

Chemistry–A European Journal

Supporting Information

Chemical Synthesis of Alpha-Synuclein Proteins via Solid-Phase Peptide Synthesis and Native Chemical Ligation

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Table of contents

1. Materials	S2
2. Supplementary methods.....	S2
3. Supplementary experimental section	S6
3.1. Supplementary general procedures	S6
3.2. Peptide and protein synthesis	S8
3.2.1. Synthesis of peptide fragments	S8
3.2.2. Synthesis of thioester conversion products.....	S9
3.2.3. Synthesis of products of first ligation	S10
3.2.4. Synthesis of products of Thz conversion.....	S11
3.2.5. Synthesis of products of second ligation	S11
3.2.6. Synthesis of desulfurized products.....	S13
4. Supplementary analytical data	S15
4.1. UHPLC data of desulfurized products.....	S15
4.2. ESI-HRMS data of desulfurized products	S17
5. References	S20

1. Materials

All chemicals were used as purchased. 2-chlorotrityl chloride resin (2-CTC) resin (100-200 mesh, 1.60 mmol/g) for solid-phase peptide synthesis (SPPS) was obtained from Iris Biotech GmbH (Marktredwitz, Germany). Fluorenylmethoxycarbonyl (Fmoc)- and side-chain protected amino acids (AAs) were acquired from Carbolution Chemicals GmbH (St. Ingbert, Germany), Iris Biotech GmbH, GL Biochem Ltd. (Shanghai, China), Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), Fluorochem Ltd. (Hadfield, United Kingdom), and BLD Pharmatech Ltd. (Shanghai, China). Piperidine for peptide synthesis was obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). *N,N'*-Diisopropylcarbodiimide (DIC) was purchased from Iris Biotech GmbH, ethyl (2*E*)-2-cyano-2-hydroxyiminoacetate (Oxyma Pure) and [dimethylamino(triazolo[4,5-*b*]pyridin-3-yloxy)methylidene]-dimethylazanium hexafluorophosphate (HATU) from Carl Roth GmbH & Co. KG. 3-Hydroxytriazolo[4,5-*b*]pyridine (HOAt) was supplied by Chemtour Biotech Co., Ltd. (Suzhou Industrial Park, Jiangsu Province, China). *N,N*-Dimethylformamide (DMF) for peptide synthesis in laboratory reagent grade was supplied by Thermo Fisher Scientific GmbH (Schwerte, Germany). Acetonitrile (ACN) was used in high-performance liquid chromatography (HPLC) grade quality and obtained from Sigma-Aldrich. Dichloromethane (DCM) and methanol (MeOH) were used in HPLC grade quality and supplied by Thermo Fisher Scientific GmbH. Deionized water (H₂O) was purified with an arium® mini water purification system from Sartorius (Göttingen, Germany) before use in HPLC. *N,N*-Diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), acetic acid anhydride (Ac₂O), sodium chloride (NaCl), and disodium hydrogen phosphate (Na₂HPO₄) were purchased from Carl Roth GmbH & Co. KG. Tri(propan-2-yl)silane (TIPS), mercaptophenylacetic acid (MPAA), guanidine hydrochloride (Gn*HCl), trisodium phosphate (Na₃PO₄), 2-methyl-2-propanethiol (*t*-BuSH), hydrazine hydrate, diethyl ether (Et₂O), and hydrochloric acid (HCl) 37% were obtained from Sigma-Aldrich. Ethane-1,2-dithiol (EDT) was acquired from Merck KGaA (Darmstadt, Germany). Thioanisole and 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044*2HCl) were supplied by TCI Co., Ltd. (Taipeh, Taiwan). Acetylacetone (acac) was purchased from abcr GmbH (Karlsruhe, Germany), Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP*HCl) and 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) from Fluorochem Ltd., O-methylhydroxylamine hydrochloride from Acros Organics (Thermo Fisher Scientific, Schwerte, Germany), and sodium hydroxide (NaOH) from Grüssing GmbH (Filsum, Germany). Potassium chloride (KCl) was obtained from AppliChem GmbH (Darmstadt, Germany), and potassium dihydrogen phosphate (KH₂PO₄) from VWR International (Darmstadt, Germany).

2. Supplementary methods

Determination of resin loading for hydrazine modified 2-CTC resin and Fmoc-Ala-preloaded 2-CTC resin. The resin loading was determined on a Jasco (Pfungstadt, Germany) V-650 spectrophotometer. A defined amount (5.00 mg) of Fmoc-Ala-NH-NH-2-CTC resin or Fmoc-Ala-O-2-CTC resin was incubated with 20% piperidine in DMF (500 µL, r.t., 10 min). The solution was transferred to another tube and the resin was treated again following the same protocol, then solutions were

combined. The absorbance of the dibenzofulvene-piperidine adduct^[1] in a dilution series of the collected solution was measured at a wavelength of 300 nm. The measurement was performed at 25 °C in a SUPRASIL quartz cuvette from Hellma Analytics (Müllheim, Germany) with a volume of 3500 µL and a path length of 10 mm. Blank measurement was performed with 20% piperidine in DMF and subtracted. The concentration of the adduct was calculated via Lambert-Beer's law $A = \epsilon \cdot c \cdot d$ with A = absorbance, ϵ = molar extinction coefficient ($\epsilon_{300 \text{ nm}}$ (dibenzofulvene-piperidine adduct) = 7800 L·mol⁻¹·cm⁻¹)^[2], c = concentration in mol/L and d = path length in cm. The resin loading in mmol/g was calculated considering the mass of the weighed in resin.

MW-SPPS. All peptides were synthesized in a CEM (Kamp-Lintfort, Germany) Liberty Blue™ peptide synthesizer in 0.05 mmol scale. DMF in peptide grade quality was used as solvent for AAs (0.2 M). AAs were used *N*-terminally Fmoc-protected (except from Boc-protected thiazolidine-4-carboxylic acid (Boc-Thz-OH) used as Cys precursor) and carried acid-labile protecting groups in the side-chains (Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-His(Boc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ser(PO(OBzl)OH)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, other AAs were not side-chain protected). Piperidine (20% in DMF) containing 0.1 M Oxyma was used as deprotection solution. DIC (0.5 M in DMF) was used as activator and Oxyma (0.5 M in DMF) containing 0.1 M DIPEA was used as activator base. For the synthesis of peptide hydrazides, hydrazine modified 2-CTC resin was used and for the synthesis of C-terminal peptide acids, Fmoc-Ala-preloaded 2-CTC resin was used. Resins were swollen in DMF for at least 20 min right before synthesis. The automated peptide synthesis was done in alternating cycles of double deprotection (2x3 mL deprotection solution), washing (4x3 mL DMF), double coupling (for each coupling: 1.25 mL amino acid solution (5 equiv), 1 mL activator solution (10 equiv) and 0.5 mL activator base solution (5 equiv Oxyma+1 equiv DIPEA)), and washing (4x3 mL DMF). Conditions for deprotections and couplings are shown in Table S1. After the synthesis was finished, final deprotection was carried out (1x3 mL deprotection solution) followed by washing (4x4 mL DMF). If final deprotection was not needed, only washing (4x4 mL DMF) was performed. As the synthesis was finished, the resin was transferred to a BD (Heidelberg, Germany) syringe with a frit, washed with DMF (5x5 mL) and DCM (5x5 mL), and dried under vacuum.

Table S1. Methods used for MW-SPPS.

Method	Conditions		
	Temperature [°C]	Power [W]	Time [s]
Deprotection A (CTC-Standard)	60	60	60
Deprotection B (Room temperature (r.t.) deprotection)	25	0	300
Final deprotection A ^[a] (Standard)	75	160	15
	90	30	50

Final deprotection B ^[b] (Conventional final deprotection)	Deprotection 1	25	0	300
	Deprotection 2	25	0	600
Coupling A ^[a] (Hydrazides)		60	75	30
		70	50	330
Coupling B ^[a] (His, phosphopeptides)		25	0	120
		50	35	600

[a] Given conditions belong to one deprotection or one coupling step in a cycle, respectively, and were repeated in case of double deprotection or double coupling in this cycle. [b] Final deprotection B was exceptionally carried out as double deprotection with different conditions for each deprotection step.

Semi-preparative HPLC purification. Purification was done in a semi-preparative scale either on a Jasco HPLC system with two semi-preparative pumps PU-4086, the UV/Vis detector UV-2075, the column oven CO-4060, and the fraction collector Advantec CHF122SC or on a Jasco HPLC system with the pumps PU-2080 Plus, the detectors UV-2075 Plus, the degasser DG-2080-53, and the column oven Pharmacia LKB HPLC Column Oven 2155. Crude peptides were prepared either in H₂O+0.1% TFA (*N*-terminal peptide hydrazides) or in H₂O/HFIP (2:1)+0.07% TFA and sonicated before injection. Ligation, thiazolidine (Thz) conversion and desulfurization reaction mixtures were diluted with H₂O+0.1% TFA before injection. Purification was done using a C4 reverse-phase column (Nucleodur® 300-5 C4ec from Macherey-Nagel (Düren, Germany), column size: 250x10 mm, pore size: 300 Å, particle size: 5 µM), and H₂O+0.1% TFA as solvent A and ACN+0.1% TFA as solvent B. Different linear gradients over 30 min (method A: 20-45% B, method B: 25-50% B, method C: 15-35% B) were used at a flow rate of 5 mL/min and a temperature of 50 °C. Detection was performed at 215 and 254 nm. Data were recorded applying the software ChromNAV Control Center. After HPLC purification, collected product fractions were lyophilized, and stored at -20 °C.

