Chemical Synthesis of site-selective advanced glycation endproducts in α-synuclein and its fragments

Electronic Supporting Information (ESI)

Contents

1. Reagents and Solvents

All reagents and chemicals were used as supplied. Dry solvents were obtained from *Acros Organics* (now *Thermo Fisher Scientific*, Nidderau, Germany) and stored over molecular sieves (4 Å). Solvents for HPLC were obtained from *Sigma-Aldrich Chemie GmbH* (Taufkirchen, Germany) in HPLC grade (99.9%). Dimethylformamide (DMF) for peptide synthesis was purchased from *Fisher Scientific GmbH* (Schwerte, Germany) in peptide grade (99.8%). Other solvents were used from *Carl Roth GmbH + Co. KG* (Karlsruhe, Germany), *VWR International GmbH* (Darmstadt, Germany), and *Sigma-Aldrich Chemie GmbH* in technical grade. Demineralized water was purified before use by using the arium® mini from *Sartorius AG* (Göttingen, Germany) to get ultra-pure Milli-Q® water. Fmoc- or Boc-protected amino acids (AA) with standard or special side protecting groups were obtained from *Carbolution Chemicals GmbH* (St. Ingbert, Germany), *BLD Pharmatech GmbH* (Kaiserslautern, Germany), *Bachem AG* (Bubendorf, Switzerland), *Sigma-Aldrich Chemie GmbH* and *GL Biochem* (Shanghai, China). Resins and coupling reagents were purchased from *Novabiochem, Merck KgaA* (Darmstadt, Germany), *Iris Biotech GmbH* (Marktredwitz, Germany), *Sigma-Aldrich Chemie GmbH*, *Carl Roth GmbH + Co. KG*, *Carbolution Chemicals GmbH* and *Fisher Scientific GmbH*. Other chemicals were purchased from *Sigma-Aldrich Chemie GmbH*, *Fisher Scientific GmbH, chemPUR GmbH* (Karlsruhe, Germany) and *abcr GmbH* (Karlsruhe, Germany). Liquid nitrogen for freezing samples and cooling reactions as well as argon was provided by *Air Liquide* (Düsseldorf, Germany).

2. General Methods

2.1 Reactions

Reactions sensitive to water and air were executed with SCHLENK technique. Therefore, air was removed with a vacuum pump from the apparatus and the line was ventilated with argon gas (> 99.996%). In between, the used glassware was heated over 100 °C under reduced pressure to remove any attached water. The process was repeated twice.

2.2 Lyophilization

To remove water from samples and gently dry peptides, a freeze-dryer model *Alpha 2-4 LD plus* lyophilizer with an attached centrifuge, model *AVC 2-18 CD plus* from *Christ* (Osterode am Harz, Germany) was used. Samples were frozen in liquid nitrogen and dried under reduced

pressure. Fractions collected from HPLC containing small amounts of acetonitrile (MeCN) were diluted with water and freeze-dried directly.

2.3 Storage

Crude and pure peptides could be kept at room temperature for up to one week. For long-term storage, they were stored in a freezer at –20 °C. Non-preloaded resins were stored in a fridge at 8 °C, preloaded resins were kept in a desiccator under vacuum at room temperature. Sensitive chemicals were layered with argon or nitrogen and stored at room temperature or, if not moisture sensitive, in the fridge. Buffers were stored in the fridge. All other chemicals were handled as recommended by the supplier.

3. Chromatographic Methods

3.1 Flash Chromatography

The purification of organic compounds was performed using a packed glass column with Geduran® silica gel 60, 230–450 mesh from *Merck KgaA* at an overpressure of 0.5 to 0.8 bar. The sample was applied after absorption to silica gel. For larger scales, an automated flash chromatography system called Isolera One™ from *Biotage* (Uppsala, Sweden) was used. Hereby, the sample was prepacked on the commercially available *Sfär* silica columns and collected with an autosampler after detection at 254 nm. Used solvents and gradients are explained in detail at the respective compounds.

3.2 Thin Layer Chromatography (TLC)

For tracing of reaction progression, purity, and identity of ultraviolet (UV)-active probes, TLC was employed on aluminium coated plates with silica gel 60 F_{254} from *Merck KgaA* by running in a TLC chamber with suited mobile phases. The compounds were detected via fluorescence quenching at 254 nm. If necessary, the compounds were stained with dyeing reagents like cerium ammonium molybdate solution (CAM) stain after separation.

3.3 High-performance liquid chromatography (HPLC)

The purification of peptides was executed on several HPLC systems from *Jasco Deutschland GmbH* (Pfungstadt, Germany). Therefore, either a pump model PU2080 Plus or model 4086 with in-line degassers was used. For the mobile phase, H_2O + 0.1% trifluoroacetic acid (TFA) served as component A and MeCN + 0.1% TFA as component B. For analytic or preparative

purposes, a C18 column from *KNAUER* (Berlin, Germany) (250 × 4.6 mm, 5 µm, 300 Å) or a C4 column (250 × 10 mm, 5 µm, 300 Å) from *MACHERY-NAGEL GmbH & Co. KG* (Düren, Germany) or *Avantor* (Ismaning, Germany), respectively, was installed. The columns were placed in a column oven model 4060 or an external oven model 2155 from *Pharmacia LKB* (formerly Uppsala, Sweden). Detection of peptidic compounds was performed at 215 and 254 nm using an MD2010 Plus Multiwavelength Detector, an MD4015 photodiode array detector or a UV 4075 detector for analytical or preparative scale. The HPLC system was attached to an automated fraction collector CHF122 SCV from *Advantech* (Munich, Germany). The exact procedures for peptide purification can be found in the standard operating procedures (SOP7).

3.4 Ultra High-performance Liquid Chromatography (UHPLC)

For purity determination, peptide product fractions from HPLC were analysed on an UltiMate® 3000 UHPLC system from *Thermo Fisher Scientific* and injected by an autosampler with an injection volume of 10 µL. Samples were filtered before use. As the mobile phase, H_2O + 0.1% TFA (solvent A) and MeCN + 0.1% TFA (solvent B) were used. The UHPLC was equipped with a C18 column (10 × 2.1 mm, 2 µm, 100 Å) from *Avantor,* and peptides were detected with a diode array detector at 215 and 245 nm. As a linear gradient, 5-75% B in 15 min (UHPLC method A) or 0-70%B (UHPLC method B) at 50 °C with a total running time of 20 min and a flow of 0.3 mL min^{-1} was used. The analysis was performed with Chromeleon® 7 program.

4. Characterization

4.1 Mass Spectrometry (MS)

Mass spectra were recorded on a coupled LTQ Orbitrap XL® spectrometer from *Thermo Fisher Scientific GmbH*. For high-resolution (HR) mass spectra, samples were measured on a maXis® (ESI-QTOF-MS) from *Bruker Daltonik GmbH* (Bremen, Germany) and reported in mass-to-charge ratio (*m/z*). Samples were analysed directly from HPLC or lyophilized and dissolved in HFIP/MeCN/H_2O + 0.1% TFA prior to measurement (depending on solubility).

4.2 Liquid Chromatography – Mass Spectrometry (LC-MS)

As alternative identification method, UHPLC was combined with MS. High-resolution mass spectra were obtained by a *Thermo Fisher* Accela™ UHPLC system connected to an LTQ Orbitrap XL MS system. As eluents, H_2O + 0.05% FA (A) and MeCN + 0.05% FA (B) at a

flow rate of 0.2 mL/min were used. The gradient was set to 20-100% B over 15 min at 40 °C. 10 µL of the sample were injected into the Kinetex C18 (150 × 2.1 mm, 5 µm, 100 Å) column from *Phenomenex Ltd.* (Aschaffenburg, Germany) and measured in ESI(+) mode.

4.3 Nuclear Magnetic Resonance (NMR)

^{1}H- and ^{13}C-NMR spectra were recorded on an Avance III HD device with 300 MHz from *Bruker BioSpin GmbH* (Rheinstetten, Germany) at 25 °C. Samples were dissolved in $CDCl_3$ or deuterated DMSO with a concentration of 20 mg mL^{-1} (indicated for each compound individually). NMR spectra were evaluated with MestReNova® (version 14.3.2). The chemical shifts are given in parts per million (ppm) and coupling constants *J* are given in Hertz (Hz). Abbreviations for multiplicities are: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad).

4.4 Ultraviolet (UV) Absorption Spectroscopy

The density of resin loadings (see also SOP5.1 and 5.2 for preloading protocols) was measured with a *Jasco* V-650 UV spectrometer attached to a *Julabo* F250 cooling system (Seelbach, Germany). The sample was inserted into a *Hellma Optik GmbH* (Jena, Germany) quartz cell with a path length of 1 cm. Spectra were recorded at the following settings:

Start-end wavelength: 230-180 nm
Bandwidth: 2.0 nm
Data interval: 0.5 nm
Scanning speed: 200 nm min^{-1}
Temperature: 20 °C
Accumulations: 3.

Also, a NanoDrop™ One C from *Thermo Fisher Scientific* was used for peptide concentration measurements with automatically set parameters for 280 nm absorption by the device. The concentration of the sample was calculated according to Lambert-Beer's law. The respective extinction coefficients are given in Table 1. For the peptide fragments, ε at 280 nm was predicted using eq. S.1 taking the molar extinction coefficients ε of the contributing amino acids (W, Y, C in disulfide bonds) into account.[1]

$$\varepsilon_{280} = (nW\ 5500) + (nY\ 1490) + (nC\ 125)\ [\text{L mol}^{-1}\ \text{cm}^{-1}] \quad \text{(S.1)}$$

Table 1: Molar extinction coefficients at given wavelength λ.

	λ [nm]	ε [L mol^{-1} cm^{-1}]	Reference
Dibenzofulvene-piperidine adduct	290	6089	[2]
Dibenzofulvene-DBU adduct	304	7624	[3]
N-Term wt (**15**)	280	1490	[1], eq. S1
N-Term E46K (**8**)	280	1490	[1], eq. S1
N-Term E46CEL (9)	280	1490	[1], eq. S1
aSyn wt (**16**)	280	5960	[1], eq. S1
aSyn E46K (**13**)	280	5960	[1], eq. S1
aSyn E46CEL (**14**)	280	5960	[1], eq. S1

4.5 Circular Dichroism (CD) Spectroscopy

CD measurements were performed on a *Jasco* J-1500 CD device in a 1 mm quartz cell from *Hellma*. The instrument was used with the following settings:

Start-end wavelength: 260-185 nm
Data pitch: 1 nm
Scanning speed: 50 nm min^{-1}
CD scale: 200 mdeg / 1.0 dOD
Band width: 1 nm
Response: 1 s.

The samples were dissolved in water to a final concentration of 10 µM. Measurements were accumulated five times and blank corrected. The measured ellipticity Θ [mdeg] was converted into the molar ellipticity Θ_{M} [deg cm^2 dmol^{-1}] using eq. S.2:

$$\Theta_M = \frac{\Theta\, M}{10\, N\, l\, c} \qquad \text{(S.2)}$$

with M [g mol^{-1}] being the molar mass of the respective peptide, N the number of amino acid residues in the peptide, l [cm] the path length of the cuvette and c [mg mL^{-1}] the concentration of the sample.

5. Standard Operating Procedures (SOPs)

5.1 SOP1: Resin Preloading

5.1.1 SOP1a: Wang Resin with Fmoc-Lys(Alloc)-OH

Wang resin low loading (LL) (1.00 g, 0.44 mmol, initial loading: 0.44 mmol/g) was swollen in DMF (10 mL, rt, 10 min). Fmoc-Lys(Alloc)-OH (1.99 g, 4.40 mmol, 10 eq.) and DMAP (5.40 mg, 0.04 mmol, 0.1 eq.) were dissolved in DMF (8 mL). Subsequently, *N,N′*-diisopropylcarbodiimide (DIC) (345 µL, 2.20 mmol, 5 eq.) was added to the mixture and the resin was incubated with the reaction solution at rt overnight. Then, the resin was washed with DMF and dichloromethane (DCM) (3 × 10 mL each) and dried *in vacuo*. The loading of the pale-yellow resin determined following SOP2a was 0.27 mmol g^{-1}.

5.1.2 SOP1b: 2-CTC Resin with Hydrazine

2-CTC resin (1.00 g, 1.60 mmol, initial loading: 1.60 mmol g^{-1}) was swollen in 5 mL DCM/DMF (50:50 *v/v*) at rt for 20 min. Subsequently, a solution of hydrazine hydrate in DMF (10% *v/v*, 7 mL) was added to the resin and agitated at rt for 1 h. The solution was drained, and the incubation was repeated twice. The resin was then washed with DMF, DCM, and again DMF (3 × 5 mL each). Afterward, the remaining free amino groups were capped with a solution of MeOH in DMF (5% *v/v*, 3 × 5 mL, 15 min, rt). The resin was again washed with DMF, DCM, DMF, and DCM (3 × 5 mL each), dried, and stored under vacuum.

For loading determination, a small sample of the resin was preloaded with Fmoc-Ala-OH. Hydrazine-modified 2-CTC resin (31.30 mg, 0.05 mmol, 1 eq., estimated loading: 1.60 mmol g^{-1}) was swollen in 1 mL DMF (rt, 20 min). A solution of Fmoc-Ala-OH (62.30 mg, 0.20 mmol, 4 eq.), hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU) (72.20 mg, 0.19 mmol, 3.8 eq.), 1-hydroxy-7-azabenzotriazole (HOAt) (27.20 mg, 0.20 mmol, 4 eq.) and *N,N′*-diisopropylethylamine (DIPEA) (70 µL, 0.40 mmol, 8 eq.) in 0.5 mL DMF was added to the resin and agitated overnight at rt. The resin was then washed with DMF, DCM, DMF and DCM (3 × 2 mL each) and dried before loading was determined according to the standard procedure described in SOP2b. Determined loadings ranged between 0.30 and 0.35 mmol g^{-1}.

5.1.3 SOP1c: 2-CTC Resin with Fmoc-Ala-OH

2-CTC resin (0.50 mg, 0.80 mmol, 1 eq., initial loading: 1.60 mmol g^{-1}) was swollen in DCM (3 mL, rt, 20 min). Next, a solution of Fmoc-Ala-OH (0.75 g, 2.40 mmol, 3 eq.) and DIPEA (1.26 mL, 7.20 mmol, 9 eq.) in 3 mL DCM was added to the resin and agitated at rt for 2.5 h. The resin was then washed with DCM, DMF and DCM (3 × 5 mL each), followed by capping with a solution of DCM/MeOH/DIPEA (85:15:5 *v/v*, 2 × 5 mL, 15 min, rt). The resin was again

washed with DCM, DMF and DCM (3 × 5 mL each), dried and stored under vacuum. Loading was determined as described in SOP2b to be 0.89 mmol g^{-1}.

5.2 SOP2: Determination of Resin Loading

5.2.1 SOP2a: Fmoc Cleavage with Piperidine

The resin loading of Wang resins was determined via Fmoc cleavage with 20% piperidine in DMF. Therefore, three resin samples of around 5 mg were weighed in and 1 mL of 20% piperidine in DMF was added. A blank probe was prepared in parallel. The samples were shaken for 3 min before diluting to 10 mL with the piperidine solution. Subsequently, the absorbance of the samples was measured in a UV spectrophotometer (see section 4.4) at 290 nm. The resin loading was calculated with equation S.3.

$$L = \frac{1}{m(resin)} \left(\frac{(A(sample) - A(blank)\, V\, f}{\varepsilon\, d}\, 10^6 \right) \tag{S.3}$$

With the extinction coefficient ε being 6089 L mol^{-1} cm^{-1} in the case of the dibenzofulvene-piperidine adduct, the volume V being 0.001 L, the dilution factor f being 10 and the path length of the cuvette d being 1 cm, eq. S.3 reduces to eq. S.4:

$$L = \frac{\big(A(sample) - A(blank)\big)\, 1.64}{m(resin)} \tag{S.4}$$

where L is the respective loading in [mmol g^{-1}], A is the absorbance and m is the mass of the resin in [mg].

5.2.2 SOP2b: Fmoc Cleavage with DBU

The resin loading of CTC resins was determined via Fmoc cleavage with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). Therefore, three resin samples with approximately 10 mg were weighed in falcons. 2 mL of 2% DBU in DMF (*v/v*) were added and the resins were gently shaken for 30 min. In parallel, a blank probe was prepared similarly without resin. Afterwards, 8 mL of MeCN was added to each falcon and the sample was further diluted by taking 2 mL of the solution and diluting it to 25 mL with MeCN. The absorbance of the samples was measured in a UV spectrophotometer (see section 5.4) at 304 nm. Loading was calculated with eq. S.3.

With the extinction coefficient ε being 7624 L mol^{-1} cm^{-1} in the case of the dibenzofulvene-DBU adduct, the volume V being 0.01 L, the dilution factor f being 12.5 and the path length of the cuvette d being 1.0 cm, eq. S.3 reduces to eq. S.5:

$$L = \frac{\big(A(sample) - A(blank)\big)\,16.4}{m(resin)} \qquad \text{(S.5)}$$

where L is the respective loading in [mmol g^{-1}], A is the absorbance and m is the mass of the resin in [mg].

5.3 SOP3: Automated Microwave-assisted SPPS

Automated SPPS was performed with a Liberty Blue™ peptide synthesizer from *CEM* (Kamp-Lintfort, Germany) attached to a *CEM* Discover microwave. The synthesis scale was applied at 0.05 mmol. The respective amount of resin (see also SOP1 for resin preloading protocols) was weighed in and pre-swollen for half an hour in a *Beckton Dickinson BD* (Vaud, Switzerland) syringe with frit. The resin was transferred to the reaction vessel and placed in the microwave. Before, all needed reagents were weighed in and dissolved in DMF for peptide synthesis grade. All amino acid building blocks were dissolved to a final concentration of 0.2 M. As activator, 0.5 M DIC in DMF and as activator base 0.5 M ethyl cyanohydroxyiminoacetate (Oxyma) in DMF were used. Fmoc deprotection was carried out with 20% piperidine in DMF. DIPEA (0.05 M) was added to the activator base (CarboMAX™)[4] and oxyma (0.05 M) to the deprotection solution to prevent aspartimide formation. For all AA, double couplings were employed. After each deprotection and coupling step, the resin was washed four times with DMF. The specific microwave conditions of the two used methods are listed in table 2.

Table 2: Microwave settings for used methods at the synthesizer.

Method	Stage	Coupling	Deprotection
A (Standard)	1	25 °C, 0 W, 120 s	60 °C, 60 W, 60 s
	2	50 °C, 35 W, 480 s	--
B (His coupling)	1	60 °C, 75 W, 30 s	60 °C, 60 W, 60 s
	2	70 °C, 50 W, 330 s	--

Once the peptide coupling was completed, the resin was transferred back to a *BD* syringe and washed with DMF, MeOH and DCM (5 × each) and dried *in vacuo* before further modifications or cleavage (please refer to SOP 5 and 6).

5.4 SOP4: Manual Microwave-assisted SPPS

All manual peptide syntheses were carried out at 0.05 mmol scale using a *CEM Discover* microwave. Hydrazine modified 2-CTC resin (0.05 mmol, 1 eq.) was swollen in 2 mL DMF. A coupling solution containing the Fmoc-AA building block (0.20 mmol, 4 eq.), (1-Cyano-2-

ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) (85.65 mg, 0.20 mmol, 4 eq.) and 2,2,6,6-tetramethylpiperidine (TMP) (50.60 µL, 0.30 mmol, 6 eq.) in 1 mL DMF was added to the resin and coupling conducted at 60 °C, 20 W, 45 min. The coupling solution was drained, and the resin washed with DMF five times. For Fmoc-deprotection, 2 mL of a deprotection solution containing 1% hydroxybenzotriazole (HOBt) (*w/v*) and 2% DBU (*v/v*) in DMF was added and stirred for 60 s at rt. Deprotection was repeated twice, followed by washing with DMF five times. This coupling-deprotection cycle was repeated for each amino acid. Following coupling of the last amino acid, capping of all free amino groups using a solution of 10% acetic anhydride (*v/v*), 5% DIPEA (*v/v*) and 85% DMF (2 × 2 mL, 10 min, rt) was carried out instead of Fmoc-deprotection. Capping was again followed by washing with DMF five times. The prepared resin was either used in automated peptide synthesis directly or was washed with DCM five times and stored *in vacuo*.

5.5 SOP5: Modifications on Resin

5.5.1 SOP5a: Alloc Deprotection

The Alloc-protected peptide on resin was swollen in a *BD* syringe with frit in DCM for half an hour. The syringe was emptied, and DCM was added to achieve minimum covering of the resin with solvent. 20 eq. of phenylsilane were added and the resin was swollen for 5 min. In the meantime, 1 eq. of tetrakis(triphenylphosphine) palladium ($Pd(PPh)_3)_4$) was weighed in and added to the resin. The syringe was placed in a *CEM* Discover microwave and heated for 5 min at 15 W and 38 °C. After washing rapidly with DCM, the procedure was repeated two times to ensure complete deprotection. The resin was washed 10 times with DCM and dried *in vacuo*.

5.5.2 SOP5b: CML Glycation

The resin-bound peptide with Alloc-deprotected Lys residues and other side-chain protected AA was swollen in DMF for 30 min. *tert*-Butyloxoacetate **6** (15-20 eq. per Lys residue in the peptide) was dissolved in DMF to a final concentration of 1 M, added to the resin and agitated for 1 h at rt. The solution was drained, and the resin rapidly washed with DMF and DCM (2 × 2 mL each). A fresh solution of DCM/MeOH (3:1 *v/v*) was added to the resin. $NaBH_4$ (10 eq. per Lys residue) was added carefully, and reduction took place open to the atmosphere and was indicated by effervescence. After 30 min, the solution was drained, and the reducing agent quenched by washing with MeOH until bubbling ceased. This was followed by washing with DCM, DMF and DCM (5 × 3 mL each). The resin was dried *in vacuo*.

5.5.3 SOP5c: CEL Glycation

The resin carrying the peptide with side-chain protected AA except for the Lys residues was swollen in NMP/2-Prop (3:1) + 5% AcOH. Then *tert*-butyl 2-oxopropanoate **7** (15 eq. per Lys residue in the peptide) was dissolved in NMP/2-Prop (3:1) + 5% AcOH (0.4 M) and $NaBH_3CN$ (15 eq. per Lys residue in the peptide) was added. The resin was incubated with the reaction solution at rt overnight. Subsequently, the resin was washed with NMP, DMF and DCM (5 × 5 mL each) and dried *in vacuo*.

