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Synthesis and Semi-Synthesis of Alpha-Synuclein: Insight into the Chemical Complexity of Synucleinopathies

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The chemical rules governing protein folding have intrigued generations of researchers for decades. With the advent of artificial intelligence (AI), prediction of protein structure has improved tremendously. However, there is still a level of analysis that is only possible through wet laboratory experiments, especially in respect to the investigation of the pathological effect of mutations and posttranslational modifications (PTMs) on proteins of interest. This requires the availability of pure peptides and proteins in sufficient quantities for biophysical, biochemical, and functional studies. In this context, chemical protein synthesis and semi-synthesis are powerful tools in protein research, which help to enlighten the role of protein modification in the physiology and pathology of proteins. A protein of high interest in the field of biomedicine is alpha-synuclein (aSyn), a protein deeply associated with several devastating neurodegenerative disorders such as Parkinson's disease (PD), dementia with Lewy bodies (DLB), or multiple systems atrophy (MSA). Here, we describe several methods and pathways to synthesize native or modified aSyn, and discuss how these approaches enable us to address pathological mechanisms that may open novel perspectives for therapeutic intervention.

Introduction

After more than 25 years of the association of the protein alpha-synuclein (aSyn) in Parkinson's disease and other diseases known as synucleinopathies, the interest in understanding the molecular mechanisms connecting aSyn with disease is as great as ever. aSyn is a 140-amino acid (AA) protein highly abundant in the human brain, and is involved in neurotransmitter release.[1,2] However, aSyn is also present in non-neuronal cells, such as erythrocytes and platelets, where it performs unknown functions.[3–7,8] aSyn is encoded by the *SNCA* gene and is an intrinsically disordered protein.[1,5–7,9] In the presence of lipids, detergents, or membranes, it can also adopt alpha-helical regions, and form soluble tetramers that appear to resist aggregation.[6,10] Physiologically, aSyn occurs in a dynamic equilibrium between a functional synaptic vesicle membrane-bound α-helical multimer and the cytosolic unstructured monomer.[5,11] Pathologically, aSyn converts into β-sheet-rich conformations upon aggregation forming oligomers, protofibrils and amyloid fibrils.[12] In the last decades, a lot of research was devoted to identifying the toxic species of aSyn. Therefore, numerous types of aggregates, their fibril morphology, aggregation kinetics and cytotoxicity have been studied using microscopic techniques, biochemical assays and several *in vitro* and *in vivo* models. Although there is still controversy in the field, oligomeric species of aSyn are thought to be more toxic than fibrillar forms, and to be associated with neuronal dysfunction and neurodegeneration.[3–5,13,14]

The propensity of aSyn 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 contribute to its pathogenicity.[15] To study the physiological functions of aSyn and its aggregation behavior, sufficient amounts of wild type (WT) and/or modified protein are required. Usually, recombinant protein expression is applied to produce pure aSyn, but biological expression is limited in the synthesis of modified protein variants. In recombinant protein synthesis, protein modification mostly is performed after protein expression by utilizing enzymes or chemical reagents to introduce different kinds of modifications. This is very efficient, but lacks site-selectivity and control, yielding mixtures of modified proteins.[16] Although these heterogenous modified proteins may reflect the biological complexity and diversity of naturally occurring aSyn modifications, they make it impossible

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to determine effects of specific modifications defined by their kind, number and position in the protein sequence. However, this is required to clarify the structure-function and structure-toxicity relationships of these modifications as well as to investigate effects of defined combined modifications. Thus, continuous research efforts are needed in order to elucidate the role of modifications in the biology and pathobiology of the protein. To overcome the limitations of biological protein expression, total chemical and semi-synthetic approaches have been investigated and enable the site-selective introduction of a single or multiple custom-made modifications into peptide and protein sequences with an immense flexibility in the kind, number and position of the modifications.[16,17] They facilitate the synthesis of protein variants carrying native or non-native modifications, including non-coded AAs, isotopes, fluorophores and PTMs, to study their effects as well as the crosstalk between multiple modifications, helping to clarify the relationship between structure, function and dysfunction of proteins.[18] In detail, the site-specific introduction of PTMs into the aSyn sequence can give insights into effects of modifications on the protein structure and function, membrane binding, oligomerization and fibrillization kinetics, aggregate morphology and neurotoxicity. Therefore, chemical protein synthesis is extremely useful alone or in combination with biological expression to better understand how modifications influence the physiology and pathology of aSyn.

Total Chemical Synthesis

To overcome limitations in protein expression and enable the synthesis of site-selectively modified aSyn proteins, three approaches for the total chemical synthesis of WT aSyn, also applicable to several biologically relevant protein variants, have been developed.[19–21] As aSyn consists of 140 AAs, chemical synthesis using solid-phase peptide synthesis (SPPS) alone would not deliver sufficient amounts of protein due to decreasing product yields with increasing number of coupling steps. Thus, 9-fluorenylmethoxycarbonyl (Fmoc)-based SPPS in combination with ligation-desulfurization approaches have been applied to the chemical synthesis of aSyn. This strategy is based on the synthesis of peptide fragments by SPPS and subsequent peptide fragment ligation using native chemical ligation (NCL) as a well-established technique to link unprotected peptide thioesters and Cys peptides in a chemoselective condensation reaction, thereby significantly extending the size of proteins accessible via chemical synthesis.[16,22,23] Radical desulfurization of Cys into Ala is performed to obtain the native full-length proteins, as aSyn lacks Cys residues in its sequence.[24] With the proteins at hand, the secondary structure and aggregation behavior of the WT protein and its variants have been investigated. The names of aSyn peptide fragments and proteins are given as H-aSyn-x-y-OH or as their *N*- or *C*-terminally functionalized analogues, with x and y being the numbers of the AA positions in the AA sequence of aSyn.

Tiago Outeiro graduated in Biochemistry at the University of Porto and did his PhD thesis at the Whitehead Institute for Biomedical research–MIT. He then was a Postdoctoral Research Fellow in the Department of Neurology of the Massachusetts General Hospital–Harvard Medical School. He is currently Full Professor and the Director of the Department of Experimental Neurodegeneration at the University Medical Center Goettingen, in Germany. Tiago holds a joint Professor position at Newcastle University in the UK. Tiago has authored >320 research articles in international journals and participates in various international boards.

Approach Used by Fauvet et al.[19]

Fauvet et al. synthesized the three peptide fragments H-aSyn-1-29-SR, H-aSyn-30-68-SR [A30Thz] and H-aSyn-69-140-OH [A69C] via automated Fmoc-SPPS. The allyloxycarbonyl (Alloc)-protected 3,4-diaminobenzoyl (Dbz) linker (*N*-acyl urea approach) was used to obtain peptide thioesters after peptide chain elongation, Alloc deprotection using phenylsilane and a palladium catalyst, Nbz activation using *p*-nitrophenyl chloroformate, cleavage from resin and subsequent thiolysis with 2-mercaptoethanesulfonate sodium (MesNa).[25] Peptides carried Thz or Cys point mutations, which were required in NCL.[26] Initially, four peptide fragments were synthesized, namely H-aSyn-1-29-SR, H-aSyn-30-68-SR [A30Thz], H-aSyn-69-106-SR [A69Thz] and H-aSyn-107-140-OH [A107C], with low yields for H-aSyn-30-68-SR [A30Thz] and H-aSyn-69-106-SR [A69Thz]. The introduction of β-sheet-disrupting Lys-Thr pseudoproline dipeptides at the positions 43–44 and 58–59 prevented aggregation of the growing peptide chain and the use of the more efficient coupling with *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N′*,*N′*-tetramethyluronium hexafluorophosphate (HATU) for AAs 30–44 led to a good yield of 10% for H-aSyn-30-68-SR [A30Thz]. In contrast, the yield of H-aSyn-69-106-SR [A69Thz] could not be improved, as it is part of the hydrophobic aggregation-prone NAC domain. Given the good solubility of the *C*-terminal domain, the longer peptide fragment H-aSyn-69-140-OH [A69C] was synthesized in 14% yield using HATU for the coupling of AAs 69–85 and the Lys-Thr pseudoproline dipeptide at the position 81–82. The three peptide fragments required two ligation steps, which were all carried out in one pot. Ligations were performed in *C*-to-*N* direction using the Thz moiety to mask the Cys residue of the central peptide fragment temporarily.[26] The protecting group was removed using *O*-methylhydroxylamine at pH 4 after the first ligation to activate the ligation product for the second ligation. After ligations were completed, 4-mercaptophenylacetic acid (MPAA) was removed by desalting, followed by radical desulfurization using 2,2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) without previous purification of ligation products. Fauvet *et al.* synthesized aSyn in a one-pot chemical synthesis approach in a total yield of 21% over all steps (Scheme 1). Synthesized and recombinant WT were studied using circular dichroism (CD) spectroscopy, and