Ultra high-performance liquid chromatography (UHPLC) analysis. Analysis of product purity and reaction monitoring was done by UHPLC using a Thermo Fisher Scientific UltiMate 3000 system with Dionex UltiMate 3000 pump, autosampler, column compartment and diode array detector. 10 µL of purified product in H₂O/ACN were injected right after semi-preparative HPLC purification. In terms of reaction monitoring, 10 µL of a mixture of 1 µL aliquot diluted with 49 µL phosphate-buffered saline (PBS) buffer and 50 µL H₂O+0.1%TFA were injected. A C18 reverse-phase column (ACE® Excel™ 100-2 C18 from Advanced Chromatography Technologies Ltd. (Aberdeen, United Kingdom), column size: 100x2.1 mm, pore size: 100 Å, particle size: 2 µM) was used with H₂O+0.1% TFA as solvent A and ACN+0.085% TFA as solvent B, running a method with a linear gradient of 5-75% B over 15 min. Flow rate was set to 0.3 mL/min and temperature to 50 °C. Detection was performed at 215 and 254 nm. Data were recorded and analyzed applying the software Chromeleon 7. The peak purity was calculated as area under the curve in %.

Electrospray ionization (high-resolution) mass spectrometry (ESI-(HR)MS). For characterization of the peptides, ESI-HR mass spectra were measured on a maXis (ESI-QTOF-MS) spectrometer from

Bruker Daltonik GmbH (Bremen, Germany) in positive ion mode. The detected ion masses were reported in mass-to-charge ratio (m/z).

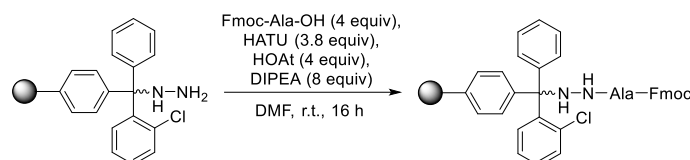
Liquid chromatography coupled to mass spectrometry (LC-MS). For characterization of products, HPLC coupled to ESI-HRMS was performed. LC was done on a Thermo Scientific Accela HPLC system with Accela pump and autosampler, and a Thermo Surveyor PDA detector (200-600 nm). 10 μ L of purified product in H₂O/ACN were injected. A C18 reverse-phase column (Kinetex® 100-5 C18 from Phenomenex (Aschaffenburg, Germany), column size: 150x2.1 mm, pore size: 100 Å, particle size: 5 μ M) was used with H₂O+0.05% formic acid as solvent A and MeOH+0.05% formic acid as solvent B, running a linear gradient of 20-100% B over 15 min at a flow rate of 0.2 mL/min, and at a temperature of 40 °C. Mass spectra were recorded on a LC-coupled LTQ Orbitrap XL™ device from Thermo Fisher Scientific GmbH in positive ion mode and reported in m/z .

Concentration determination. Concentration of protein samples was determined on a Thermo Fisher Scientific NanoDrop™ One^C spectrophotometer. H₂O (2 μ L) was used as blank. Samples were dissolved in water (mass concentration: 1 mg/mL) and 2 μ L of each sample were applied. Absorptions were measured at 280 nm and the concentrations were then calculated by Lambert-Beer's law $A = \epsilon \cdot c \cdot d$ with A = measured absorbance, ϵ = molar extinction coefficient in $L \cdot mol^{-1} \cdot cm^{-1}$, c = concentration in mol/L (was converted in mg/mL by including the molecular weights of the proteins) and d = path length in cm ($d = 1$ cm). The molar extinction coefficient was assumed as ϵ (α -Synuclein) = $(n(Trp) \cdot 5500) + (n(Tyr) \cdot 1490) + (n(Cys) \cdot 125) = 4 \cdot 1490 = 5960 L \cdot mol^{-1} \cdot cm^{-1}$ for all synthesized α -Synuclein variants with n being the quantity of the corresponding AA residue in the protein and 5500, 1490, and 125 being the AA molar absorptivities at 280 nm.^[3]

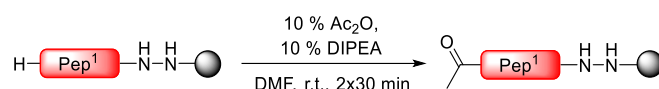
Circular dichroism (CD) spectroscopy. CD spectra were recorded on a Jasco J-1500 CD spectrometer with a Haake WKL 26 cooling system. Bidistilled H₂O was used as solvent for the samples. Each protein sample (volume: 200 μ L, concentration: 5 μ mol/L) was measured from 260 to 185 nm at 20 °C in a quartz cuvette from Hellma Analytics (QS quartz SUPRASIL) with a path length of 1 mm. CD scale was set to 200 mdeg/1.0 dOD. A bandwidth of 1 nm, a DIT of 1 s, a data pitch of 1 nm, and a scanning speed of 50 nm/min were used. Five accumulations were done for each experiment. Bidistilled H₂O served as blank and blank measurement was subtracted from sample measurements. Measured ellipticity θ in mdeg was converted into molar ellipticity θ_M in $deg \cdot cm^2 \cdot dmol^{-1}$ via the formula: $\theta_M = (\theta \cdot MW) / (10 \cdot N \cdot d \cdot c)$ ^[4] with MW = molecular weight of the protein in g/mol, N = number of amide bonds in the protein, d = path length in cm and c = concentration in mg/mL.

3. Supplementary experimental section

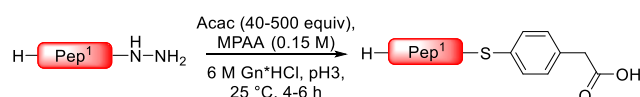
3.1. Supplementary general procedures



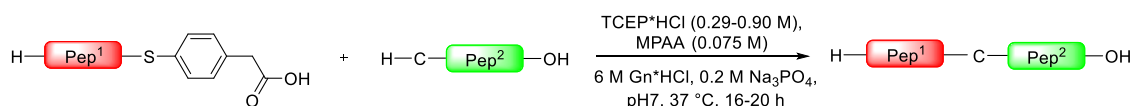
Scheme S1. Fmoc-Ala-preloading of hydrazine modified 2-CTC resin for the photometrical determination of the initial resin loading. Fmoc-protected Ala coupled to the hydrazine modified resin under use of the AA activating reagents HATU and HOAt was used to estimate the extent of hydrazine modification.



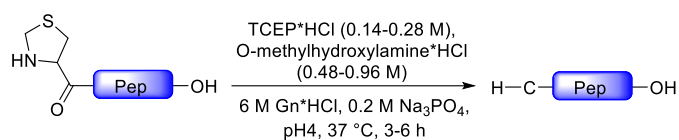
Scheme S2. Acetylation of *N*-terminal peptide hydrazides on resin using Ac₂O as acetylating reagent. After acetylation, peptide hydrazides were cleaved from resin and used in the synthesis of *N*-terminally acetylated protein variants.



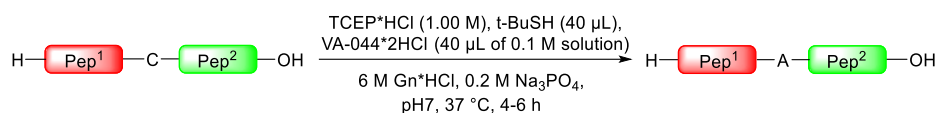
Scheme S3. Thioester conversion of a peptide hydrazides using acac to form a pyrazole intermediate via Knorr pyrazole synthesis and MPAA for the thioester formation. Thioester conversions were performed in guanidine-containing thioester conversion buffer at a pH of 3 for 4-6 h.



Scheme S4. Native chemical ligation (NCL) of peptide thioesters and Cys peptides applying TCEP as reducing agent and MPAA as NCL catalyst. NCLs were performed in guanidine-containing ligation buffer at a pH of 7 for 16-20 h.



Scheme S5. Thz conversion of peptides carrying *N*-terminal Thz moieties into Cys peptides as activation for a further ligation step applying O-methylhydroxylamine. Thz conversions were performed in guanidine-containing ligation buffer at a pH of 4 for 3-6 h.



Scheme S6. Radical desulfurization of Cys residues, which were introduced during synthesis to facilitate NCL, into the native Ala residues of the peptide or protein of interest. Desulfurizations were performed in guanidine-containing ligation buffer at a pH of 7 for 4-6 h.

3.2. Peptide and protein synthesis

3.2.1. *Synthesis of peptide fragments*

H- α -Syn-1-68-NH-NH₂: H- α -Syn-1-68-NH-NH₂ was prepared via MW-SPPS with Deprotection A, Coupling A, and Final deprotection A on hydrazine modified 2-CTC resin (0.05 mmol, 0.35 mmol/g). Coupling B was used for His coupling. Cleavage was done according to General procedure D. Purification was performed via semi-preparative HPLC using method A. The product (5.8 mg, 0.83 μ mol, 2%) was obtained as a white solid in a purity of 99%. UHPLC: t_R = 12.377 min. MS (ESI) m/z : 1737.7 [M+4H]⁴⁺, 1390.4 [M+5H]⁵⁺, 1159.0 [M+6H]⁶⁺, 993.5 [M+7H]⁷⁺, 869.5 [M+8H]⁸⁺, 773.1 [M+9H]⁹⁺. HR-MS (ESI) m/z : calc. for C₃₀₂H₅₁₇N₈₆O₉₆S₂ ([M+5H]⁵⁺): 1390.5547, found: 1390.5619; calc. for C₃₀₂H₅₁₈N₈₆O₉₆S₂ ([M+6H]⁶⁺): 1158.9635, found: 1158.9671; calc. for C₃₀₂H₅₁₉N₈₆O₉₆S₂ ([M+7H]⁷⁺): 993.5412, found: 993.5445; calc. for C₃₀₂H₅₂₀N₈₆O₉₆S₂ ([M+8H]⁸⁺): 869.4744, found: 869.4778.