5.6 SOP6: Cleavages

For peptide cleavage, the dry resin was transferred into a *BD* syringe. The cleavage solution consisting of 82.5% TFA, 5% H_2O, 5% thioanisole, 5% TIPS and 2.5% EDT (*v/v/v/v/v*; 1 mL of cleavage solution per 10 µmol of peptide) was drawn up in the syringe and shaken for 2 h. Afterwards, the solution containing the peptide was collected in a falcon or an *Eppendorf* tube and dried in a nitrogen stream. Precipitation of the crude peptide was obtained upon the addition of ice-cold ether, centrifugation of the pellet, and removal of the supernatant. This was repeated five times. The pellet was dried *in vacuo* or dissolved in water and dried on the lyophilizer.

5.7 SOP7: HPLC Purification

The general HPLC setup is described in chapter 3.3. Depending on their solubility, peptides were dissolved in H_2O + 0.1% TFA only or in HFIP / H_2O + 0.1% TFA 0.3:1 or 1:1 (*v/v*) and sonicated and filtrated prior to injection. For semi-preparative scale, up to 40 mg of peptide were dissolved in 1.5 mL of solvent. The flow was set to 3 mL min^{-1}. All purifications were performed at 50 °C. The respective gradients are shown in table 3:

Table 3: Gradients for HPLC purification.

Gradient	Conditions
A	3-50% B in 35 min as step gradient with steps of 10% B, total time 40 min
B	3-70% B in 45 min as step gradient with steps of 10% B, total time 50 min
C	15-40% B in 30 min as linear gradient, total time 40 min
D	20-45% B in 30 min as linear gradient, total time 40 min
E	25-50% B in 30 min as linear gradient, total time 40 min

Before each run, the HPLC system was purged in with the respective starting gradient for 10 min. After collecting, fractions containing the product were pooled, frozen with liquid nitrogen and dried in the lyophilizer. Purity was confirmed with UHPLC (chapter 3.4).

5.8 SOP8: Ligations and Related Reactions

5.8.1 SOP8a: Hydrazide Thioester Conversion

The conversion of peptide hydrazides to the corresponding thioesters was carried out in *Eppendorf* protein LoBind® tubes. Peptide hydrazide (6.25-20 mg mL^{-1}, 1 eq.) was dissolved in thioester conversion buffer, consisting of 6 M guanidine hydrochloride (Gnd • HCl) and 0.15 M MPAA at pH 3. The solution was bubbled with argon for a few minutes and acetylacetone (120 eq.) added. The solution was mixed thoroughly and then agitated at 37 °C for 4-6 h. Completion of the reaction was monitored with UHPLC analysis. The conversion product was used directly in NCL without purification.

5.8.2 SOP8b: Native Chemical Ligation

Cys peptide (7.5-20 mg mL^{-1}, 1 eq.) was dissolved in ligation buffer, consisting of 6 M Gnd · HCl, 0.2 M Na_3PO_4, 50 mM MPAA and 0.1 M tris(2-carboxyethyl)phosphine hydrochloride (TCEP • HCl) at pH 7. This solution was combined with the thioester conversion mixture and adjusted to pH 7. The solution was bubbled with argon for a few minutes, before being agitated at 37 °C for 24-40 h. Completion of the reaction was monitored with UHPLC analysis. The ligation product was either used directly in consequent Thz conversion, without purification, or diluted fourfold with H_2O + 0.1 % TFA and purified using HPLC (SOP7).

5.8.3 SOP8c: Thz Deprotection

TCEP • HCl (70 mM) and *O*-methylhydroxylamine (0.24 M) were added to the NCL reaction mixture and the pH value was adjusted to 4. The solution was bubbled with argon for a few minutes, before being agitated at 37 °C for 17 h. Completion of the reaction was monitored with UHPLC analysis. The conversion product was diluted fourfold with H_2O + 0.1% TFA and purified using HPLC.

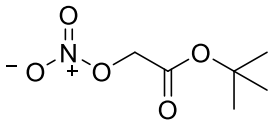
5.8.4 SOP8d: Desulfurization

The desulfurization of full-length, ligated proteins was carried out in *Eppendorf* protein LoBind® tubes. The protein (4-9 mg mL^{-1}, 1 eq.) was dissolved in desulfurization buffer, consisting of 6 M Gnd • HCl, 0.2 M Na_3PO_4 and 0.5 M TCEP • HCl at pH 7, and bubbled with argon for a few minutes. *tert*-Butylthiol (*t*-BuSH) (40 µL) and 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) (40 µL of a 0.1 M solution in H_2O) were added and

the reaction mixture agitated at 37 °C for 6 h. Completion of the reaction was monitored with UHPLC analysis. The reaction mixture was diluted tenfold with H_2O + 0.1% TFA and purified using semi-prep. HPLC.

6. Organic Syntheses of Carbonyl Compounds

tert-Butoxycarbonylmethyl nitrate (**5**)



$C_6H_{11}NO_5$
177.16 g/mol
5

tert-Butyl bromoacetate (14.3 mL, 96.8 mmol, 1.0 eq.) was introduced to a flame-dried flask under argon atmosphere and dissolved in dry MeCN (80 mL). Upon treatment with $AgNO_3$ (30.0 g, 176.6 mmol, 1.8 eq.), the reaction proceeded at room temperature in the dark and vigorous stirring for 48 h. The solvent was removed *in vacuo* and the resulting residue was extracted with Et_2O (3 × 100 mL). The organic phase was washed with brine, dried over $MgSO_4$ and evaporated, yielding a colorless oil (15.0 g, 84.7 mmol, 87.5%), which was used directly in the next step without further purification. The product (**5**) was visible on TLC with CAM stain (R_f = 0.91, hexane/EtOAc 8:2).

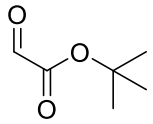
^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 4.76 (s, 2H, CH_2), 1.48 (s, 9H, 3 × CH_3).

^{13}C-NMR (75MHz, $CDCl_3$): δ (ppm) = 164.9, 83.6, 67.7. 28.0.

MS (ESI+) *m/z:* 200.1 $[M+Na]^+$.

HR-MS (ESI+) *m/z:* calc. for $C_6H_{11}NO_5Na$ ($[M+Na]^+$): 200.0529, found: 200.0533.

tert-Butyloxoacetate (**6**)



$C_6H_{10}O_3$
130.14 g/mol
6

A solution of *tert*-butoxycarbonylmethyl nitrate (**5**) (17.1 g, 96.5 mmol, 1.0 eq.) in anhydrous DMSO (200 mL) was treated with anhydrous sodium acetate (6.8 g, 83.0 mmol, 0.9 eq.) under argon atmosphere for 20 min. The reaction mixture was poured into a mixture of brine and ice (500 mL) and extracted with cold ether (5 x 300 mL). The organic layer was separated, washed with sat. aqueous Na_2CO_3 and concentrated. The resulting oil was taken up with DCM, washed with brine and the phases were separated. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude mixture was purified via flash chromatography using a gradient of petroleum ether/EtOAc 100:0 to 90:10 (*v/v*). The product (2.6 g, 20.1 mmol, 21.0%) appeared as a colourless oil (TLC with CAM stain, R_f = 0.09, hexane/EtOAc 8:2).

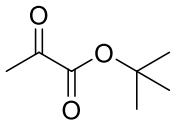
^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 9.30 (s, 1H), 1.57 (s, 9H, 3 × CH_3).

^{13}C-NMR (75MHz, $CDCl_3$): δ (ppm) = 185.4, 129.0, 67.7, 28.1.

MS (ESI+) *m/z:* 130.1 $[M]^+$.

GC-MS (EI+) *m/z:* calc. for C_4H_9 ($[t\text{Bu}]^+$): 57.0699, found: 57.0700.

tert-Butyl oxopropanoate (**7**)



$C_7H_{12}O_3$
144.17 g/mol
7

Pyruvic acid (8.8 g, 100.0 mmol, 1.0 eq.) and *tert*-butanol (14.8 g, 200.0 mmol, 2.0 eq.) were dissolved in THF (100.0 mL). Pyridine (20.1 mL, 250.0 mmol, 2.5 eq.) was added and the mixture was cooled to 0 °C followed by dropwise addition of mesyl chloride (9.3 mL, 120.0 mmol, 1.2 eq.) over 10 min. After stirring for 30 min at 0 °C, the reaction mixture was allowed to warm to room temperature and stirred overnight. Then, the reaction was quenched by addition of H_2O (200 mL) and extracted with Et_2O (3 x 200 mL). The organic phase was

dried over $MgSO_4$ and concentrated under reduced pressure. Purification of the crude product was performed by flash column chromatography (petroleum ether/EtOAc 100:0 to 96:4) and product *tert*-butyl 2-oxopropanoate **7** (7.9 g, 54.5 mmol, 54.0 %) was obtained as a yellow oil (R_f = 0.25, petroleum ether/EtOAc 40:1).

^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 2.41 (s, 3H, CH_3), 1.54 (s, 9H, 3 × CH_3).

^{13}C-NMR (75 MHz, $CDCl_3$): δ (ppm) = 193.3, 160.5, 84.1, 28.1, 26.6.

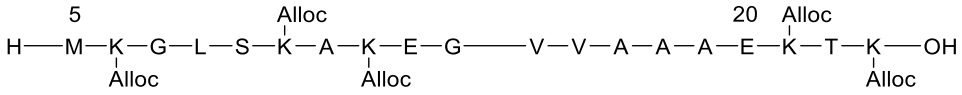
HR-MS (ESI+) *m/z*: calc. for $C_7H_{12}O_3Na$ ($[M+Na]^+$): 167.0679, found: 167.0685.

7. Peptide Syntheses and Related Reactions

General remark: Peptides are represented in one letter scheme for the sake of clarity. Special modifications are added in chemical formulas or standard abbreviations. The numbers above or below the AA sequences are depicting the position in the native sequence of aSyn for orientation. Mutations are shown in red.

7.1 Pep1 and Pep2

Pep1-Alloc



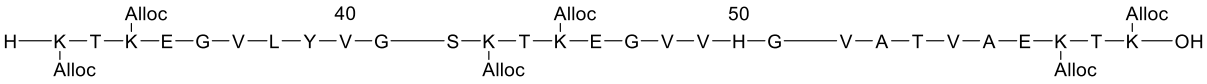
$C_{104}H_{172}N_{24}O_{36}S$
2366.71 g/mol

Pep1-Alloc was synthesized with automated SPPS as described in SOP3 on Fmoc-Lys(Alloc)-OH preloaded Wang resin (SOP1a, 185 mg). The four remaining Lys residues were introduced as Fmoc-Lys(Alloc)-OH. The final *N*-terminal AA was introduced as Boc-Met-OH. To verify successful coupling, a test cleavage with a small amount of resin was performed after SPPS. The crude peptide was analysed with MS(ESI) and not used further.

MS (ESI+) *m/z:* 804.4 $[M+H+2Na]^{3+}$, 1206.1 $[M+2Na]^{2+}$, 2409.2 [M+2Na].

HR-MS (ESI+) *m/z:* calc. for $C_{104}H_{173}N_{24}O_{36}SNa_2$ ($[M+H+2Na]^{3+}$): 804.3991, found: 804.3970; calc. for $C_{104}H_{172}N_{24}O_{36}SNa_2$ ($[M+2Na]^{2+}$): 1206.0950, found: 1206.0926.

Pep2-Alloc



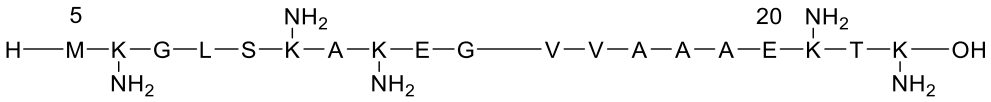
$C_{159}H_{255}N_{37}O_{54}$
3548.99 g/mol

Pep2-Alloc was synthesized with automated SPPS as described in SOP3 on Fmoc-Lys(Alloc)-OH preloaded Wang resin (SOP1a, 185 mg). The five remaining Lys residues were introduced as Fmoc-Lys(Alloc)-OH. The final *N*-terminal AA was introduced as Boc-Lys(Alloc)-OH. To verify successful coupling, a test cleavage with a small amount of resin was performed after SPPS. The crude peptide was analysed with MS(ESI) and not used further.

MS (ESI+) *m/z:* 899.2 $[M+2H+2Na]^{4+}$, 1198.6 $[M+H+2Na]^{3+}$, 3590.8 [M+2Na].

HR-MS (ESI+) *m/z:* calc. for $C_{159}H_{258}N_{37}O_{54}Na$ ($[M+3H+Na]^{4+}$): 893.7130, found: 893.7111; calc. for $C_{159}H_{257}N_{37}O_{54}Na_2$ ($[M+2H+2Na]^{4+}$): 899.2085, found: 899.2083; calc. for $C_{159}H_{256}N_{37}O_{54}Na_3$ ($[M+H+3Na]^{4+}$): 904.7040, found: 904.7038; calc. for $C_{159}H_{257}N_{37}O_{54}Na$ ($[M+2H+Na]^{3+}$): 1191.2816, found: 1191.2806; calc. for $C_{159}H_{256}N_{37}O_{54}Na_2$ ($[M+H+2Na]^{3+}$): 1198.6089, found: 1198.6081; calc. for $C_{159}H_{255}N_{37}O_{54}Na_3$ ($[M+3Na]^{3+}$): 1205.9362, found: 1205.9358.

Pep1 Alloc Deprotected



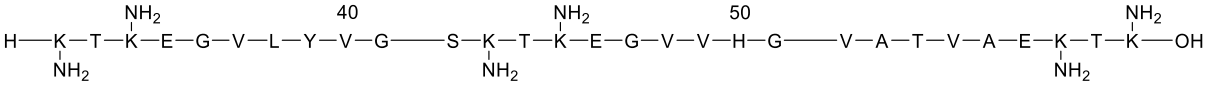
$C_{84}H_{152}N_{24}O_{26}S$
1946.34 g/mol

Resin-bound Pep1-Alloc (0.05 mmol) was Alloc deprotected following SOP5a using palladium catalyst (57.78 mg, 0.05 mmol, 1 eq.) and phenylsilane (0.12 mL, 1 mmol, 20 eq.). To verify successful deprotection, a test cleavage with a small amount of resin was performed. The crude peptide was analysed with MS(ESI) and not used further.

MS (ESI+) *m/z:* 649.7 $[M+3H]^{3+}$, 974.0 $[M+2H]^{2+}$, 1945.1 $[M]^{+}$.

HR-MS (ESI+) *m/z:* calc. for $C_{84}H_{156}N_{24}O_{26}S$ ($[M+4H]^{4+}$): 487.2838, found: 487.2830; calc. for $C_{84}H_{155}N_{24}O_{26}S$ ($[M+3H]^{3+}$): 649.7094, found: 649.7091; calc. for $C_{84}H_{154}N_{24}O_{26}S$ ($[M+2H]^{2+}$): 973.5588, found: 973.5580.

Pep2 Alloc Deprotected



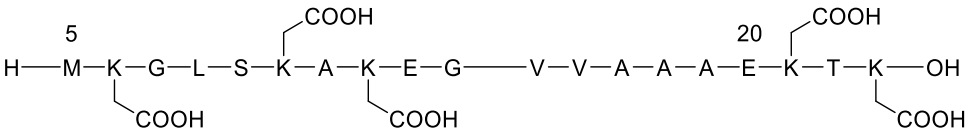
$C_{135}H_{231}N_{37}O_{42}$
3044.55 g/mol

Resin-bound Pep2-Alloc (0.05 mmol) was Alloc deprotected following SOP5a using palladium catalyst (57.78 mg, 0.05 mmol, 1 eq.) and phenylsilane (0.12 mL, 1 mmol, 20 eq.). To verify successful deprotection, a test cleavage with a small amount of resin was performed. The crude peptide was analysed with MS(ESI) and not used further.

MS (ESI+) *m/z:* 609.8 $[M+5H]^{5+}$, 761.9 $[M+4H]^{4+}$, 1015.6 $[M+3H]^{3+}$, 3042.7 $[M]^{+}$.

HR-MS (ESI+) *m/z:* calc. for $C_{135}H_{236}N_{37}O_{42}$ ($[M+5H]^{5+}$): 609.9501, found: 609.9518; calc. for $C_{135}H_{235}N_{37}O_{42}$ ($[M+4H]^{4+}$): 761.9349, found: 761.9362; calc. for $C_{135}H_{234}N_{37}O_{42}$ ($[M+3H]^{3+}$): 1015.5775, found: 1015.5785.

Pep1-CML (1)



$C_{94}H_{162}N_{24}O_{36}S$
2236.52 g/mol
1

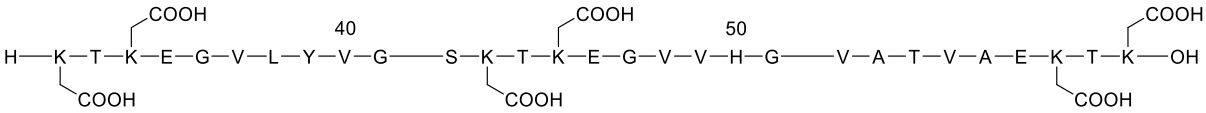
Resin-bound Pep1 Alloc deprotected (200 mg, 0.02 mmol) was glycated following SOP5b using aldehyde **6** (195.21 mg, 1.50 mmol, 75 eq.) in DMF (1.5 mL) and $NaBH_4$ (37.83 mg, 1.00 mmol, 50 eq.). The crude peptide was cleaved from the resin (SOP6). Purification was performed with HPLC method B as described in SOP7. The fractions containing the product **1** (t_R = 14.5 min) were pooled, lyophilized and frozen to obtain a white powder (3.8 mg, 8.4%, 1.68 µmol, >99% pure).

UHPLC (method B): t_R = 10.4 min

MS (ESI+) *m/z:* 746.4 $[M+3H]^{3+}$, 1119.1 $[M+2H]^{2+}$, 2235.1 $[M]^{+}$.

HR-MS (ESI+) *m/z:* calc. for $C_{94}H_{166}N_{24}O_{36}S$ ($[M+4H]^{4+}$: 559.7899, found: 559.7894, calc. for $C_{94}H_{165}N_{24}O_{36}S$ ($[M+3H]^{3+}$: 746.3851, found: 746.3890, calc. for $C_{94}H_{164}N_{24}O_{36}S$ ($[M+2H]^{2+}$: 1118.5725, found: 1118.5720.

Pep2-CML (2)



$C_{147}H_{243}N_{37}O_{54}$
3392.77 g/mol
2

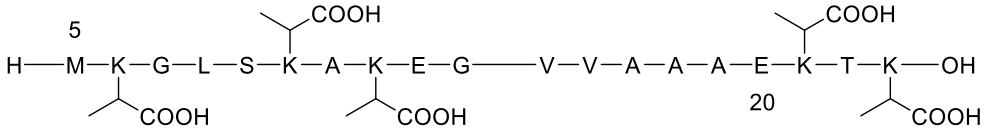
Resin-bound Pep2 Alloc deprotected (200 mg, 0.02 mmol) was glycated following SOP5b using aldehyde **6** (234.25 mg, 1.8 mmol, 90 eq.) in DMF (1.8 mL) and $NaBH_4$ (45.40 mg, 1.20 mmol, 60 eq.). The crude peptide was cleaved from the resin (SOP6). Pure peptide **2** was obtained upon purification with HPLC method A (SOP7) at t_R = 22.8 min as a white powder (2.9 mg, 0.86 µmol, 4.3%, 95% pure).

UHPLC (method B): t_R = 11.2 min

MS (ESI+) *m/z:* 679.4 $[M+5H]^{5+}$, 848.9 $[M+4H]^{4+}$, 1131.6 $[M+3H]^{3+}$.

HR-MS (ESI+) *m/z:* calc. for $C_{147}H_{248}N_{37}O_{54}$ ($[M+5H]^{5+}$): 679.3560, found: 679.3577; calc. for $C_{147}H_{247}N_{37}O_{54}$ ($[M+4H]^{4+}$): 848.9432, found: 848.9456; calc. for $C_{147}H_{246}N_{37}O_{54}$ ($[M+3H]^{3+}$): 1131.5885, found: 1131.5923.

Pep1-CEL (3)



$C_{99}H_{172}N_{24}O_{36}S$
2306.66 g/mol
3

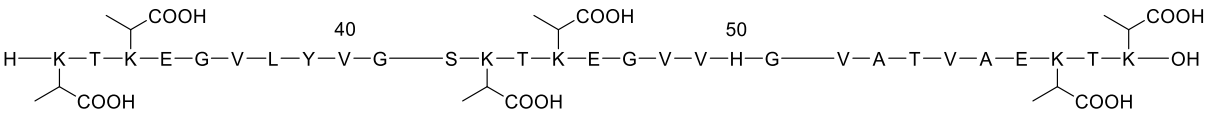
Resin-bound Pep1 Alloc deprotected (0.15 g, 0.04 mmol) was glycated following SOP5c using ketone **7** (432.51 mg 3.00 mmol, 75 eq.) in NMP/2-Prop (3:1) + 5% AcOH (7.5 mL) and $NaBH_3CN$ (188.52 mg, 3.00 mmol, 75 eq.). The crude peptide was cleaved from the resin (SOP6). Purification was performed with HPLC method C as described in SOP7. The fractions containing the product **3** (t_R = 10.3 min) were pooled, lyophilized and frozen to obtain a white powder (13.1 mg, 5.68 µmol, 14%, 95% pure).

UHPLC (method A): t_R = 9.8 min

MS (ESI+) *m/z*: 2305.2 $[M+H]^+$, 2327.2 $[M+Na]^+$, 1154.1 $[M+2H]^{2+}$, 769.7 $[M+3H]^{3+}$, 577.6 $[M+4H]^{4+}$.

HR-MS (ESI+) *m/z*: calc. for $C_{99}H_{174}N_{24}O_{36}S$ ($[M+2H]^{2+}$): 1154.1131, found 1154.1138; calc. for $C_{99}H_{173}N_{24}O_{36}SNa$ ($[M+H+Na]^{2+}$): 1165.1040, found 1165.1015; calc. for $C_{99}H_{175}N_{24}O_{36}S$ ($[M+3H]^{3+}$): 769.7445, found 769.7451; calc. for $C_{99}H_{174}N_{24}O_{36}SNa$ ($[M+2H+Na]^{3+}$): 777.0718, found 777.0700; calc. for $C_{99}H_{176}N_{24}O_{36}S$ ($[M+4H]^{4+}$): 577.5602, found 577.5613; calc. for $C_{99}H_{175}N_{24}O_{36}SNa$ ($[M+3H+Na]^{4+}$): 583.0557, found 583.0542; calc. for $C_{99}H_{177}N_{24}O_{36}S$ ($[M+5H]^{5+}$): 462.2496, found 462.2499.