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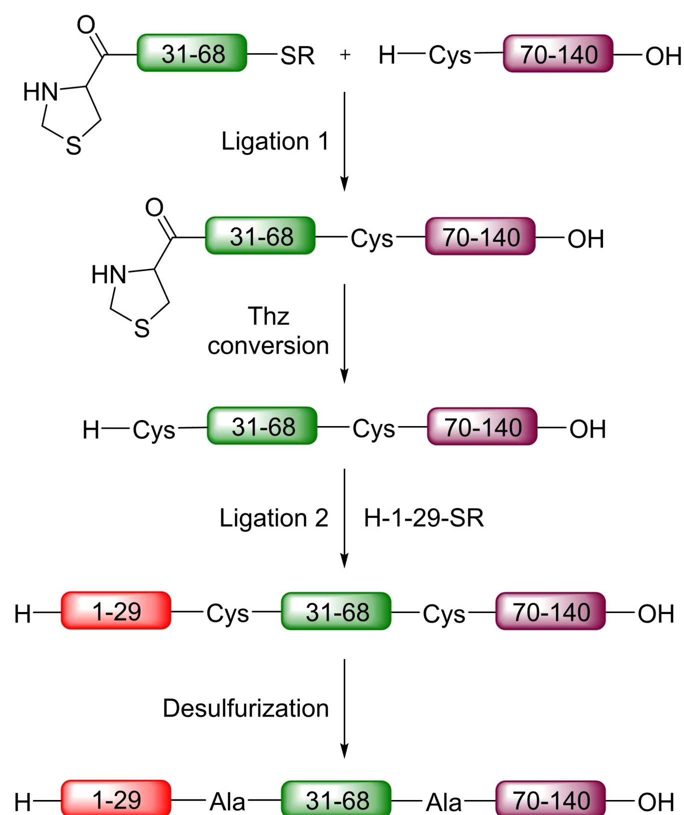


Scheme 1. Total chemical synthesis of aSyn reported by Fauvet *et al.*[19] (−SR: thioester, Thz: thiazolidine)

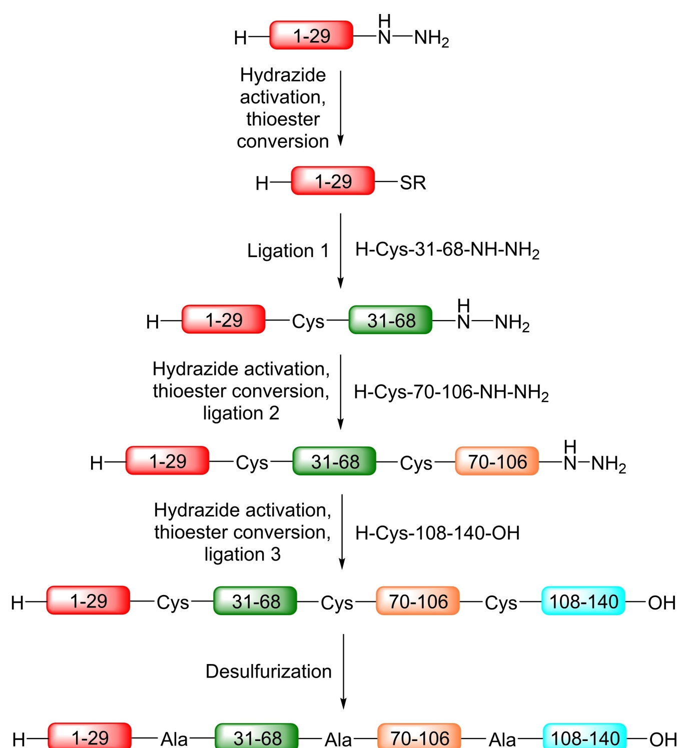
both showed a random coil secondary structure as well as α-helices upon addition of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (POPG) vesicles. Additionally, no significant differences in the fibrillization, monitored by Thioflavin T (ThT) assay, and a similar loss of soluble protein during aggregation, monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were observed. Transmission electron microscopy (TEM) showed that both form similar fibrillar structures during aggregation. Therefore, synthesized and recombinant WT were demonstrated to have similar membrane binding and aggregation properties.

Approach Used by Zheng et al.[20]

Zheng *et al.* synthesized the four peptide fragments H-aSyn-1-29-NH-NH_2, H-aSyn-30-68-NH-NH_2 [A30C], H-aSyn-69-106-NH-NH_2 [A69C] and H-aSyn-107-140-OH [A107C] of nearly equal length via Fmoc-SPPS in yields between 18 and 30%. Hydrazine-modified 2-chlorotrityl chloride (2-CTC) resin was used to prepare peptide hydrazides as thioester precursors, which were activated into peptide azides at −15 °C using $NaNO_2$, followed by thiolysis at pH 3 using MPAA and NCL with Cys peptides at pH 7 in a one-pot reaction. The four peptide fragments required three ligation steps. Since ligations were performed in *N*-to-*C* direction, a masking of *N*-terminal Cys was not necessary. Further, a protection of the free *C*-terminal hydrazide functionality during protein synthesis was not required, because it is not reactive in NCL before activation. Products were purified after each ligation step. Radical desulfurization of Cys using VA-044 was performed at the end to obtain WT aSyn in an overall yield of 11% (Scheme 2). High product purity was shown by high-performance liquid chromatography (HPLC), while electrospray ionization (ESI) mass analysis and SDS-PAGE confirmed the product identity. The structural characterization using CD spectroscopy showed a random coil secondary structure and the ThT assay showed aggregation, as expected.

Approach Used by Gatzemeier et al.[21]

Gatzemeier *et al.* synthesized the three peptide fragments H-aSyn-1-68-NH-NH_2, H-aSyn-69-106-NH-NH_2 [A30Thz] and H-aSyn-107-140-OH [A107C] and their modified variants via Fmoc-SPPS in yields between 2 and 25%. Hydrazine-modified 2-CTC resin was used to prepare peptide hydrazides as thioester precursors, which were activated into acyl pyrazoles using acetylacetone (Acac) and converted into thioesters at pH 3 using MPAA, followed by NCL with Cys peptides at pH 7 in a one-pot reaction. The three peptide fragments required two ligation steps. Since ligations were performed in *C*-to-*N* direction, a masking of the *N*-terminal Cys residue of the central peptide fragment using the Thz moiety was necessary and the protecting group was removed using *O*-methylhydroxylamine at pH 4 after the first ligation to activate the ligation product for the second ligation. Purifications were performed after Thz



Scheme 2. Total chemical synthesis of aSyn reported by Zheng *et al.*[20]

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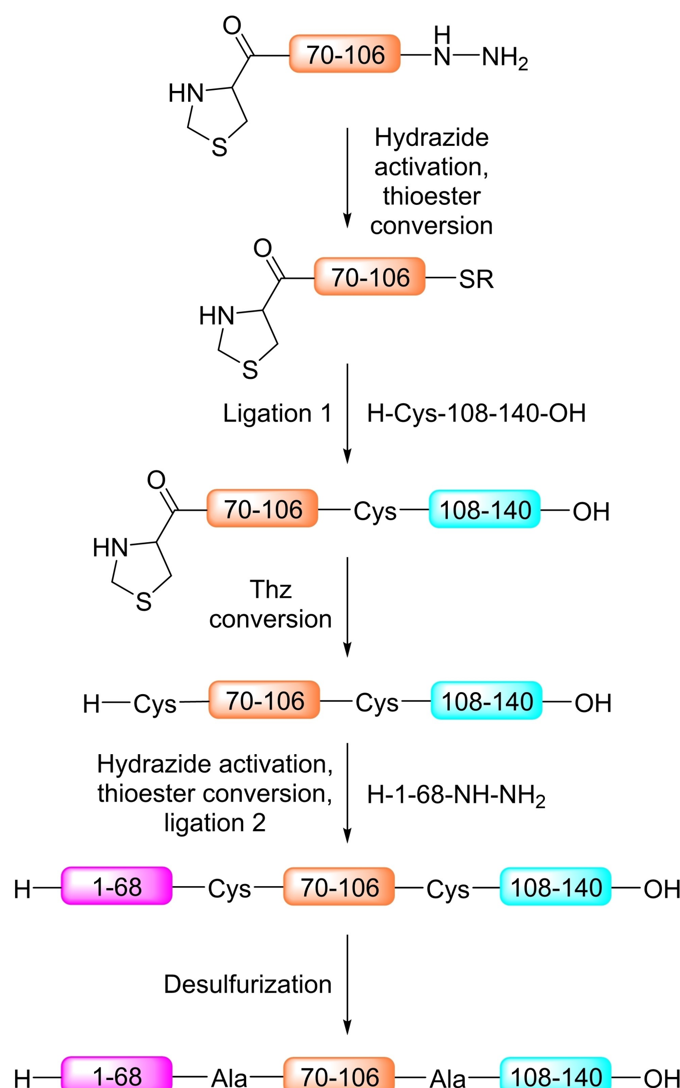
conversion and after the second ligation. Radical desulfurization of Cys using VA-044 was performed at the end to obtain aSyn and several of its protein variants in overall yields of 1.3–2.4% (Scheme 3). High product purities were shown by HPLC, and ESI mass analysis confirmed the product identities. The structural characterization using CD spectroscopy showed random coil secondary structures for WT aSyn and the synthesized variants.