Acetyl- α -Syn-1-68-NH-NH₂: Acetyl- α -Syn-1-68-NH-NH₂ was prepared via MW-SPPS with Deprotection A, Coupling A, and Final deprotection A on hydrazine modified 2-CTC resin (0.05 mmol, 0.35 mmol/g). Coupling B was used for His coupling. Acetylation of the *N*-terminus was carried out on resin following General procedure E. Cleavage was done according to General procedure D. Purification was performed via semi-preparative HPLC using method B. The product (8.3 mg, 1.19 μ mol, 2%) was obtained as a white solid in a purity of 96%. UHPLC: t_R = 13.147 min. MS (ESI) m/z : 1748.5 [M+4H]⁴⁺, 1399.0 [M+5H]⁵⁺, 1166.0 [M+6H]⁶⁺, 999.5 [M+7H]⁷⁺, 874.7 [M+8H]⁸⁺. HR-MS (ESI) m/z : calc. for C₃₀₄H₅₁₉N₈₆O₉₇S₂ ([M+5H]⁵⁺): 1398.9569, found: 1398.9616; calc. for C₃₀₄H₅₁₈N₈₆O₉₇S₂Na ([M+4H+Na]⁵⁺): 1403.3532, found: 1403.3571; calc. for C₃₀₄H₅₂₀N₈₆O₉₇S₂ ([M+6H]⁶⁺): 1165.9653, found: 1165.9692; calc. for C₃₀₄H₅₁₉N₈₆O₉₇S₂Na ([M+5H+Na]⁶⁺): 1169.6289, found: 1169.6315; calc. for C₃₀₄H₅₂₁N₈₆O₉₇S₂ ([M+7H]⁷⁺): 999.5427, found: 999.5453; calc. for C₃₀₄H₅₂₀N₈₆O₉₇S₂Na ([M+6H+Na]⁷⁺): 1002.6830, found: 1002.6856.

H- α -Syn-1-68-NH-NH₂ [A53T]: H- α -Syn-1-68-NH-NH₂ [A53T] was prepared via MW-SPPS with Deprotection A, Coupling A, and Final deprotection A on hydrazine modified 2-CTC resin (0.05 mmol, 0.35 mmol/g). Coupling B was used for His coupling. Cleavage was done according to General procedure D. Purification was performed via semi-preparative HPLC using method A. The product (8.2 mg, 1.18 μ mol, 2%) was obtained as a white solid in a purity >99%. UHPLC: t_R = 12.250 min. MS (ESI) m/z : 1745.7 [M+4H]⁴⁺, 1396.6 [M+5H]⁵⁺, 1164.0 [M+6H]⁶⁺, 1001.0 [M+6H+Na]⁷⁺, 877.8 [M+7H+K]⁸⁺. HR-MS (ESI) m/z : calc. for C₃₀₃H₅₁₉N₈₆O₉₇S₂ ([M+5H]⁵⁺): 1396.5568, found: 1396.5592; calc. for C₃₀₃H₅₂₀N₈₆O₉₇S₂ ([M+6H]⁶⁺): 1163.9653, found: 1163.9648.

H- α -Syn-69-106-NH-NH₂ [A69Thz]: H- α -Syn-69-106-NH-NH₂ [A69Thz] was prepared via MW-SPPS with Deprotection A, Coupling A, and without final deprotection on hydrazine modified 2-CTC resin (0.05 mmol, 0.35 mmol/g) because Boc-Thz-OH was used as *N*-terminal residue. Removal of *N*-terminal Boc group and cleavage were done in one step according to General procedure D. Purification was performed via semi-preparative HPLC using method A. The product (26.0 mg, 6.91 μ mol, 14%) was obtained as a white solid in a purity of 96%. UHPLC: t_R = 12.260 min. MS (ESI) m/z : 1254.7 [M+3H]³⁺,

946.7 [M+3H+Na]⁴⁺, 765.2 [M+3H+Na+K]⁵⁺. HR-MS (ESI) *m/z*: calc. for C₁₆₁H₂₇₄N₄₇O₅₄S ([M+3H]³⁺): 1254.6636, found: 1254.6664; calc. for C₁₆₁H₂₇₃N₄₇O₅₄SNa ([M+2H+Na]³⁺): 1261.9909, found: 1261.9915; calc. for C₁₆₁H₂₇₅N₄₇O₅₄S ([M+4H]⁴⁺): 941.2495, found: 941.2506; calc. for C₁₆₁H₂₇₄N₄₇O₅₄SNa ([M+3H+Na]⁴⁺): 946.7450, found: 946.7444; calc. for C₁₆₁H₂₇₅N₄₇O₅₄SNa ([M+4H+Na]⁵⁺): 757.5974, found: 757.5958.

H-α-Syn-107-140-OH [A107C]: H-α-Syn-107-140-OH [A107C] was prepared via MW-SPPS with Deprotection A, Coupling A, and Final deprotection A on Fmoc-Ala-preloaded 2-CTC resin (0.05 mmol, 0.54 mmol/g). Cleavage was done according to General procedure D. Purification was performed via semi-preparative HPLC using method A. The product (49.2 mg, 12.64 μmol, 25%) was obtained as a white solid in a purity of 92%. UHPLC: *t_R* = 11.560 min. MS (ESI) *m/z*: 1312.6 [M+H+2Na]³⁺, 990.2 [M+H+3Na]⁴⁺. HR-MS (ESI) *m/z*: calc. for C₁₆₅H₂₃₉N₃₇O₆₆S₃Na ([M+2H+Na]³⁺): 1305.1863, found: 1305.1869; calc. for C₁₆₅H₂₃₈N₃₇O₆₆S₃Na₂ ([M+H+2Na]³⁺): 1312.5136, found: 1312.5144; calc. for C₁₆₅H₂₃₉N₃₇O₆₆S₃Na₂ ([M+2H+2Na]⁴⁺): 984.6371, found: 984.6361; calc. for C₁₆₅H₂₃₈N₃₇O₆₆S₃Na₃ ([M+H+3Na]⁴⁺): 990.1325, found: 990.1312; calc. for C₁₆₅H₂₃₇N₃₇O₆₆S₃Na₄ ([M+4Na]⁴⁺): 995.6280, found: 995.6269.

H-α-Syn-107-140-OH [A107C, P-S129]: H-α-Syn-107-140-OH [A107C, P-S129] was prepared via MW-SPPS from AA 139-130 with Deprotection A and Coupling A and for AA 129-107 with Deprotection B, Coupling B, and Final deprotection B on Fmoc-Ala-preloaded 2-CTC resin (0.05 mmol, 0.69 mmol/g). Cleavage was done according to General procedure D. Purification was performed via semi-preparative HPLC using method C. The product (30.8 mg, 7.76 μmol, 16%) was obtained as a white solid in a purity of 98%. UHPLC: *t_R* = 11.337 min. HR-MS (ESI) *m/z*: calc. for C₁₆₅H₂₄₁N₃₇O₆₉PS₃ ([M+3H]³⁺): 1324.5141, found: 1324.5129; calc. for C₁₆₅H₂₄₀N₃₇O₆₉PS₃Na ([M+2H+Na]³⁺): 1331.8414, found: 1331.8375; calc. for C₁₆₅H₂₃₉N₃₇O₆₉PS₃Na₂ ([M+H+2Na]³⁺): 1339.1687, found: 1339.1623; calc. for C₁₆₅H₂₄₀N₃₇O₆₉PS₃Na₂ ([M+2H+2Na]⁴⁺): 1004.6283, found: 1004.6214.

3.2.2. Synthesis of thioester conversion products

H-α-Syn-1-68-MPAA: H-α-Syn-1-68-MPAA (quantitative conversion: 7.08 mg, 1.00 μmol, 100%) was prepared from H-α-Syn-1-68-NH-NH₂ (6.95 mg, 1.00 μmol, 1 equiv) via General procedure F. After UHPLC monitoring showed completion of the reaction, the reaction mixture was used without further purification for ligation reactions with H-α-Syn-69-140-OH [A69C, A107C] and H-α-Syn-69-140-OH [A69C, A107C, P-S129]. UHPLC: *t_R* = 12.547 min. MS (ESI) *m/z*: 1772.2 [M+4H]⁴⁺, 1417.8 [M+5H]⁵⁺, 1181.6 [M+6H]⁶⁺, 1016.1 [M+6H+Na]⁷⁺, 892.0 [M+6H+2Na]⁸⁺, 795.5 [M+6H+3Na]⁹⁺. HR-MS (ESI) *m/z*: calc. for C₃₁₀H₅₂₁N₈₄O₉₈S₃ ([M+5H]⁵⁺): 1417.7521, found: 1417.7490; calc. for C₃₁₀H₅₂₂N₈₄O₉₈S₃ ([M+6H]⁶⁺): 1181.6280, found: 1181.6277; calc. for C₃₁₀H₅₂₃N₈₄O₉₈S₃ ([M+7H]⁷⁺): 1012.9679, found: 1012.9669; calc. for C₃₁₀H₅₂₂N₈₄O₉₈S₃Na ([M+6H+Na]⁷⁺): 1016.1081, found: 1016.1067; calc. for C₃₁₀H₅₂₃N₈₄O₉₈S₃Na ([M+7H+Na]⁸⁺): 889.2205, found: 889.2242.

Acetyl-α-Syn-1-68-MPAA: Acetyl-α-Syn-1-68-MPAA (quantitative conversion: 4.70 mg, 0.66 μmol, 100%) was prepared from Acetyl-α-Syn-1-68-NH-NH₂ (4.61 mg, 0.66 μmol, 1 equiv) via General

procedure F (in 0.1 mL of thioester conversion buffer). After UHPLC monitoring showed completion of the reaction, the reaction mixture was used without further purification for ligation reactions with H- α -Syn-69-140-OH [A69C, A107C] and H- α -Syn-69-140-OH [A69C, A107C, P-S129]. UHPLC: t_R = 13.333 min. MS (ESI) m/z : 1787.7 [M+3H+Na]⁴⁺, 1434.9 [M+3H+2Na]⁵⁺, 1196.0 [M+4H+2Na]⁶⁺, 1028.4 [M+4H+3Na]⁷⁺. HR-MS (ESI) m/z : calc. for C₃₁₂H₅₂₁N₈₄O₉₉S₃Na₂ ([M+3H+2Na]⁵⁺): 1434.9470, found: 1434.9487; calc. for C₃₁₂H₅₂₂N₈₄O₉₉S₃Na₂ ([M+4H+2Na]⁶⁺): 1195.9570, found: 1195.9590; calc. for C₃₁₂H₅₂₃N₈₄O₉₉S₃Na₂ ([M+5H+2Na]⁷⁺): 1025.2499, found: 1025.2466.

