

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

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**Abstract:** Alpha-Synuclein ( $\alpha$ -Synuclein) is a 140 amino acid protein implicated in neurodegenerative disorders known as synucleinopathies, where it accumulates in proteinaceous inclusions in the brain. The normal physiological function of  $\alpha$ -Synuclein remains obscure, as it exists in several non-neuronal cells in which its function has not been studied. Given the tremendous interest in studying  $\alpha$ -Synuclein, and the existing limitations in the production of modified forms of the protein, we developed a method for the chemical synthesis of  $\alpha$ -Synuclein by combining peptide fragment

synthesis via automated microwave-assisted solid-phase peptide synthesis and ligation strategies. Our synthetic pathway enables the synthesis of protein variants of interest, carrying either mutations or posttranslational modifications, for further investigations of the effects on the structure and aggregation behavior of the protein. Ultimately, our study forms the foundation for future syntheses and studies of other custom-made  $\alpha$ -Synuclein variants with a single or several modifications, as necessary.

## Introduction

In neuronal cells, the 140 amino acid (AA) protein  $\alpha$ -Synuclein is thought to play a role in neurotransmitter release.<sup>[1–3]</sup> However, the protein exists also in non-neuronal cells, such as erythrocytes, where it performs other roles, given the distinct function of these cells.<sup>[4]</sup> While the native structure of  $\alpha$ -Synuclein is thought to be intrinsically disordered, it is also known to occur in aggregated forms in the brains of patients with Parkinson's disease (PD) and other related synucleinopathies. Currently, oligomeric forms of  $\alpha$ -Synuclein are thought to cause neuronal cell death and neurodegeneration.<sup>[1–3,5,6]</sup> The propensity of the protein to aggregate is influenced by changes

in the native AA sequence of the protein as well as by posttranslational modifications (PTMs) implying that mutations and PTMs can contribute to the protein pathogenicity.<sup>[7]</sup> On one hand, several  $\alpha$ -Synuclein point mutations have been associated with familial forms of PD, like A30G, A30P, E46K, H50Q, G51D, A53E, A53T, and A53V.<sup>[5,8–10]</sup> A30P increases the oligomerization propensity of  $\alpha$ -Synuclein.<sup>[2,8]</sup> E46K, H50Q, and A53T cause increased  $\alpha$ -Synuclein aggregation, while G51D and A53E attenuate it.<sup>[8]</sup> A53V accelerates  $\alpha$ -Synuclein fibrillation.<sup>[10]</sup> Interestingly, these mutations are located in the membrane binding N-terminal domain, suggesting alterations in this region of the protein are more likely to cause pathology.<sup>[11]</sup> On the other hand, several  $\alpha$ -Synuclein PTMs, like serine and tyrosine

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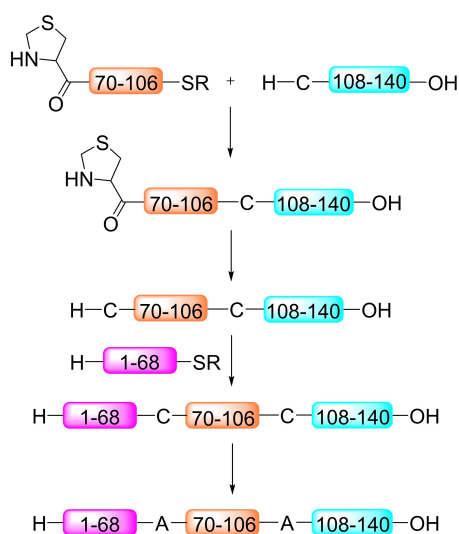
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phosphorylation, tyrosine nitration, ubiquitination, acetylation, or lysine glycation, are reported to influence the protein's aggregation in terms of promoting or inhibiting aggregate formation.<sup>[6–8,12,13]</sup> These examples are just but a small sample of the possible number of PTMs that may occur in  $\alpha$ -Synuclein, and do not even include combinations thereof, indicating that extensive research efforts are necessary in order to elucidate the role of PTMs in the biology and pathobiology of the protein. Since many PTMs are not accessible by recombinant expression in bacteria, it is advantageous to produce  $\alpha$ -Synuclein with specific chemical modifications to investigate their impact on  $\alpha$ -Synuclein. The advantage in chemical protein synthesis is an immense flexibility regarding the kinds of protein modifications, their quantities, and positions in the protein. To overcome the limitations in expression, we developed a new method for the chemical synthesis of wild type (WT)  $\alpha$ -Synuclein and demon-

strate that this method is suitable for the synthesis of several biologically relevant protein variants. Therefore, we first synthesized three peptide fragments by microwave-assisted solid-phase peptide synthesis (MW-SPPS) and subsequently ligated them using the widely spread native chemical ligation (NCL), which is a well-established technique to link peptide thioesters and cysteine (Cys) peptides,<sup>[14,15]</sup> followed by radical desulfurization<sup>[16]</sup> to yield the full-length proteins (Scheme 1).

Previous reports on the chemical synthesis of  $\alpha$ -Synuclein relied on the synthesis of either three or four peptide fragments via Fluorenylmethoxycarbonyl (Fmoc)-based SPPS followed by NCL and final desulfurization.<sup>[17,18]</sup> These studies demonstrated the total chemical synthesis of WT  $\alpha$ -Synuclein, but not of modified  $\alpha$ -Synuclein variants. The new synthetic pathway described here is applicable for the chemical synthesis of WT  $\alpha$ -Synuclein, of physiologically relevant *N*-terminally acetylated  $\alpha$ -Synuclein, of the  $\alpha$ -Synuclein mutant A53T, and of S129 phosphorylated  $\alpha$ -Synuclein with and without *N*-terminal acetylation (Figure 1). The well explored mutant A53T known to expedite fibril formation<sup>[19]</sup> was selected to show the general applicability of this method to  $\alpha$ -Synuclein mutations. By synthesizing the mentioned mutant, we showed that this method is robust to alterations in the *N*-terminal peptide fragment. Furthermore, by introducing the S129 phosphorylation in the C-terminal peptide fragment, we demonstrated that this method also tolerates the introduction of modifications at sites outside of the *N*-terminal peptide fragment. Our new synthetic strategy will enable the introduction of further mutations or PTMs and combinations thereof in the AA sequence of  $\alpha$ -Synuclein.



Scheme 1. Our pathway for the chemical synthesis of  $\alpha$ -Synuclein.

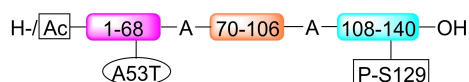


Figure 1. Synthesized  $\alpha$ -Synuclein derivatives with mutation (circular edge) and posttranslational modifications (rectangular edge).