Pep2-CEL (4)



$C_{9153}H_{255}N_{37}O_{54}$
3476.93 g/mol
4

Resin-bound Pep2 Alloc deprotected (0.15 g, 0.04 mmol) was glycated following SOP5c using ketone **7** (519.01 g, 3.60 mmol, 90 eq.) in NMP/2-Prop (3:1) + 5% AcOH (9.0 mL) and $NaBH_3CN$ (226.22 mg, 3.60 mmol, 90 eq.). The crude peptide was cleaved from the resin (SOP6). Pure peptide **4** was obtained upon purification with HPLC method C (SOP7) at t_R = 16.0 min as a white powder (3.6 mg, 1.04 µmol, 3.0%, 95% pure).

UHPLC (method A): t_R = 10.6 min

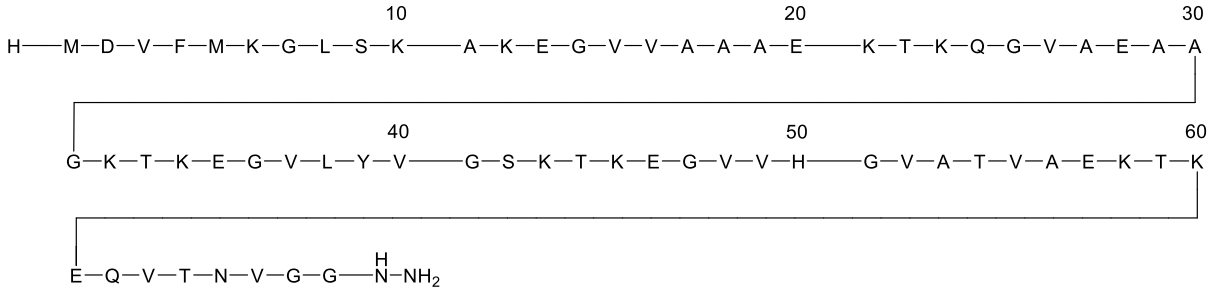
MS (ESI+) *m/z*: 3474.8 $[M+H]^+$, 1160.0 $[M+3H]^{3+}$, 870.0 $[M+4H]^{4+}$, 696.4 $[M+5H]^{5+}$.

HR-MS (ESI+) *m/z*: calc. for $C_{153}H_{258}N_{37}O_{54}$ ($[M+3H]^{3+}$): 1159.6198, found 1159.6230; calc. for $C_{153}H_{259}N_{37}O_{54}$ ($[M+4H]^{4+}$): 869.9666, found 869.9689; calc. for $C_{153}H_{260}N_{37}O_{54}$

([M+5H]$^{5+}$): 696.1748, found 696.1768; calc. for $C_{153}H_{261}N_{37}O_{54}$ ([M+6H]$^{6+}$): 580.3135, found 580.3155.

7.2 aSyn Fragments and Ligation Products

aSyn *N*Term [1-68] wt (15)



$C_{302}H_{512}N_{86}O_{96}S_2$
6948.04 g/mol
16

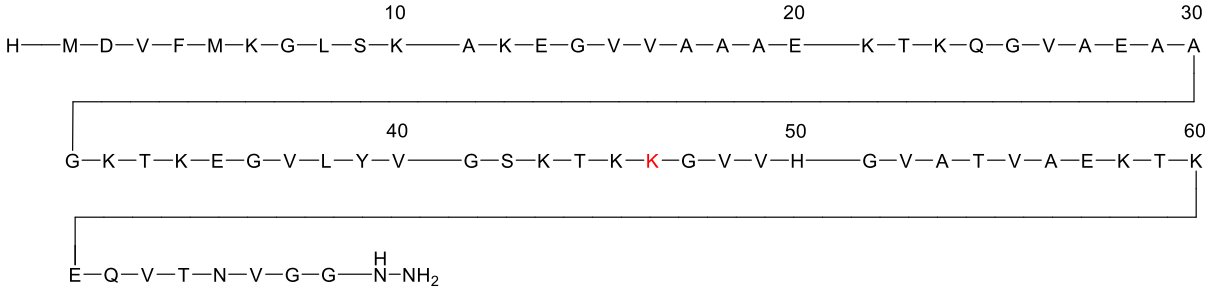
Peptide **16** was synthesized on hydrazine modified 2-CTC resin (SOP1b, 143 mg, 0.05 mmol) according to the automated SPPS protocol described in SOP3 and cleaved according to SOP6. The crude peptide was purified using HPLC (SOP7) method D (t_R = 22.1 min). Pure peptide **15** (1.60 mg, 0.23 µmol, < 1%) was obtained as white powder in 99% purity.

UHPLC (method A): t_R = 12.2 min.

MS (ESI+) *m/z*: 733.0 [M+9H]$^{9+}$, 869.5 [M+8H]$^{8+}$, 993.5 [M+7H]$^{7+}$, 1158.8 [M+6H]$^{6+}$.

HR-MS (ESI+) *m/z*: calc. for $C_{302}H_{520}N_{86}O_{96}S_2$ ([M+8H]$^{8+}$): 869.4744, found: 869.4771; calc. for $C_{302}H_{519}N_{86}O_{96}S_2$ ([M+7H]$^{7+}$): 993.5412, found: 993.5456; calc. for $C_{302}H_{518}N_{86}O_{96}S_2$ ([M+6H]$^{6+}$): 1158.9635, found: 1158.9654; calc. for $C_{302}H_{512}N_{86}O_{96}S_2$ ([M]$^{+}$): 6947.7, found: 6943.7.

aSyn *N*Term [1-68] E46K (8)



$C_{303}H_{517}N_{87}O_{94}S_2$
6947.10 g/mol
8

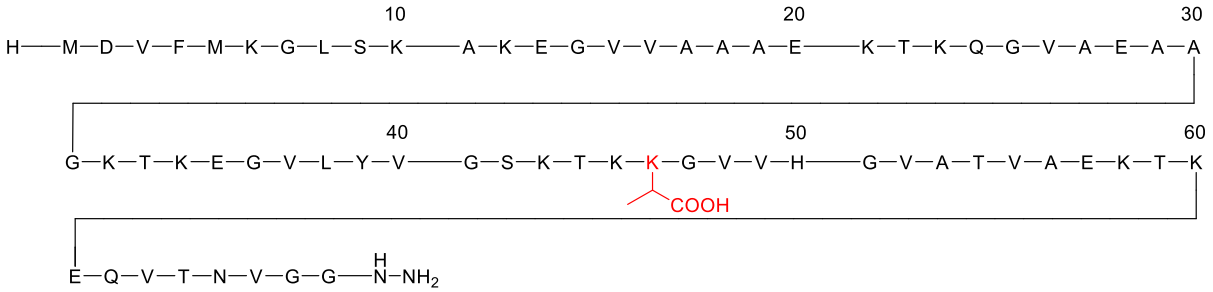
Peptide **8** was synthesized on hydrazine modified 2-CTC resin (SOP1b, 143 mg, 0.05 mmol) using first manual peptide synthesis (SOP4) for the residues AA 61-68, followed by automated peptide synthesis (SOP3) for the remaining residues AA 1-60. AA 46 was introduced as Fmoc-Lys(Boc)-OH point mutation. The peptide was cleaved according to SOP6. The crude peptide was purified using HPLC (SOP7) method D (t_R = 23.8 min). Pure peptide **8** (5.30 mg, 0.76 µmol, 2%) was obtained as white powder in 99% purity.

UHPLC (method A): t_R = 11.9 min.

MS (ESI+) *m/z*: 632.5 $[M+11H]^{11+}$, 695.7 $[M+10H]^{10+}$, 772.9 $[M+9H]^{9+}$, 869.4 $[M+8H]^{8+}$, 993.4 $[M+7H]^{7+}$, 1158.8 $[M+6H]^{6+}$.

HR-MS (ESI+) *m/z*: calc. for $C_{303}H_{526}N_{87}O_{94}S_2$ $([M+9H]^{9+})$: 772.8728, found: 772.8759; calc. for $C_{303}H_{525}N_{87}O_{94}S_2$ $([M+8H]^{8+})$: 869.3560, found: 869.3589; calc. for $C_{303}H_{524}N_{87}O_{94}S_2$ $([M+7H]^{7+})$: 993.4058, found: 993.4092; calc. for $C_{303}H_{523}N_{87}O_{94}S_2$ $([M+6H]^{6+})$: 1158.8056, found: 1158.8100.

aSyn *N*Term [1-68] E46CEL (9)



$C_{306}H_{521}N_{87}O_{96}S_2$
7019.17 g/mol
10

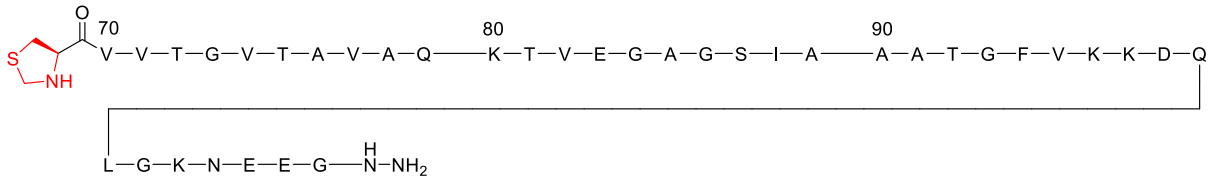
Peptide **10** was prepared on hydrazine modified 2-CTC resin (SOP1b, 143 mg, 0.05 mmol) using first manual peptide synthesis (SOP4) for the residues 61-68, followed by automated synthesis (SOP3) of the remaining residues 1-60. Alloc deprotection was carried out on resin as described in SOP5a with $Pd(PPh_3)_4$ (57.78 mg, 0.05 mmol, 1 eq.) and phenylsilane (123.40 µL, 1.00 mmol, 20 eq.) in DCM. The synthesized CEL building block, compound **7**, was introduced via reductive amination as explained in SOP5c. Compound **7** (108.13 mg, 0.75 mmol, 15 eq.) was dissolved in 1.88 mL NMP/2-Prop (3:1 *v/v*) with 5% acetic acid. $NaBH_3CN$ (47.13 mg, 0.75 mmol, 15 eq.) was added and the solution added to the resin-bound peptide. Cleavage was performed according to SOP6, followed by HPLC with method E (SOP7), t_R = 18.5 min. The pure product **9** (3.80 mg, 0.54 µmol, 1%) was obtained as white powder in 86% purity.

UHPLC (method A): t_R = 12.1 min.

MS (ESI+) *m/z*: 780.9 $[M+9H]^{9+}$, 878.4 $[M+8H]^{8+}$, 1003.7 $[M+7H]^{7+}$, 1170.7 $[M+6H]^{6+}$.

HR-MS (ESI+) *m/z*: calc. for $C_{306}H_{529}N_{87}O_{96}S_2$ ($[M+8H]^{8+}$): 878.3586, found: 878.3615; calc. for $C_{306}H_{528}N_{87}O_{96}S_2$ ($[M+7H]^{7+}$): 1003.6945, found: 1003.6982; calc. for $C_{306}H_{527}N_{87}O_{96}S_2$ ($[M+6H]^{6+}$): 1170.8091, found: 1170.8124.

aSyn [69-106] A69Thz (10)



$C_{161}H_{271}N_{47}O_{54}S_2$
3761.27 g/mol
11

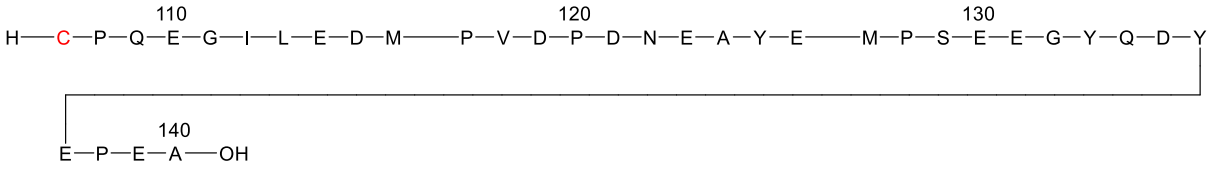
Peptide **11** was synthesized on hydrazine modified 2-CTC resin (SOP1b, 143 mg, 0.05 mmol) according to the automated synthesis protocol described in SOP3. The last AA was introduced using a Boc-Thz-OH building block. Cleavage of the peptide was achieved according to SOP6. The crude peptide was purified using HPLC (SOP7, method D, t_R = 21.2 min) and the pure product **10** (42.70 mg, 11.35 µmol, 23 %) obtained as a white powder in purity of 96%.

UHPLC (method A): t_R = 12.1 min.

MS (ESI+) *m/z*: 760.8 $[M+4H+Na]^{5+}$, 941.3 $[M+4H]^{4+}$, 1254.7 $[M+3H]^{3+}$.

HR-MS (ESI+) *m/z*: calc. for $C_{161}H_{275}N_{47}O_{54}SNa$ ($[M+4H+Na]^{5+}$): 757.5974, found: 757.6005; calc. for $C_{161}H_{275}N_{47}O_{54}S$ ($[M+4H]^{4+}$): 941.2495, found: 941.2526; calc. for $C_{161}H_{274}N_{47}O_{54}SNa$ ($[M+3H]^{3+}$): 1254.6636, found: 1254.6693.

aSyn [107-140] A107C (11)



$C_{165}H_{237}N_{37}O_{66}S_3$
3891.08 g/mol
12

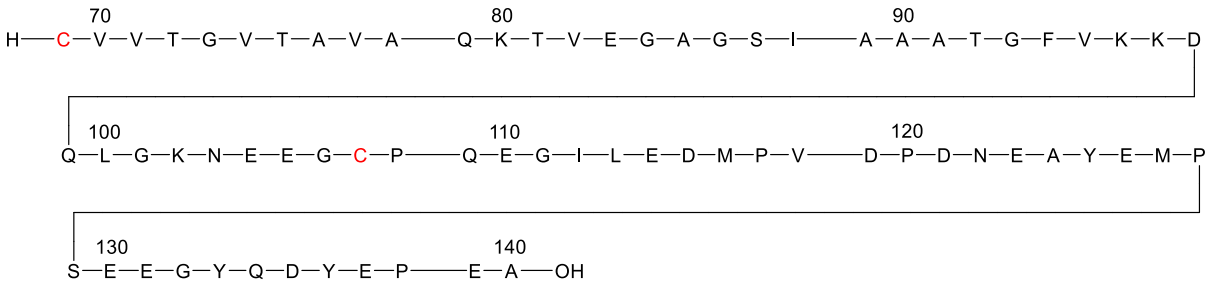
Peptide **12** was synthesized on Fmoc-Ala preloaded 2-CTC resin (SOP1c, 56.18 mg, 0.05 mmol) according to the automated synthesis protocol described in SOP3. As last AA building block, Fmoc-Cys(Trt)-OH was used for Cys point mutation. The peptide was cleaved (SOP6). Crude peptide was purified with HPLC method D as explained in SOP7 (t_R = 19.3 min). Pure peptide **11** (14.10 mg, 3.62 µmol, 8%) was obtained as white powder in 95% purity.

UHPLC (method A): t_R = 11.3 min.

MS (ESI+) *m/z*: 988.6 $[M+2H+2Na]^{4+}$, 1312.5 $[M+H+2Na]^{3+}$, 1957.3 $[M+H+Na]^{2+}$.

HR-MS (ESI+) *m/z*: calc. for $C_{165}H_{239}N_{37}O_{66}S_3Na_2$ ($[M+2H+2Na]^{4+}$): 984.6371, found: 984.6330; calc. for $C_{165}H_{240}N_{37}O_{66}S_3$ ($[M+3H]^{3+}$): 1297.8590, found: 1297.8608; calc. for $C_{165}H_{239}N_{37}O_{66}S_3Na$ ($[M+2H+Na]^{3+}$): 1305.1863, found: 1305.1853; calc. for $C_{165}H_{238}N_{37}O_{66}S_3Na_2$ ($[M+H+2Na]^{3+}$): 1312.5136, found: 1312.5140.

aSyn [69-140] A69C, A107C (12)



$C_{325}H_{504}N_{82}O_{120}S_4$
7608.30 g/mol
13

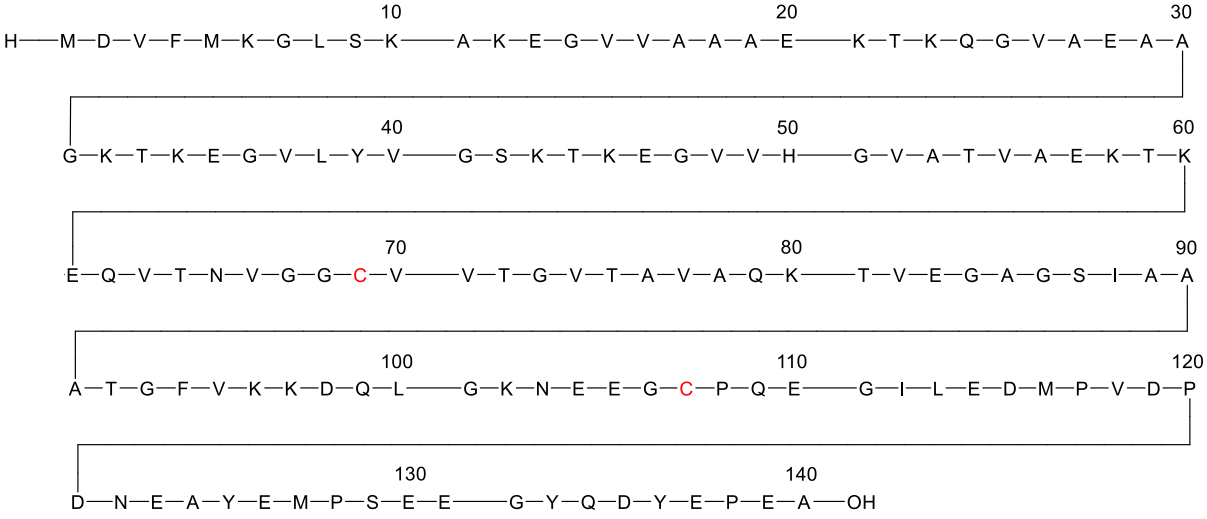
Thioester conversion as described in SOP8a was carried out on Thz peptide **11** (8.00 mg, 2.13 µmol, 1 eq.), followed by ligation (SOP8b) with peptide **12** (8.29 mg, 2.13 µmol, 1 eq.) and then Thz conversion (SOP8c). The reaction product was purified via HPLC method D (t_R = 25.2 min) to yield pure peptide **12** (3.00 mg, 0.39 µmol, 19% over three steps) as white powder in > 99% purity.

UHPLC (method A): t_R = 12.4 min.

MS (ESI+) *m/z*: 1094.1 $[M+5H+2Na]^{7+}$, 1276.2 $[M+4H+2Na]^{6+}$, 1526.9 $[M+4H+Na]^{5+}$.

HR-MS (ESI+) *m/z*: calc. for $C_{325}H_{511}N_{82}O_{120}S_4$ ($[M+7H]^{7+}$): 1087.7907, found: 1087.7908; calc. for $C_{325}H_{510}N_{82}O_{120}S_4Na$ ($[M+6H+Na]^{7+}$): 1090.9310, found: 1090.9317; calc. for $C_{325}H_{510}N_{82}O_{120}S_4$ ($[M+6H]^{6+}$): 1268.9213, found: 1268.9215; calc. for $C_{325}H_{509}N_{82}O_{120}S_4Na$ ($[M+5H+Na]^{6+}$): 1272.5850, found: 1272.5861; calc. for $C_{325}H_{509}N_{82}O_{120}S_4$ ($[M+5H]^{5+}$): 1522.5041, found: 1522.5083; calc. for $C_{325}H_{508}N_{82}O_{120}S_4$ ($[M+4H+Na]^{5+}$): 1526.9005, found: 1526.9020.

aSyn [1-140] wt, A69C, A107C



$C_{627}H_{1012}N_{166}O_{216}S_6$
14524.30 g/mol

Thioester conversion (SOP8a) was carried out with *N*-terminal fragment **15** (2.50 mg, 0.36 µmol, 1 eq.), followed by ligation (SOP8b) with peptide **12** (2.74 mg, 0.36 µmol, 1 eq.).

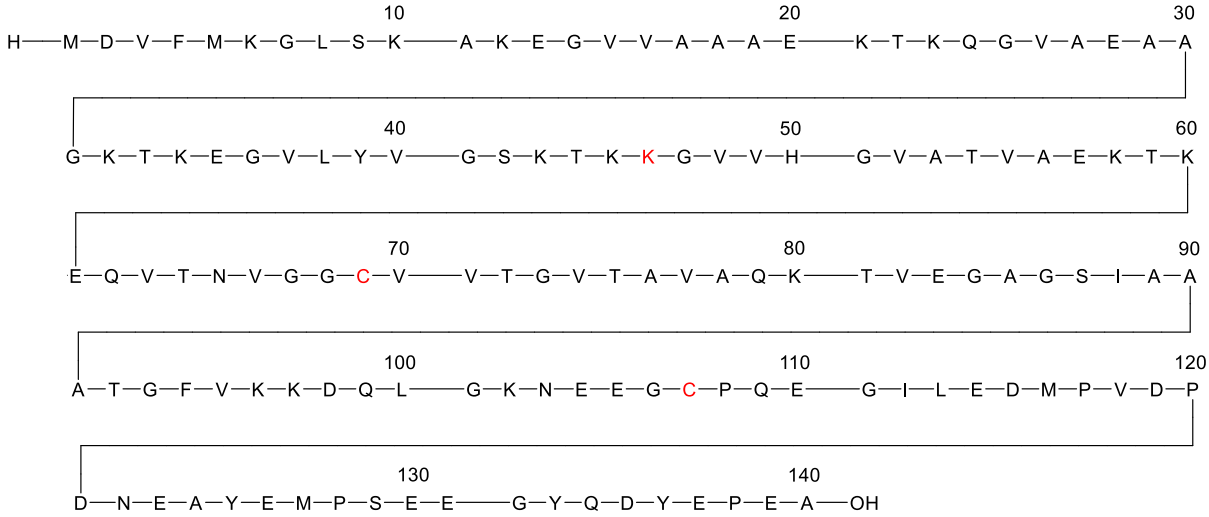
HPLC was carried out according to SOP7 method E (t_R = 27.5 min) to obtain pure protein **aSyn [1-140] wt A69C, A107C** (0.90 mg, 61.97 nmol, 18% over two steps) as white powder in 95% purity.