H- α -Syn-1-68-MPAA [A53T]: H- α -Syn-1-68-MPAA [A53T] (quantitative conversion: 3.13 mg, 0.44 μ mol, 100%) was prepared from H- α -Syn-1-68-NH-NH₂ [A53T] (3.07 mg, 0.44 μ mol, 1 equiv) via General procedure F. After UHPLC monitoring showed completion of the reaction, the reaction mixture was used without further purification for ligation reaction with H- α -Syn-69-140-OH [A69C, A107C]. UHPLC: t_R = 12.457 min. HR-MS (ESI) m/z : calc. for C₃₁₁H₅₂₅N₈₄O₉₉S₃ ([M+7H]⁷⁺): 1017.2551, found: 1017.2555; calc. for C₃₁₁H₅₂₆N₈₄O₉₉S₃ ([M+8H]⁸⁺): 890.2241, found: 890.2241; calc. for C₃₁₁H₅₂₇N₈₄O₉₉S₃ ([M+9H]⁹⁺): 791.4222, found: 791.4222; calc. for C₃₁₁H₅₂₈N₈₄O₉₉S₃ ([M+10H]¹⁰⁺): 712.3807, found: 712.3805.

H- α -Syn-69-106-MPAA [A69Thz]: H- α -Syn-69-106-MPAA [A69Thz] (quantitative conversion: 12.47 mg, 3.20 μ mol, 100%) was prepared from H- α -Syn-69-106-NH-NH₂ [A69Thz] (12.04 mg, 3.20 μ mol, 1 equiv) via General procedure F. After UHPLC monitoring showed completion of the reaction, the reaction mixture was used without further purification for ligation reactions with H- α -Syn-107-140-OH [A107C] and H- α -Syn-107-140-OH [A107C, P-S129]. UHPLC: t_R = 12.510 min. MS (ESI) m/z : 1300.0 [M+3H]³⁺, 975.2 [M+4H]⁴⁺, 780.4 [M+5H]⁵⁺. HR-MS (ESI) m/z : calc. for C₁₆₉H₂₇₈N₄₅O₅₆S₂ ([M+3H]³⁺): 1299.9926, found: 1299.9923; calc. for C₁₆₉H₂₇₉N₄₅O₅₆S₂ ([M+4H]⁴⁺): 975.2463, found: 975.2465; calc. for C₁₆₉H₂₈₀N₄₅O₅₆S₂ ([M+5H]⁵⁺): 780.3985, found: 780.3985.

3.2.3. Synthesis of products of first ligation

H- α -Syn-69-140-OH [A69Thz, A107C]: H- α -Syn-69-140-OH [A69Thz, A107C] (quantitative conversion: 24.38 mg, 3.20 μ mol, 100%) was obtained from thioester H- α -Syn-69-106-MPAA [A69Thz] (12.47 mg, 3.20 μ mol, 1 equiv) and Cys peptide H-107-140-OH [A107C] (12.45 mg, 3.20 μ mol, 1 equiv) via NCL according to General procedure G. After UHPLC monitoring showed completion of the reaction, the reaction mixture was used without further purification in Thz conversion reaction. UHPLC: t_R = 12.537 min. MS (ESI) m/z : 1542.5 [M+H+4Na]⁵⁺, 1292.9 [M+6Na]⁶⁺, 1102.1 [M+3H+4Na]⁷⁺, 972.8 [M+H+7Na]⁸⁺. HR-MS (ESI) m/z : calc. for C₃₂₆H₅₀₇N₈₂O₁₂₀S₄Na₂ ([M+3H+2Na]⁵⁺): 1533.6969, found: 1533.6921; calc. for C₃₂₆H₅₀₆N₈₂O₁₂₀S₄Na₃ ([M+2H+3Na]⁵⁺): 1538.0933, found: 1538.0915; calc. for C₃₂₆H₅₀₅N₈₂O₁₂₀S₄Na₄ ([M+H+4Na]⁵⁺): 1542.4897, found: 1542.4899; calc. for C₃₂₆H₅₀₈N₈₂O₁₂₀S₄Na₂ ([M+4H+2Na]⁶⁺): 1278.2486, found: 1278.2458; calc. for C₃₂₆H₅₀₇N₈₂O₁₂₀S₄Na₃ ([M+3H+3Na]⁶⁺): 1281.9123, found: 1281.9131; calc. for C₃₂₆H₅₀₆N₈₂O₁₂₀S₄Na₄ ([M+2H+4Na]⁶⁺): 1285.5759, found: 1285.5754; calc. for C₃₂₆H₅₀₉N₈₂O₁₂₀S₄Na₂ ([M+5H+2Na]⁷⁺): 1095.7856, found: 1095.7824; calc. for C₃₂₆H₅₀₈N₈₂O₁₂₀S₄Na₃ ([M+4H+3Na]⁷⁺): 1099.2122, found: 1099.2121; calc. for C₃₂₆H₅₀₇N₈₂O₁₂₀S₄Na₄

($[M+3H+4Na]^7+$): 1102.0661, found: 1102.0646; calc. for $C_{326}H_{508}N_{82}O_{120}S_4Na_4$ ($[M+4H+4Na]^8+$): 964.4338, found: 964.4340.

H- α -Syn-69-140-OH [A69Thz, A107C, P-S129]: H- α -Syn-69-140-OH [A69Thz, A107C, P-S129] (quantitative conversion: 24.64 mg, 3.20 μ mol, 100%) was obtained from thioester H- α -Syn-69-106-MPAA [A69Thz] (12.47 mg, 3.20 μ mol, 1 equiv) and Cys peptide H-107-140-OH [A107C, P-S129] (12.71 mg, 3.20 μ mol, 1 equiv) via NCL according to General procedure G. After UHPLC monitoring showed completion of the reaction, the reaction mixture was used without further purification in Thz conversion reaction. UHPLC: t_R = 12.467 min. HR-MS (ESI) m/z : calc. for $C_{326}H_{510}N_{82}O_{123}PS_4$ ($[M+5H]^5+$): 1540.8974, found: 1540.8970; calc. for $C_{326}H_{511}N_{82}O_{123}PS_4$ ($[M+6H]^6+$): 1284.2490, found: 1284.2494; calc. for $C_{326}H_{512}N_{82}O_{123}PS_4$ ($[M+7H]^7+$): 1100.9288, found: 1100.9291.

3.2.4. Synthesis of products of Thz conversion

H- α -Syn-69-140-OH [A69C, A107C]: H- α -Syn-69-140-OH [A69Thz, A107C] (24.38 mg, 3.20 μ mol, 1 equiv) was treated with TCEP*HCl and O-methylhydroxylamine*HCl following General procedure H, and purified by semi-preparative HPLC using method A to yield H- α -Syn-69-140-OH [A69C, A107C] (6.10 mg, 0.80 μ mol, 25% over 3 steps) as a white solid in a purity of 98%. UHPLC: t_R = 12.403 min. MS (ESI) m/z : 1529.3 $[M+4H+K]^5+$, 1274.8 $[M+5H+K]^6+$, 1096.1 $[M+5H+Na+K]^7+$, 959.2 $[M+6H+Na+K]^8+$. HR-MS (ESI) m/z : calc. for $C_{325}H_{508}N_{82}O_{120}S_4Na$ ($[M+4H+Na]^5+$): 1526.9005, found: 1526.9031; calc. for $C_{325}H_{510}N_{82}O_{120}S_4$ ($[M+6H]^6+$): 1268.9213, found: 1268.9191; calc. for $C_{325}H_{509}N_{82}O_{120}S_4Na$ ($[M+5H+Na]^6+$): 1272.5850, found: 1272.5846; calc. for $C_{325}H_{511}N_{82}O_{120}S_4$ ($[M+7H]^7+$): 1087.7907, found: 1087.7938; calc. for $C_{325}H_{510}N_{82}O_{120}S_4Na$ ($[M+6H+Na]^7+$): 1090.9310, found: 1090.9287; calc. for $C_{325}H_{511}N_{82}O_{120}S_4Na$ ($[M+7H+Na]^8+$): 954.6905, found: 954.6927.

H- α -Syn-69-140-OH [A69C, A107C, P-S129]: H- α -Syn-69-140-OH [A69Thz, A107C, P-S129] (24.64 mg, 3.20 μ mol, 1 equiv) was treated with TCEP*HCl and O-methylhydroxylamine*HCl following General procedure H, and purified by semi-preparative HPLC using method A to yield H- α -Syn-69-140-OH [A69C, A107C, P-S129] (4.80 mg, 0.62 μ mol, 19% over 3 steps) as a white solid in a purity of 95%. UHPLC: t_R = 12.373 min. MS (ESI) m/z : 1552.1 $[M+2H+3Na]^5+$, 1296.9 $[M+2H+4Na]^6+$, 1114.9 $[M+2H+5Na]^7+$. HR-MS (ESI) m/z : calc. for $C_{325}H_{507}N_{82}O_{123}PS_4Na_3$ ($[M+2H+3Na]^5+$): 1551.6865, found: 1551.6921; calc. for $C_{325}H_{509}N_{82}O_{123}PS_4Na_2$ ($[M+4H+2Na]^6+$): 1289.5763, found: 1289.5811; calc. for $C_{325}H_{508}N_{82}O_{123}PS_4Na_3$ ($[M+3H+3Na]^6+$): 1293.2400, found: 1293.2407; calc. for $C_{325}H_{510}N_{82}O_{123}PS_4Na_2$ ($[M+5H+2Na]^7+$): 1105.4950, found: 1105.4997; calc. for $C_{325}H_{509}N_{82}O_{123}PS_4Na_3$ ($[M+4H+3Na]^7+$): 1108.6353, found: 1108.6378.