To conclude, these three approaches enabling the total chemical synthesis of aSyn utilized common strategies of modern chemical protein synthesis. The target protein was obtained by combining SPPS and NCL. The three approaches differ in the choice of the ligation sites, the number of peptide fragments and ligations as well as the methods used in thioester preparation. Ligation sites were chosen in a way that enabled the synthesis of peptide fragments of about 30 to 70 AAs in length, as the yield of longer peptide fragments would decrease dramatically. In SPPS, manual or automated coupling was applied, different resins and different AA activation reagents were utilized. To obtain peptide thioesters, Dbz peptides or peptide hydrazides were used as thioester precursors and activated in different ways. The ligation direction and thus the protection strategies in the sequential ligations differed. Whereas in *C*-to-*N* direction a temporary protection strategy for Cys was necessary, a protection strategy was not required in *N*-to-*C* direction using peptide hydrazides as masked thioesters. NCLs were carried out in guanidine (Gn) buffer at pH 7 using MPAA as catalyst and tris(2-carboxyethyl)phosphine (TCEP) as reducing agent. Desulfurizations were performed using VA-044 as radical initiator. One-pot reaction approaches were utilized in different extends. All three methods demonstrated the successful total chemical synthesis of aSyn in mg scale. Pure products were obtained and biologically characterized using established assays in the field. However, the overall yields differed between the methods, from 1 to 21%. The differences in yields are most likely due to different synthetic parameters and purification conditions. While *Fauvet et al.* obtained a very high overall yield of 21% perfoming a one-pot synthetic approach with only one purification step at the end of the ligation-desulfurization reaction sequence, the yield obtained by *Gatzemeier et al.* was only 1–2% of full-length proteins carrying out a purification step after each ligation, which may be caused by loss of sample during purification. This suggests an advantage of one-pot reaction approaches. However, the approach of *Gatzemeier et al.* included the synthesis of modified variants besides WT protein, which may also change the yield. Another aspect to consider is that performing total one-pot approaches increases the risk of undesired side-reactions of excess reagents during ligations. Altogether, these methods demonstrate the importance and opportunities of total chemical protein synthesis in the investigation of the biological and pathological properties of aSyn, which is highly relevant in the context of the study of PD pathogenesis and for the development of possible treatment strategies. Additionally, the methods utilized are not limited to aSyn and open a wide range of possibilities for the chemical synthesis of other proteins of interest. *Zheng et al.* reported a very high workload of about 30 working days to obtain aSyn in mg scale, which is very time-consuming, while the other approaches do not give an exact workload, but are also highly costly. Thus, total chemical synthesis for producing large proteins carrying tailor-made site-specific modifications can be challenging and time-consuming. Alternatively, protein semi-synthesis can be applied to combine advantages of biological expression and chemical synthesis.



Scheme 3. Total chemical synthesis of aSyn reported by Gatzemeier *et al.*[21]

Semi-Synthesis

Chemical ligation strategies have been extended to the combined use of chemically synthesized and biologically-expressed peptide fragments in the so-called expressed protein ligation (EPL) approach.[16,27] The use of chemically synthesized peptide fragments enables the site-specific introduction of well-defined modifications inaccessible via expression, such as unnatural AAs, PTMs and biophysical probes, and in combination with expressed peptide fragments allows access to large

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proteins, thus making EPL a powerful tool in protein engineering.[23,27–29]

To enable EPL, a thioester and a Cys peptide are required as in NCL. Thioesters and Cys peptides are easily accessible via chemical methods. While thioesters can be prepared via Fmoc-SPPS using different linker and activation strategies, Cys peptides are obtained by replacing an Ala residue by Cys during SPPS. As necessary, modifications can be introduced via different ways, such as the exchange of AAs during SPPS in order to introduce point mutations, the use of modified AAs as building blocks in SPPS or site-selective introduction of modifications after SPPS at resin-bound peptides following orthogonal protection group strategies.[21,30,31] In recombinant protein synthesis, peptide thioesters can be produced by intein technology.[18,23,27] The peptide fragment is expressed *N*-terminally to an intein domain, followed by thiolysis of the intein fusion protein, resulting in the formation of a *C*-terminal protein thioester, which allows the semi-synthesis of complex proteins.[28] Hydrazides as thioester precursors can also be produced by recombinant techniques via hydrazinolysis of intein fusion products or by hydrazinolysis of

C-terminally incorporated α-hydroxy acids, thereby having a potential in the sequential assembly of large proteins.[18,32] Further, *C*-terminal peptide fragments carrying an *N*-terminal Cys residue can be biologically expressed. Subsequently, they usually are treated with *O*-methylhydroxyl-amine to cleave unreactive cyclic Thz adducts, which are formed under biological conditions by reaction with aldehydes or ketones abundant in bacteria, and purified prior to use in ligation.[33–35]

Numerous semi-synthetic approaches for aSyn variants carrying site-selective modifications are reported, aiming for the investigation of the effects of these modifications on the protein structure and aggregation. Some of them are discussed below.

Phosphorylation

Under physiological conditions, the level of phosphorylated (p) aSyn is low, whereas pS87 and pS129 as well as pY125, pY133 and pY136 have been detected in pathologically aggregated aSyn species.[15,36,37] Chemically, phosphorylation is introduced by incorporation of phosphorylated AA building blocks, such as Fmoc-Tyr(PO(OBzl)OH)-OH and Fmoc-Ser(PO(OBzl)OH)-OH, at the respective AA position during SPPS (Figure 1).

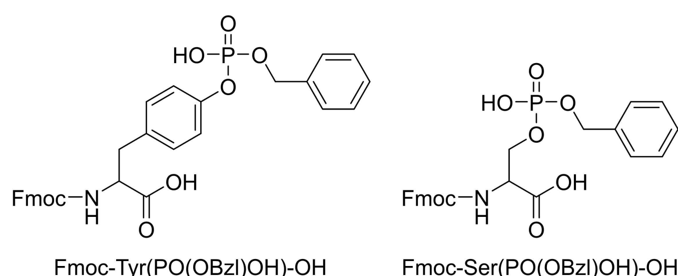

Figure 1. Examples of phosphorylated AA building blocks for use in SPPS.

Dikiy *et al.* synthesized semi-synthetic phosphorylated H-aSyn-1-140-OH [pY39] from chemically synthesized H-aSyn-1-29-SR and H-aSyn-30-55-SR [A30Thz, pY39] as well as recombinant H-aSyn-56-140-OH [A56C] (Scheme 4A). Fmoc-SPPS was carried out automatically, except from manual coupling of the phosphorylated Tyr at position 39 in form of Fmoc-Tyr(PO-(OBzl)OH)-OH. Thioester preparation was performed by the *N*-acylurea approach. *C*-To-*N*-directed ligation using the Thz protection strategy for the central peptide fragment was applied in a one-pot manner, followed by radical desulfurization.[38]

Zhao *et al.* used a similar semi-synthetic approach to synthesize phosphorylated H-aSyn-1-140-OH [pY39] from chemically synthesized H-aSyn-1-29-NH-NH_2 and H-aSyn-30-52-NH-NH_2 [A30C, pY39] as well as recombinant H-aSyn-53-140-OH [A53C] (Scheme 4B). Peptide hydrazides were prepared by manual Fmoc-SPPS and hydrazides were converted into thioesters oxidatively via peptide azides. *N*-To-*C*-directed ligation was applied, followed by radical desulfurization.[39]

Pan *et al.* used two different semi-synthetic approaches (and a chemoenzymatic approach, which is not discussed here) to generate H-aSyn-1-140-OH [SC9, pY39], which was doubly labeled with fluorophores at position 9 and position 72 or 136, respectively. Either synthetic H-aSyn-1-29-NH-NH_2 [S9C], synthetic H-aSyn-30-55-NH-NH_2 [A30C, pY39] and recombinant H-aSyn-56-140-OH [A56C], or recombinant H-aSyn-1-36-SR [S9C], synthetic H-aSyn-37-55-NH-NH_2 [V37penicillamine (Pen), pY39] and recombinant H-aSyn-56-140-OH [A56C] were used (Scheme 4C). For labeling, either a Cys mutation was introduced and the peptide fragment was treated with the fluorophore-maleimide to attach the fluorophore, or unnatural AA mutagenesis in the *C*-terminal peptide fragment, followed by labeling, was applied. Peptide hydrazides were prepared by Fmoc-SPPS and converted into thioesters oxidatively via peptide azides. *N*-To-*C*-directed ligation was performed, followed by radical desulfurization of Pen into Val and Cys into Ala.[40]

Hejjaoui *et al.* synthesized semi-synthetic phosphorylated H-aSyn-1-140-OH [pY125] from recombinant H-aSyn-1-106-SR and chemically synthesized H-aSyn-107-140-OH [A107C, pY125] by Fmoc-SPPS using phosphorylated Tyr as building block at position 125, followed by ligation and radical desulfurization (Scheme 4D).[41]

Similarly, Fauvet *et al.* and Ma *et al.* synthesized semi-synthetic phosphorylated H-aSyn-1-140-OH [pS129] from the same peptide fragments but using side-chain benzyl-protected phosphorylated Ser (pSer) as building block at position 129 in Fmoc-SPPS (Scheme 4E).[30,42]

Nitration

In some cases, aSyn has found to be nitrated (n) at Y39, 125, 133 and/or 136.[15,43] Chemically, nitration is introduced by incorporation of the nitrated AA building block Fmoc-3-nitro tyrosine during SPPS at the respective AA position (Figure 2).

