheses were carried out on a 0.2 mmol scale and coupling steps were performed with 5 eq. Fmoc-amino acid, 5 eq. HBTU, and 10 eq. DIPEA in DMF. The specifications for the requirement of double couplings, HATU coupling reagent, or pseudoproline dipeptides are described in the text. N-terminal amino acids were attached with Boc protecting groups on their N-terminus. Fmoc deprotections were carried out with 20% piperidine in DMF. Peptide side chain deprotection and cleavage from the Wang resin was performed by treatment with 95:2.5:2.5 TFA:TIPS:water under inert gas for 3-4h to generate peptides with a C-terminal acid functionality. For the synthesis of α-syn(A69C-140) which contains oxidation-prone methionine residues, the cleavage cocktail known as Reagent H was used [2] specifically, 82.5% TFA, 2.5% ethanedithiol, 5% thioanisole, 5% dimethylsulfide, containing 75 mg phenol, 50 mg ammonium iodide, and 100 µl TIPS per mL of cleavage solution. The crude products were subsequently precipitated with excess volume of cold ether, dissolved in 50% aqueous acetonitrile containing 0.1% TFA, and lyophilized. Peptides were then purified by RP-HPLC using either a Vydac or a Phenomenex C4 preparative column and a linear gradient of 0-50% B where solvent A was 95:5 water:acetonitrile, 0.1% TFA and solvent B was 95:5 acetonitrile:water, 0.1% TFA. Peptide elutions were monitored at both 214 nm and 280 nm. Peptide and protein masses were confirmed by MALDI-TOF analysis operating linear negative, linear positive, or reflector positive modes. The purity of purified peptides was assessed by re-injection on a Vydac C4 analytical column.

Synthesis of peptide thioesters

Peptide thioesters were generated based on the Nbz method developed by Blanco-Canosa et al. [3]. 3-Fmoc-3,4-diaminobenzoic acid (Fmoc-Dbz-OH) was prepared by reacting 500 mg (3.3 mmol) of 3,4-diaminobenzoic acid with 1 eq (1.1 g, 3.3 mmol) Fmoc-OSu in 10 mL of 1:1 acetonitrile:0.1M $NaHCO_3$ pH 7.9 at RT with stirring for 12-16h. Then, the mixture was acidified until pH dropped to about 1.0 and filtered through a sintered glass funnel. The filtrate was washed multiple times (at least 3x each) with diethyl ether, hexanes, and MeOH, and finally transferred into a glass flask and vacuum-dried. Synthesis of peptide thioesters started with the loading of 0.2 mmol of Rink amide MBHA resin with Fmoc-Dbz-OH. The resin was treated with 5eq Fmoc-Dbz-OH, 5 eq HATU and 10 eq DIPEA twice for 2h each at RT. Then, the 4-amino position on the loaded Dbz linker was protected as described by Mahto and colleagues [4] by treating the resin with 350 mM allyl chloroformate and 1 eq DIPEA in dry DCM for 24h at RT and then washed with DCM and DMF. After deprotection of the 3-Fmoc group on the Alloc-protected Dbz linker, SPPS was continued as described above. After completion of the peptide elongation, the resin was swelled in argon-sparged DCM and the Alloc group was removed by adding 20 eq phenylsilane and 0.35 eq tetrakis(triphenylphosphine)palladium(0). The deprotection was done for 30 min and repeated as needed. After deprotection of the 4-amino position, the Nbz group was activated by sequential treatments with 5 eq 4-nitrophenylchloroformate in DCM followed by 0.5 M DIPEA in DMF. Nbz activation cycles were also repeated if needed. Peptides were then cleaved from the solid support and deprotected using Reagent H as described above; precipitated in cold diethyl ether and lyophilized. Treatment with 50 mM MESNA in 6.0M GdnHCl, 200 mM sodium phosphate pH 7.5 displaced the C-terminal Nbz group to obtain a thioester. Peptides were purified by RP-HPLC on a C18 preparative column (Vydac 218MS, 20mm ID x 250 mm length, 5 µm), lyophilized and stored at -20°C until use.