3.2.5. Synthesis of products of second ligation

H- α -Syn-1-140-OH [A69C, A107C]: H- α -Syn-1-68-MPAA (7.08 mg, 1.00 μ mol, 1 equiv) and H- α -Syn-69-140-OH [A69C, A107C] (7.61 mg, 1.00 μ mol, 1 equiv) were ligated according to General procedure

G. After UHPLC monitoring showed completion of the reaction, the mixture was purified by semi-preparative HPLC following method B. H- α -Syn-1-140-OH [A69C, A107C] (3.40 mg, 0.45 μ mol, 23% over 2 steps) was obtained as a white solid in a purity of 99%. UHPLC: t_R = 13.633 min. MS (ESI) m/z : 2076.2 [M+7H]⁷⁺, 1816.8 [M+8H]⁸⁺, 1614.9 [M+9H]⁹⁺, 1453.5 [M+10H]¹⁰⁺, 1321.4 [M+11H]¹¹⁺, 1211.5 [M+12H]¹²⁺, 1118.3 [M+13H]¹³⁺, 1039.0 [M+14H]¹⁴⁺, 969.9 [M+15H]¹⁵⁺, 908.6 [M+16H]¹⁶⁺. HR-MS (ESI) m/z : calc. for C₆₂₇H₁₀₂₀N₁₆₆O₂₁₆S₆ ([M+8H]⁸⁺): 1816.4052, found: 1816.4101; calc. for C₆₂₇H₁₀₂₁N₁₆₆O₂₁₆S₆ ([M+9H]⁹⁺): 1614.6944, found: 1614.6881; calc. for C₆₂₇H₁₀₂₂N₁₆₆O₂₁₆S₆ ([M+10H]¹⁰⁺): 1453.3257, found: 1453.3246; calc. for C₆₂₇H₁₀₂₃N₁₆₆O₂₁₆S₆ ([M+11H]¹¹⁺): 1321.2967, found: 1321.2993; calc. for C₆₂₇H₁₀₂₄N₁₆₆O₂₁₆S₆ ([M+12H]¹²⁺): 1211.2726, found: 1211.2760; calc. for C₆₂₇H₁₀₂₅N₁₆₆O₂₁₆S₆ ([M+13H]¹³⁺): 1118.1753, found: 1118.1805; calc. for C₆₂₇H₁₀₂₆N₁₆₆O₂₁₆S₆ ([M+14H]¹⁴⁺): 1038.3775, found: 1038.3779.

Acetyl- α -Syn-1-140-OH [A69C, A107C]: Acetyl- α -Syn-1-68-MPAA (4.70 mg, 0.66 μ mol, 1 equiv) and H- α -Syn-69-140-OH [A69C, A107C] (5.02 mg, 0.66 μ mol, 1 equiv) were ligated according to General procedure G (0.1 mL of thioester conversion buffer + 0.1 mL ligation buffer). After UHPLC monitoring showed completion of the reaction, the mixture was purified by semi-preparative HPLC following method B. Acetyl- α -Syn-1-140-OH [A69C, A107C] (1.81 mg, 0.12 μ mol, 18% over 2 steps) was obtained as a white solid in a purity of 99%. UHPLC: t_R = 14.150 min. HR-MS (ESI) m/z : calc. for C₆₂₉H₁₀₂₄N₁₆₆O₂₁₇S₆ ([M+10H]¹⁰⁺): 1457.5267, found: 1457.5269; calc. for C₆₂₉H₁₀₂₅N₁₆₆O₂₁₇S₆ ([M+11H]¹¹⁺): 1325.1159, found: 1325.1172; calc. for C₆₂₉H₁₀₂₆N₁₆₆O₂₁₇S₆ ([M+12H]¹²⁺): 1214.7735, found: 1214.7700; calc. for C₆₂₉H₁₀₂₇N₁₆₆O₂₁₇S₆ ([M+13H]¹³⁺): 1121.4068, found: 1121.4076; calc. for C₆₂₉H₁₀₂₈N₁₆₆O₂₁₇S₆ ([M+14H]¹⁴⁺): 1041.3783, found: 1041.3786; calc. for C₆₂₉H₁₀₂₉N₁₆₆O₂₁₇S₆ ([M+15H]¹⁵⁺): 972.0202, found: 972.0214; calc. for C₆₂₉H₁₀₃₀N₁₆₆O₂₁₇S₆ ([M+16H]¹⁶⁺): 911.3319, found: 911.3334.

H- α -Syn-1-140-OH [A53T, A69C, A107C]: H- α -Syn-1-68-MPAA [A53T] (3.13 mg, 0.44 μ mol, 1 equiv) and H- α -Syn-69-140-OH [A69C, A107C] (3.35 mg, 0.44 μ mol, 1 equiv) were ligated according to General procedure G. After UHPLC monitoring showed completion of the reaction, the mixture was purified by semi-preparative HPLC following method B. H- α -Syn-1-140-OH [A53T, A69C, A107C] (0.92 mg, 0.06 μ mol, 14% over 2 steps) was obtained as a white solid in a purity of 95%. UHPLC: t_R = 13.570 min. HR-MS (ESI) m/z : calc. for C₆₂₈H₁₀₂₅N₁₆₆O₂₁₇S₆ ([M+11H]¹¹⁺): 1324.0249, found: 1324.0242; calc. for C₆₂₈H₁₀₂₆N₁₆₆O₂₁₇S₆ ([M+12H]¹²⁺): 1213.7735, found: 1213.7703; calc. for C₆₂₈H₁₀₂₇N₁₆₆O₂₁₇S₆ ([M+13H]¹³⁺): 1120.4838, found: 1120.4790; calc. for C₆₂₈H₁₀₂₈N₁₆₆O₂₁₇S₆ ([M+14H]¹⁴⁺): 1040.4496, found: 1040.4451.

H- α -Syn-1-140-OH [A69C, A107C, P-S129]: H- α -Syn-1-68-MPAA (4.86 mg, 0.69 μ mol, 1 equiv) and H- α -Syn-69-140-OH [A69C, A107C, P-S129] (5.30 mg, 0.69 μ mol, 1 equiv) were ligated according to General procedure G (0.2 mL of thioester conversion buffer + 0.2 mL ligation buffer). After UHPLC monitoring showed completion of the reaction, the mixture was purified by semi-preparative HPLC following method B. H- α -Syn-1-140-OH [A69C, A107C, P-S129] (1.32 mg, 0.09 μ mol, 13% over 2 steps) was obtained as a white solid in a purity >99%. UHPLC: t_R = 13.553 min. HR-MS (ESI) m/z : calc. for C₆₂₇H₁₀₂₄N₁₆₆O₂₁₉PS₆ ([M+11H]¹¹⁺): 1328.5664, found: 1328.5633; calc. for C₆₂₇H₁₀₂₅N₁₆₆O₂₁₉PS₆ ([M+12H]¹²⁺): 1217.9364, found: 1217.9342.

Acetyl- α -Syn-1-140-OH [A69C, A107C, P-S129]: Acetyl- α -Syn-1-68-MPAA (4.63 mg, 0.65 μ mol, 1 equiv) and H- α -Syn-69-140-OH [A69C, A107C, P-S129] (5.00 mg, 0.65 μ mol, 1 equiv) were ligated according to General procedure G (0.1 mL of thioester conversion buffer + 0.1 mL ligation buffer). After UHPLC monitoring showed completion of the reaction, the mixture was purified by semi-preparative HPLC following method B. Acetyl- α -Syn-1-140-OH [A69C, A107C, P-S129] (1.32 mg, 0.09 μ mol, 14% over 2 steps) was obtained as a white solid in a purity >99%. UHPLC: t_R = 14.080 min. HR-MS (ESI) m/z : calc. for $C_{629}H_{1025}N_{166}O_{220}PS_6$ ($[M+10H]^{10+}$): 1465.5233, found: 1465.5252; calc. for $C_{629}H_{1026}N_{166}O_{220}PS_6$ ($[M+11H]^{11+}$): 1332.3855, found: 1332.3894; calc. for $C_{629}H_{1027}N_{166}O_{220}PS_6$ ($[M+12H]^{12+}$): 1221.3538, found: 1221.3538; calc. for $C_{629}H_{1028}N_{166}O_{220}PS_6$ ($[M+13H]^{13+}$): 1127.4810, found: 1127.4819; calc. for $C_{629}H_{1029}N_{166}O_{220}PS_6$ ($[M+14H]^{14+}$): 1047.0902, found: 1047.0909; calc. for $C_{629}H_{1030}N_{166}O_{220}PS_6$ ($[M+15H]^{15+}$): 977.3513, found: 977.3520; calc. for $C_{629}H_{1031}N_{166}O_{220}PS_6$ ($[M+16H]^{16+}$): 916.3298, found: 916.3299; calc. for $C_{629}H_{1032}N_{166}O_{220}PS_6$ ($[M+17H]^{17+}$): 862.4284, found: 862.4288.