Burai *et al.* synthesized semi-synthetic H-aSyn-1-140-OH [nY39/nY125] carrying a site-selective nitration at Y39 or Y125.

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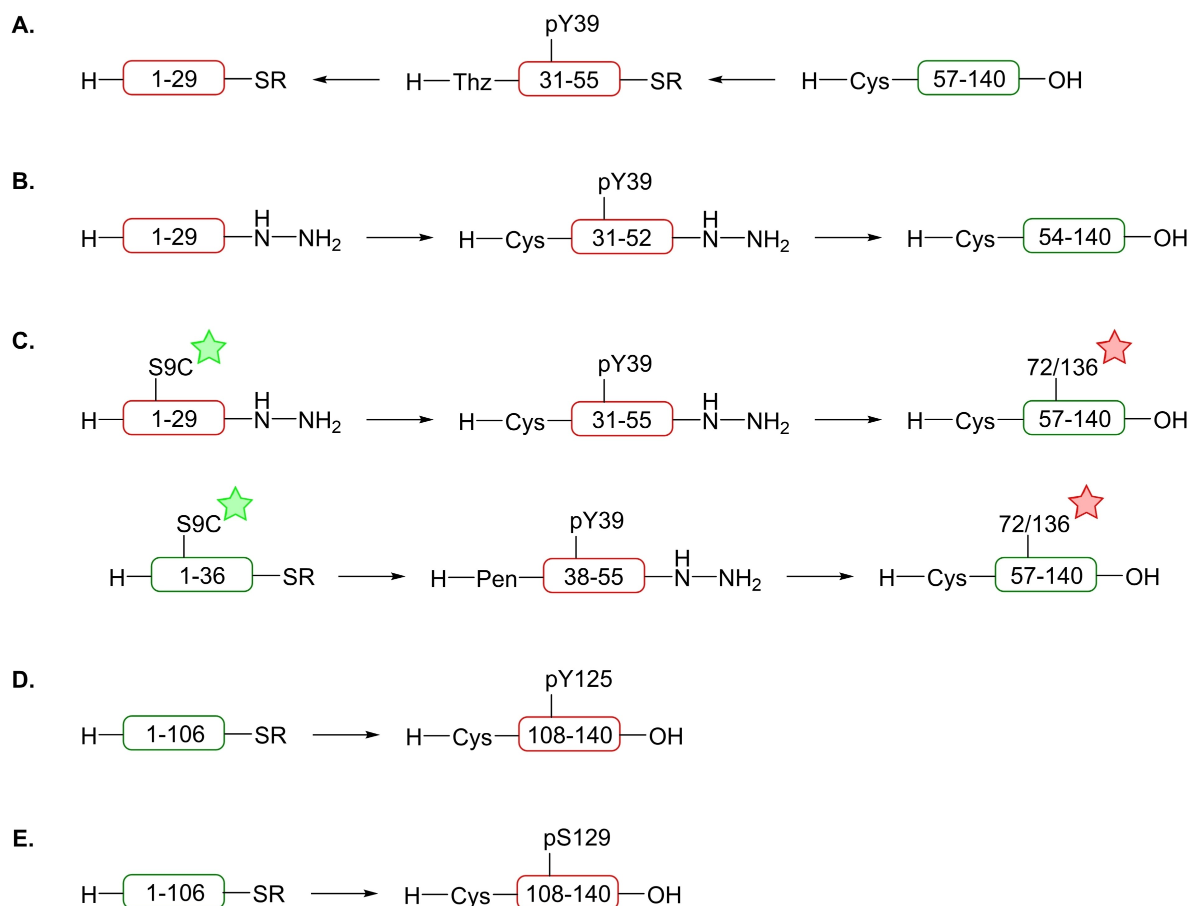


Scheme 4. Semi-synthetic approaches for phosphorylated aSyn proteins. Chemically synthesized peptide fragments are depicted in red frames and biologically expressed peptide fragments in green frames. Labeling is indicated by colored stars.

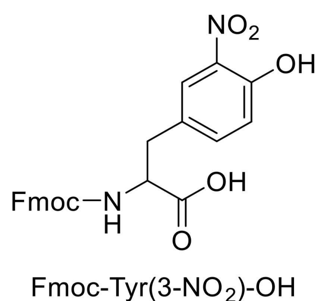
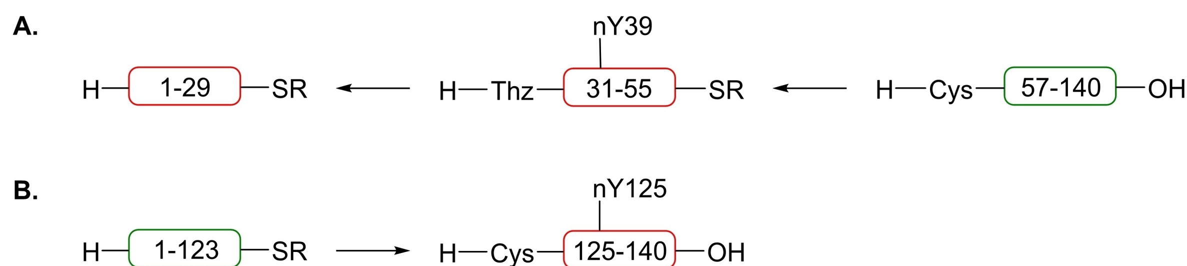


Figure 2. Nitrated Tyr building block for use in SPPS.

In the synthesis of Y39-nitrated aSyn, chemically synthesized H-aSyn-1-29-SR, chemically synthesized nitrated H-aSyn-30-55-SR [A30Thz, nY39] and recombinant H-aSyn-56-140-OH [A56C] were applied (Scheme 5A). In the synthesis of Y125-nitrated aSyn, recombinant H-aSyn-1-123-SR and chemically synthesized H-aSyn-124-140-OH [A124C, nY125] were ligated and desulfurized (Scheme 5B). Fmoc-SPPS of synthetic peptide fragments was performed with commercially available Fmoc-3-nitro tyrosine as building block at positions 39 or 125, respectively. In the synthesis of Y39-nitrated aSyn, thioester preparation of the *N*-terminal and central peptide fragments was performed by



Scheme 5. Semi-synthetic approaches for nitrated aSyn proteins. Chemically synthesized peptide fragments are depicted in red frames and biologically expressed peptide fragments in green frames.

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exploiting the *N*-acylurea approach. *C*-To-*N*-directed ligation using the Thz protection strategy for the central peptide fragment was applied in a one-pot manner, followed by a modified desulfurization. Due to nitro-reduction under standard radical desulfurization conditions, 2-nitrobenzylamine was added to the desulfurization mixture in order to suppress the nitro-reduction and enable Cys desulfurization in presence of 3-nitro tyrosine.[43]

Ubiquitination

The Lys residues 6, 10 and 12 are the major ubiquitination sites in aSyn.[44,45] Monoubiquitination affects aSyn aggregation depending on the modification site.[44] Furthermore, the length of ubiquitin chains plays an important role in regulating aSyn fibril formation.[14,46] Ubiquitination of aSyn with ubiquitin (ub) ligases results in heterogenous ubiquitination. However, site-specific modification is necessary to determine precise effects of ubiquitination. Chemically, ubiquitination is introduced via ligation of an ubiquitin thioester and a side-chain thiolated AA in aSyn.

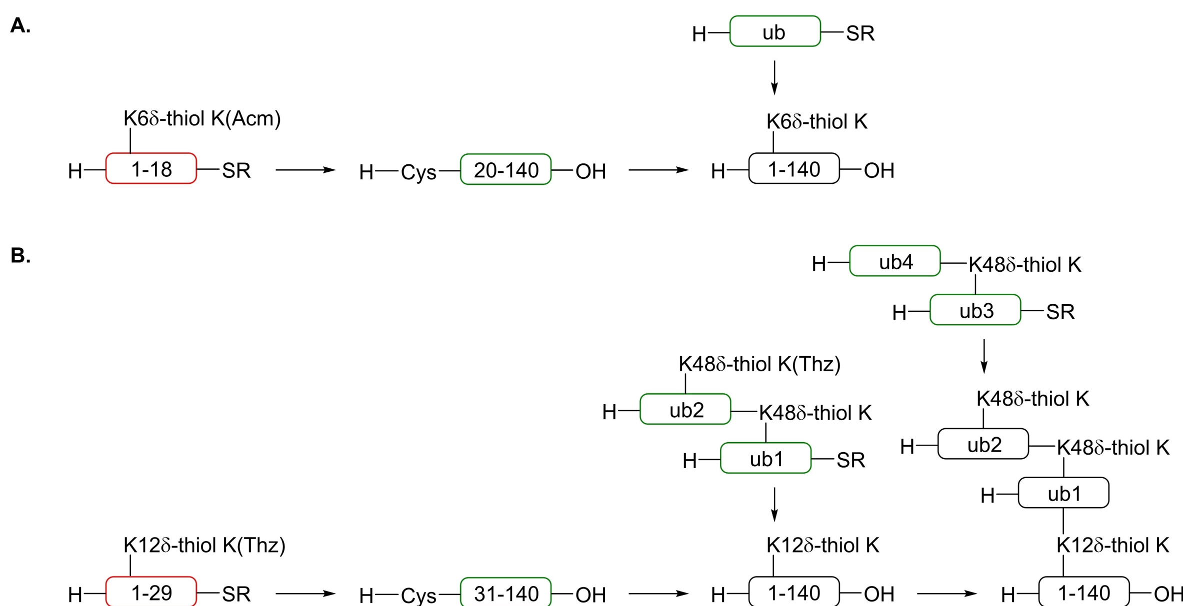
Hejjaoui *et al.* synthesized semi-synthetic H-aSyn-1-140-OH [ubK6], ubiquitinated at K6, from synthesized H-aSyn-1-18-SR [K6δ-thiol K] and recombinant H-aSyn-19-140-OH [A19C]. *tert*-Butyloxycarbonyl (Boc)-SPPS was used to synthesize H-aSyn-1-18-SR [K6δ-thiol K] carrying the acetamidomethyl (Acm)-protected δ-thiol Lys instead of K6.[47] After ligation, the Acm group was deprotected using $Hg(OAc)_2$ and dithiothreitol. Then, the recombinant ubiquitin thioester ub-SR, was ligated to the side-chain of δ-thiol Lys, followed by radical desulfurization of C19 and δ-thiol Lys (Scheme 6A).[28,33,48,49]