UHPLC (method A): t_R = 13.4 min.

MS (ESI+) *m/z*: 1041.5 $[M+12H+2Na]^{14+}$, 1121.7 $[M+11H+2Na]^{13+}$, 1213.3 $[M+11H+Na]^{12+}$, 1323.6 $[M+10H+Na]^{11+}$, 1453.3 $[M+10H]^{10+}$, 1614.7 $[M+9H]^{9+}$, 1816.4 $[M+8H]^{8+}$.

HR-MS (ESI+) *m/z*: calc. for $C_{627}H_{1026}N_{166}O_{216}S_6$ ($[M+14H]^{14+}$): 1038.3775, found: 1038.3736; calc. for $C_{627}H_{1025}N_{166}O_{216}S_6$ ($[M+13H]^{13+}$): 1118.1753, found: 1118.1747; calc. for $C_{627}H_{1024}N_{166}O_{216}S_6$ ($[M+12H]^{12+}$): 1211.2726, found: 1211.2697; calc. for $C_{627}H_{1023}N_{166}O_{216}S_6$ ($[M+11H]^{11+}$): 1321.2967, found: 1321.2984; calc. for $C_{627}H_{1022}N_{166}O_{216}S_6$ ($[M+10H]^{10+}$): 1453.3257, found: 1453.3277; calc. for $C_{627}H_{1021}N_{166}O_{216}S_6$ ($[M+9H]^{9+}$): 1614.6944, found: 1614.7027.

aSyn [1-140] E46K, A69C, A107C



$C_{628}H_{1017}N_{167}O_{214}S_6$
14523.36 g/mol

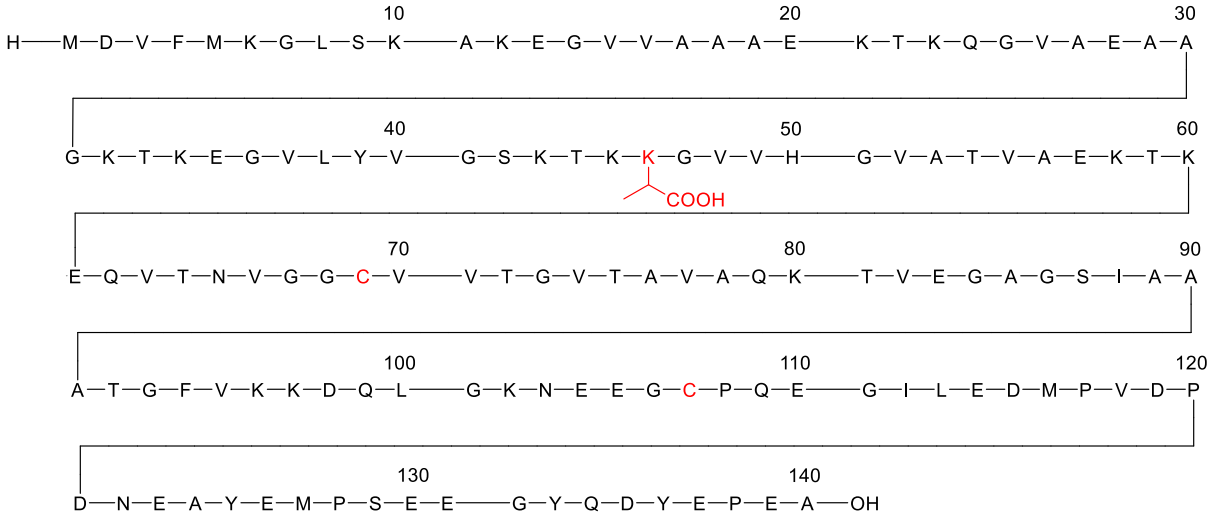
Thioester conversion (SOP8a) was carried out with *N*-terminal fragment **8** (2.50 mg, 0.36 µmol, 1 eq.), followed by ligation (SOP8b) with peptide **12** (2.74 mg, 0.36 µmol, 1 eq.). HPLC was carried out according to SOP7 method E (t_R = 27.5 min) to obtain pure protein **aSyn [1-140] E46K, A69C, A107C** (0.80 mg, 55.08 nmol, 16% over two steps) as white powder in 93% purity.

UHPLC (method A): t_R = 13.2 min.

MS (ESI+) *m/z*: 911.5 $[M+14H+2Na]^{16+}$, 973.3 $[M+13H+2Na]^{15+}$, 1041.9 $[M+12H+2Na]^{14+}$, 1121.4 $[M+11H+2Na]^{13+}$, 1211.5 $[M+12H]^{12+}$, 1321.4 $[M+11H]^{11+}$, 1453.3 $[M+10H]^{10+}$, 1614.8 $[M+9H]^{9+}$, 1816.6 $[M+8H]^{8+}$.

HR-MS (ESI+) *m/z*: calc. for $C_{628}H_{1031}N_{167}O_{214}S_6$ ($[M+14H]^{14+}$): 1038.31, found: 1038.30; calc. for C628H1030N167O214S6Na ($[M+13H+Na]^{14+}$): 1039.88, found: 1039.88; calc. for $C_{628}H_{1030}N_{167}O_{214}S_6$ ($[M+13H]^{13+}$): 1118.1024, found: 1118.1032; calc. for $C_{628}H_{1029}N_{167}O_{214}S_6$ ($[M+12H]^{12+}$): 1211.1936, found: 1211.1900; calc. for $C_{628}H_{1028}N_{167}O_{214}S_6$ ($[M+11H]^{11+}$): 1321.2106, found: 1321.2096; calc. for $C_{628}H_{1027}N_{167}O_{214}S_6$ ($[M+10H]^{10+}$): 1453.2309, found: 1453.2295; calc. for $C_{628}H_{1026}N_{167}O_{214}S_6$ ($[M+9H]^{9+}$): 1614.59, found: 1614.59.

aSyn [1-140] E46CEL, A69C, A107C



$C_{631}H_{1021}N_{167}O_{216}S_6$
14595.42 g/mol

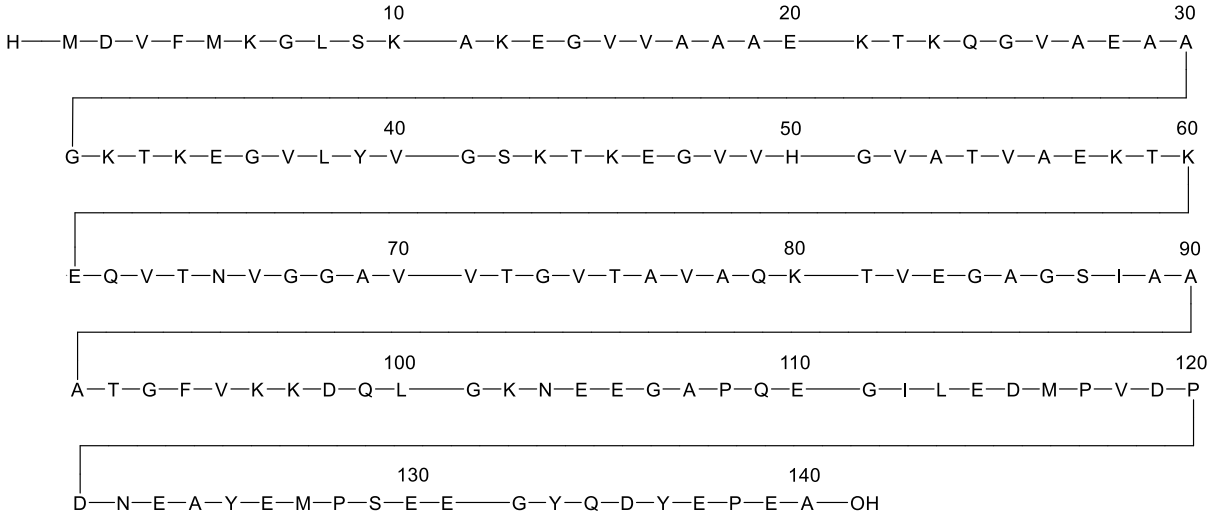
Thioester conversion (SOP8a) was carried out with *N*-terminal fragment **9** (2.50 mg, 0.36 µmol, 1 eq.), followed by ligation (SOP8b) with peptide **12** (2.74 mg, 0.36 µmol, 1 eq.). HPLC was carried out according to SOP7 method E (t_R = 26.2 min) to obtain pure protein **aSyn [1-140] E46K, A69C, A107C** (0.40 mg, 27.41 nmol, 8% over two steps) as white powder in 98% purity.

UHPLC (method A): t_R = 13.2 min.

MS (ESI+) *m/z*: 1052.1 $[M+9H+5Na]^{14+}$, 1128.6 $[M+10H+3Na]^{13+}$, 1221.0 $[M+10H+2Na]^{12+}$, 1340.2 $[M+5H+6Na]^{11+}$, 1469.3 $[M+6H+4Na]^{10+}$, 1634.7 $[M+4H+5Na]^{9+}$, 1833.4 $[M+5H+3Na]^{8+}$.

HR-MS (ESI+) *m/z*: calc. for $C_{631}H_{1033}N_{167}O_{216}S_6$ $([M+12H]^{12+})$: 1217.1954, found: 1217.1951; calc. for $C_{631}H_{1032}N_{167}O_{216}S_6$ $([M+11H]^{11+})$: 1327.7579, found: 1327.7541.

aSyn [1-140] wt (16)



$C_{627}H_{1012}N_{166}O_{216}S_4$
14460.18 g/mol
17

Desulfurization as described in SOP8d was carried out on protein **aSyn [1-140] wt, A69C, A107C** (0.90 mg, 61.97 nmol, 1 eq.), followed by purification with HPLC (SOP7) using method E (t_R = 28.1 min). Pure protein **16** (0.40 mg, 27.66 nmol, 45%) was obtained as white powder in 78% purity.

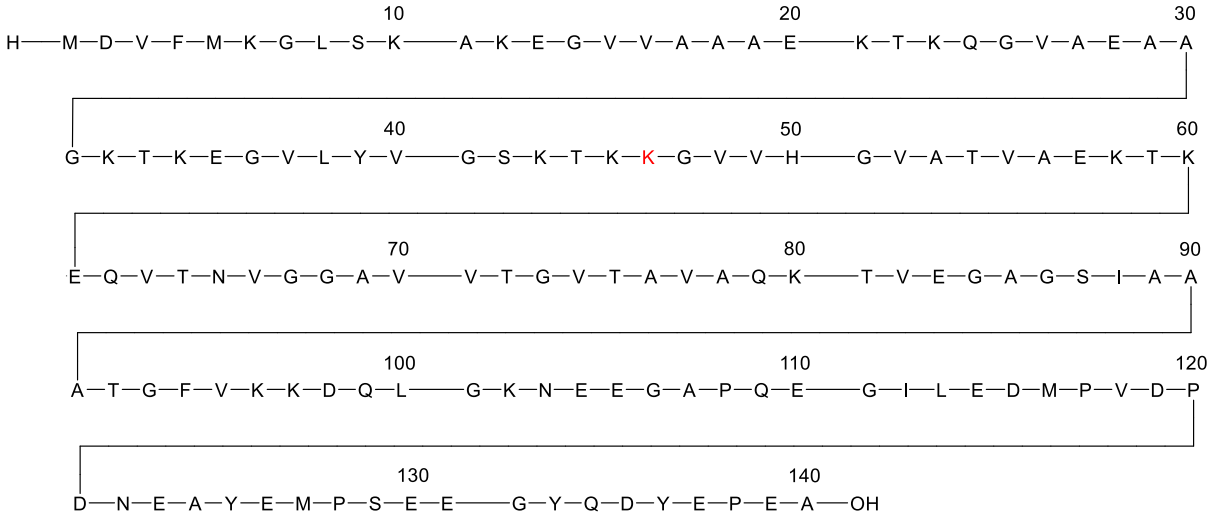
UHPLC (method A): t_R = 13.6 min.

MS (ESI+) *m/z*: 851.5 $[M+17H]^{17+}$, 904.7 $[M+16H]^{16+}$, 965.0 $[M+15H]^{15+}$, 1033.8 $[M+14H]^{14+}$, 1113.3 $[M+13H]^{13+}$, 1206.0 $[M+12H]^{12+}$, 1315.5 $[M+11H]^{11+}$, 1447.0 $[M+10H]^{10+}$.

HR-MS (ESI+) *m/z*: calc. for $C_{627}H_{1027}N_{166}O_{216}S_4$ $([M+15H]^{15+})$: 964.9566, found: 964.9543; calc. for $C_{627}H_{1026}N_{166}O_{216}S_4$ $([M+14H]^{14+})$: 1033.8102, found: 1033.8072; calc. for $C_{627}H_{1025}N_{166}O_{216}S_4$ $([M+13H]^{13+})$: 1113.2565, found: 1113.2536; calc. for

$C_{627}H_{1024}N_{166}O_{216}S_4$ ($[M+12H]^{12+}$): 1205.9440, found: 1205.9403; calc. for $C_{627}H_{1023}N_{166}O_{216}S_4$ ($[M+11H]^{11+}$): 1315.4837, found: 1315.4801.

aSyn [1-140] E46K (13)



$C_{628}H_{1017}N_{167}O_{214}S_4$
14459.24 g/mol
14

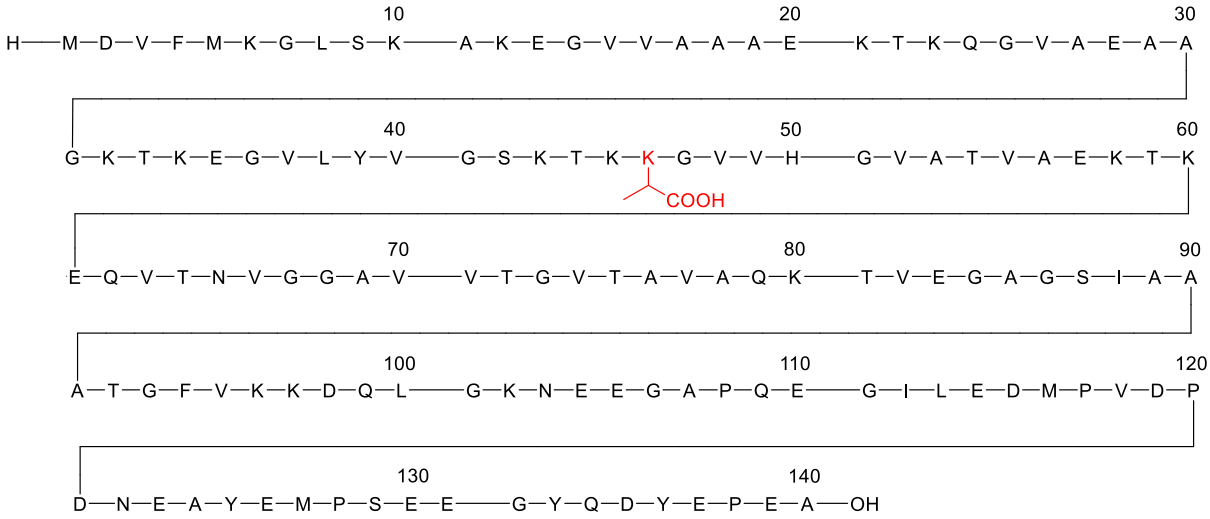
Desulfurization as described in SOP8d was carried out on protein **aSyn [1-140] E46K, A69C, A107C** (0.80 mg, 55.08 nmol, 1 eq.), followed by purification with HPLC (SOP7) using method E (t_R = 26.3 min). Pure protein **13** (0.40 mg, 27.66 nmol, 51%) was obtained as white powder in 80% purity.

UHPLC (method A): t_R = 13.3 min.

MS (ESI+) *m/z*: 851.5 $[M+17H]^{17+}$, 904.7 $[M+16H]^{16+}$, 964.9 $[M+15H]^{15+}$, 1033.8 $[M+14H]^{14+}$, 1113.2 $[M+13H]^{13+}$, 1205.9 $[M+12H]^{12+}$, 1315.4 $[M+11H]^{11+}$, 1446.8 $[M+10H]^{10+}$.

HR-MS (ESI+) *m/z*: calc. for $C_{628}H_{1033}N_{167}O_{214}S_4$ ($[M+16H]^{16+}$): 904.6504, found: 904.6471; calc. for $C_{628}H_{1032}N_{167}O_{214}S_4$ ($[M+15H]^{15+}$): 964.8933, found: 964.8900; calc. for $C_{628}H_{1031}N_{167}O_{214}S_4$ ($[M+14H]^{14+}$): 1033.7427, found: 1033.7386; calc. for $C_{628}H_{1030}N_{167}O_{214}S_4$ ($[M+13H]^{13+}$): 1113.1838, found: 1113.1793; calc. for $C_{628}H_{1029}N_{167}O_{214}S_4$ ($[M+12H]^{12+}$): 1205.8651, found: 1205.8603; calc. for $C_{628}H_{1028}N_{167}O_{214}S_4$ ($[M+11H]^{11+}$): 1315.3976, found: 1315.3917.

aSyn [1-140] E46CEL (14)



$C_{631}H_{1021}N_{167}O_{216}S_4$
14531.30 g/mol
15

Desulfurization as described in SOP8d was carried out on protein **aSyn [1-140] E46CEL, A69C, A107C** (0.40 mg, 27.41 nmol, 1 eq.), followed by purification with HPLC (SOP7) using method E (t_R = 26.9 min). Pure protein **14** (0.2 mg, 13.76 nmol, 51%) was obtained as white powder in 89% purity.

UHPLC (method A): t_R = 13.4 min.

MS (ESI+) *m/z*: 909.2 $[M+16H]^{16+}$, 969.8 $[M+15H]^{15+}$, 1038.9 $[M+14H]^{14+}$, 1118.7 $[M+13H]^{13+}$, 1212.0 $[M+12H]^{12+}$, 1322.0 $[M+11H]^{11+}$, 1454.1 $[M+10H]^{10+}$.

HR-MS (ESI+) *m/z*: calc. for $C_{631}H_{1037}N_{167}O_{216}S_4$ ($[M+16H]^{16+}$): 909.1519, found: 909.1482; calc. for $C_{631}H_{1036}N_{167}O_{216}S_4$ ($[M+15H]^{15+}$): 969.6949, found: 969.6913; calc. for $C_{631}H_{1035}N_{167}O_{216}S_4$ ($[M+14H]^{14+}$): 1038.8868, found: 1038.8829; calc. for $C_{631}H_{1034}N_{167}O_{216}S_4$ ($[M+13H]^{13+}$): 1118.7237, found: 1118.7193; calc. for $C_{631}H_{1033}N_{167}O_{216}S_4$ ($[M+12H]^{12+}$): 1211.8668, found: 1211.8617; calc. for $C_{631}H_{1032}N_{167}O_{216}S_4$ ($[M+11H]^{11+}$): 1321.9449, found: 1321.9395; calc. for $C_{631}H_{1031}N_{167}O_{216}S_4$ ($[M+10H]^{10+}$): 1454.0387, found: 1454.0329.