3.2.6. Synthesis of desulfurized products

H- α -Syn-1-140-OH: Desulfurization of H- α -Syn-1-140-OH [A69C, A107C] (2.90 mg, 0.20 μ mol, 1 equiv) was performed following General procedure I. The product was purified by semi-preparative HPLC using method B to yield H- α -Syn-1-140-OH (1.22 mg, 0.08 μ mol, 42%) as a white solid in a purity >99%. UHPLC: t_R = 13.767 min. HR-MS (ESI) m/z : calc. for $C_{627}H_{1022}N_{166}O_{216}S_4$ ($[M+10H]^{10+}$): 1446.9313, found: 1446.9358; calc. for $C_{627}H_{1023}N_{166}O_{216}S_4$ ($[M+11H]^{11+}$): 1315.4837, found: 1315.4917; calc. for $C_{627}H_{1024}N_{166}O_{216}S_4$ ($[M+12H]^{12+}$): 1205.9440, found: 1205.9489; calc. for $C_{627}H_{1025}N_{166}O_{216}S_4$ ($[M+13H]^{13+}$): 1113.2565, found: 1113.2596; calc. for $C_{627}H_{1026}N_{166}O_{216}S_4$ ($[M+14H]^{14+}$): 1033.8102, found: 1033.8132; calc. for $C_{627}H_{1027}N_{166}O_{216}S_4$ ($[M+15H]^{15+}$): 964.9566, found: 964.9585; calc. for $C_{627}H_{1028}N_{166}O_{216}S_4$ ($[M+16H]^{16+}$): 904.7098, found: 904.7123; calc. for $C_{627}H_{1029}N_{166}O_{216}S_4$ ($[M+17H]^{17+}$): 851.5508, found: 851.5520; calc. for $C_{627}H_{1030}N_{166}O_{216}S_4$ ($[M+18H]^{18+}$): 804.2984, found: 804.3005.

Acetyl- α -Syn-1-140-OH: Desulfurization of Acetyl- α -Syn-1-140-OH [A69C, A107C] (1.75 mg, 0.12 μ mol, 1 equiv) was performed following General procedure I. The product was purified by semi-preparative HPLC using method B to yield Acetyl- α -Syn-1-140-OH (0.82 mg, 0.06 μ mol, 47%) as a white solid in a purity >99%. UHPLC: t_R = 14.343 min. HR-MS (ESI) m/z : calc. for $C_{629}H_{1025}N_{166}O_{217}S_4$ ($[M+11H]^{11+}$): 1319.3028, found: 1319.3058; calc. for $C_{629}H_{1026}N_{166}O_{217}S_4$ ($[M+12H]^{12+}$): 1209.4448, found: 1209.4469; calc. for $C_{629}H_{1027}N_{166}O_{217}S_4$ ($[M+13H]^{13+}$): 1116.4881, found: 1116.4911; calc. for $C_{629}H_{1028}N_{166}O_{217}S_4$ ($[M+14H]^{14+}$): 1036.8109, found: 1036.8139; calc. for $C_{629}H_{1029}N_{166}O_{217}S_4$ ($[M+15H]^{15+}$): 967.7573, found: 967.7599; calc. for $C_{629}H_{1030}N_{166}O_{217}S_4$ ($[M+16H]^{16+}$): 907.3355, found: 907.3376; calc. for $C_{629}H_{1031}N_{166}O_{217}S_4$ ($[M+17H]^{17+}$): 854.0220, found: 854.0240.

H- α -Syn-1-140-OH [A53T]: Desulfurization of H- α -Syn-1-140-OH [A53T, A69C, A107C] (0.50 mg, 0.03 μ mol, 1 equiv) was performed following General procedure I. The product was purified by semi-preparative HPLC using method B to yield H- α -Syn-1-140-OH [A53T] (0.26 mg, 0.02 μ mol, 52%) as a

white solid in a purity of 99%. UHPLC: t_R = 13.583 min. HR-MS (ESI) m/z : calc. for $C_{628}H_{1024}N_{166}O_{217}S_4$ ($[M+10H]^{10+}$): 1449.9324, found: 1449.9313; calc. for $C_{628}H_{1025}N_{166}O_{217}S_4$ ($[M+11H]^{11+}$): 1318.2119, found: 1318.2115; calc. for $C_{628}H_{1026}N_{166}O_{217}S_4$ ($[M+12H]^{12+}$): 1208.4448, found: 1208.4442; calc. for $C_{628}H_{1027}N_{166}O_{217}S_4$ ($[M+13H]^{13+}$): 1115.5650, found: 1115.5647; calc. for $C_{628}H_{1028}N_{166}O_{217}S_4$ ($[M+14H]^{14+}$): 1035.9538, found: 1035.9534; calc. for $C_{628}H_{1029}N_{166}O_{217}S_4$ ($[M+15H]^{15+}$): 966.9573, found: 966.9565; calc. for $C_{628}H_{1030}N_{166}O_{217}S_4$ ($[M+16H]^{16+}$): 906.5855, found: 906.5849; calc. for $C_{628}H_{1031}N_{166}O_{217}S_4$ ($[M+17H]^{17+}$): 853.3161, found: 853.3161.

H- α -Syn-1-140-OH [P-S129]: Desulfurization of H- α -Syn-1-140-OH [A69C, A107C, P-S129] (0.69 mg, 0.05 μ mol, 1 equiv) was performed following General procedure I. The product was purified by semi-preparative HPLC using method B to yield H- α -Syn-1-140-OH [P-S129] (0.37 mg, 0.03 μ mol, 54%) as a white solid in a purity >99%. UHPLC: t_R = 13.733 min. HR-MS (ESI) m/z : calc. for $C_{627}H_{1025}N_{166}O_{219}PS_4$ ($[M+12H]^{12+}$): 1212.6078, found: 1212.6057; calc. for $C_{627}H_{1026}N_{166}O_{219}PS_4$ ($[M+13H]^{13+}$): 1119.4078, found: 1119.4047.

Acetyl- α -Syn-1-140-OH [P-S129]: Desulfurization of Acetyl- α -Syn-1-140-OH [A69C, A107C, P-S129] (0.78 mg, 0.05 μ mol, 1 equiv) was performed following General procedure I. The product was purified by semi-preparative HPLC using method B to yield Acetyl- α -Syn-1-140-OH [P-S129] (0.49 mg, 0.03 μ mol, 63%) as a white solid in a purity >99%. UHPLC: t_R = 14.180 min. HR-MS (ESI) m/z : calc. for $C_{629}H_{1025}N_{166}O_{220}PS_4$ ($[M+10H]^{10+}$): 1459.1290, found: 1459.1318; calc. for $C_{629}H_{1026}N_{166}O_{220}PS_4$ ($[M+11H]^{11+}$): 1326.5725, found: 1326.5744; calc. for $C_{629}H_{1027}N_{166}O_{220}PS_4$ ($[M+12H]^{12+}$): 1216.1087, found: 1216.1108; calc. for $C_{629}H_{1028}N_{166}O_{220}PS_4$ ($[M+13H]^{13+}$): 1122.6394, found: 1122.6410; calc. for $C_{629}H_{1029}N_{166}O_{220}PS_4$ ($[M+14H]^{14+}$): 1042.5228, found: 1042.5243; calc. for $C_{629}H_{1030}N_{166}O_{220}PS_4$ ($[M+15H]^{15+}$): 973.0884, found: 973.0901; calc. for $C_{629}H_{1031}N_{166}O_{220}PS_4$ ($[M+16H]^{16+}$): 912.3333, found: 912.3350.

4. Supplementary analytical data

4.1. UHPLC data of desulfurized products

UHPLC analysis of purified desulfurized products was carried out using a C18 reverse-phase column with a linear gradient of 5-75% ACN+0.085% TFA in H₂O+0.1% TFA over 15 min at a temperature of 50 °C. The flow rate was set to 0.3 mL/min and detection was performed at 215 nm.

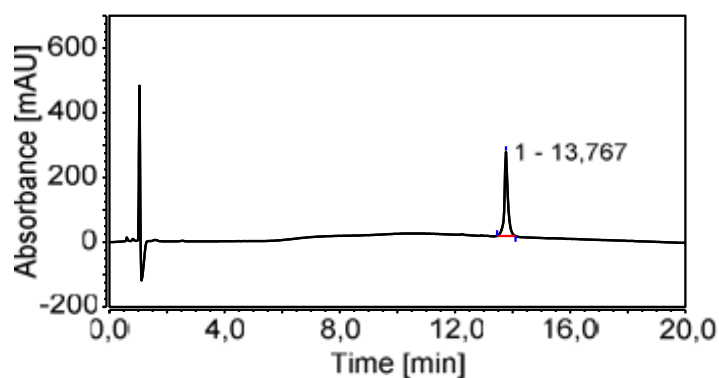


Figure S1. UHPLC chromatogram of H-a-Syn-1-140-OH.

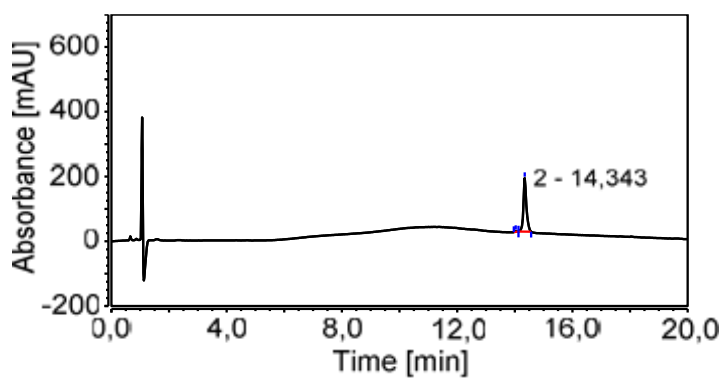


Figure S2. UHPLC chromatogram of Acetyl-a-Syn-1-140-OH.

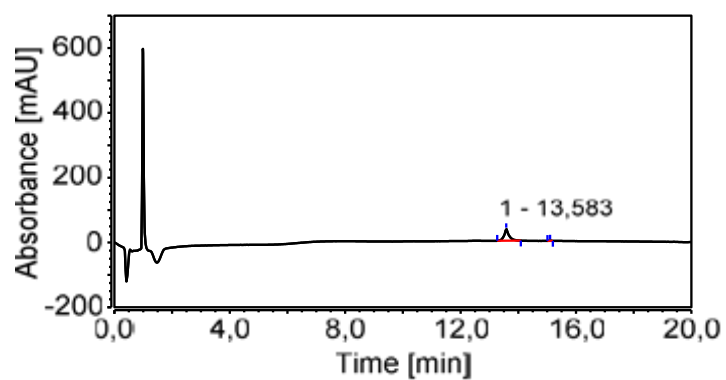


Figure S3. UHPLC chromatogram of H-a-Syn-1-140-OH [A53T].