Haj-Yahya *et al.* prepared semi-synthetic polyubiquitinated aSyn. This method enabled the generation of K12 di- and tetraubiquitinated protein from chemically synthesized H-aSyn-1-29-SR [K12δ-thiol K] and recombinant H-aSyn-30-140-OH [A30C]. Fmoc-SPPS was used to synthesize H-aSyn-1-29-SR [K12δ-thiol K] carrying the Thz-protected δ-thiol Lys instead of K12. Thioester preparation was performed by *N*-acylurea approach. After ligation, the Thz group was cleaved to yield free δ-thiol Lys accessible for the attachment of the K48-linked diubiquitin thioester ub21-SR under ligation conditions. This ligation was followed by Thz-unmasking of the δ-thiol Lys residue of the incorporated diubiquitin and the next ligation with the K48-linked diubiquitin thioester ub43-SR.Finally, radical desulfurization of C30 and the δ-thiol Lys residues was performed (Scheme 6B).[46,50]

Glycosylation

O-Glc*N*Acylation, an attachment of *N*-acetyl-D-glucosamine at Ser or Thr residues, is introduced chemically via incorporation of glycosylated AA building blocks during SPPS (Figure 3).

Marotta *et al.* synthesized semi-synthetic glycosylated (g) H-aSyn-1-140-OH [gT72], carrying *O*-linked *O*-Glc*N*Ac at T72, from recombinant H-aSyn-1-68-SR, synthetic H-aSyn-69-75-SR [A69Thz, gT72] and recombinant H-aSyn-76-140-OH [A76C] (Scheme 7A). Fmoc-SPPS was carried out manually and thioester preparation was achieved via the *N*-acylurea approach. For incorporation of gT72, Fmoc-protected pentafluorophenyl-activated glycosylated Thr, carrying acetyl groups (Ac) at the sugar hydroxyl groups, was used and *O*-acetyl groups were removed after peptide synthesis by treatment with hydrazine hydrate. Then, linker activation with *p*-nitrophenyl chloroformate, cleavage and thiolysis were performed, followed by *C*-to-*N*-directed ligation using the Thz protection strategy for the central peptide fragment and radical desulfurization.[51,52]



Scheme 6. Semi-synthetic approaches for ubiquitinated aSyn proteins. Chemically synthesized peptide fragments are depicted in red frames and biologically expressed peptide fragments in green frames.

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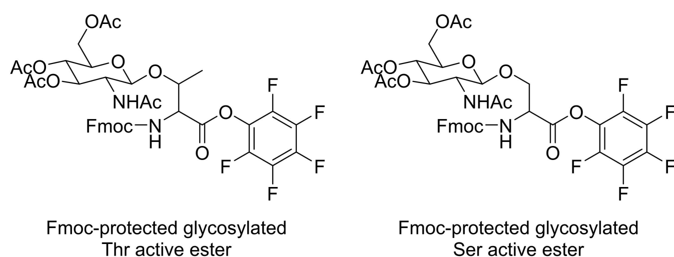

Figure 3. Examples of glycosylated AA building blocks for use in SPPS.

Lewis *et al.* synthesized semi-synthetic glycosylated H-aSyn-1-140-OH [gS87], carrying *O*-linked *O*-GlcNAc at S87, from recombinant H-aSyn-1-75-SR, synthetic H-aSyn-76-90-SR [A76Thz, gS87] and recombinant H-aSyn-91-140-OH [A91C] by a similar approach as Marotta *et al.* (Scheme 7B). Fmoc-protected pentafluorophenyl-activated *O*-Glc*N*Ac Ser, carrying acetyl groups at the sugar hydroxyl groups, was applied to incorporate the glycosylation.[51,53]

Levine *et al.* prepared different site-specifically glycosylated aSyn variants carrying a glycosylation at T72, T75, T81 or S87, and a triply glycosylated protein bearing glycosylations at T72, T75 and T81 (Scheme 7C). *N*-Terminal peptide thioesters and *C*-terminal peptide fragments were obtained recombinantly. The central peptide thioesters, carrying the glycosylation and an *N*-terminal Thz moiety, were synthesized chemically via Fmoc-SPPS and utilizing the *N*-acylurea approach in thioester preparation. The glycosylated AA building block was introduced during SPPS, as described above. Ligations were performed *C*-to-*N*-directed under use of the Thz protection strategy for the central peptide fragment, followed by radical desulfurization.[51–54]

A.

gT72
H—[1-68]—SR ← H—Thz—[70-75]—SR ← H—Cys—[77-140]—OH

B.

gS87
H—[1-75]—SR ← H—Thz—[77-90]—SR ← H—Cys—[92-140]—OH

C.

gT72
H—[1-68]—SR ← H—Thz—[70-75]—SR ← H—Cys—[77-140]—OH

gT75
H—[1-68]—SR ← H—Thz—[70-90]—SR ← H—Cys—[92-140]—OH

gT81
H—[1-75]—SR ← H—Thz—[77-90]—SR ← H—Cys—[92-140]—OH

gS87
H—[1-84]—SR ← H—Thz—[86-90]—SR ← H—Cys—[92-140]—OH

gT72,75,81
H—[1-68]—SR ← H—Thz—[70-90]—SR ← H—Cys—[92-140]—OH

Scheme 7. Semi-synthetic approaches for glycosylated aSyn proteins. Chemically synthesized peptide fragments are depicted in red frames and biologically expressed peptide fragments in green frames.

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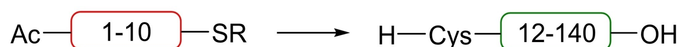
Acetylation

N-Terminal acetylation is prevalent for the physiological form of aSyn and modulates its binding to lipid membranes.[37,44,55]

Fauvet *et al.* produced semi-synthetic *N*-terminally acetylated (ac) WT protein from chemically synthesized Ac-aSyn-1-10-SR and recombinant H-aSyn-11-140-OH [A11C] (Scheme 8). Fmoc-SPPS of the *N*-terminal peptide fragment was performed using *N*-acetyl Met as *N*-terminal AA and the peptide thioester was generated via the *N*-acylurea approach. Acetylation of the peptide on resin using acetic anhydride (Ac_2O) was not possible in this case, as it would cause acetylation of the free amine of the Dbz linker, necessary for *C*-terminal activation. Ligation of the peptide fragments was then followed by radical desulfurization.[35]

Arginylation

Glutamate arginylation is achieved via incorporation of an arginylated Glu building block during SPPS (Figure 4).



Scheme 8. Semi-synthetic approach for *N*-terminally acetylated aSyn. The chemically synthesized peptide fragment is depicted in a red frame and the biologically expressed peptide fragment in a green frame.

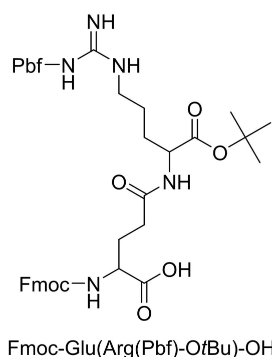


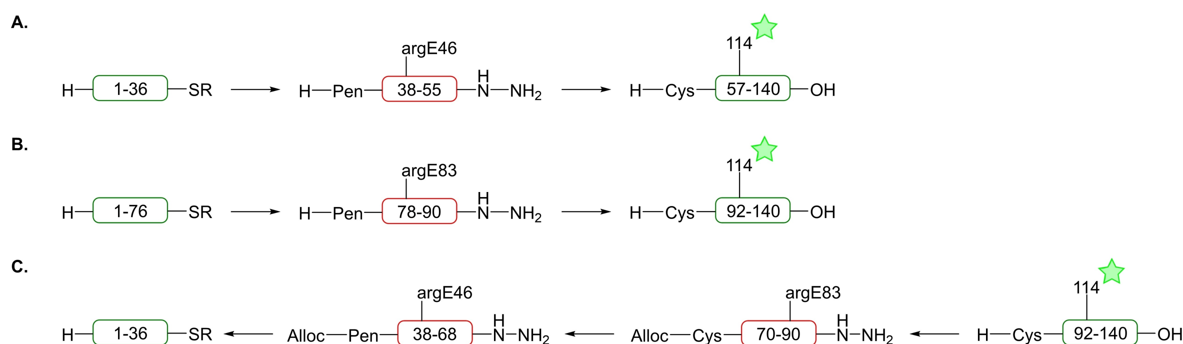
Figure 4. Arginylated Glu building block for use in SPPS.