8. Appendix

8.1 NMR Spectra

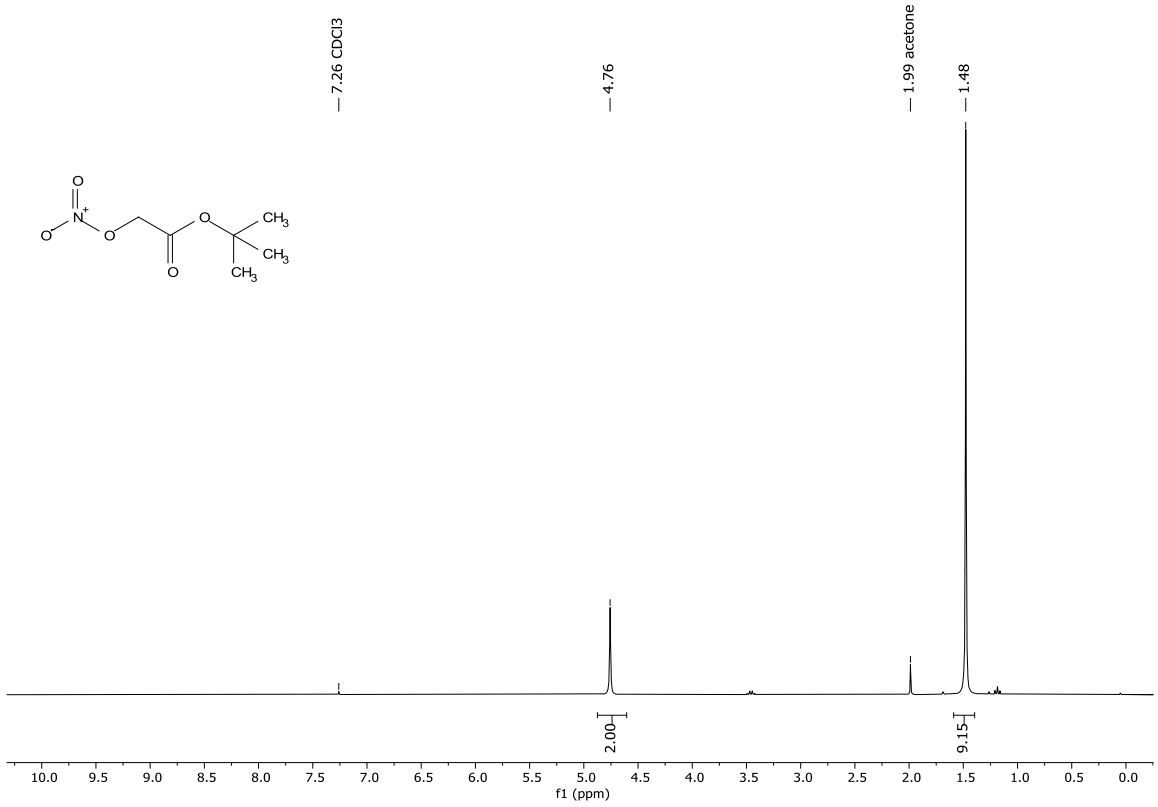


Figure S1: ^{1}H-NMR spectra of *tert*-butoxycarbonylmethyl nitrate (**5**)

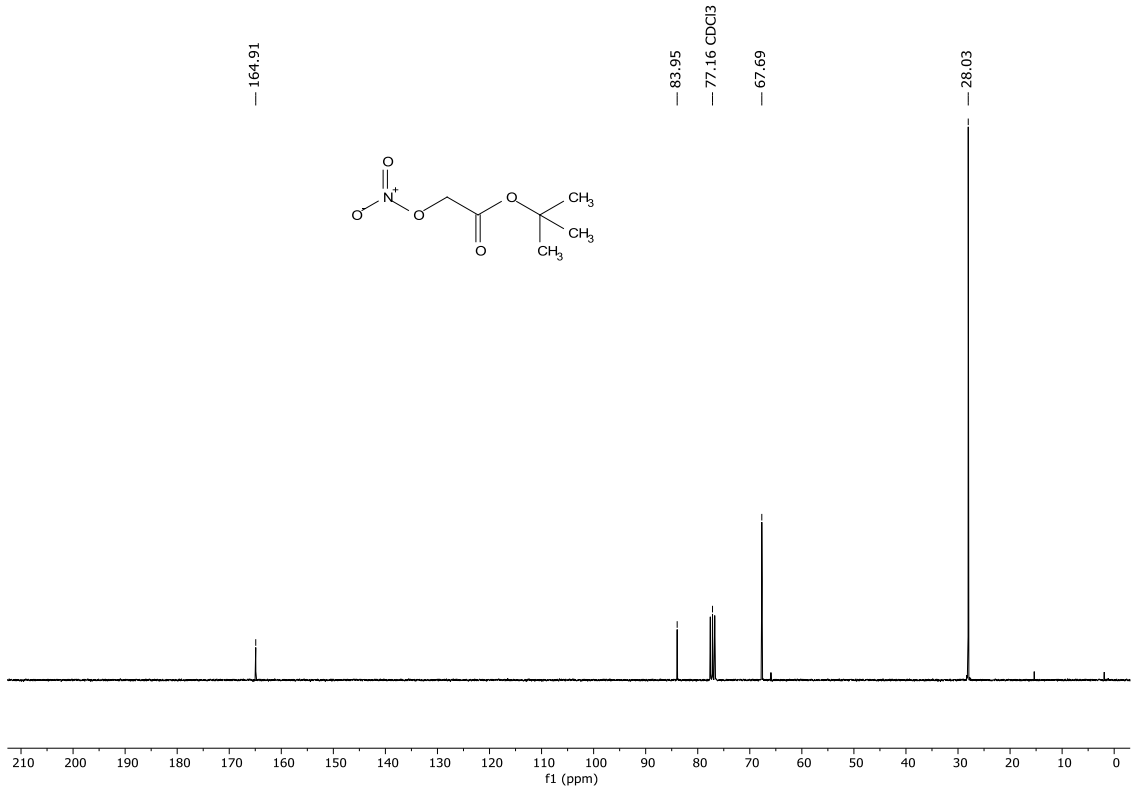


Figure S2: ^{13}C-NMR spectra of *tert*-butoxycarbonylmethyl nitrate (**5**)

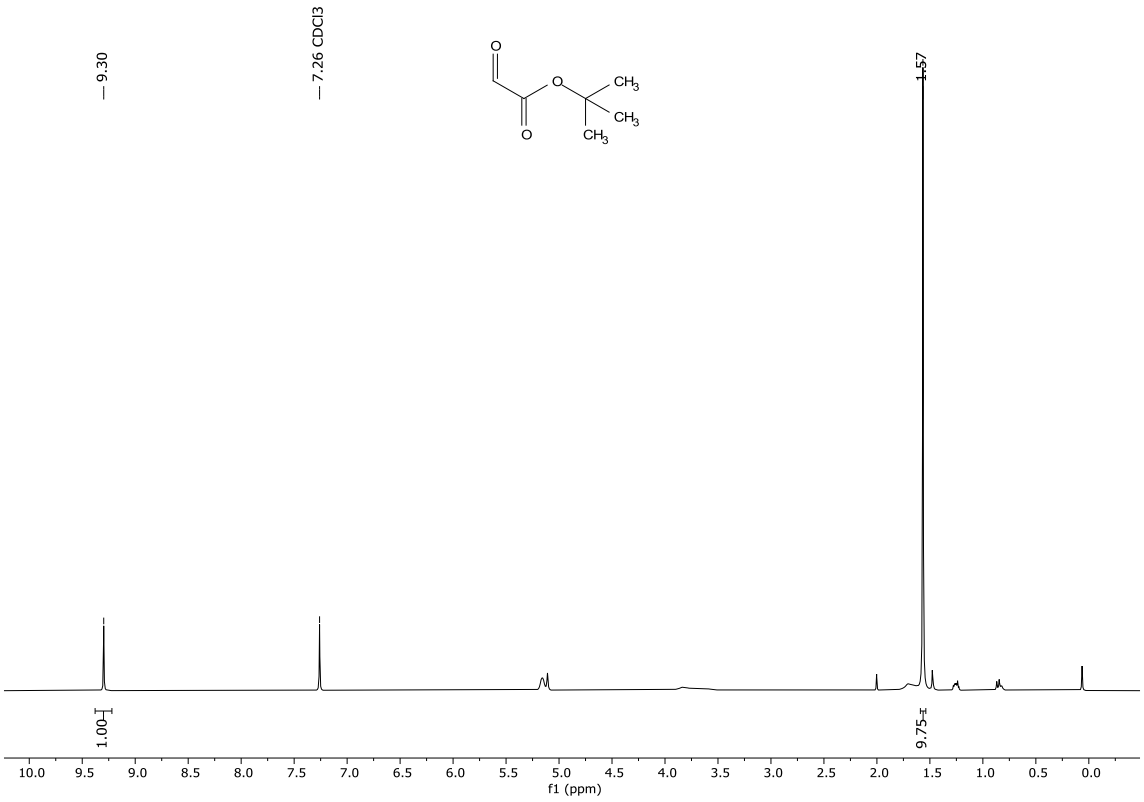


Figure S3: ^{1}H-NMR spectra of *tert*-butyloxoacetate (**6**)

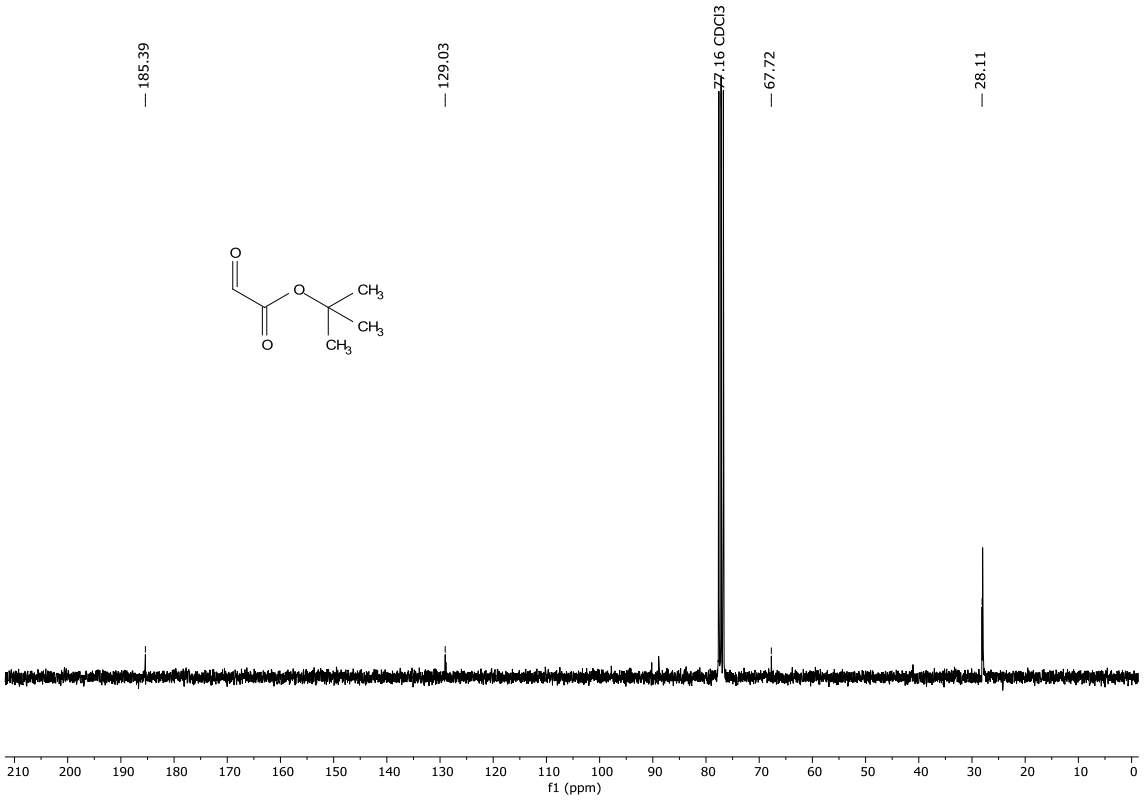


Figure S4: ^{13}C-NMR spectra of *tert*-butyloxoacetate (**6**)

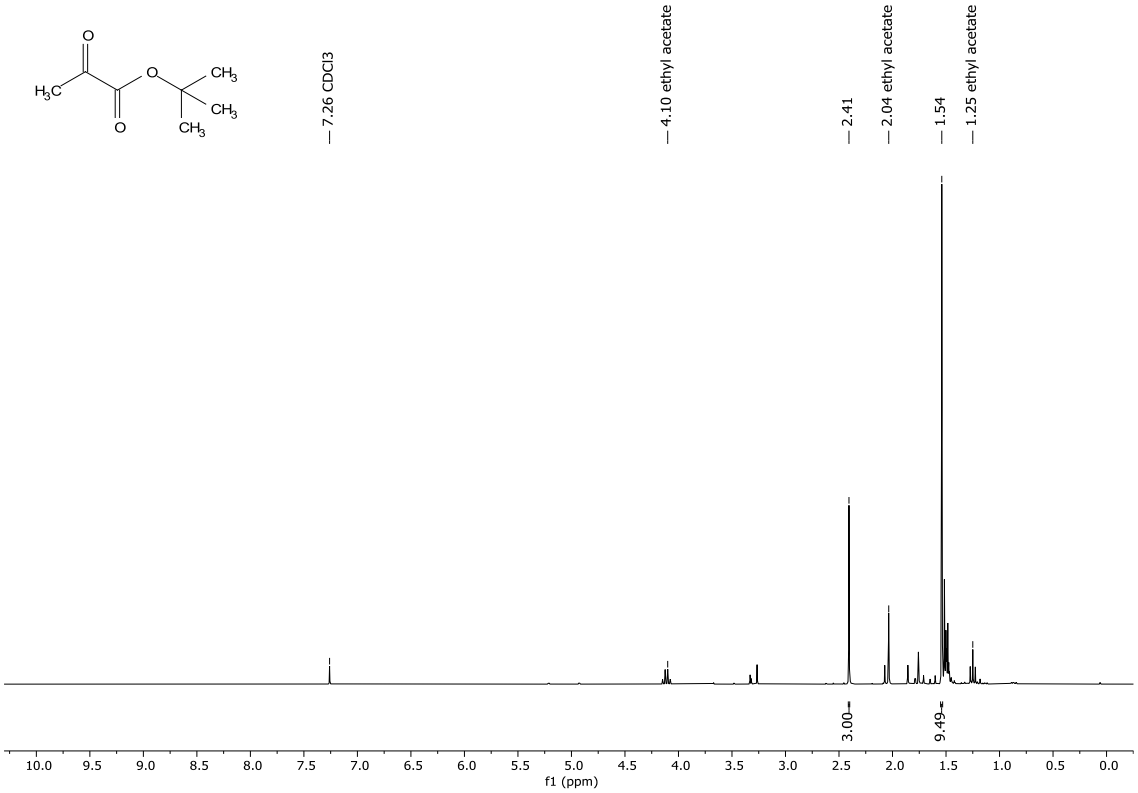


Figure S5: ^{1}H-NMR spectra of *tert*-butyl oxopropanoate (**7**)

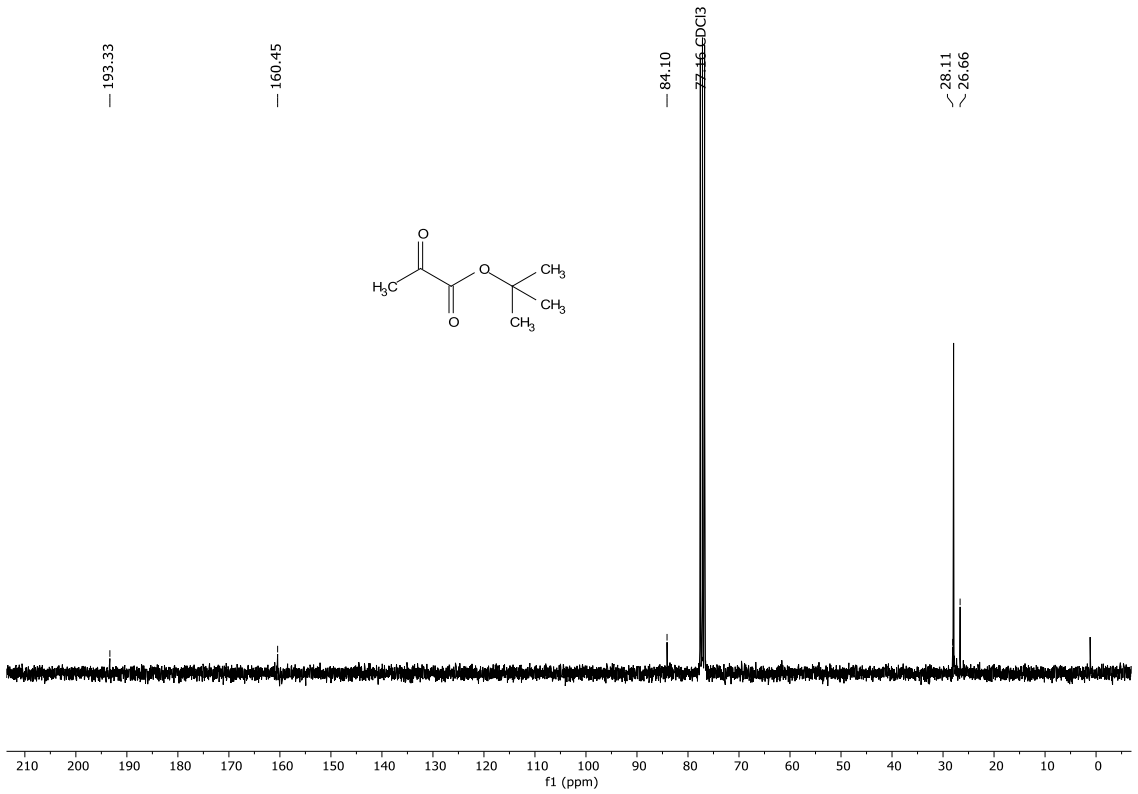


Figure S6: ^{13}C-NMR spectra of *tert*-butyl oxopropanoate (**7**)

8.2 HR-MS Spectra

Note: Some HR-MS spectra were recorded with ESI(MS), some with LC-ESI(MS), therefore, the layout of the spectra may vary. For each peptide, one exemplary HR-MS spectrum for a certain *m/z* ratio is given, with the calculated values in the bottom of the picture and the measured values on top in case of ESI(MS) spectra. For LC-ESI(MS) spectra, several HR-MS *m/z* ratio peaks are shown, without calculated values. For full-length aSyn proteins with two Cys mutations, additionally, an ESI(MS) spectrum without HR is given.

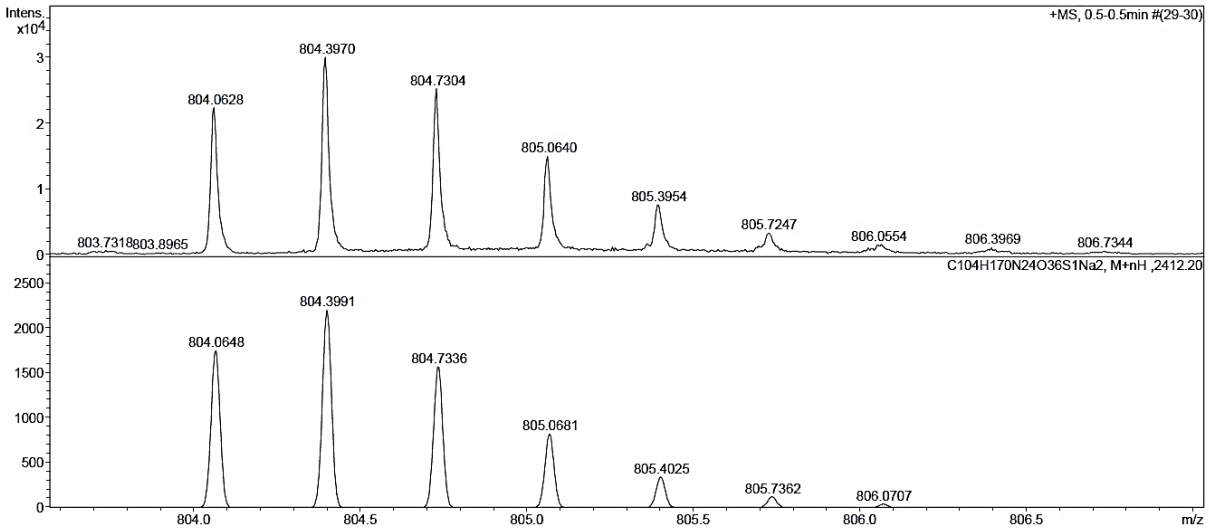


Figure S7: HR-MS spectrum of Pep1-Alloc ($[M+H+2Na]^{3+}$).

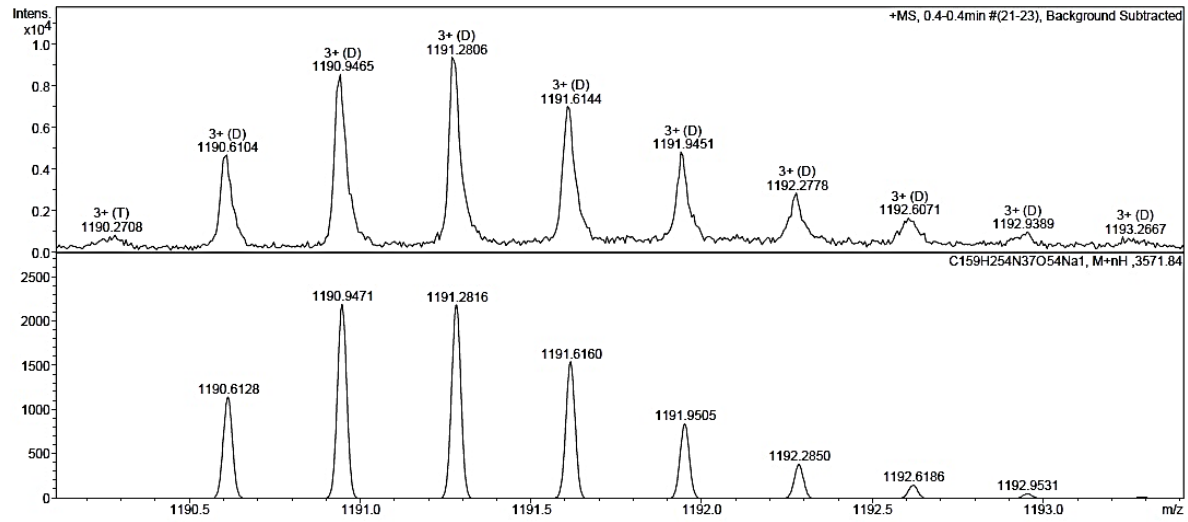


Figure S8: HR-MS spectrum of Pep2-Alloc ($[M+2H+Na]^{3+}$).

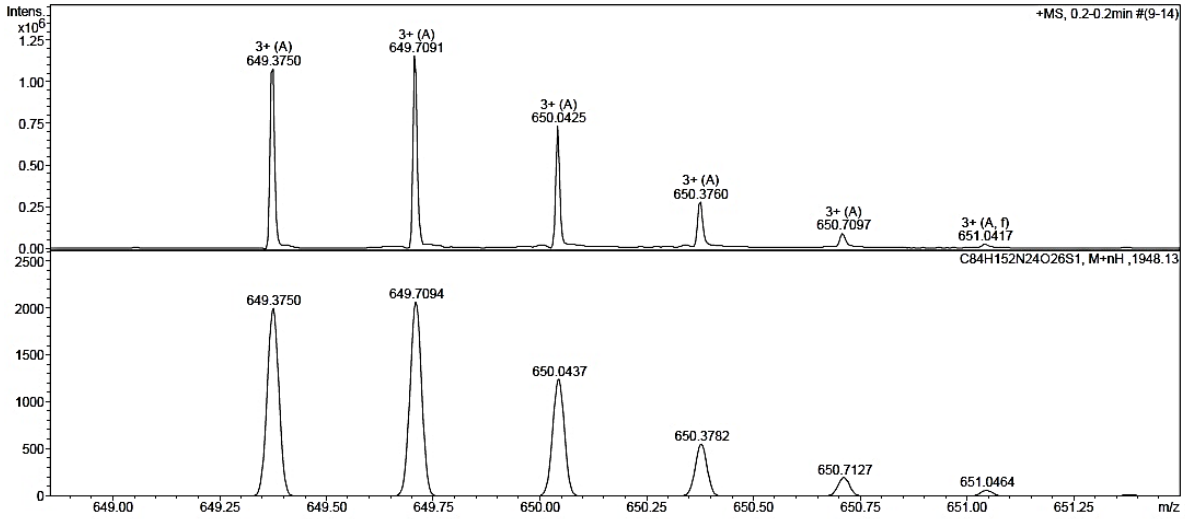


Figure S9: HR-MS spectrum of Pep1 Alloc deprotected ($[M+3H]^{3+}$).