## Results and Discussion

### Synthetic strategy

Since the AA sequence of  $\alpha$ -Synuclein is too long for MW-SPPS in one step, we decided to synthesize three peptide fragments (Figure 2) that were subsequently ligated from C- to *N*-terminus by NCL. We first synthesized the pink colored peptide fragment (AA 1–68, Figure 2) carrying a C-terminal hydrazide function as thioester precursor,<sup>[20]</sup> the orange colored peptide fragment (AA 69–106, Figure 2) carrying a *N*-terminal thiazolidine (Thz) moiety

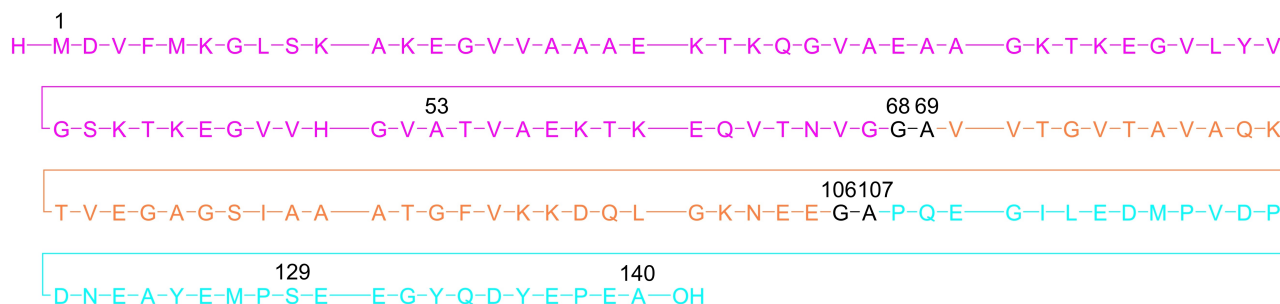
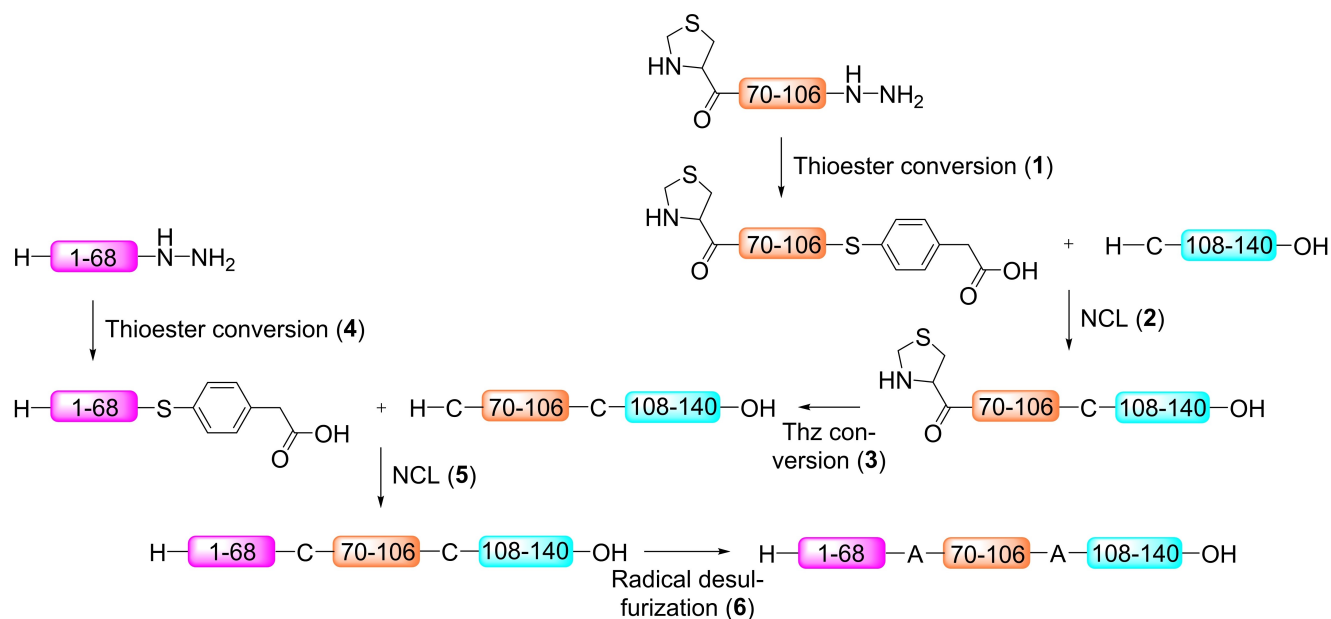


Figure 2. AA sequence of native  $\alpha$ -Synuclein with the three peptide fragments for MW-SPPS marked in pink, orange and blue. Ligation sites are marked in black (both Ala residues were replaced by Cys or Cys precursor temporarily).



**Scheme 2.** Strategy for the chemical synthesis of WT  $\alpha$ -Synuclein (Conditions: Thioester conversions (1), (4): acetylacetone (acac) (40–500 equiv), 4-mercatophenylacetic acid (MPAA) (0.15 M), 6 M Gn\*HCl, pH 3, 25 °C, 4–6 h; NCLs (2), (5): Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP\*HCl) (0.29–0.90 M), MPAA (0.075 M), 6 M Gn\*HCl, 0.2 M Na<sub>3</sub>PO<sub>4</sub>, pH 7, 37 °C, 16–20 h; Thz conversion (3): TCEP\*HCl (0.14–0.28 M), O-methylhydroxylamine\*HCl (0.48–0.96 M), 6 M Gn\*HCl, 0.2 M Na<sub>3</sub>PO<sub>4</sub>, pH 4, 37 °C, 3–6 h; Desulfurization (6): TCEP\*HCl (1.00 M), 2-methyl-2-propanethiol (t-BuSH) (40  $\mu$ L), 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA044\*2HCl) (40  $\mu$ L of 0.1 M solution), 6 M Gn\*HCl, 0.2 M Na<sub>3</sub>PO<sub>4</sub>, pH 7, 37 °C, 4–6 h).