Pan *et al.* presented the first synthetic strategy for incorporating glutamate arginylation into proteins by synthesizing semi-synthetic arginylated (arg) H-aSyn-1-140-OH [argE46], H-aSyn-1-140-OH [argE83] and H-aSyn-1-140-OH [argE46,83], optionally fluorophore-labeled at position 114 utilizing unnatural AA mutagenesis. In the synthesis of H-aSyn-1-140-OH [argE46], recombinant H-aSyn-1-36-SR, chemically synthesized H-aSyn-37-55-NH-NH_2 [V37Pen, argE46] and recombinant H-aSyn-56-140-OH [A56C, optionally fluorophore-labeled] were applied (Scheme 9A). In the synthesis of H-aSyn-1-140-OH [argE83], recombinant H-aSyn-1-76-SR, chemically synthesized H-aSyn-77-90-NH-NH_2 [V77Pen, argE83] and recombinant H-aSyn-91-140-OH [A91C, optionally fluorophore-labeled] were used (Scheme 9B). An arginylated glutamate was synthesized as Fmoc-Glu(Arg(Pbf)-O*t*Bu)-OH (Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) and introduced during Fmoc-SPPS, while Pen was introduced as Fmoc-Pen(Trt)-OH. Peptide hydrazides were used as thioester precursors. Ligations were performed in *N*-to-*C* direction, followed by radical desulfurization of Pen and Cys at the ligation sites. The doubly arginylated variant was synthesized from recombinant H-aSyn-1-36-SR, chemically synthesized Alloc-aSyn-37-68-NH-NH_2 [V37Pen, argE46] and Alloc-aSyn-69-90-NH-NH_2 [A69C, argE83] as well as recombinant H-aSyn-91-140-OH [A91C, optionally fluorophore-labeled] in *C*-to-*N* direction using Alloc as temporary protecting group for Cys and Pen (Scheme 9C).[56,57]

Labeling

Batjargal *et al.* synthesized semi-synthetic thioamide-labeled aSyn in order to track conformational changes during aggregation via distance-dependent quenching of Trp fluorescence by thioamides. The respective thioamide building block (Figure 5) was introduced during SPPS.

Chemically synthesized H-aSyn-1-8-R [V3thioamide Val (thio V)], masked with a linker for activation into the thioester, and recombinant H-aSyn-9-140-OH [S9C, F94 W], carrying a Trp mutation, were used. The thioamide Val monomer for position 3 was synthesized in form of its Fmoc-protected thiovaline-benzotriazolide and introduced during manual Fmoc-SPPS. Ligation of the thioamide-containing peptide and the Cys



Scheme 9. Semi-synthetic approaches for arginylated aSyn proteins. Chemically synthesized peptide fragments are depicted in red frames and biologically expressed peptide fragments in green frames. Labeling is indicated by colored stars.

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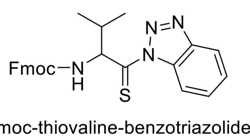
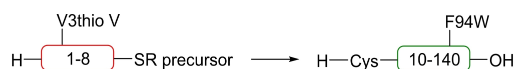


Figure 5. Val thioamide building block for use in SPPS.

peptide was performed successfully, while Cys was not desulfurized to maintain the thioamide (Scheme 10).[58]

Later, Wissner *et al.* synthesized semi-synthetic aSyn labeled with a fluorophore/thioamide Förster resonance energy transfer (FRET) pair. Chemically synthesized Ac-aSyn-1-4-OH [D2thio D] and recombinant H-aSyn-6-140-OH [Y39*p*-cyanophenylalanine (Cnf)] were applied. Asp in position 2 was introduced in form of the Fmoc-protected Asp thioamide precursor Asp(*t*Bu) thiocarboxybenzotriazole (Figure 6) during Fmoc-SPPS.

Thioester conversion of the chemically synthesized protected peptide fragment was performed by treatment with methyl 3-mercaptopropionate and benzotriazol-1-yloxytri(pyrrolidino)phosphonium hexafluorophosphate (PyBOP)/*N*,*N*-diisopropylethylamine (DIPEA), followed by global deprotection using trifluoroacetic acid (TFA). *S*-(Thiomethyl)homocysteine (Hcm) was attached enzymatically to the *N*-terminus of the Cnf-containing peptide fragment. Then, the disulfide moiety of Hcm was cleavedto convert it into homocysteine (Hcs). Subsequently, ligation between the thioester peptide and the Hcs-containing peptide was performed.



Scheme 10. Semi-synthetic approach for thioamide-labeled aSyn. The chemically synthesized peptide fragment is depicted in a red frame and the biologically expressed peptide fragment in a green frame.

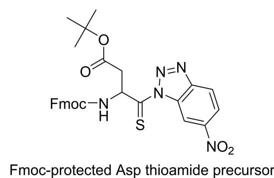
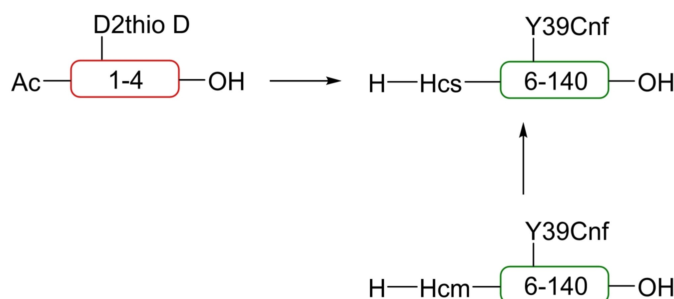


Figure 6. Asp thioamide building block for use in SPPS.



Scheme 11. Semi-synthetic approach for fluorophore/thioamide-labeled aSyn. The chemically synthesized peptide fragment is depicted in a red frame and the biologically expressed peptide fragment in a green frame.

Finally, conversion of Hcs into Met was carried out by selective methylation to form Ac-aSyn-1-140-OH [D2thio D, Y39Cnf] (Scheme 11). In this case, Hcs, which can be easily converted into Met, was used at the ligation site instead of classical NCL with a Cys peptide. The latter requires radical desulfurization to yield the native protein, which would also desulfurize the thioamide.[59]

In general, different semi-synthetic approaches to produce modified aSyn variants use different ligation sites to keep the chemically synthesized peptide fragments as short as possible, as larger peptides can dramatically reduce yields in SPPS. Nevertheless, an Ala junction which can be temporarily replaced by Cys is needed for the ligation. As aSyn is rich of Ala junctions, there is an immense flexibility in choosing the ligation sites, which facilitates specific adaption of each approach to obtain as high yields as possible for the chemically synthesized peptide fragments. It is known that ligation at sterically hindered AAs like Val is difficult and that especially Asn, Asp, Gln and Glu are prone to side-reactions during thioester formation, which negatively affect the ligation outcome. However, the reported methods do not include information about ligation sites that failed. Due to the lack of steric hindrance at Ala-Ala and Gly-Ala junctions, these sites often were used in the mentioned semi-synthetic approaches for chemical ligation obtaining good yields. For example, Ala-Ala at position 29 and 30 or position 90 and 91, as well as Gly-Ala at position 68 and 69 or position 106 and 107 were utilized in several approaches.[30,35,38–41,43,46,52–54,56] However, ligations at sterically hindered Val-Ala positions (52 and 53, 55 and 56) or Thr-Ala positions (75 and 76) were also shown to be successful. Ligation products were obtained with good yields after overnight incubation. Thus, ligation reactions take longer at these positions, while ligations at Gly or Ala often are completed in several hours.[38–40,43,52–54,56] Furthermore, ligations at Glu or Lys can be carried out successfully, as well as ligations between Gly and Pen or between Phe and Hcs instead of classical chemical ligation with Cys.[35,40,43,56,59] In addition to the localization of the ligation sites and steric hindrance, ligation yields can also be negatively influenced by the formation of unreactive side-products. For example, hydrolysis of peptide thioesters has been reported even at lower pH during NCL. This lowers ligation yields and causes problems during purification, because the hydrolyzed thioester peptide H-aSyn-1-106-OH coelutes with the full-length proteins, and requires optimized purification conditions.[41,42] A higher resistance to hydrolysis has been reported for H-aSyn-1-123-SR compared to H-aSyn-1-106-SR.[43] Thus, thioester stability might be also sequence-dependent.