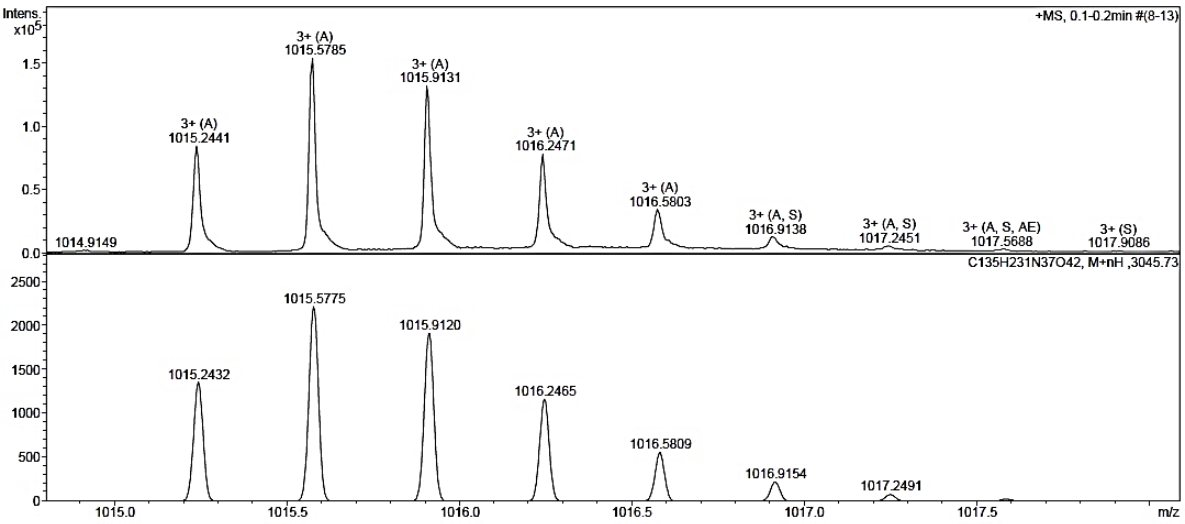


Figure S10: HR-MS spectrum of Pep2 Alloc deprotected ($[M+3H]^{3+}$).

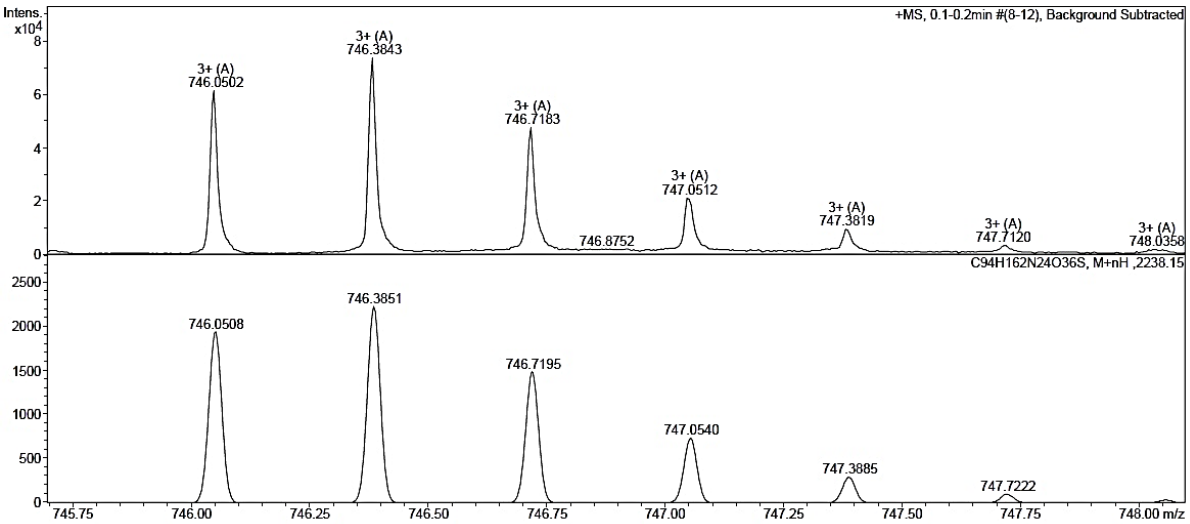


Figure S11: HR-MS spectrum of Pep1-CML (**1**) ($[M+3H]^{3+}$).

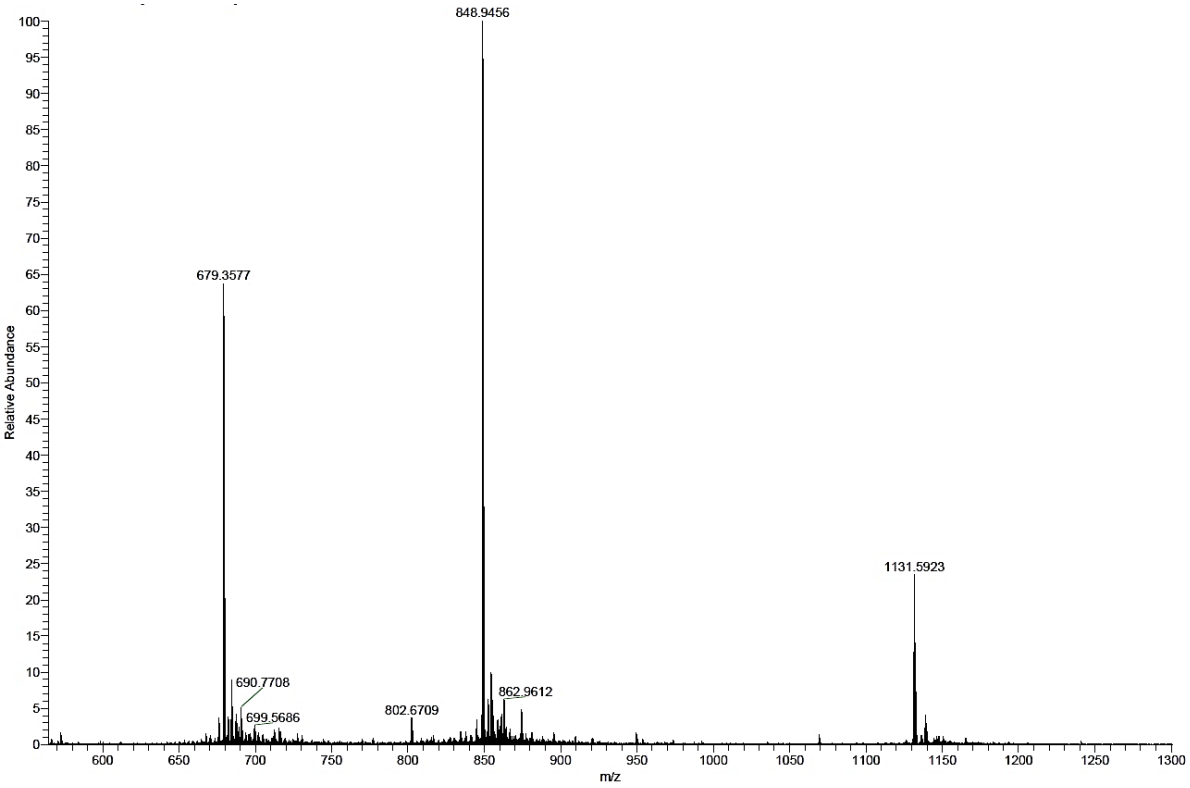


Figure S12: HR-MS spectrum of Pep2-CML (**2**) (679.3577 $[M+5H]^{5+}$, 849.9456 $[M+4H]^{4+}$, 1131.5923 $[M+3H]^{3+}$).

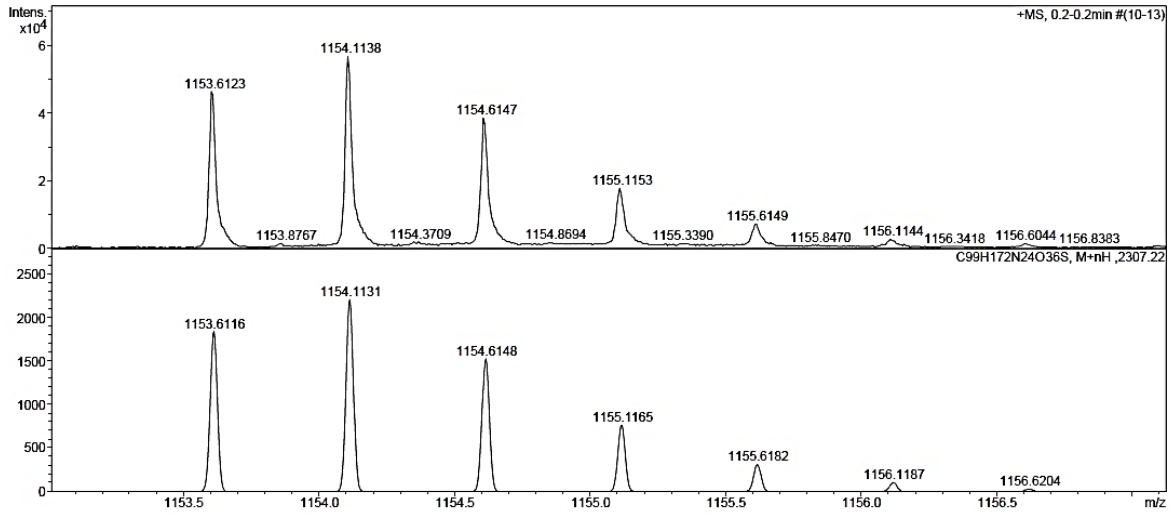


Figure S13: HR-MS spectrum of Pep1-CEL (**3**) ($[M+2H]^{2+}$).

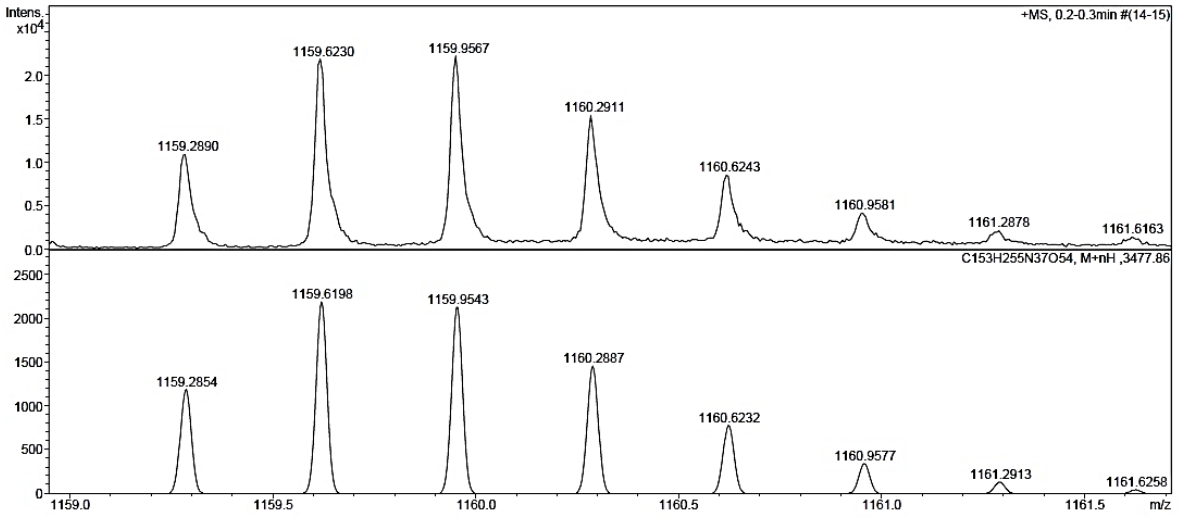


Figure S14: HR-MS spectrum of Pep2-CEL (**4**) ([M+2H]$^{2+}$).

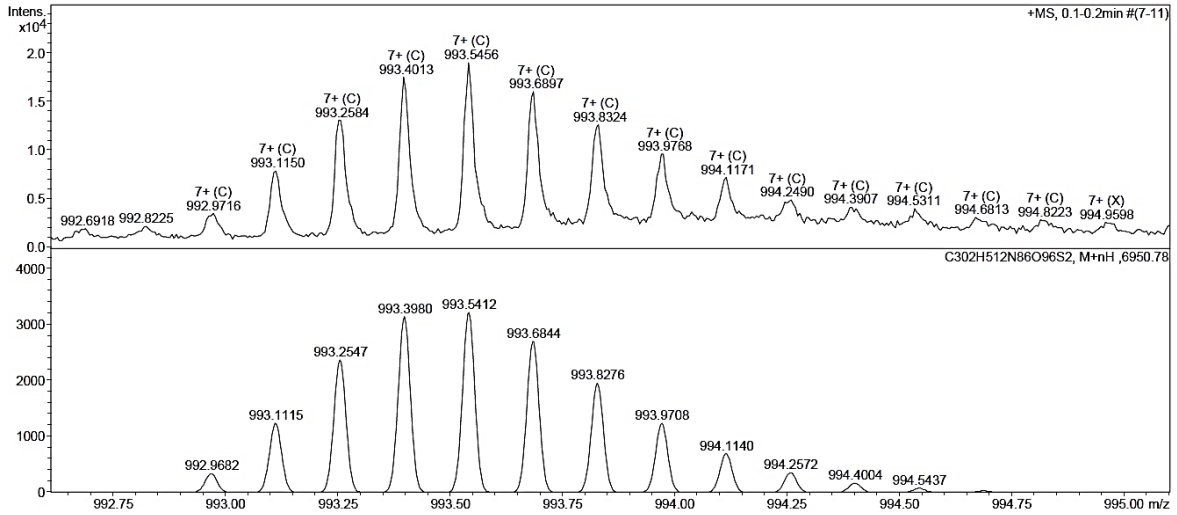


Figure S15: HR-MS spectrum of aSyn *N*Term [1-68] wt (**15**) ([M+7H]$^{7+}$).

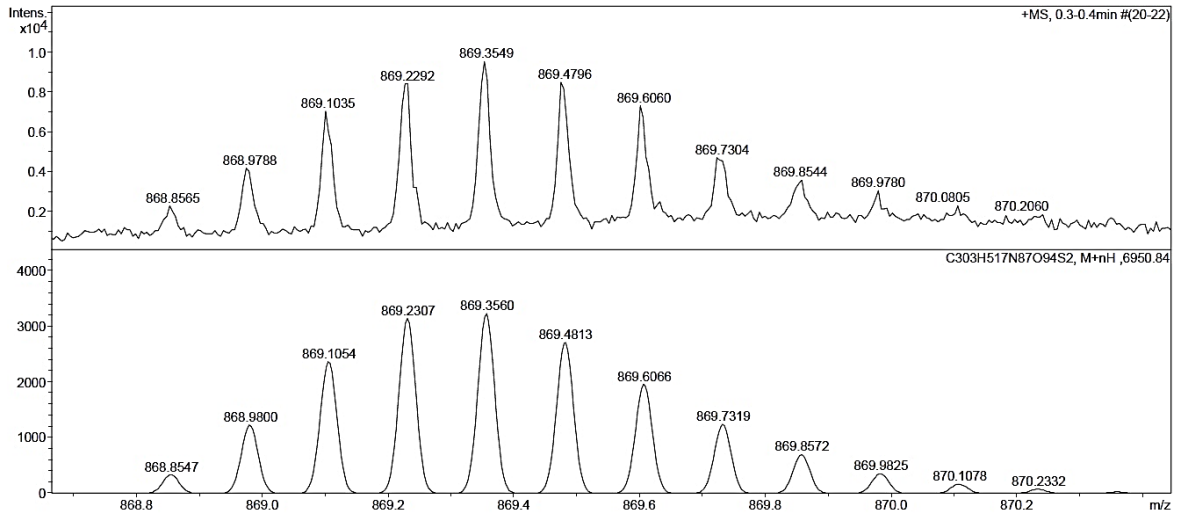


Figure S16: HR-MS spectrum of aSyn *N*Term [1-68] E46K (**8**) ([M+8H]$^{8+}$).

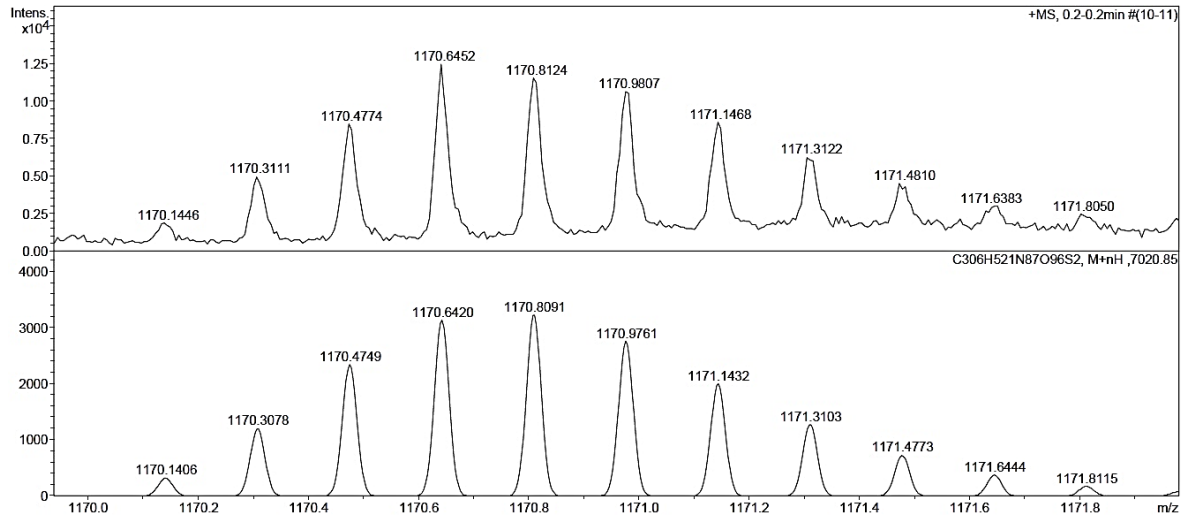


Figure S17: HR-MS spectrum of aSyn *N*Term [1-68] E46CEL (**9**) ([M+6H]$^{6+}$).

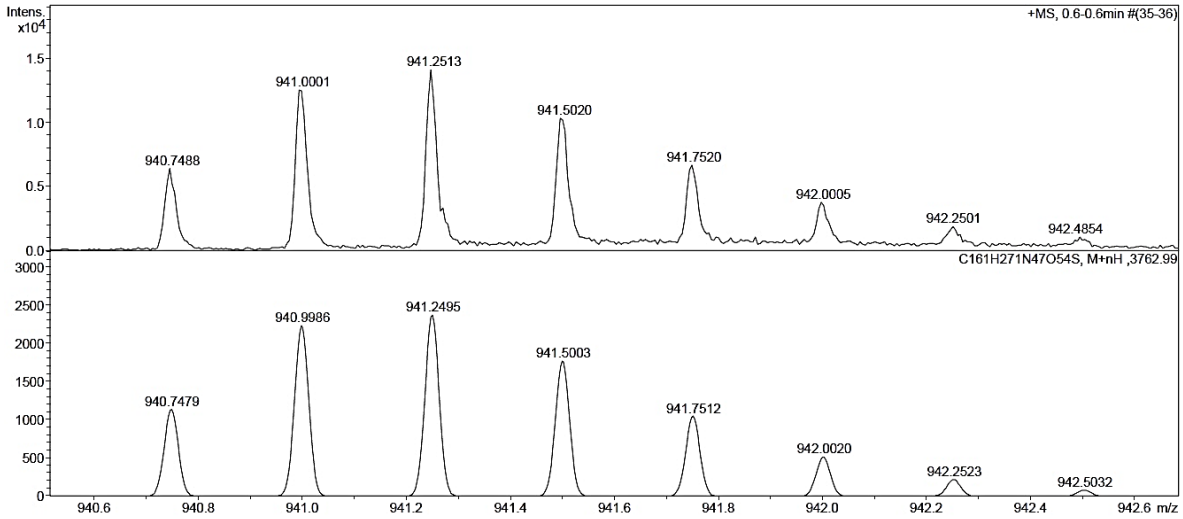


Figure S18: HR-MS spectrum of aSyn [69-106] A69Thz (**10**) ([M+4H]$^{4+}$).

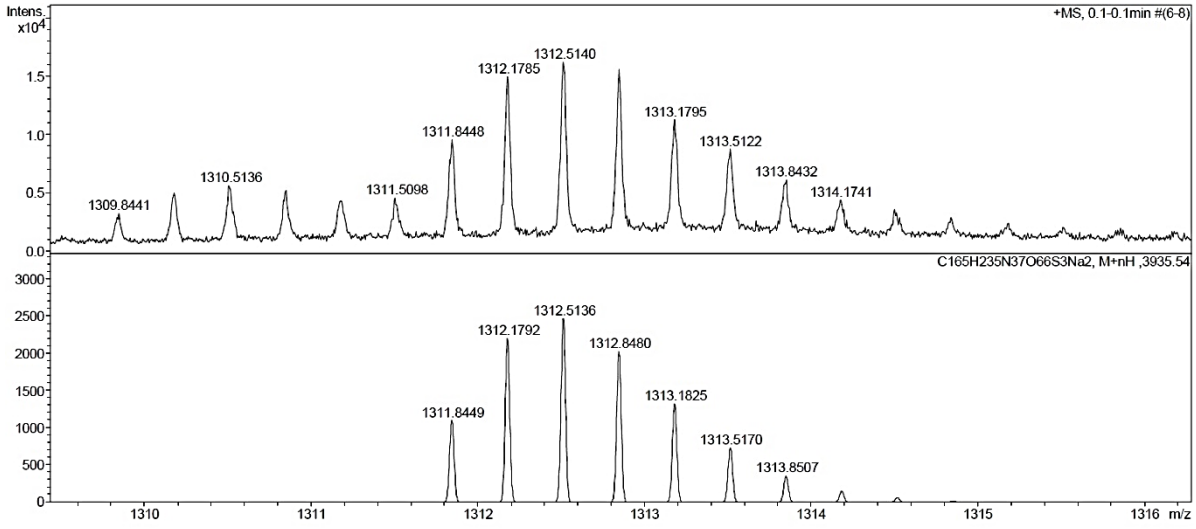


Figure S19: HR-MS spectrum of aSyn [107-140] A107C (**11**) ([M+H+2Na]$^{3+}$).