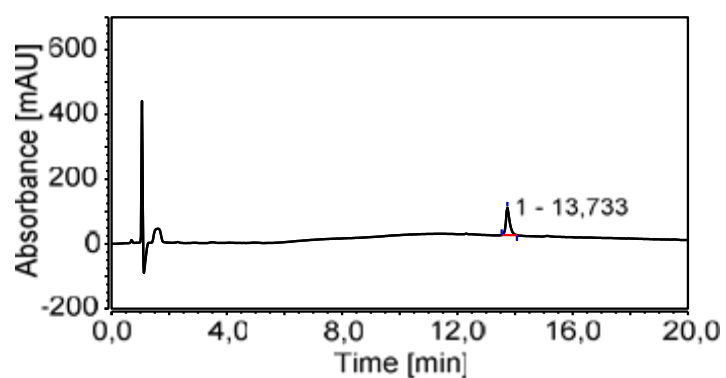


Figure S4. UHPLC chromatogram of H-a-Syn-1-140-OH [P-S129].

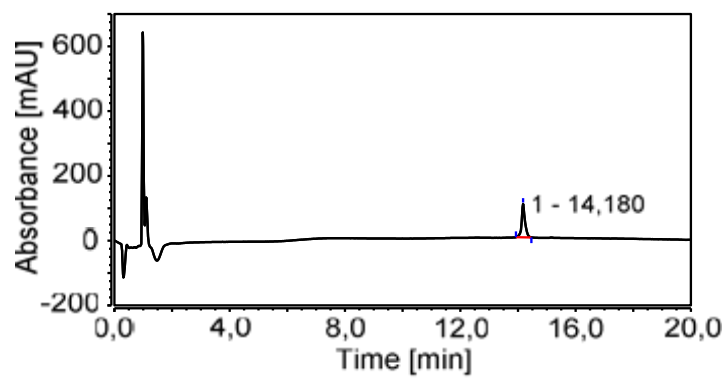


Figure S5. UHPLC chromatogram of Acetyl-a-Syn-1-140-OH [P-S129].

4.2. ESI-HRMS data of desulfurized products

Mass analysis of purified desulfurized products was performed by HPLC coupled to ESI-MS. LC was carried out on a C18 column using a linear gradient of 20-100% MeOH+0.05% formic acid in H₂O+0.05% formic acid over 15 min at a temperature of 40 °C. The flow rate was set to 0.2 mL/min. Mass spectra were recorded in positive ion mode.

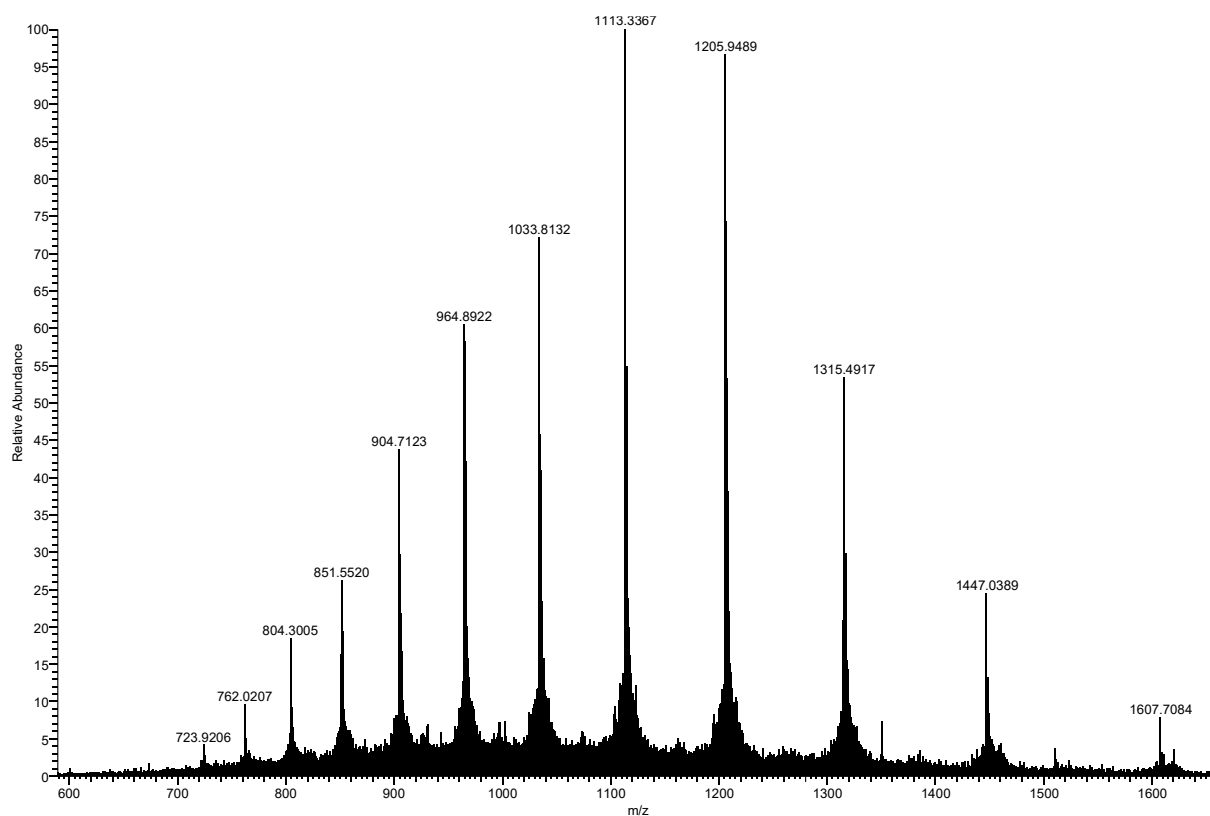


Figure S6. ESI-HRMS spectrum of H-a-Syn-1-140-OH.

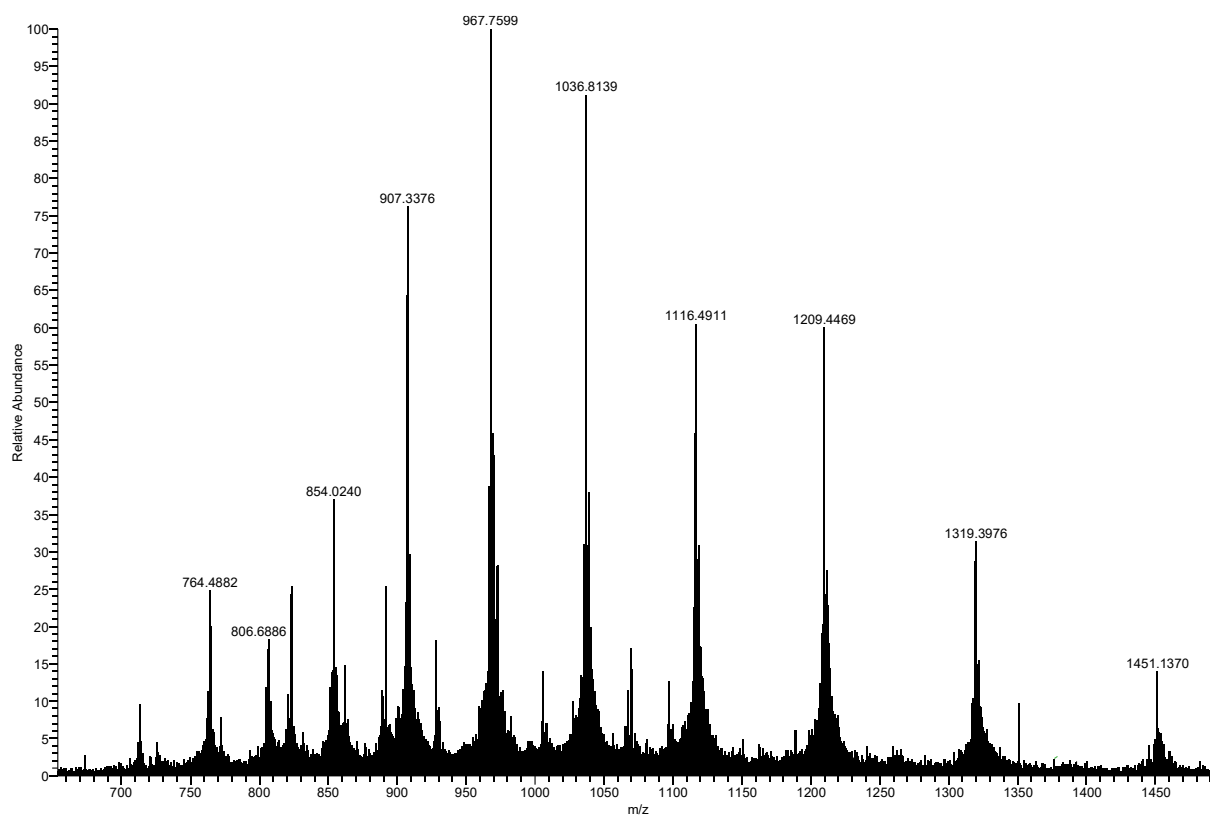


Figure S7. ESI-HRMS spectrum of Acetyl- α -Syn-1-140-OH.