[A69Thz] as Cys precursor,<sup>[21]</sup> and a C-terminal hydrazide for thioester conversion, and the blue colored peptide fragment (AA 107–140, Figure 2) carrying a N-terminal Cys residue [A107C] for ligation. Since there is a Cys residue necessary for NCL, but  $\alpha$ -Synuclein lacks Cys residues, both alanine (Ala) residues at the ligation sites were replaced by Cys during the synthesis and desulfurized at the end to receive the desired WT protein.<sup>[14,22]</sup> Glycine (Gly)-Ala junctions served as ligation sites because Gly lacks steric hindrance which is crucial for the success of the ligation and Ala is needed for the temporary exchange with Cys facilitating a reverse conversion to the native Ala residue at the end.  $\alpha$ -Synuclein and its variants were synthesized by the combination of three peptide fragments via thioester conversion<sup>[20]</sup> (see supplementary general procedures Scheme S3), NCL<sup>[17,18]</sup> (see supplementary general procedures Scheme S4), Thz conversion<sup>[18]</sup> (see supplementary general procedures Scheme S5) and desulfurization<sup>[16]</sup> (see supplementary general procedures Scheme S6) following the strategy shown in Scheme 2. For the synthesis of the  $\alpha$ -Synuclein variants the N-terminal peptide fragment was either acetylated<sup>[23]</sup> before cleavage from the resin (see supplementary general procedures Scheme S2) and/or selected modifications were introduced during MW-SPPS at specific locations in the AA sequence of the N- or C-terminal peptide fragment, respectively (Table 1).

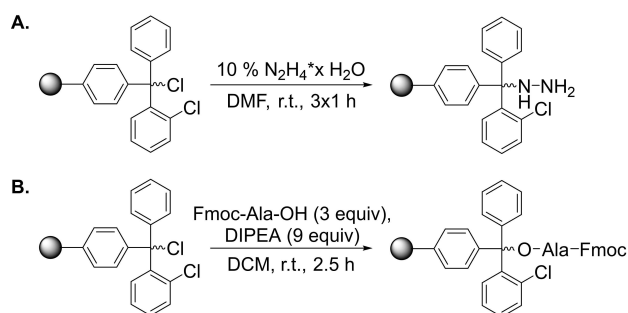
**Table 1.** Synthesized  $\alpha$ -Synuclein derivatives and modifications in the synthesis.

$\alpha$ -Synuclein derivative	Modification
H- $\alpha$ -Syn-1-140-OH	–
Acetyl- $\alpha$ -Syn-1-140-OH	acetylation <sup>[23]</sup> of N-terminal peptide fragment before cleavage
H- $\alpha$ -Syn-1-140-OH [A53T]	point mutation in synthesis of N-terminal peptide fragment [threonine instead of Ala in position 53]
H- $\alpha$ -Syn-1-140-OH [P-S129]	AA exchange in synthesis of C-terminal peptide fragment [phosphorylated serine (P-Ser) instead of Ser in position 129]
Acetyl- $\alpha$ -Syn-1-140-OH [P-S129]	acetylation <sup>[23]</sup> of N-terminal peptide fragment before cleavage and AA exchange in synthesis of C-terminal peptide fragment [P-Ser instead of Ser in position 129]

### Synthesis of $\alpha$ -Synuclein peptide fragments by automated MW-SPPS on 2-chlorotrityl chloride (2-CTC) resin

To facilitate the synthesis of peptide hydrazides that are reported to be easily converted into thioesters for the use in NCL,<sup>[20]</sup> we decided to use 2-CTC resin, although it is not very common for automated peptide synthesis. Prior to use, the 2-CTC resin was modified manually either with hydrazine for the synthesis of peptide hydrazides<sup>[17]</sup> or with Ala as the first AA<sup>[24]</sup> for the automated synthesis of peptide acids used as C-terminal peptide fragments (Scheme 3).

The hydrazine-modified resin was used directly in automated SPPS to produce the peptide hydrazides after loading was determined by preloading a small amount of the hydrazine modified 2-CTC resin with Fmoc-Ala-OH<sup>[23]</sup> (see supplementary

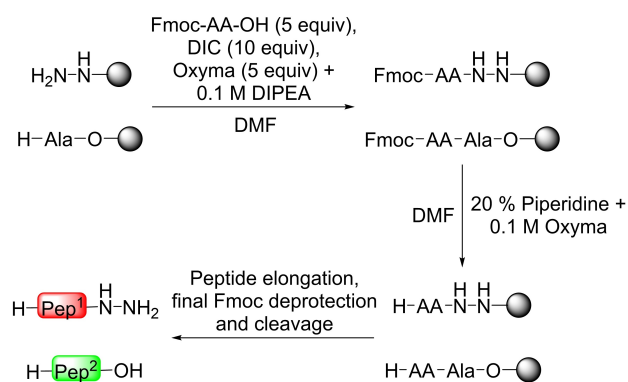


**Scheme 3.** Initial modification of 2-CTC resin. **A.** Hydrazine modification of 2-CTC resin using hydrazine hydrate ( $\text{N}_2\text{H}_4 \cdot x \text{H}_2\text{O}$ ) in *N,N*-dimethylformamide (DMF).<sup>[17]</sup> **B.** Fmoc-Ala-preloading of 2-CTC resin with Fmoc-Ala-OH and *N,N*-diisopropylethylamine (DIPEA) in dichloromethane (DCM).<sup>[24]</sup>

general procedures Scheme S1) and detecting the cleaved dibenzofulvene-piperidine adduct<sup>[25]</sup> photometrically. Manual preloading of the first AA was not necessary because the conditions in automated peptide synthesis led to an efficient coupling of the first AA to the hydrazine-modified 2-CTC resin.

The peptide fragment synthesis of peptide hydrazides and peptide acids was performed using fully-automated and microwave-assisted Fmoc-SPPS procedures in a CEM Liberty Blue<sup>TM</sup> peptide synthesizer utilizing alternating cycles of AA couplings and Fmoc deprotections under application of *N,N'*-diisopropylcarbodiimide (DIC) and ethyl (2*E*)-2-cyano-2-hydroxyiminoacetate (Oxyma) (the latter containing DIPEA) as activator and activator base solutions for AA couplings and piperidine (containing Oxyma) for Fmoc deprotection (Scheme 4).