To conclude, the reported methods enable the production of pure modified proteins in mg scale, which is sufficient for biophysical and biochemical studies. There is less information regarding the time-expense of the semi-synthetic approaches, but it can be assumed that these strategies reduce the time needed to obtain pure protein, while at the same time increase overall protein yields compared to total chemical synthetic approaches. The reported overall yields of semi-synthetic approaches vary strongly, between 2.4 and 37.7 %, but are mostly in the range of 11–26 %, which is generally higher than

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in total chemical synthesis of aSyn. The differences might be explained by the variation of the number of ligation and purification steps in the different synthetic strategies. Presumably, the tendency of higher yields in semi-synthesis compared to total synthetic approaches is caused by the reduction of the number of peptide fragments to be ligated from 3 to 4 in total synthesis to often 2 to 3 in semi-synthesis. Additionally, a strategy using methyl thioglycolate instead of MPAA in the ligation step prior to desulfurization has been reported to save the purification step prior to desulfurization, as it does not inactivate the radical initiator during reaction, which is an easy way to avoid product loss due to a high number of purification steps.[56]

Taken together, all these semi-synthetic approaches enable the production of a variety of protein variants that are important in deciphering the effects of several modifications, occurring in aSyn, on its membrane binding properties and aggregation propensity. Specific effects of the modifications are summarized in Table 1. Additionally, labeling strategies facilitate the tracking of conformational changes during aggregation.

Table 1. Effects of site-selective modifications in aSyn on its membrane binding affinity and aggregation behavior determined under use of semi-synthetic aSyn proteins. Arrows pointing down indicate a decrease and arrows pointing up an increase.[48,60]

PTM	Site of modification	Effect on membrane binding	Effect on aggregation
Phosphorylation[30,38–41]	Y39	↓	↓& altered fibril morphology
	Y125	No effect	No effect
	S129	↓	↑& altered fibril morphology
Nitration[43]	Y39 or Y125	↓	Altered kinetics and fibril morphology
Ubiquitination[33,46]	K6	No effect	↓
	K12 (tetra-ubiquitinated)	Not determined	↓
Glycosylation[52–54]	T72	No effect	↓& altered fibril morphology
	T75	No effect	↓& altered fibril morphology
	T81	No effect	↓& altered fibril morphology
	T72/75/81	↓ (slight decrease)	↓
	S87	No effect	↓& altered fibril morphology
Acetylation[35]	*N*-Terminus	No effect	No effect
Arginylation[56]	E46	No effect	No effect
	E83	No effect	↓
	E46 and E83	No effect	↓

Discussion

Several synthetic and semi-synthetic approaches have been used to produce wild type aSyn and modified protein variants. As aSyn is known to undergo several posttranslational modifications, it is important to study the effects of these modifications on the physiology and pathology of the protein. Modifications can extensively influence the behavior of the protein, including membrane binding, aggregation, and morphology of the aggregates (Table 1). Thus, studying the role of protein modifications as well as mutations provides insight into the chemical complexity of synucleinopathies and can help to better understand the molecular mechanisms leading to pathology. In addition to the production of the protein in biological systems, such as bacteria, the synthetic and semi-synthetic approaches discussed here are indispensable tools for producing specific variants of aSyn. Considering the low yields in total chemical synthesis of aSyn, the combination of biological expression and chemical synthesis in semi-synthetic approaches seems to be superior for obtaining site-specifically modified proteins. Applying the reported methods to produce modified aSyn, it has been shown that phosphorylation of S129 induces the formation of an aSyn strain with different fibril structure and higher toxicity indicating the regulation of the levels of S129 phosphorylation as a potential strategy in treating synucleinopathies.[30] Furthermore, studies of Y39 phosphorylated aSyn indicated that this modification influences membrane binding, resembling effects of the PD-associated G51D mutation.[38] On the other hand, the same modification has been reported to enhance propagation and cytotoxicity while being more resistant to protease cleavage by forming fibrils where the entire *N*-terminus is included in the fibril core.[39] These are just a few examples of how the production of modified aSyn using semi-synthetic approaches has given insight into pathological mechanisms associated with aSyn modifications. At present, these studies are only possible due to the use of synthetic approaches that enable the introduction of mutations and modifications at specific sites, and serve as examples that can be applied to the production of other disease-related proteins.

Summary and Outlook

Chemical synthesis and semi-synthesis enable the specific production of proteins of interest, with or without modifications. In the case of aSyn, these methods are important as they enable the introduction of modifications at any site of aSyn, offering immense possibilities for clarifying the structural and functional effects of protein variants carrying single or multiple modifications. Although the amounts of peptides and proteins produced using chemical synthesis can limit the types of studies and applications, these approaches offer a unique

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advantage over biological systems that do not afford selectivity in the introduction of certain posttranslational modifications. Therefore, we consider it will be important to continue to optimize chemical synthesis approaches as they may transform our understanding of the complex world of protein posttranslational modifications in biology and in pathological conditions, then paving the way for the development of novel therapeutic approaches.

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Conflict of Interests

The authors declare no conflict of interest.

Keywords: Alpha-synuclein · Posttranslational modification · Chemical protein synthesis · Solid-phase peptide synthesis · Native chemical ligation · Parkinson's disease