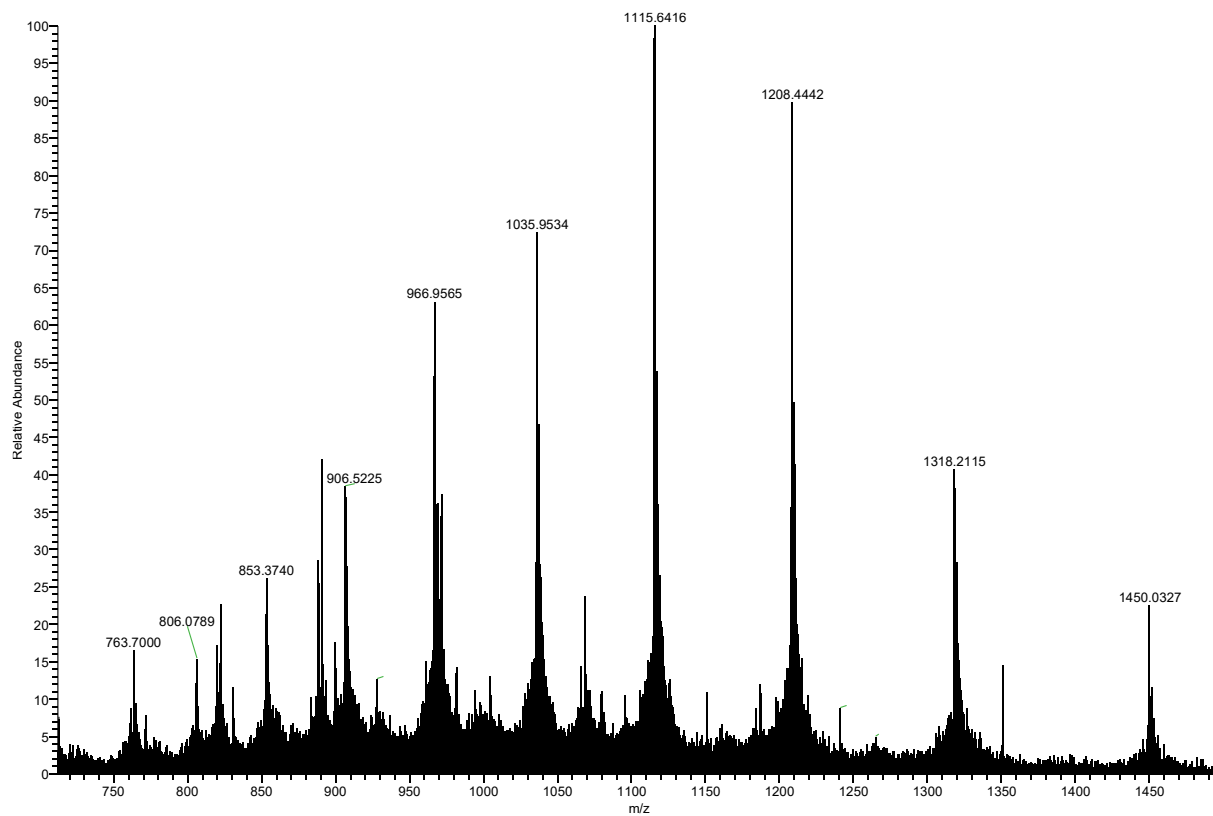


Figure S8. ESI-HRMS spectrum of H- α -Syn-1-140-OH [A53T].

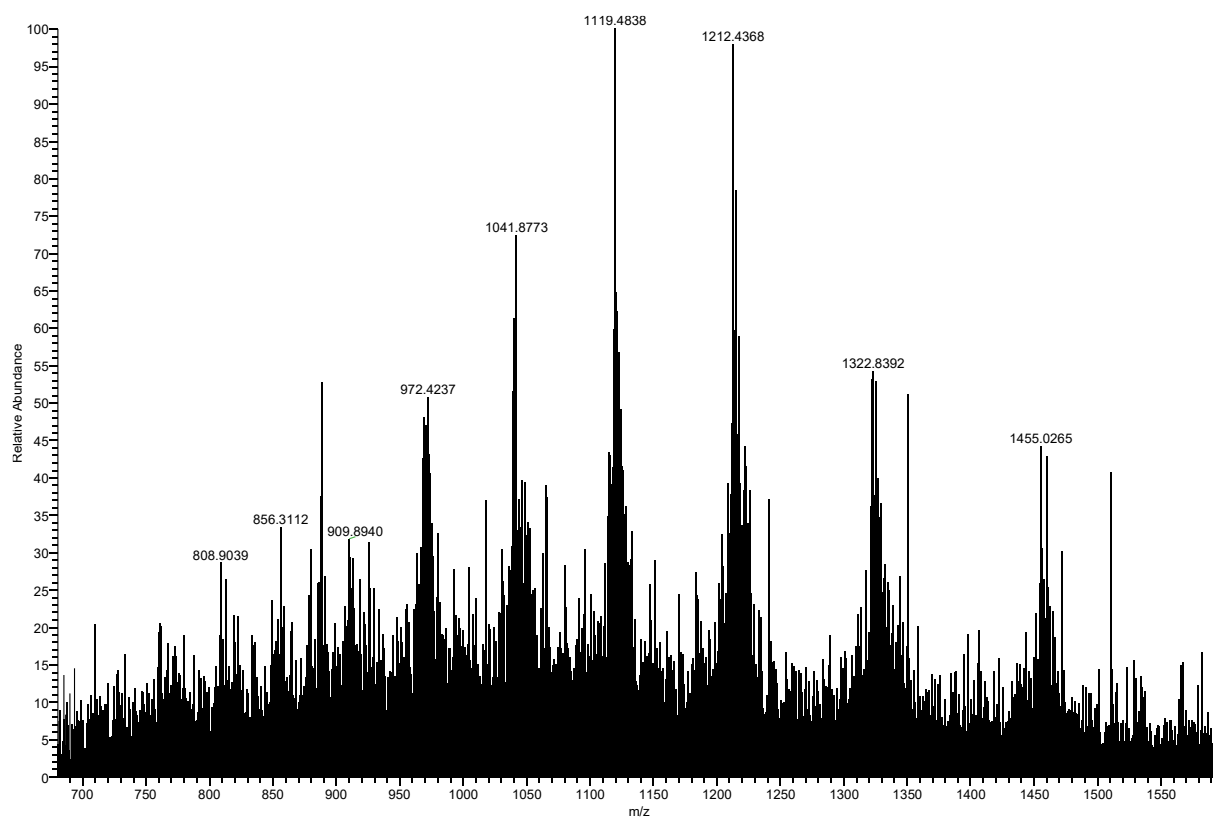


Figure S9. ESI-HRMS spectrum of H-a-Syn-1-140-OH [P-S129].

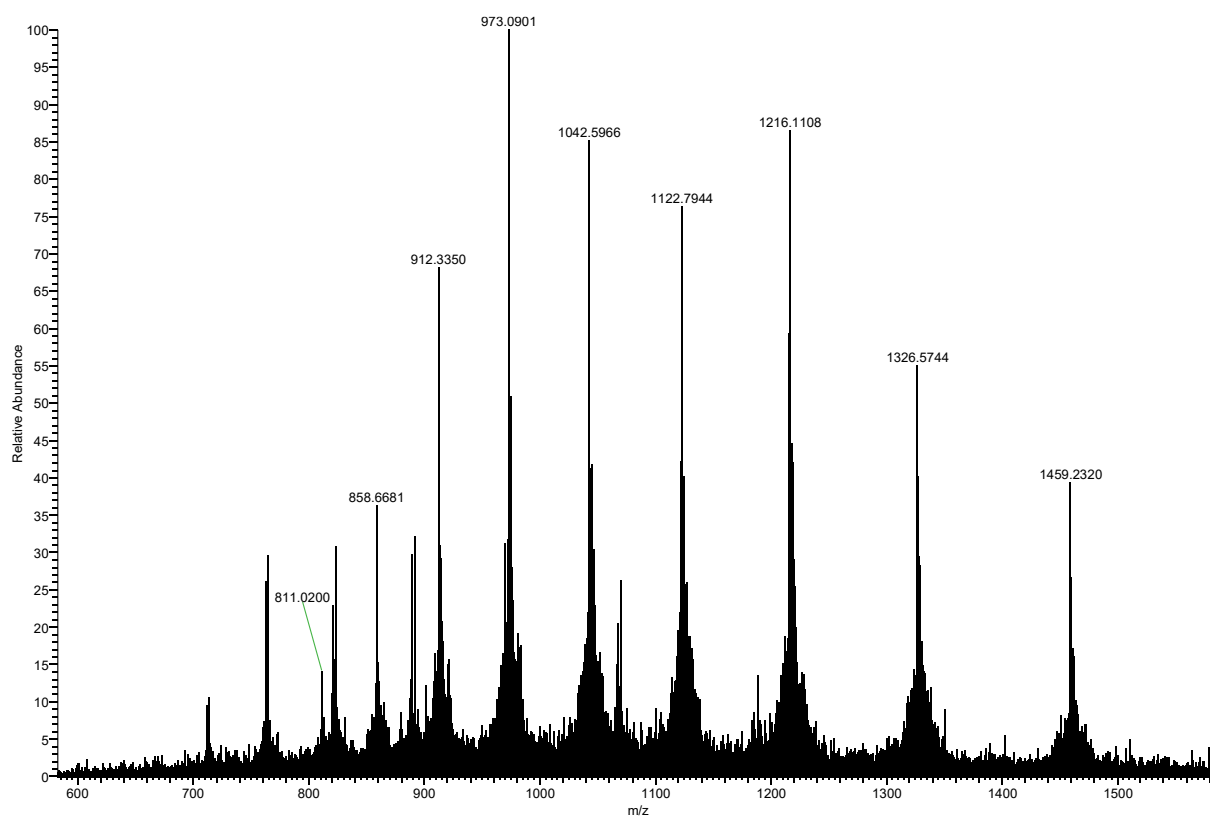


Figure S10. ESI-HRMS spectrum of Acetyl-a-Syn-1-140-OH [P-S129].

5. References

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