Since the 2-CTC resin is very acid labile,<sup>[24,26]</sup> standard synthesis protocols for automated synthesis were adapted.<sup>[24,27]</sup> In comparison to standard conditions, temperatures as well as the microwave power were decreased during the cycles, whereas deprotection and coupling times were prolonged. For synthesis details see supplementary methods Table S1. As the desired peptide fragments were longer than 25 AAs, resin with medium loading was applied and every AA was introduced by double coupling. To avoid premature cleavage under acidic



**Scheme 4.** Automated MW-SPPS of peptide hydrazides and peptide acids using DIC and Oxyma (containing DIPEA) for AA activation and piperidine (containing Oxyma) for Fmoc deprotection.

conditions, DIPEA was added to the activator base solution containing Oxyma to increase the pH during the coupling.<sup>[28]</sup> Furthermore, Oxyma was added to the deprotection solution to suppress aspartimide formation of aspartic acids under basic conditions,<sup>[29]</sup> but nevertheless the pH during deprotection was high enough, as premature cleavage of the peptide fragments was not observed and Fmoc deprotection occurred properly. The couplings of histidine (His) and P-Ser required special attention to avoid known side-reactions. Milder conditions regarding coupling temperature and microwave irradiation were used for their couplings compared to other AA couplings (see supplementary methods Table S1). We applied Fmoc-His(Boc)-OH instead of Fmoc-His(Trt)-OH to suppress epimerization at elevated temperatures.<sup>[30]</sup> In the synthesis of the phosphorylated peptides for the coupling of Fmoc-Ser(PO-(OBzl)OH)-OH and the following AAs, room temperature deprotection without microwave irradiation and mild couplings again with DIPEA in the Oxyma solution were used to avoid dephosphorylation of the acid-sensitive phosphoester linkage at high temperatures during carbodiimide coupling<sup>[27]</sup> and to prevent  $\beta$ -elimination of the protected phosphate group, which is enhanced under microwave irradiation during Fmoc deprotection using piperidine.<sup>[27,31]</sup> Despite  $\beta$ -piperidinyl-alanyl formation<sup>[31]</sup> was observed, the product yield was high enough to proceed with the following reactions.

All desired peptide fragments were obtained in acceptable yields ranging from 2%–25% (Table 2), showing that our optimized automated Fmoc-SPPS protocols are suitable for the synthesis of  $\alpha$ -Synuclein peptide fragments on 2-CTC resin.

*N*-terminal peptide fragments were synthesized as peptide hydrazides in moderate yields of 2% (Table 2), which is caused by their exceptional length of 68 AAs. Since the coupling efficiency is less than 100% for each step and this effect is potentiating cycle by cycle, we expected a decrease in yield with increasing peptide fragment length. Nevertheless, in each case pure peptide was isolated in a sufficient amount for the following reactions. Interestingly, the modified or mutated *N*-terminal peptide fragments did not show a decreased yield compared to the WT *N*-terminal peptide fragment, meaning that *N*-terminal acetylation after SPPS before resin cleavage or the change of one AA in the AA sequence of this peptide fragment in SPPS are well-tolerated to the protocol. As expected, the syntheses of the shorter peptide fragments delivered higher yields (14%–25%, Table 2). The yield of the phosphorylated C-terminal peptide fragment (16%, Table 2) was lower than for the same peptide fragment without

**Table 2.** Yields of the purified peptide hydrazides and peptide acids (scale: 0.05 mmol).

Peptide fragment	Yield [%]	Yield [mg]	Purity [%]
H- $\alpha$ -Syn-1-68-NH-NH <sub>2</sub>	2	5.8	99
Acetyl- $\alpha$ -Syn-1-68-NH-NH <sub>2</sub>	2	8.3	96
H- $\alpha$ -Syn-1-68-NH-NH <sub>2</sub> [A53T]	2	8.2	> 99
H- $\alpha$ -Syn-69-106-NH-NH <sub>2</sub> [A69Thz]	14	26.0	96
H- $\alpha$ -Syn-107-140-OH [A107C]	25	49.2	92
H- $\alpha$ -Syn-107-140-OH [A107C, P-S129]	16	30.8	98

phosphorylation (25%, Table 2) because of the mentioned  $\beta$ -piperidiny-alanyl formation during the synthesis. The purities of the peptide fragments were at least 95%, except from H- $\alpha$ -Syn-107-140-OH [A107C] with a purity of 92%, which made them good starting materials for ligation reactions.

### Synthesis of WT $\alpha$ -Synuclein and $\alpha$ -Synuclein variants by ligation reaction sequence

The reaction sequence of thioester conversion (1) – first NCL (2) – Thz conversion (3) – thioester conversion (4) – second NCL (5) – desulfurization (6) (Scheme 2) was applied successfully to WT  $\alpha$ -Synuclein and the  $\alpha$ -Synuclein variants as described below.

#### Thioester conversion (1) and first one-pot NCL (2)

After peptide hydrazides and peptide acids were synthesized on 2-CTC resin, cleaved and purified, the first step in the following reaction sequence was the thioester conversion. For the synthesis of thioesters from peptide hydrazides two different methods are known, either via an azide<sup>[17]</sup> or via Knorr pyrazole synthesis with an acyl pyrazole intermediate.<sup>[20]</sup>

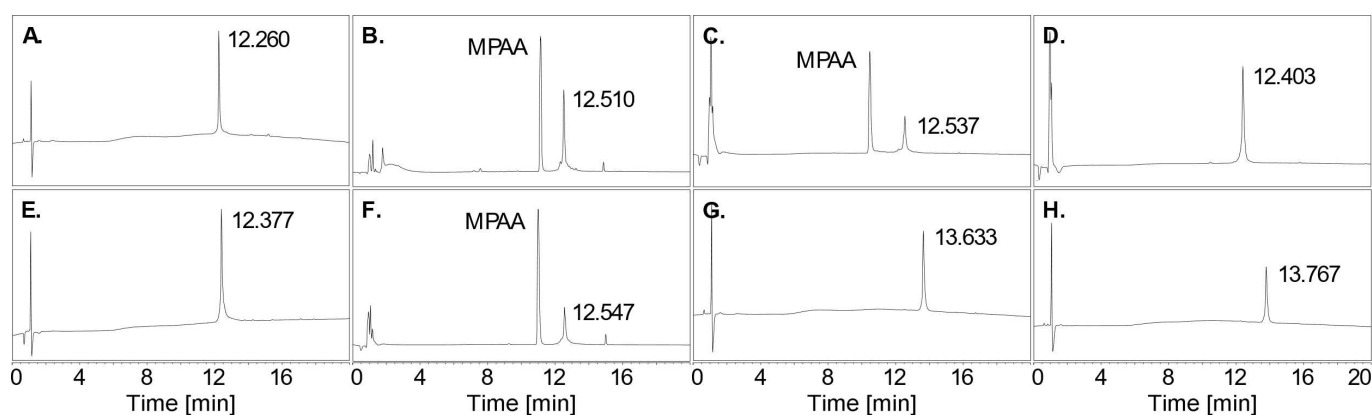
We decided to use the method via the acyl pyrazole even if longer reaction times were needed, because handling in terms of maintaining the correct reaction temperature is easier and the acyl pyrazole intermediate is not as redox-sensitive as the azide intermediate, which can be reduced by TCEP into the amide leading to inactivation for NCL.<sup>[17]</sup> To our knowledge, thioester conversion via Knorr pyrazole synthesis has not yet been applied to the chemical synthesis of  $\alpha$ -Synuclein. Acac was used to form acyl pyrazole intermediates under acidic conditions, and in presence of MPAA 3,5-dimethylpyrazole was cleaved off during thioester formation.<sup>[20]</sup> In the beginning, the peptide hydrazide H- $\alpha$ -Syn-69-106-NH-NH<sub>2</sub> [A69Thz] was con-