[1] A. Villar-Piqué, T. Lopes da Fonseca, T. F. Outeiro, *J. Neurochem.* **2016**, *139 (Suppl. 1)*, 240.
[2] a) I. C. Brás, M. Xylaki, T. F. Outeiro, *Prog. Brain Res.* **2020**, *252*, 91; b) I. C. Brás, T. F. Outeiro, *Cells* **2021**, *10*, 375.
[3] B. Butler, D. Sambo, H. Khoshbouei, *J. Chem. Neuroanat.* **2017**, *83–84*, 41.
[4] C. B. Lücking, A. Brice, *Cell. Mol. Life Sci.* **2000**, *57*, 1894.
[5] J. Burré, *J. Parkinson's Dis.* **2015**, *5*, 699.
[6] J. Burré, S. Vivona, J. Diao, M. Sharma, A. T. Brunger, T. C. Südhof, *Nature* **2013**, *498*, E4–E7.
[7] B. Fauvet, M. K. Mbefo, M.-B. Fares, C. Desobry, S. Michael, M. T. Ardah, E. Tsika, P. Coune, M. Prudent, N. Lion, et al., *J. Biol. Chem.* **2012**, *287*, 15345.
[8] a) R. Barbour, K. Kling, J. P. Anderson, K. Banducci, T. Cole, L. Diep, M. Fox, J. M. Goldstein, F. Soriano, P. Seubert, et al., *Neurodegener. Dis.* **2008**, *5*, 55; b) C. Tian, G. Liu, L. Gao, D. Soltys, C. Pan, T. Stewart, M. Shi, Z. Xie, N. Liu, T. Feng, et al., *Transl. Neurodegener.* **2019**, *8*, 15.
[9] a) M. Delenclos, J. D. Burgess, A. Lamprokostopoulou, T. F. Outeiro, K. Vekrellis, P. J. McLean, *J. Neurochem.* **2019**, *150*, 566; b) S. Chandra, X. Chen, J. Rizo, R. Jahn, T. C. Südhof, *J. Biol. Chem.* **2003**, *278*, 15313; c) P. H. Weinreb, W. Zhen, A. W. Poon, K. A. Conway, P. T. Lansbury, *Biochemistry* **1996**, *35*, 13709; d) V. N. Uversky, J. Li, A. L. Fink, *J. Biol. Chem.* **2001**, *276*, 10737.
[10] a) T. Bartels, J. G. Choi, D. J. Selkoe, *Nature* **2011**, *477*, 107; b) W. Wang, I. Perovic, J. Chittuluru, A. Kaganovich, L. T. T. Nguyen, J. Liao, J. R. Auclair, D. Johnson, A. Landeru, A. K. Simorellis et al., *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 17797.
[11] a) V. Gao, J. A. Briano, L. E. Komer, J. Burré, *J. Mol. Biol.* **2023**, *435*, 167714; b) J. Burré, M. Sharma, T. C. Südhof, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, E4274–E4283.
[12] a) I. C. Brás, A. Dominguez-Meijide, E. Gerhardt, D. Koss, D. F. Lázaro, P. I. Santos, E. Vasili, M. Xylaki, T. F. Outeiro, *J. Neurochem.* **2020**, *153*, 433; b) B. Caughey, P. T. Lansbury, *Annu. Rev. Neurosci.* **2003**, *26*, 267; c) I. Dikiy, D. Eliezer, *Biochim. Biophys. Acta* **2012**, *1818*, 1013.
[13] N. Bengoa-Vergniory, R. F. Roberts, R. Wade-Martins, J. Alegre-Abarrategui, *Acta Neuropathol.* **2017**, *134*, 819.
[14] J. Zhang, X. Li, J.-D. Li, *Front. Neurol. Neurosci.* **2019**, *13*, 381.
[15] N. d. O. Manzanza, L. Sedlackova, R. N. Kalaria, *Front. Aging Neurosci.* **2021**, *13*, 690293.
[16] S. B. H. Kent, *Chem. Soc. Rev.* **2009**, *38*, 338.
[17] X. Guan, P. K. Chaffey, C. Zeng, Z. Tan, *Top. Curr. Chem.* **2015**, *363*, 155.
[18] L. Raibaut, N. Ollivier, O. Melnyk, *Chem. Soc. Rev.* **2012**, *41*, 7001.
[19] B. Fauvet, S. M. Butterfield, J. Fuks, A. Brik, H. A. Lashuel, *Chem. Commun.* **2013**, *49*, 9254.
[20] J.-S. Zheng, S. Tang, Y.-K. Qi, Z.-P. Wang, L. Liu, *Nat. Protoc.* **2013**, *8*, 2483.
[21] L. M. Gatzemeier, F. Meyer, U. Diederichsen, T. F. Outeiro, *Chem. Eur. J.* **2023**, *29*, e202300649.
[22] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. Kent, *Science* **1994**, *266*, 776.
[23] V. Agouridas, O. El Mahdi, V. Diemer, M. Cargoët, J.-C. M. Monbaliu, O. Melnyk, *Chem. Rev.* **2019**, *119*, 7328.
[24] Q. Wan, S. J. Danishefsky, *Angew. Chem. Int. Ed.* **2007**, *46*, 9248.
[25] a) J. B. Blanco-Canosa, P. E. Dawson, *Angew. Chem. Int. Ed. Engl.* **2008**, *47*, 6851; b) S. K. Mahto, C. J. Howard, J. C. Shimko, J. J. Ottesen, *ChemBioChem* **2011**, *12*, 2488.
[26] D. Bang, S. B. H. Kent, *Angew. Chem. Int. Ed. Engl.* **2004**, *43*, 2534.
[27] T. W. Muir, D. Sondhi, P. A. Cole, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6705.
[28] M. R. Pratt, T. Abeywardana, N. P. Marotta, *Biomol. Eng.* **2015**, *5*, 1210.
[29] A. C. Conibear, E. E. Watson, R. J. Payne, C. F. W. Becker, *Chem. Soc. Rev.* **2018**, *47*, 9046.
[30] M.-R. Ma, Z.-W. Hu, Y.-F. Zhao, Y.-X. Chen, Y.-M. Li, *Sci. Rep.* **2016**, *6*, 37130.
[31] P. Gruber, T. Hofmann, *J. Pept. Res.* **2005**, *66*, 111.
[32] a) Y.-C. Huang, G.-M. Fang, L. Liu, *Natl. Sci. Rev.* **2016**, *3*, 107; b) G.-M. Fang, Y.-M. Li, F. Shen, Y.-C. Huang, J.-B. Li, Y. Lin, H.-K. Cui, L. Liu, *Angew. Chem.* **2011**, *123*, 7787; c) Y.-M. Li, M.-Y. Yang, Y.-C. Huang, Y.-T. Li, P. R. Chen, L. Liu, *ACS Chem. Biol.* **2012**, *7*, 1015.
[33] M. Hejjaoui, M. Haj-Yahya, K. S. A. Kumar, A. Brik, H. A. Lashuel, *Angew. Chem. Int. Ed.* **2011**, *50*, 405.
[34] I. E. Gentle, D. P. de Souza, M. Baca, *Bioconjugate Chem.* **2004**, *15*, 658.
[35] B. Fauvet, M.-B. Fares, F. Samuel, I. Dikiy, A. Tandon, D. Eliezer, H. A. Lashuel, *J. Biol. Chem.* **2012**, *287*, 28243.
[36] a) H. Fujiwara, M. Hasegawa, N. Dohmae, A. Kawashima, E. Masliah, M. S. Goldberg, J. Shen, K. Takio, T. Iwatsubo, *Nat. Cell Biol.* **2002**, *4*, 160; b) M. Okochi, J. Walter, A. Koyama, S. Nakajo, M. Baba, T. Iwatsubo, L. Meijer, P. J. Kahle, C. Haass, *J. Biol. Chem.* **2000**, *275*, 390.
[37] J. P. Anderson, D. E. Walker, J. M. Goldstein, R. de Laat, K. Banducci, R. J. Caccavello, R. Barbour, J. Huang, K. Kling, M. Lee, et al., *J. Biol. Chem.* **2006**, *281*, 29739.
[38] I. Dikiy, B. Fauvet, A. Jovičić, A.-L. Mahul-Mellier, C. Desobry, F. El-Turk, A. D. Gitler, H. A. Lashuel, D. Eliezer, *ACS Chem. Biol.* **2016**, *11*, 2428.
[39] K. Zhao, Y.-J. Lim, Z. Liu, H. Long, Y. Sun, J.-J. Hu, C. Zhao, Y. Tao, X. Zhang, D. Li, et al. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 20305.
[40] B. Pan, E. Rhoades, E. J. Petersson, *ACS Chem. Biol.* **2020**, *15*, 640.
[41] M. Hejjaoui, S. Butterfield, B. Fauvet, F. Vercruysse, J. Cui, I. Dikiy, M. Prudent, D. Olschewski, Y. Zhang, D. Eliezer, et al., *J. Am. Chem. Soc.* **2012**, *134*, 5196.
[42] B. Fauvet, H. A. Lashuel, *Methods Mol. Biol.* **2016**, *1345*, 3.
[43] R. Burai, N. Ait-Bouziad, A. Chiki, H. A. Lashuel, *J. Am. Chem. Soc.* **2015**, *137*, 5041.
[44] L. Breydo, J. W. Wu, V. N. Uversky, *Biochim. Biophys. Acta* **2012**, *1822*, 261.
[45] T. Nonaka, T. Iwatsubo, M. Hasegawa, *Biochemistry* **2005**, *44*, 361.
[46] M. Haj-Yahya, B. Fauvet, Y. Herman-Bachinsky, M. Hejjaoui, S. N. Bavikar, S. V. Karthikeyan, A. Ciechanover, H. A. Lashuel, A. Brik, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 17726.
[47] M. Haj-Yahya, K. S. Ajish Kumar, L. A. Erlich, A. Brik, *Biopolymers* **2010**, *94*, 504.
[48] H. Chen, Y.-F. Zhao, Y.-X. Chen, Y.-M. Li, *ACS Chem. Neurosci.* **2019**, *10*, 910.
[49] K. S. Ajish Kumar, M. Haj-Yahya, D. Olschewski, H. A. Lashuel, A. Brik, *Angew. Chem. Int. Ed. Engl.* **2009**, *48*, 8090.
[50] K. S. Ajish Kumar, S. N. Bavikar, L. Spasser, T. Moyal, S. Ohayon, A. Brik, *Angew. Chem. Int. Ed. Engl.* **2011**, *50*, 6137.
[51] N. P. Marotta, C. A. Cherwien, T. Abeywardana, M. R. Pratt, *ChemBioChem* **2012**, *13*, 2665.
[52] N. P. Marotta, Y. H. Lin, Y. E. Lewis, M. R. Ambroso, B. W. Zaro, M. T. Roth, D. B. Arnold, R. Langen, M. R. Pratt, *Nat. Chem.* **2015**, *7*, 913.
[53] Y. E. Lewis, A. Galesic, P. M. Levine, C. A. de Leon, N. Lamiri, C. K. Brennan, M. R. Pratt, *ACS Chem. Biol.* **2017**, *12*, 1020.
[54] P. M. Levine, A. Galesic, A. T. Balana, A.-L. Mahul-Mellier, M. X. Navarro, C. A. de Leon, H. A. Lashuel, M. R. Pratt, *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 1511.

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[55] a) M. Runfola, A. de Simone, M. Vendruscolo, C. M. Dobson, G. Fusco, *Sci. Rep.* **2020**, *10*, 204; b) P. Zabrocki, I. Bastiaens, C. Delay, T. Bammens, R. Ghillebert, K. Pellens, C. de Virgilio, F. van Leuven, J. Winderickx, *Biochim. Biophys. Acta* **2008**, *1783*, 1767.
[56] B. Pan, N. Kamo, M. Shimogawa, Y. Huang, A. Kashina, E. Rhoades, E. J. Petersson, *J. Am. Chem. Soc.* **2020**, *142*, 21786.
[57] C. Haase, H. Rohde, O. Seitz, *Angew. Chem. Int. Ed. Engl.* **2008**, *47*, 6807.
[58] a) S. Batjargal, Y. J. Wang, J. M. Goldberg, R. F. Wissner, E. J. Petersson, *J. Am. Chem. Soc.* **2012**, *134*, 9172; b) J. M. Goldberg, S. Batjargal, E. J. Petersson, *J. Am. Chem. Soc.* **2010**, *132*, 14718.
[59] R. F. Wissner, A. M. Wagner, J. B. Warner, E. J. Petersson, *Synlett* **2013**, *24*, 2454.
[60] S. P. Moon, A. T. Balana, M. R. Pratt, *Curr. Opin. Chem. Biol.* **2021**, *64*, 76.

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CONCEPT

Alpha-synuclein (aSyn) physiologically is involved in neurotransmitter release, but pathologically aggregates into proteinaceous deposits in the brain, being associated with Parkinson's disease (PD). Herein, the various reported synthetic and semi-synthetic routes to obtain wild-type (WT) and modified aSyn are summarized in order to enlighten their importance for studying the effects of modifications on the structure and aggregation behavior of aSyn.

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Synthesis and Semi-Synthesis of Alpha-Synuclein: Insight into the Chemical Complexity of Synucleinopathies

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