One-pot total synthesis of α-syn

The one-pot synthesis of α-syn was performed according to the methods described by Bang & Kent [5]. Typical syntheses were done at a 1 µmol scale and peptide concentrations were kept at 1 mM. 7.6 mg of peptide **1** (α-syn(A69C-140)) were dissolved in NCL buffer (6.0 M GdnHCl, 0.2M sodium phosphate, 30 mM TCEP, 50 mM MPAA, pH 7.5) that had been degassed by argon bubbling (10 min) before. Afterwards, reactions were kept under inert gas at all times, due to the tendency of α-syn to undergo methionine oxidation when NCL reactions are not carried out under inert atmosphere. Following dissolution of peptide **1**, 1 eq (4.2 mg) of peptide **2** (Thz-α-syn(30-68)SR) were added and the reaction was incubated at 37°C with orbital agitation at 950 rpm and monitored regularly by withdrawing 5 µL aliquots for RP-UHPLC, SDS-PAGE, and mass spectrometry analyses. Aliquots were immediately diluted 20X in 95:5 water:acetonitrile + 0.1 % TFA, centrifuged (20000g, 4°C, 5 min) and put on ice to quench the reaction. For RP-UHPLC analysis, 4 µL of the diluted aliquots were loaded on a Waters Acquity UPLC BEH300 C4 column (2.1x100mm, 1.7 µm) and eluted with a 10% to 90%B gradient over 9.75 min at 0.6 mL/min where A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile. For SDS-PAGE analysis, diluted aliquots were mixed with an equal volume of 2X Laemmli sample buffer and 20 µL were loaded on a 15% polyacrylamide SDS gel. For analysis by mass spectrometry, the aliquots were further diluted 10X and analyzed by LC-ESI-MS (Thermo LTQ instrument) or by MALDI-TOF MS (AB Sciex 4700). Once the NCL reaction was complete, with a conversion yield of about 94% (measured by RP-UHPLC), 30 mM fresh TCEP and 100 mM methoxyamine were added and the pH was decreased to about 4.0. The reaction was then put back at 37°C under agitation and monitored by mass spectrometry to follow the -12 Da mass shift indicative of the removal of the Thz protection in the intermediate **4** (Thz-α-syn(31-140) A69C) to yield the intermediate product **5** (α-syn(30-140) A30C A69C). Following completion of the Thz deprotection as measured by mass spectrometry, the pH was adjusted back to 7.5 and 30 mM fresh TCEP and 1 eq (3.1 mg) of peptide **3** (α-syn(1-29)SR) were added and the second NCL proceeded to yield product **6** (α-syn(1-140)

A30C A69C), with a reaction yield of 91% as determined by RP-UHPLC analysis. Desulfurization was then carried out as described below to obtain the final product **7** (wt α-syn(1-140).

Desulfurization

Free-radical desulfurization of the ligated α-syn constructs was carried out following the method described by Wan et al. [6]. The ligation reactions were done in presence of an excess of MPAA, which quenches the desulfurization reaction. Thus, a prior desalting step was performed using a PD-10 column (GE Healthcare) equilibrated with 50 mM ammonium bicarbonate. Desalted protein fractions were lyophilized, and then redissolved in argon-sparged 6.0M GdnHCl, 0.2 M sodium phosphate, pH 7.5. Then, TCEP, 2-2'-Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044), and 2-methyl-2-propanethiol were added to final concentrations of 200 mM, 5.4 mM, and 840 mM respectively. The reaction tube was sealed and placed at 37°C under agitation until the reaction (as monitored by a loss of 32Da per cysteine residue desulfurized) was determined to be complete by mass spectrometry. The mixture was desalted (using a PD-10 column) in order to separate the protein from the thiol, and then purified by RP-HPLC on a semi-preparative InertSil C8 column (7.6x250 mm, 5 µm) using a gradient from 20% to 70%B gradient over 40 min at 5 mL/min. Fractions containing pure protein (verified by RP-UHPLC and mass spectrometry) were lyophilized and kept at -20°C until futher use. The combined desulfurization and purification yield was 21%, with 0.21 µmol of pure product **7** (~3 mg) obtained. This final yield was measured by UV spectroscopy of the purified protein before lyophilization, using an extinction coefficient of 5974 $M^{-1}cm^{-1}$ at 275 nm.