verted into the thioester H- $\alpha$ -Syn-69-106-MPAA [A69Thz] (Scheme 2, (1)) and reaction progress was monitored by UHPLC analysis, which showed a slightly higher retention time of the thioester (12.510 min, Figure 3, B) after 5.5 h of reaction compared to the peptide hydrazide (12.260 min, Figure 3, A). First attempts of thioester conversions were purified after completion of the reaction and electrospray ionization high-resolution mass spectrometry (ESI-HRMS) was used to confirm thioester formation. Later on, only UHPLC monitoring was done to trace the reaction progress.

After completion of the thioester conversion, a one-pot NCL reaction<sup>[18]</sup> (Scheme 2, (2)) of thioester H- $\alpha$ -Syn-69-106-MPAA [A69Thz] with Cys peptide H- $\alpha$ -Syn-107-140-OH [A107C] was performed at pH 7 in presence of MPAA as a catalyst for the ligation, and with TCEP as reducing agent to prevent formation of disulfide bonds between two Cys residues or between Cys and the excess MPAA.<sup>[15]</sup> The buffer solution was bubbled with argon before use in order to avoid oxidation of methionines during NCL.<sup>[18]</sup> Reaction monitoring showed a peak at 12.537 min (Figure 3, C) after 16 h, which was assumed to be the ligation product H- $\alpha$ -Syn-69-140-OH [A69Thz, A107C] while the Cys peptide H- $\alpha$ -Syn-107-140-OH [A107C] with a retention time of 11.560 min disappeared completely after overnight reaction. In the synthesis of phosphorylated  $\alpha$ -Synuclein, H- $\alpha$ -Syn-107-140-OH [A107C, P-S129] instead of H- $\alpha$ -Syn-107-140-OH [A107C] was used as Cys peptide for the first NCL with the thioester H- $\alpha$ -Syn-69-106-MPAA [A69Thz] to obtain H- $\alpha$ -Syn-69-140-OH [A69Thz, A107C, P-S129]. UHPLC showed completion of the reaction after 16 h.

#### Thz conversion (3)

For the ligation of three peptide fragments, a protection strategy for the Cys residue of the central peptide fragment is necessary when ligations are performed from C- to N-terminus



**Figure 3.** Ultra high-performance liquid chromatography (UHPLC) chromatograms of reaction sequence yielding WT  $\alpha$ -Synuclein with peak retention times ( $R_t$ ) using a gradient of 5–75% acetonitrile (ACN) over 15 min at temperature of 50 °C and detection at 215 nm. A. Purified peptide hydrazide H- $\alpha$ -Syn-69-106-NH<sub>2</sub> [A69Thz] ( $R_t$  = 12.260 min). B. Thioester conversion reaction mixture at 5.5 h of reaction time with product H- $\alpha$ -Syn-69-106-MPAA [A69Thz] ( $R_t$  = 12.510 min). C. Ligation reaction mixture at 16 h of reaction time with product H- $\alpha$ -Syn-69-140-OH [A69Thz, A107C] ( $R_t$  = 12.537 min). D. Purified Thz conversion product H- $\alpha$ -Syn-69-140-OH [A69C, A107C] ( $R_t$  = 12.403 min). E. Purified peptide hydrazide H- $\alpha$ -Syn-1-68-NH-NH<sub>2</sub> ( $R_t$  = 12.377 min). F. Thioester conversion reaction mixture at 6 h of reaction time with product H- $\alpha$ -Syn-1-68-MPAA ( $R_t$  = 12.547 min). G. Purified second ligation product H- $\alpha$ -Syn-1-140-OH [A69C, A107C] ( $R_t$  = 13.633 min). H. Purified H- $\alpha$ -Syn-1-140-OH ( $R_t$  = 13.767 min).

to suppress cyclization and polymerization during ligation.<sup>[21]</sup> We used the Thz moiety as Cys precursor<sup>[18,21]</sup> at the N-terminal position of the central peptide fragment H- $\alpha$ -Syn-69-106-NH-NH<sub>2</sub> [A69Thz] during the ligation with Cys peptide H- $\alpha$ -Syn-107-140-OH [A107C] and converted the Thz moiety back into Cys (Thz conversion) to activate the peptide for the next ligation requiring a Cys peptide. The Thz conversion of H- $\alpha$ -Syn-69-140-OH [A69Thz, A107C] into H- $\alpha$ -Syn-69-140-OH [A69C, A107C] (Scheme 2, (3)) was carried out also as part of the one-pot reaction sequence directly after the first ligation using O-methylhydroxylamine at pH 4<sup>[18]</sup> followed by purification of the product. Product conversion was confirmed by a change in UHPLC retention time from 12.537 min for H- $\alpha$ -Syn-69-140-OH [A69Thz, A107C] (Figure 3, C) to 12.403 min for H- $\alpha$ -Syn-69-140-OH [A69C, A107C] (Figure 3, D). UHPLC showed completion of the reaction after 5 h.

Following this strategy of thioester conversion (1), first NCL (2), and Thz conversion (3) in a one-pot reaction, we successfully synthesized two intermediate products, which are the WT peptide fragment and a peptide fragment with phosphorylation at S129 (Table 3), both accessible for the next ligation.

The yields for the unphosphorylated and the phosphorylated peptides were 25 and 19% (Table 3), respectively, which is acceptable over three steps. The peptides were prepared in very high purities of at least 95%.

#### Thioester conversion (4) and second one-pot NCL (5)

Subsequently, the peptide hydrazide H- $\alpha$ -Syn-1-68-NH-NH<sub>2</sub> was converted into the thioester H- $\alpha$ -Syn-1-68-MPAA and ligated with the Thz conversion product H- $\alpha$ -Syn-69-140-OH [A69C, A107C] (Scheme 2, (4) and (5)) following the same procedure as described above ((1) and (2)) and monitored by UHPLC. In thioester conversion, a shift of the retention time of 12.377 min for the hydrazide (Figure 3, E) to 12.547 min for the thioester was observed (Figure 3, F) after 6 h of reaction. Second ligation was completed after 16 h, the product H- $\alpha$ -Syn-1-140-OH [A69C, A107C] was purified and showed a retention time of 13.633 min (Figure 3, G).