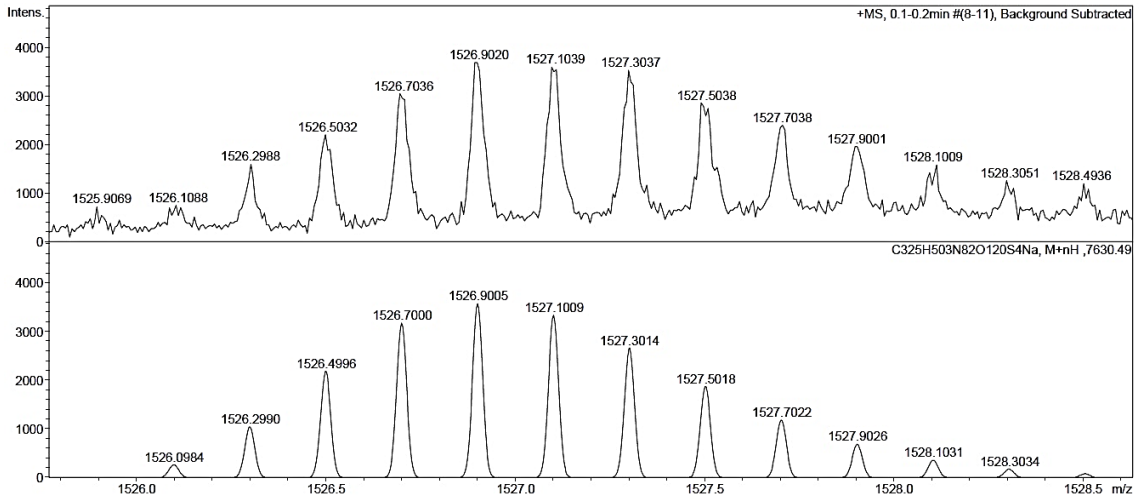


Figure S20: HR-MS spectrum of aSyn [69-140] A69C, A107C (**12**) ([M+4H+Na]$^{5+}$).

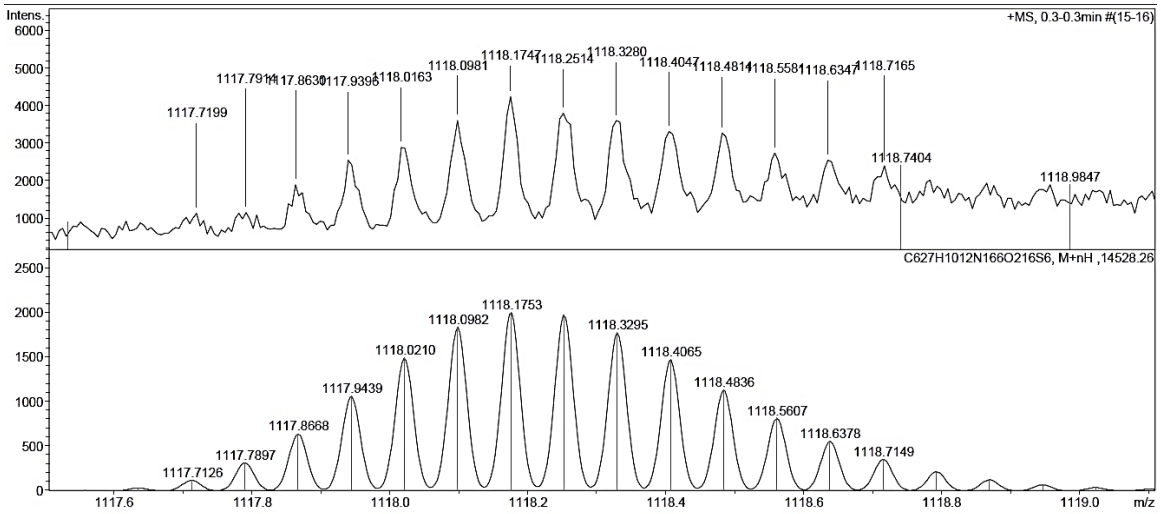


Figure S21: HR-MS spectrum of aSyn [1-140] wt, A69C, A107C ([M+13H]$^{13+}$).

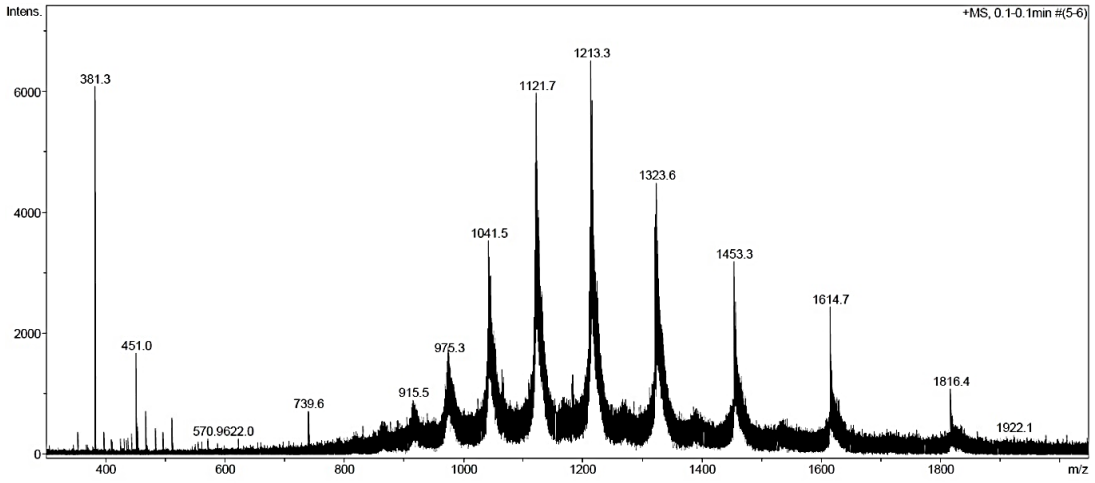


Figure S22: MS spectrum of aSyn [1-140] wt, A69C, A107C. Visible are peaks from 975.3 [M+15H]$^{15+}$ to 1816.4 [M+8H]$^{8+}$.[a]

[a] Noisiness of the mass data is a result of different waiting times between synthesis and taking the mass spectra, which may have affected the folding of the proteins and led to aggregation.

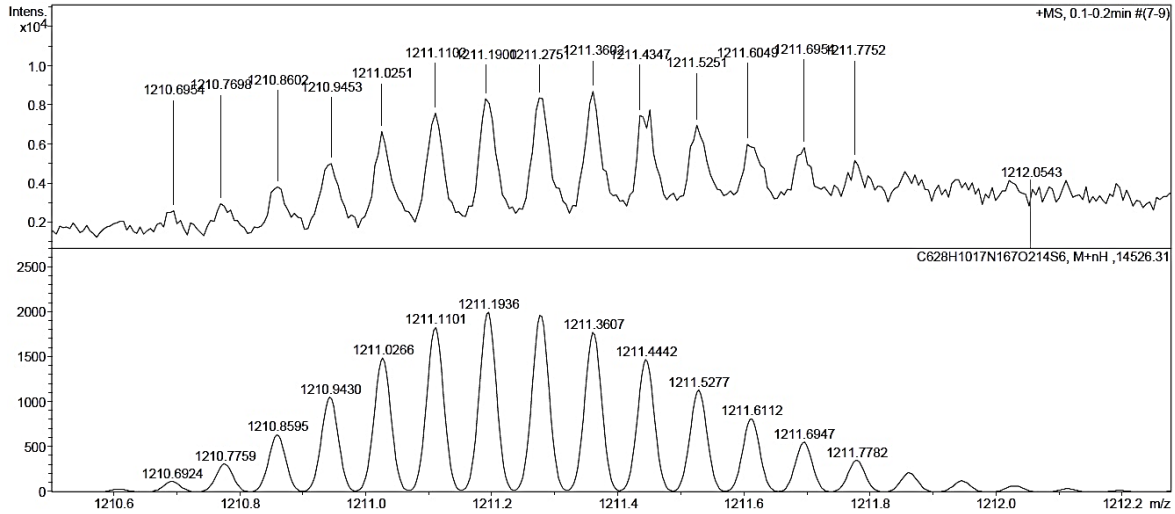


Figure S23: HR-MS spectrum of aSyn [1-140] E46K, A69C, A107C ($[M+12H]^{12+}$).

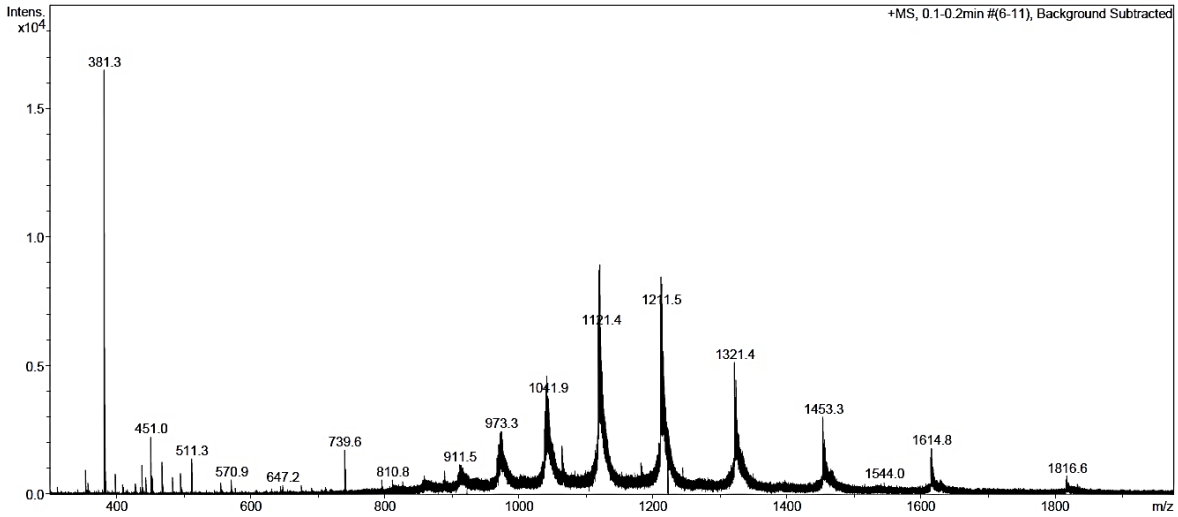


Figure S24: MS spectrum of aSyn [1-140] E46K, A69C, A107C. Visible are peaks from 973.3 ($[M+13H+2Na]^{15+}$) to 1816.6 ($[M+8H]^{8+}$).[a]

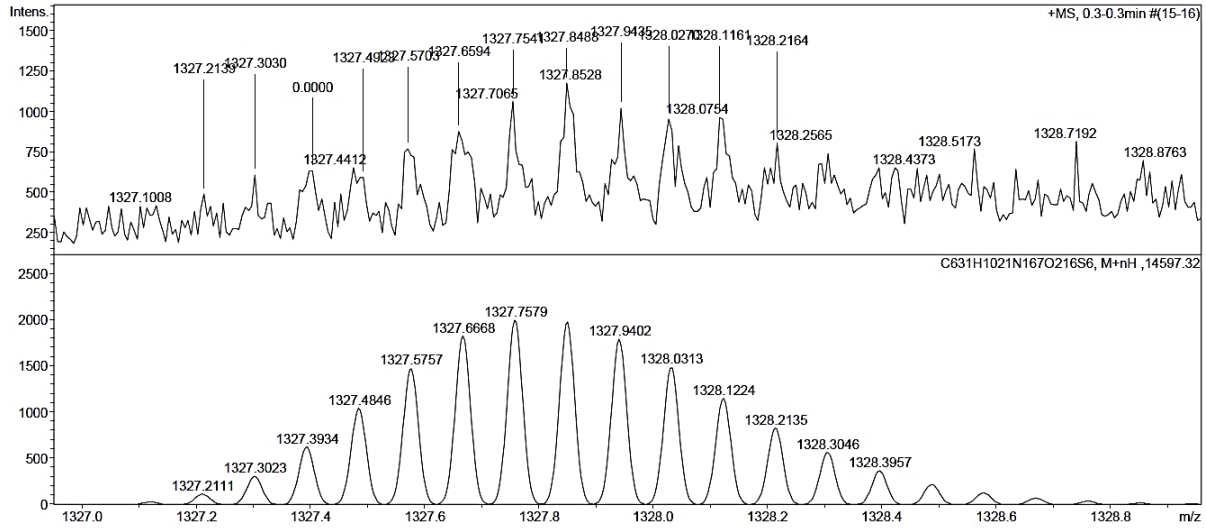


Figure S25: HR-MS spectrum of aSyn [1-140] E46CEL, A69C, A107C ($[M+11H]^{11+}$).

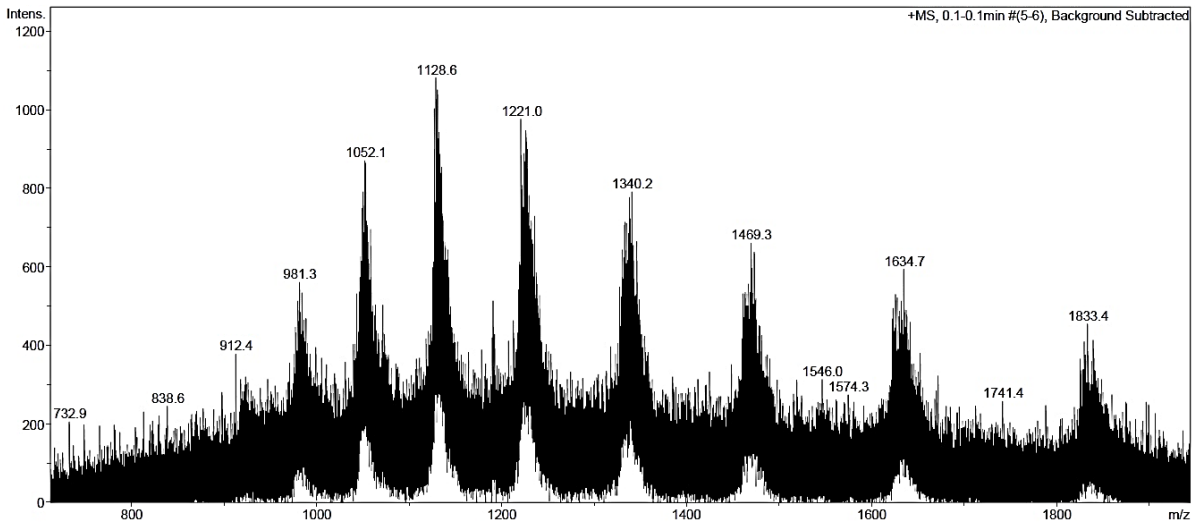


Figure S26: MS spectrum of aSyn [1-140] E46CEL, A69C, A107C. Visible are peaks from 1052.1 ($[M+9H+5Na]^{14+}$) to 1833.4 ($[M+5H+3Na]^{8+}$).[a]

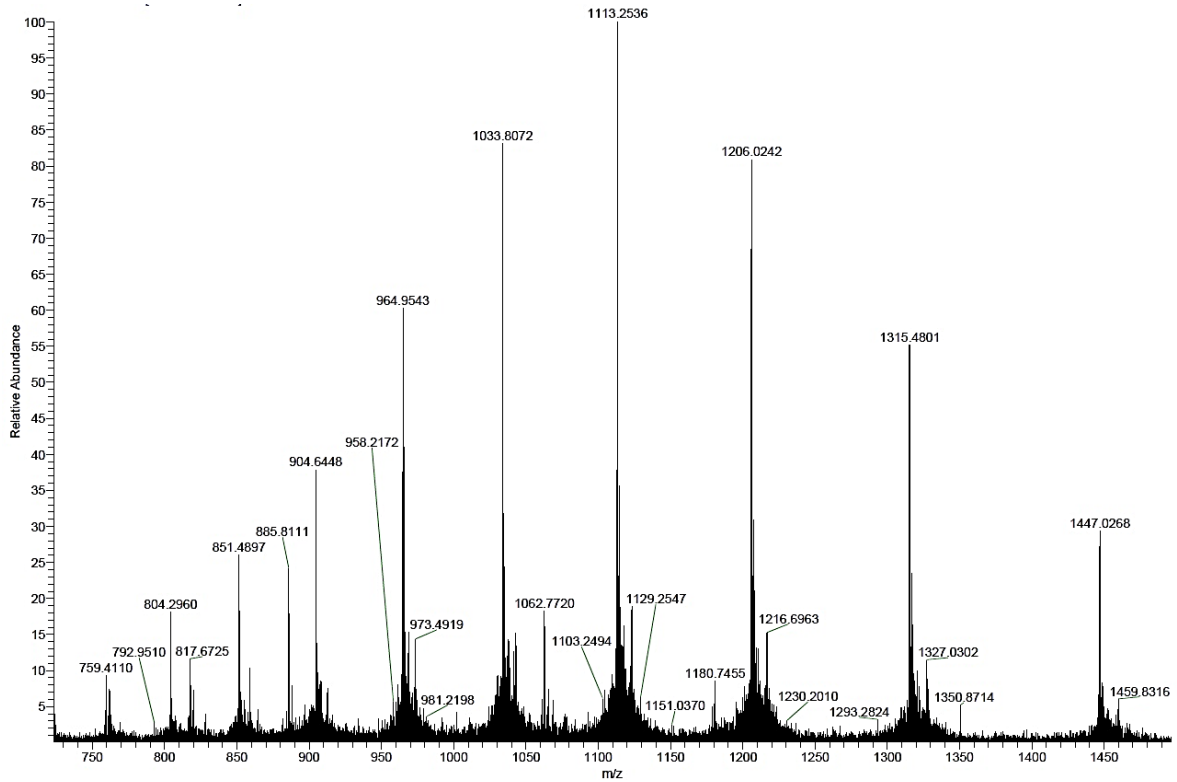


Figure S27: HR-MS spectrum of aSyn [1-140] wt (**16**). Visible are peaks from 851.4897 ($[M+17H]^{17+}$) to 1447.0268 ($[M+10H]^{10+}$).

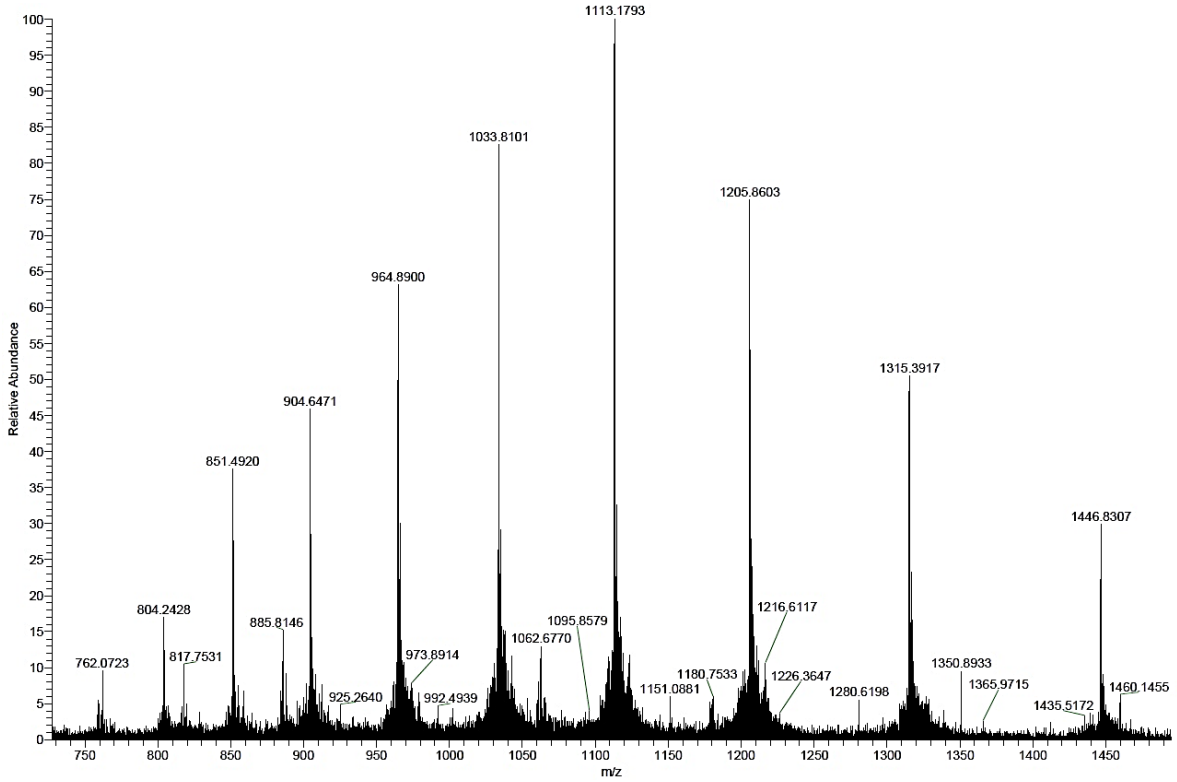


Figure S28: HR-MS spectrum of aSyn [1-140] E46K (**13**). Visible are peaks from 851.4920 ($[M+17H]^{17+}$) to 1446.8307 ($[M+10H]^{10+}$).

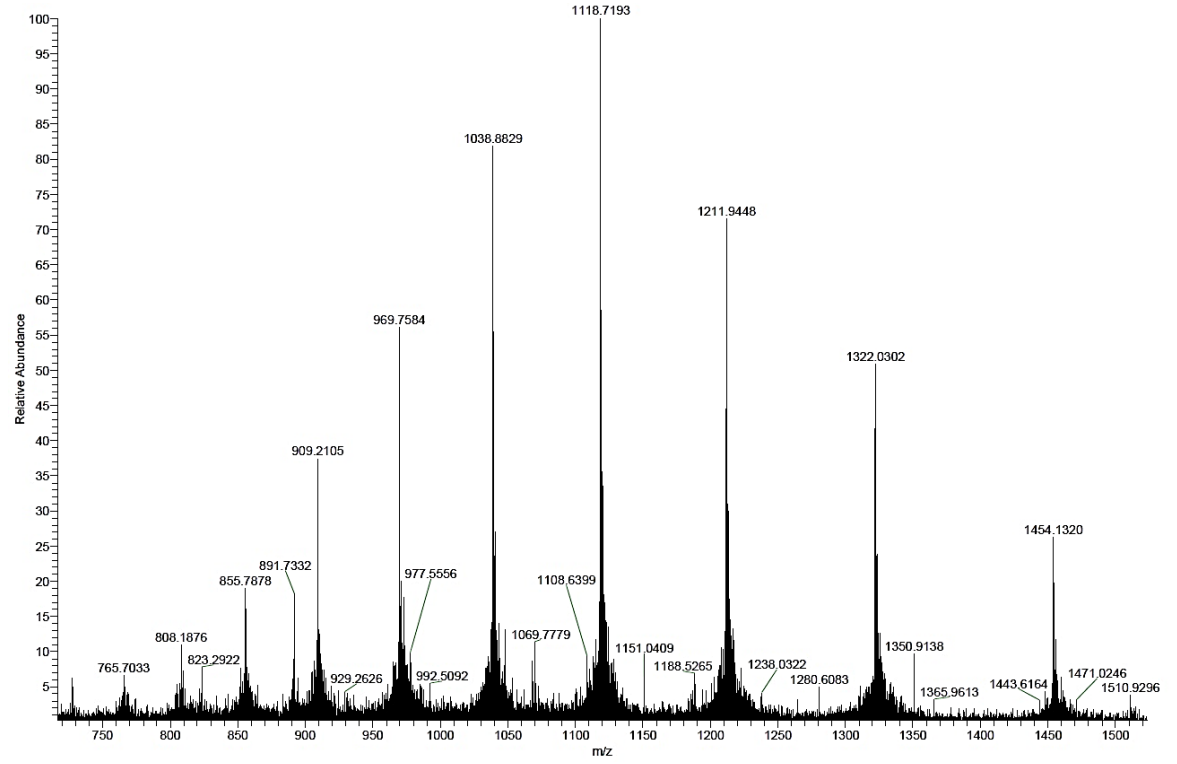


Figure S29: HR-MS spectrum of aSyn [1-140] E46CEL (**14**). Visible are peaks from 855.7878 ($[M+17H]^{17+}$) to 1454.1320 ($[M+10H]^{10+}$).