Circular Dichroism (CD) spectroscopy and membrane binding assays

CD analysis was performed at 20°C on a Jasco J-815 spectrometer. Protein samples (160 µL) at a concentration of 5 µM in CD buffer (20 mM sodium phosphate pH 7.4) were placed in a 1 mm optical path quartz cuvette for analysis. Spectra were collected from 250 nm to 195 nm with a data pitch of 0.2 nm, scanning speed 50 nm/min, digital integration time 2s, and bandwidth of 2 nm. 5 to 10 spectra were averaged to produce the data reported in the figures. For membrane binding analyses, 100 nm diameter POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt), purchased from Avanti Polar Lipids) were prepared by extrusion. A sufficient amount of POPG solution in chloroform was transferred to a small glass vial and the solvent was evaporated under a gentle stream of argon while rotating the flask, then placed under high vacuum (at RT) for 20 min. The lipid film was resolubilized by adding CD buffer to obtain a multilamellar vesicle suspension of POPG with a lipid concentration of 10 mg/mL. Ten freeze/thaw cycles between liquid nitrogen and a room-temperature waterbath were performed to enhance disruptions of the multilamellar vesicles. Large unilamellar vesicles (LUV) were then prepared by extrusion of that suspension, applying 20 passes through a polycarbonate filter with 100 nm average pore diameter (purchased from Avestin). Final LUV preparations were stored at 4°C and used within two days. Samples for membrane binding analyses were prepared by adding 0.5 or 5 mass equivalents of lipid with respect to protein concentration, and incubating protein/LUV samples at RT for 20 min before CD measurements. Protein concentration was also kept at 5µM for these experiments.

Aggregation assays

Lyophilized proteins were dissolved to about 30 µM in TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.5) that had been degassed by bubbling argon for 10 min. Protein solutions were then filtered through 100 kDa MWCO Microcon filters in order to remove any insoluble or oligomeric material at the beginning of the experiment. Filtration was achieved by centrifugation at 13 000g for 15 min at 4°C, then protein concentrations were precisely measured by UV spectroscopy (for wt α-syn, we used an extinction coefficient of $\varepsilon_{275nm} = 5974\ M^{-1}cm^{-1}$). Proteins were then diluted to a final concentration of 15µM (final volume: 500 µL) in 1.5 mL Sarstedt screw-cap tubes; the solutions were blanketed with argon and placed at 37°C with orbital shaking at 1000 rpm on a Thermo-Mixer Compact shaker (Eppendorf). 50 µL aliquots were then withdrawn every 24h during 5 days for analysis by Thioflavin T (ThT) fluorescence, SDS-PAGE, and transmission electron microscopy (TEM). For ThT analysis, a stock ThT solution was first prepared by mixing 1344 µL of 0.5M glycine pH 8.5 with 153.2 µL water and 16.8 µL ThT 1 mM in water. The stock solution was split into 216 µL aliquots. To each aliquot, 24 µL of well-mixed sample solution were added, the solution was thoroughly vortexed and loaded into three wells of a 384-well black, flat-bottom plate (Nunc, 70 µL per well). After loading all samples, the plate was agitated at room temperature for 5 min and then immediately scanned for fluorescence intensity on a Bucher Analyst AD plate reader (excitation: 450 nm, emission: 485 nm). For SDS-PAGE analysis of soluble protein content, samples were centrifuged at 20 000g for 10 min at 4°C to pellet insoluble aggregates, and then 7µL of the supernatant were mixed with 7 µL of 2X Laemmli sample buffer. 10 µL of the mixtures were loaded on 15% polyacrylamide SDS gels which were stained with a Coomassie R-450 solution. The relative amounts of soluble protein with respect to the initial conditions were determined by densitometry analysis (performed with ImageJ) of the scanned gels. For TEM analyses, 5 µL aliquots were mixed with 5 µL TBS and 5 µL were spotted on Formvar-coated 200-mesh copper grids (Electron Microscopy Sciences). Grids were washed twice with 5 µL of ultrapure water and then stained twice with 5 µL of an aqueous 2% w/v uranyl acetate solution (Electron Microscopy Sciences) and then vacuum-dried from the edges of the grids. Grids were imaged using a Tecnai Spirit BioTWIN electron microscope operated at 80 kV with a LaB_6 source. Digital micrographs were recorded with a 4k x 4k FEI Eagle CCD camera (FEI, Hillsboro, OR, USA).

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