By ligating the three thioesters H- $\alpha$ -Syn-1-68-MPAA, Acetyl- $\alpha$ -Syn-1-68-MPAA, and H- $\alpha$ -Syn-1-68-MPAA [A53T] with the two Thz conversion products H- $\alpha$ -Syn-69-140-OH [A69C, A107C] and H- $\alpha$ -Syn-69-140-OH [A69C, A107C, P-S129], we successfully synthesized five  $\alpha$ -Synuclein variants of interest (Table 4), still carrying Cys mutations at positions 69 and 107, which had to be changed back into Ala in the final desulfurization step.

**Table 3.** Yields of the purified Thz conversion products.

Thz conversion product	Yield [%]	Yield [mg]	Purity [%]
H- $\alpha$ -Syn-69-140-OH [A69C, A107C]	25 over 3 steps	6.10	98
H- $\alpha$ -Syn-69-140-OH [A69C, A107C, P-S129]	19 over 3 steps	4.80	95

**Table 4.** Yields of the purified products of second ligation.

Product of second ligation	Yield [%]	Yield [mg]	Purity [%]
H- $\alpha$ -Syn-1-140-OH [A69C, A107C]	23 over 2 steps	3.40	99
Acetyl- $\alpha$ -Syn-1-140-OH [A69C, A107C]	18 over 2 steps	1.81	99
H- $\alpha$ -Syn-1-140-OH [A53T, A69C, A107C]	14 over 2 steps	0.92	95
H- $\alpha$ -Syn-1-140-OH [A69C, A107C, P-S129]	13 over 2 steps	1.32	> 99
Acetyl- $\alpha$ -Syn-1-140-OH [A69C, A107C, P-S129]	14 over 2 steps	1.32	> 99

The yields for thioester conversions and second ligations in one-pot reaction approaches were between 13 and 23% over two steps (Table 4), which tends to be lower compared to the three-step reaction (1)–(3) described above, probably due to the growing peptide chain length. Nevertheless, purities of at least 95% were achieved for all products.

#### Desulfurization (6)

After second ligation, desulfurization of H- $\alpha$ -Syn-1-140-OH [A69C, A107C] into native H- $\alpha$ -Syn-1-140-OH (Scheme 2, (6)) was performed to convert the non-native Cys residues in positions 69 and 107 into the native Ala residues. We adapted a protocol for radical desulfurization using VA-044 as radical initiator, which is well soluble in aqueous systems and has a low decomposition temperature.<sup>[16]</sup> TCEP was used for mediating the reduction of Cys.<sup>[16]</sup> UHPLC analysis showed a slightly higher retention time for the desulfurization product H- $\alpha$ -Syn-1-140-OH (13.767 min, Figure 3, H) than for the starting material H- $\alpha$ -Syn-1-140-OH [A69C, A107C] (13.633 min, Figure 3, G) after 4 h of reaction.

All five products of the second NCL were successfully converted with moderate to good yields (Table 5).

The yields of the desulfurized products were between 42% and 63% (Table 5). The synthesis of H- $\alpha$ -Syn-1-140-OH delivered a yield of 42%, which is lower than the 72% yield reported in literature for the final desulfurization of WT  $\alpha$ -Synuclein.<sup>[17]</sup> Purities of all five products were at least 99%. The overall yields were between 1.3% and 2.4% (Table 5), with 2.4% for the WT  $\alpha$ -Synuclein. This is lower than the overall yields previously

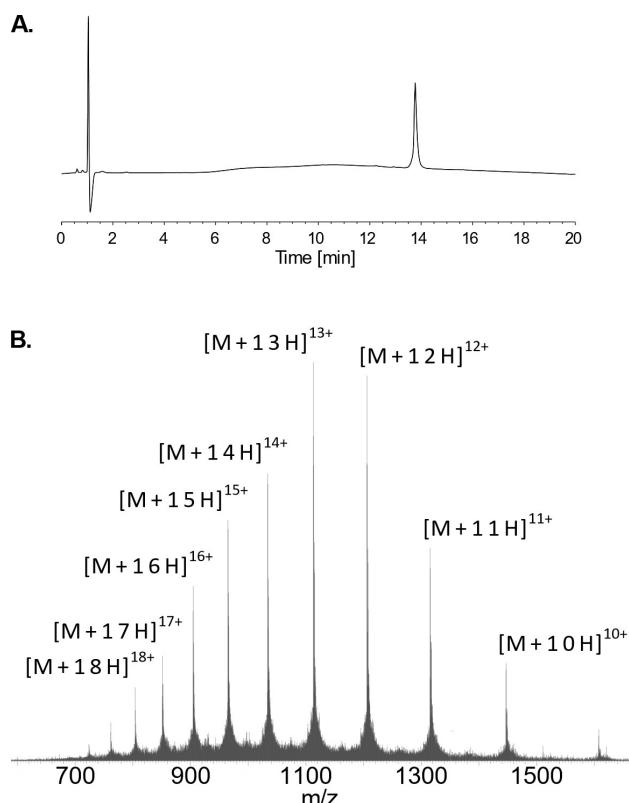
**Table 5.** Yields of purified desulfurized products.

Product	Yield [%]	Yield [mg]	Purity [%]	Overall yield over 6 steps [%]
H- $\alpha$ -Syn-1-140-OH	42	1.22	> 99	2.4
Acetyl- $\alpha$ -Syn-1-140-OH	47	0.82	> 99	2.1
H- $\alpha$ -Syn-1-140-OH [A53T]	52	0.26	99	1.8
H- $\alpha$ -Syn-1-140-OH [P-S129]	54	0.37	> 99	1.3
Acetyl- $\alpha$ -Syn-1-140-OH [P-S129]	63	0.49	> 99	1.7

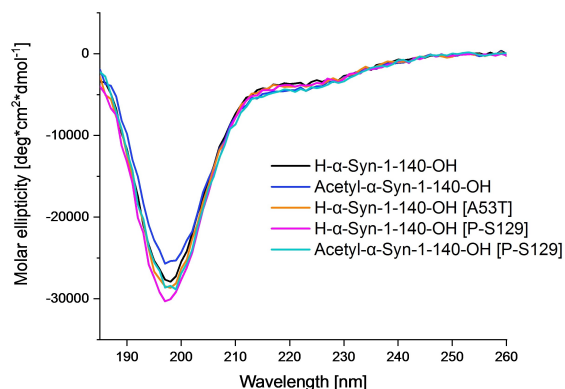
reported for the chemical synthesis of WT  $\alpha$ -Synuclein obtained via different routes,<sup>[17,18]</sup> and further optimization of the procedures will be needed to generate comparable yields via this new method. Importantly, using the present route we also synthesized modified protein variants besides the WT protein, so that a decrease in yield is acceptable.