8.3 UHPLC Chromatograms

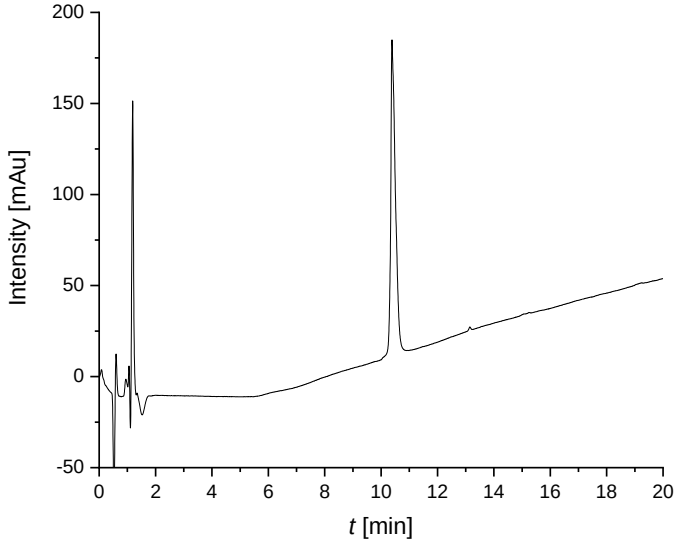


Figure S30: UHPLC chromatogram of Pep1-CML (**1**)

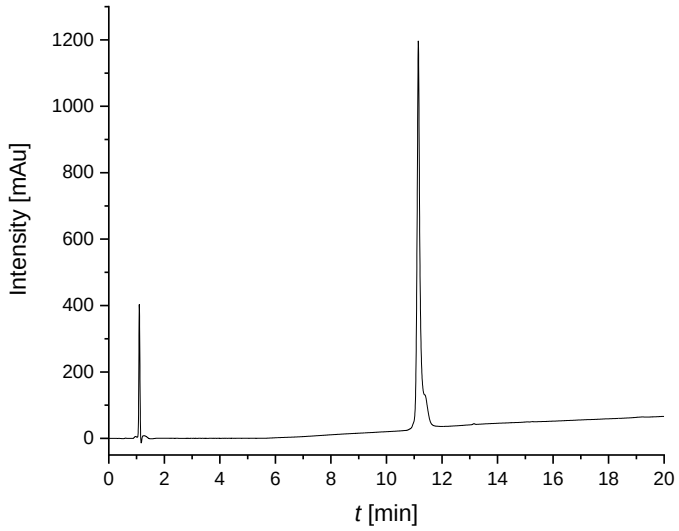


Figure S31: UHPLC chromatogram of Pep2-CML (**2**)

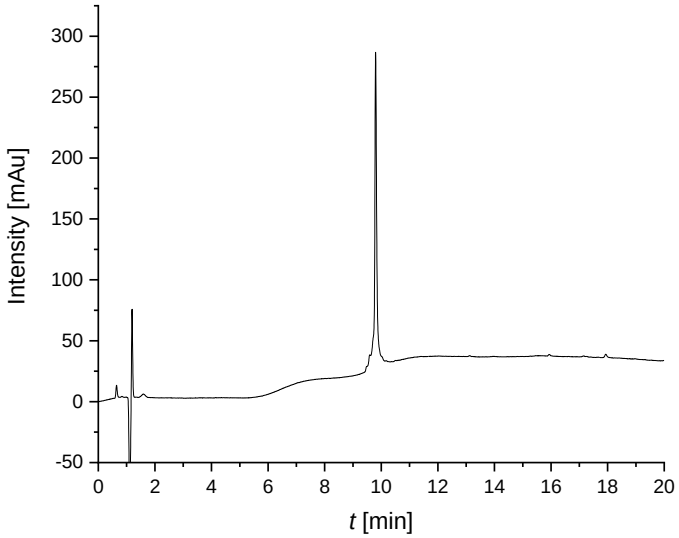


Figure S32: UHPLC chromatogram of Pep1-CEL (**3**)

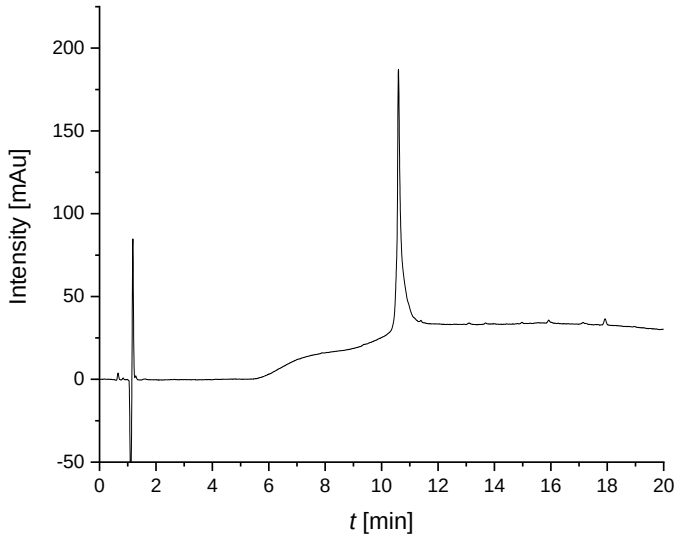


Figure S33: UHPLC chromatogram of Pep2-CEL (**4**)

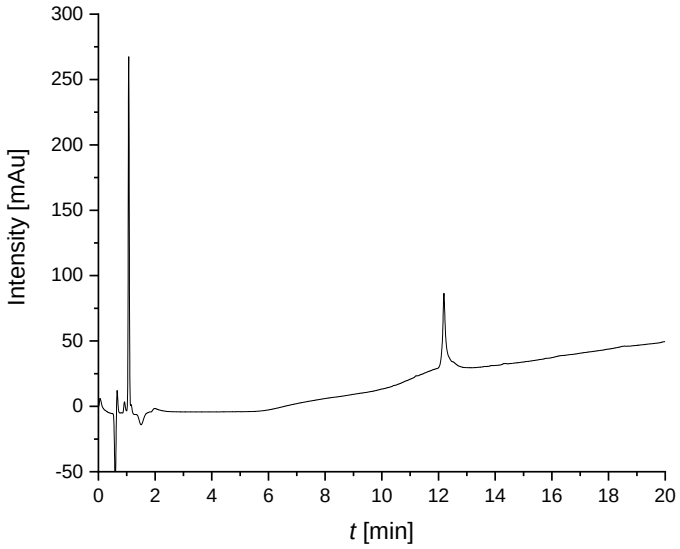


Figure S34: UHPLC chromatogram of aSyn *N*Term [1-68] wt (**15**)

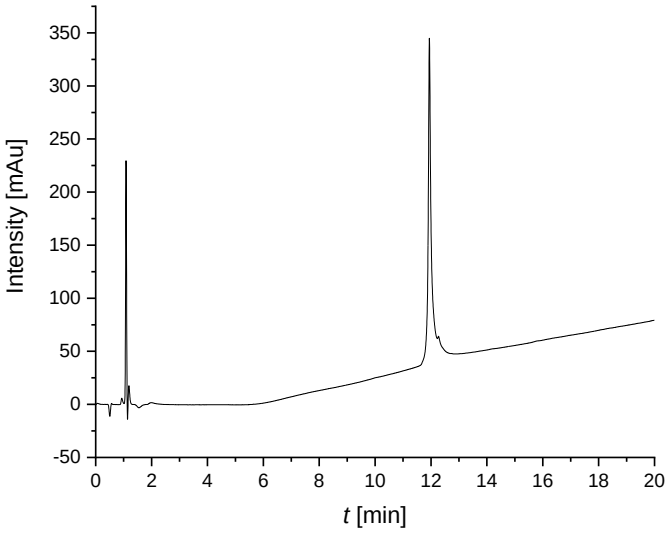


Figure S35: UHPLC chromatogram of aSyn *N*Term [1-68] E46K (**8**)

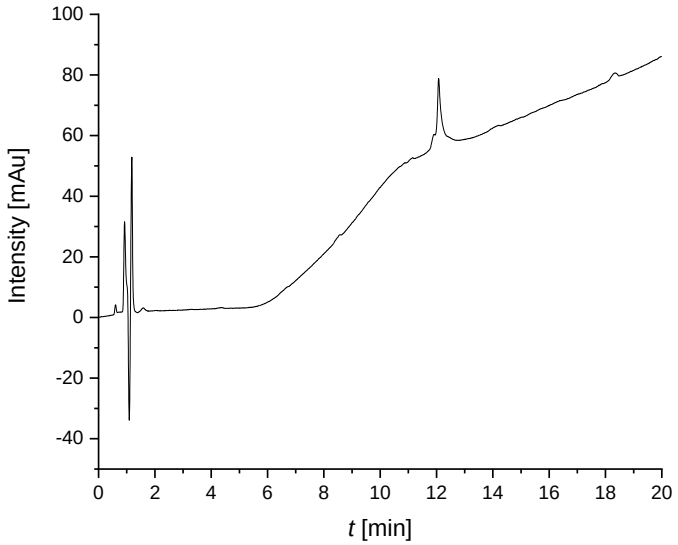


Figure S36: UHPLC chromatogram of aSyn *N*Term [1-68] E46CEL (**9**)

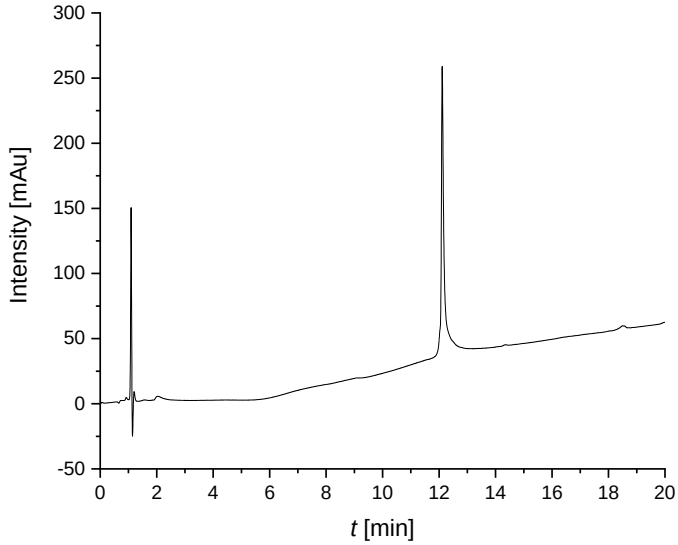


Figure S37: UHPLC chromatogram of aSyn [69-106] A69Thz (**10**)

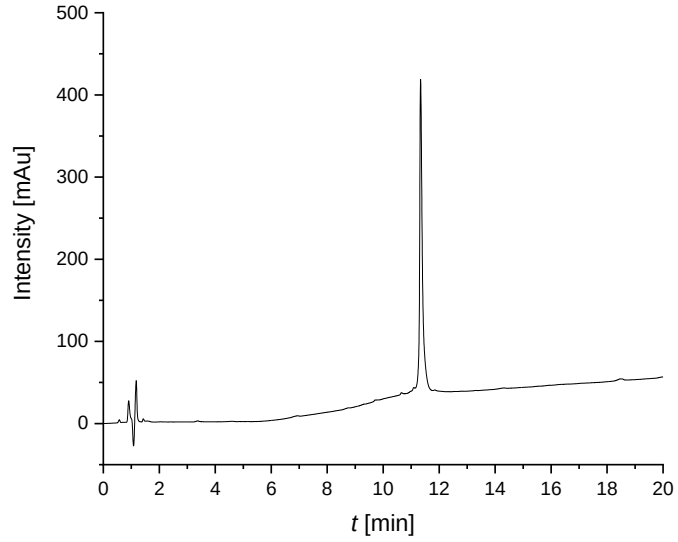


Figure S38: UHPLC chromatogram of aSyn [107-140] A107C (**11**)

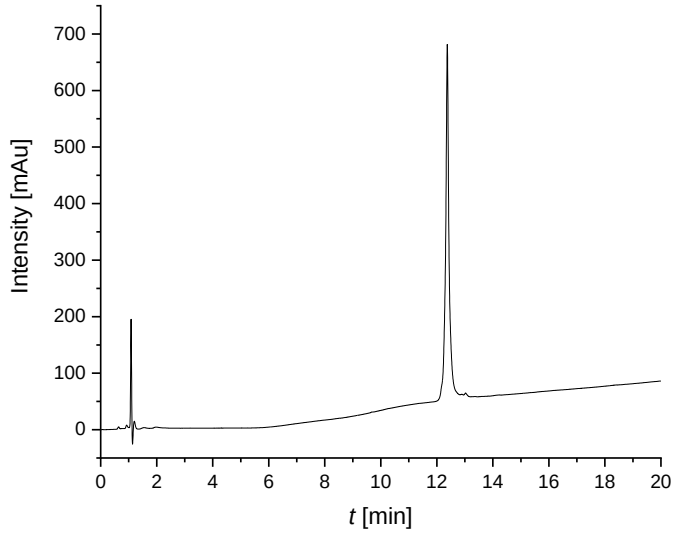


Figure S39: UHPLC chromatogram of aSyn [69-140] A69C, A107C (**12**)

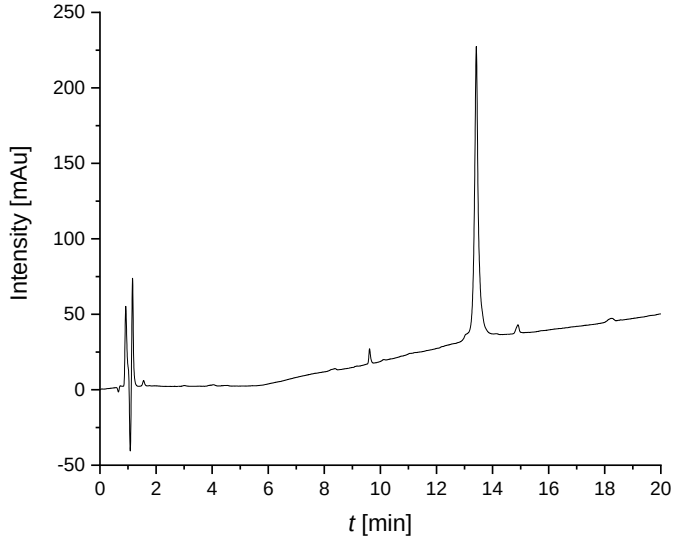


Figure S40: UHPLC chromatogram of aSyn [1-140] wt, A69C, A107C

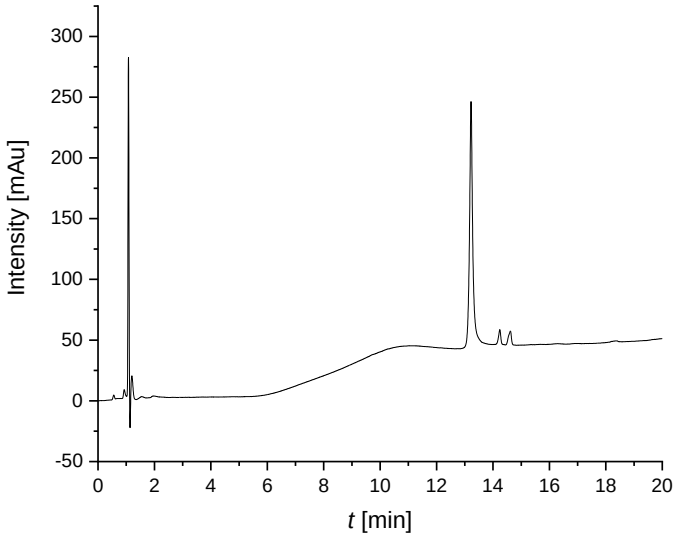


Figure S41: UHPLC chromatogram of aSyn [1-140] E46K, A69C, A107C

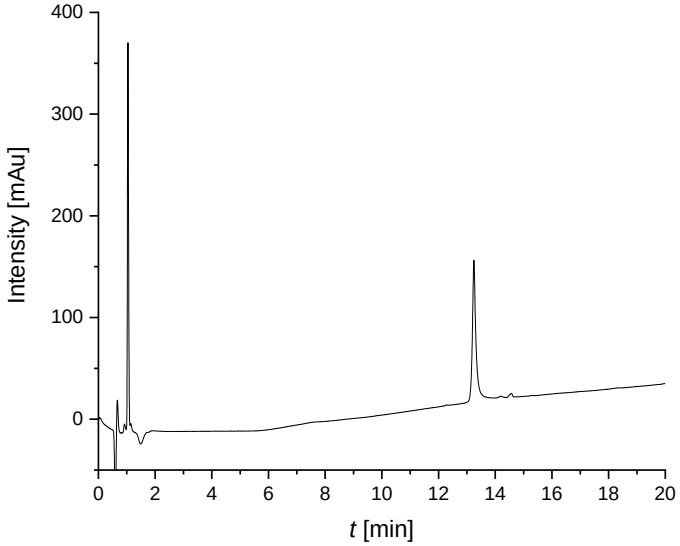


Figure S42: UHPLC chromatogram of aSyn [1-140] E46CEL, A69C, A107C

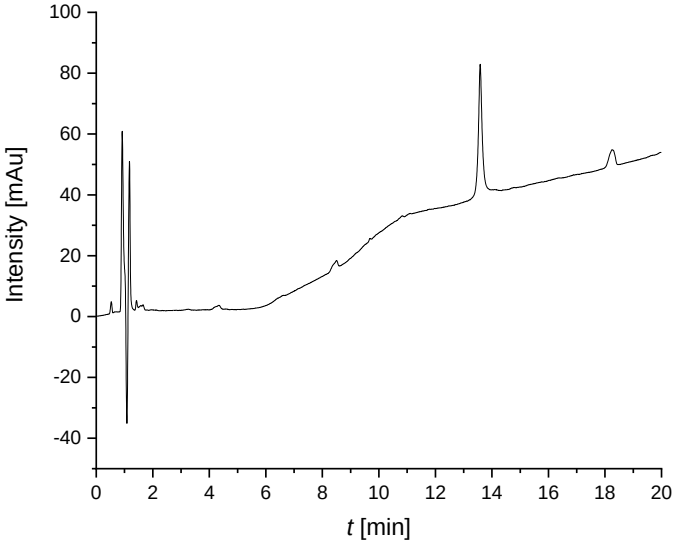


Figure S43: UHPLC chromatogram of aSyn [1-140] wt (**16**)

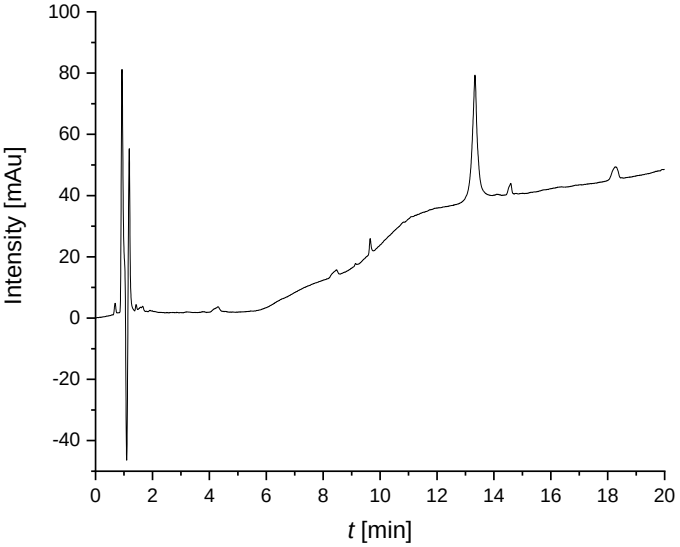


Figure S44: UHPLC chromatogram of aSyn [1-140] E46K (**13**)

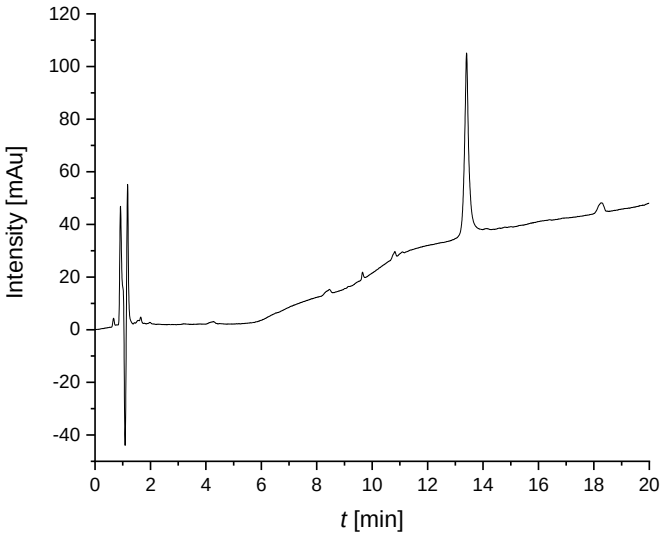


Figure S45: UHPLC chromatogram of aSyn [1-140] E46CEL (**14**)

9. References

[1] C. N. Pace, F. Vajdos, L. Fee, G. Grimsley, T. Gray, *Protein Sci.* **1995**, *4*, 2411–2423.

[2] S. Eissler, M. Kley, D. Bächle, G. Loidl, T. Meier, D. Samson, *J. Pept. Sci.* **2017**, *23*, DOI 10.1002/psc.3021.

[3] M. Gude, J. Ryf, P. D. White, *Lett. Pept. Sci.* **2002**, *9*, 203–206.

[4] S. K. Singh, J. M. Collins, *Pept. Synth. Methods Protoc.* **2020**, 95–109.