## Product characterization

For analytical characterization of the products UHPLC chromatograms and high-resolution ESI mass spectra were recorded. UHPLC analysis of WT  $\alpha$ -Synuclein showed a peak at 13.767 min with a purity over 99%, and the ESI-HR mass spectrum confirmed the product identity (Figure 4, A and B). For analytical characterization of all desulfurized products see supplementary analytical data Figures S1–S5 (UHPLC chromatograms) and Figures S6–S10 (mass spectra).



**Figure 4.** Product characterization. A. UHPLC chromatogram of WT H- $\alpha$ -Syn-1-140-OH. B. ESI-HR mass spectrum of WT H- $\alpha$ -Syn-1-140-OH.



**Figure 5.** CD spectra of WT  $\alpha$ -Synuclein and the variants were recorded in water at a concentration of 5  $\mu$ M.

## Characterization of secondary structure by circular dichroism (CD) measurements

CD spectra of WT  $\alpha$ -Synuclein and the variants were recorded to determine their secondary structure (Figure 5). The spectra showed random coil secondary structures for WT  $\alpha$ -Synuclein and for all variants with absorption minima around 198 nm indicating that the synthesized WT  $\alpha$ -Synuclein and the modified variants have a disordered secondary structure independent of the introduced modifications. This agrees with previous observations displaying a random coil structure for biologically expressed WT  $\alpha$ -Synuclein, different mutants and phosphorylated variants.<sup>[32]</sup> Nevertheless, further experiments are necessary to derive firm conclusions about differences in the protein structure and aggregation behavior of the products. More precisely, in future studies we will determine the secondary structure, membrane binding and aggregation properties of the different  $\alpha$ -Synuclein derivatives comparing chemically synthesized and biologically expressed products. Additionally, the present work allows to examine if the chemically synthesized *N*-terminally acetylated  $\alpha$ -Synuclein, A53T mutant and phosphorylated variants will show a behavior that is in agreement with previous observations visualizing that acetylation of  $\alpha$ -Synuclein's *N*-terminus enhances the affinity for membrane binding,<sup>[33]</sup> that A53T causes increased aggregation,<sup>[8]</sup> and that phosphorylation of S129 promotes formation of  $\alpha$ -Synuclein oligomers and fibrils.<sup>[12]</sup>

## Conclusions

In this study, we focused on the chemical synthesis of  $\alpha$ -Synuclein using a new synthetic pathway, in order to overcome limitations in recombinant protein expression in bacteria.  $\alpha$ -Synuclein and several of its variants were successfully synthesized by combining MW-SPPS and ligation strategies. Following an adapted protocol for automated MW-SPPS on 2-CTC resin facilitated the synthesis of  $\alpha$ -Synuclein peptide fragments. Peptide hydrazides were used as thioester precursors and linked to Cys peptides in NCL. Full-length proteins were synthesized fusing three peptide fragments by ligation from C- to *N*-terminus, and utilizing the Thz moiety to mask the *N*-terminal Cys residue of the central peptide fragment. We have shown that the combination of thioester conversion via Knorr pyrazole synthesis, NCL, Thz conversion, and desulfurization is suitable

for the syntheses of WT  $\alpha$ -Synuclein and selected modified  $\alpha$ -Synuclein variants. Compared to previously reported strategies for the chemical synthesis of  $\alpha$ -Synuclein, this method is based on fully automated peptide synthesis and the application of only three instead of four peptide fragments. Therefore, this involves two instead of three ligations, which makes it more convenient and potentially faster. Additionally, pseudoprolines are not needed to enhance efficiency in SPPS. Our new synthetic strategy enabled us to successfully synthesize in milligram scale pure WT  $\alpha$ -Synuclein as well as biologically relevant protein derivatives carrying either a point mutation or a phosphorylation, which will now allow for detailed investigations regarding their protein structure and aggregation behavior. The reported synthetic pathway can be used in the future to create further variants carrying both mutations and PTMs in the same molecule.

## Experimental Section

### General procedures

**General procedure A: Hydrazine modification of 2-CTC resin.** 2-CTC resin (2.00 g, 3.20 mmol, loading: 1.60 mmol/g) was swollen in DCM/DMF (50:50 (V/V), 10 mL, r.t., 30 min) and subsequently incubated with hydrazine hydrate in DMF (10% (V/V), 3  $\times$  13 mL, r.t., 3  $\times$  1 h). Afterwards, the resin was washed with DMF (3  $\times$  10 mL), DCM (3  $\times$  10 mL) and DMF (3  $\times$  10 mL) followed by capping with MeOH in DMF (5% (V/V), 3  $\times$  10 mL, r.t., 3  $\times$  15 min). Then, the resin was washed with DMF (3  $\times$  10 mL), DCM (3  $\times$  10 mL), DMF (3  $\times$  10 mL) and DCM (3  $\times$  10 mL), dried, and stored under vacuum.

**General procedure B: Fmoc-Ala-preloading of hydrazine modified 2-CTC resin.** This step was performed only for the determination of the resin loading. In the synthesis of peptide hydrazides, the hydrazine modified 2-CTC resin was used without preloading. For the determination of the resin loading, hydrazine modified 2-CTC resin was preloaded with Fmoc-protected Ala (see supplementary general procedures Scheme S1). The resin (31.30 mg, 0.05 mmol, estimated loading: 1.60 mmol/g) was swollen in DMF (1 mL, r.t., 10 min). Then, a solution of Fmoc-Ala-OH (62.30 mg, 0.20 mmol, 4 equiv.), HATU (72.20 mg, 0.19 mmol, 3.8 equiv.), HOAt (27.20 mg, 0.20 mmol, 4 equiv.), and DIPEA (70  $\mu$ L, 0.40 mmol, 8 equiv.) in 0.5 mL DMF was added to the resin and incubated overnight at room temperature. The resin was washed with DMF (3  $\times$  2 mL), DCM (3  $\times$  2 mL), DMF (3  $\times$  2 mL) and DCM (3  $\times$  2 mL), and dried under vacuum. The loading was determined as written in the supplementary methods section (see supporting information) and assumed as loading of the hydrazine modified 2-CTC resin. Loadings between 0.35 and 0.52 mmol/g were achieved.

**General procedure C: Fmoc-Ala-preloading of 2-CTC resin.** For the synthesis of C-terminal peptide acids, 2-CTC resin was preloaded with Fmoc-Ala-OH. 2-CTC resin (1.00 g, 1.60 mmol, loading: 1.60 mmol/g) was swollen in DCM (5 mL, r.t., 20 min). Then, a solution of Fmoc-Ala-OH (1.49 g, 4.80 mmol, 3 equiv.) and DIPEA (2.52 mL, 14.40 mmol, 9 equiv.) in 6 mL DCM was added to the resin and incubated at room temperature for 2.5 h. The resin was washed with DCM (3  $\times$  5 mL), DMF (3  $\times$  5 mL) and DCM (3  $\times$  5 mL) followed by capping with a solution of DCM/MeOH/DIPEA (85:15:5, 2  $\times$  5 mL, r.t., 2  $\times$  15 min). The resin was washed with DCM (3  $\times$  5 mL), DMF (3  $\times$  5 mL) and DCM (3  $\times$  5 mL), and dried under vacuum. Loading was determined as written in the supplementary methods section (see supporting information). Determined loadings were between 0.54 and 0.69 mmol/g.

**General procedure D: Cleavage and precipitation.** Cleavage of peptide hydrazides and peptide acids from 2-CTC resin and global deprotection of acid-labile side-chain protecting groups as well as removal of *N*-terminal *tert*-butyloxycarbonyl (Boc) group (if necessary) were performed in one step by using a cleavage mixture of TFA/H<sub>2</sub>O/TIPS/thioanisole/EDT (82.5:5:5:5:2.5). The resin (synthesis scale: 0.05 mmol) was incubated with 10 mL of the cleavage cocktail at room temperature for 2 h. Then, the solution was filtered off and TFA was removed under nitrogen stream. The peptide was precipitated in 10 mL ice cold Et<sub>2</sub>O and centrifuged (8000 rpm, 3 min, -4  $^{\circ}$ C, 3  $\times$ ). The precipitate was dried at room temperature for 30 min and stored at -20  $^{\circ}$ C.

**General procedure E: Acetylation of *N*-terminal peptide hydrazide.** After MW-SPPS on hydrazine modified 2-CTC resin, the resin loaded with *N*-terminally Fmoc-deprotected peptide hydrazide (0.05 mmol) was swollen in DMF (5 mL, r.t., 20 min). Then, a solution of Ac<sub>2</sub>O/DIPEA/DMF (1:1:8, 10 mL) was added and incubated at room temperature for 30 min. The step was repeated once followed by washing with DMF (3  $\times$  5 mL), DCM (3  $\times$  5 mL), DMF (3  $\times$  5 mL) and DCM (3  $\times$  5 mL), and drying under vacuum. Afterwards, cleavage was performed according to General procedure D.

**General procedure F: Thioester conversion.** MPAA (6.31 mg, 37.5  $\mu$ mol, 0.15 M) was added to thioester conversion buffer (6 M Gn<sup>\*</sup>HCl, 0.25 mL). The mixture was sonicated, pH was adjusted to 3 with 1 M NaOH and then the buffer was bubbled with argon for 10 min. Afterwards, peptide hydrazide (12.3–56.9 mg/mL, 1 equiv.) and acac (40–500 equiv.) were added and the solution was agitated at 25  $^{\circ}$ C for 4–6 h. Completion of the reaction was monitored by UHPLC analysis (1  $\mu$ L sample + 49  $\mu$ L PBS buffer + 50  $\mu$ L H<sub>2</sub>O + 0.1% TFA). Subsequently, NCL was carried out in one-pot reaction approach without previous thioester purification.

**General procedure G: NCL.** After TCEP<sup>\*</sup>HCl (0.29–0.90 M) was added to the ligation buffer (6 M Gn<sup>\*</sup>HCl, 0.2 M Na<sub>3</sub>PO<sub>4</sub>, 0.25 mL), the solution was adjusted to pH 7 with 1 M NaOH and bubbled with argon for 10 min. Then, Cys peptide (13.4–66.9 mg/mL, 1 equiv.) was dissolved in ligation buffer and the solution was combined with the reaction mixture of the thioester conversion, adjusted to pH 7 with 1 M NaOH, bubbled with argon again, and agitated at 37  $^{\circ}$ C for 16–20 h. No additional MPAA was added, the concentration of MPAA in the ligation reaction mixture after dilution of thioester reaction mixture was 0.075 M. Completion of the reaction was monitored by UHPLC analysis (1  $\mu$ L sample + 49  $\mu$ L PBS buffer + 50  $\mu$ L H<sub>2</sub>O + 0.1% TFA). Subsequently, either Thz conversion was carried out in one-pot reaction approach without previous purification of the ligation product or the reaction mixture was diluted with H<sub>2</sub>O + 0.1% TFA and purified by semi-preparative HPLC if isolation of ligation product was desired.

**General procedure H: Thz conversion.** TCEP<sup>\*</sup>HCl (0.14–0.28 M) and *O*-methylhydroxylamine<sup>\*</sup>HCl (0.48–0.96 M) were added to the ligation reaction mixture and pH was adjusted to 4 with 1 M NaOH. The mixture was bubbled with argon for 10 min and agitated at 37  $^{\circ}$ C for 3–6 h. Completion of the reaction was monitored by UHPLC analysis (1  $\mu$ L sample + 49  $\mu$ L PBS buffer + 50  $\mu$ L H<sub>2</sub>O + 0.1% TFA). The reaction mixture was diluted with H<sub>2</sub>O + 0.1% TFA and the product was purified by semi-preparative HPLC.

**General procedure I: Desulfurization.** After TCEP<sup>\*</sup>HCl (1.00 M) was added to the ligation buffer (6 M Gn<sup>\*</sup>HCl, 0.2 M Na<sub>3</sub>PO<sub>4</sub>, 0.1 mL), the solution was adjusted to pH 7 with 1 M NaOH and bubbled with argon for 10 min. Then, the peptide (6.9–29.0 mg/mL, 1 equiv.) was dissolved in the buffer, *t*-BuSH (40  $\mu$ L) and VA-044<sup>\*</sup>2HCl (40  $\mu$ L of 0.1 M solution in H<sub>2</sub>O) were added, and the mixture was agitated at 37  $^{\circ}$ C for 4–6 h. Completion of the reaction was monitored by UHPLC analysis (1  $\mu$ L sample + 49  $\mu$ L PBS buffer + 50  $\mu$ L H<sub>2</sub>O + 0.1% TFA). The reaction mixture was diluted with H<sub>2</sub>O + 0.1% TFA and the desulfurization product was purified by semi-preparative HPLC.



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## Conflict of Interests

All authors declare no conflicts of interest.

## Data Availability Statement

The data of this study are available within the supporting information of this article and from the corresponding authors upon reasonable request.

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