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Chemical Synthesis of Microtubule-Associated Protein Tau

Wyatt C. Powell,
Department of Chemistry, University of Colorado, Boulder, Colorado 80309, United States

Ruiheng Jing,
Department of Chemistry, University of Colorado, Boulder, Colorado 80309, United States

Maciej A. Walczak
Department of Chemistry, University of Colorado, Boulder, Colorado 80309, United States;

Abstract

Deposits of the microtubule-associated protein Tau (MAPT) serve as a hallmark of neurodegenerative diseases known as tauopathies. Numerous studies have demonstrated that in diseases such as Alzheimer's disease (AD), Tau undergoes extensive remodeling. The attachment of post-translational modifications distributed throughout the entire sequence of the protein correlates with clinical presentation. A systematic examination of these protein alterations can shed light on their roles in both healthy and diseased states. However, the ability to access these modifications in the entire protein chain is limited as Tau can only be produced recombinantly or through semisynthesis. In this article, we describe the first chemical synthesis of the longest 2N4R isoform of Tau, consisting of 441 amino acids. The 2N4R Tau was divided into 3 major segments and a total of 11 fragments, all of which were prepared via solid-phase peptide synthesis. The successful chemical strategy has relied on the strategic use of two cysteine sites (C291 and C322) for the native chemical ligations (NCLs). This was combined with modern preparative protein chemistries, such as mercaptothreonine ligation (T205), diselenide–selenoester ligation (D358), and mutations of mercaptoamino acids into native residues via homogeneous radical desulfurization (A40, A77, A119, A157, A246, and A390). The successful completion of the synthesis has established a robust and scalable route to the native protein in multimilligram quantities and high purity. In broader terms, the presented strategy can be applied to the preparation of other shorter isoforms of Tau as well as to introduce all post-translational modifications that are characteristic of tauopathies such as AD.

Graphical Abstract

Corresponding Author: **Maciej A. Walczak** – Department of Chemistry, University of Colorado, Boulder, Colorado 80309, United States; maciej.walczak@colorado.edu.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c07338.
Detailed experimental procedures, copies of NMR spectra, LC traces, and MS spectra (PDF)

Complete contact information is available at: https://pubs.acs.org/10.1021/jacs.3c07338

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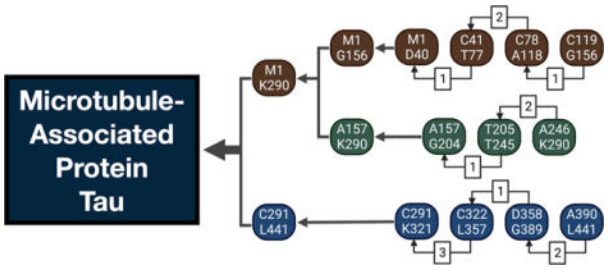

INTRODUCTION

Neurodegenerative diseases, collectively known as tauopathies,[2,3] are characterized by intraneuronal deposits of a microtubule-associated protein Tau (MAPT).[4–7] Alzheimer's disease (AD), the leading cause of dementia and a representative tauopathy, is marked by the presence of senile plaques and neurofibrillary tangles composed of hyper-phosphorylated Tau.[8–10] The protein Tau, first described by Kirschner et al.,[12] belongs to a family of microtubule-associated proteins.[14] Tau is expressed in six variants resulting from alternative presplicing of a single *MAPT* gene (Figure 1A).[16] These isoforms differ by the presence of one or two inserts in the N-terminal fragment and repeat regions (R) in the microtubule-binding domain (MTB) of the C-terminus (Figure 1B). As an intrinsically disordered protein, Tau is highly susceptible to post-translational modifications (PTMs, Figure 1C).[11,17–19] These modifications, including phosphorylation,[20–22] acetylation,[23,24] glycosylation,[25,26] ubiquitination,[27] SUMOylation,[28] and methylation,[29] play crucial roles in regulating Tau localization, protein–protein interactions, degradation/clearance, and microtubule binding. Importantly, PTMs alter the charge and hydrophobicity, potentially providing the driving force for Tau to undergo aggregation associated with neurodegenerative diseases.[30,31]

Various strategies have been employed to examine the role PTMs in Tau biology.[32] One such method is isolating Tau from post-mortem samples or from tauopathy mouse models. Another common method involves enzymatic functionalization of Tau to obtain phosphorylated, acetylated,[33] ubiquitinated,[34] and glycosylated forms, but this method lacks specificity and control over the location or extent of modifications. Furthermore, preparation of pure Tau is challenging because, as a disordered protein, Tau is vulnerable to protease degradation.[35,36] While more site-specific methods exist, they rely on the use of point mutations that mimic charge-neutralizing PTMs (S to D mutations for phosphoserine[37–40] or K to Q for acetyl lysine[41–43]) or by using bioconjugate functionalization of dehydroalanine.[44] The major disadvantage of this strategy is that these mimics may not possess the same structure, acidity, or charge as natural PTMs. Chemical synthesis, on the other hand, may overcome all these limitations because it enables the preparation of the exact same chemical structure as patients with complete specificity. Semisynthesis, which combines recombinant proteins with chemical synthesis, has been exploited by several groups,[45] including studies on hyperphosphorylation/*O*-glycosylation in the C-terminus (Hackenberger et al.),[46–49] phosphorylation and acetylation in the microtubule binding domain (Lashuel et al.),[50,51] and lysine carboxymethylation (Becker et al.).[52] These studies have provided important insights into the role of individual PTMs mostly located in

the MTB or C-terminal region and complement chemical approaches to understand the molecular mechanisms of neurodegeneration based on perturbation of protein structure/function in Parkinson's disease (α-synuclein[53–57] with various PTMs such as nitration,[58] phosphorylation,[59,60] N-terminal acetylation,[61] *O*-GlcNAc,[62] and ubiquitination[63,64]), AD (Aβ),[65–67] and Huntington's disease (huntingtin).[68–71]

Despite the above-mentioned efforts, complete chemical synthesis of full-length Tau has remained elusive. We posited that the total synthesis of Tau can overcome these limitations, enabling the production of the same chemical structure found in patients with complete specificity. Given the broad structural diversity and localization of PTMs throughout Tau (Figure 1C), a chemical strategy that can systematically evaluate each PTM individually, or in combinations, would greatly facilitate in vitro investigations into the role of site-specific PTMs. Here, we report the first total synthesis of the longest isoform of Tau (2N4R) optimized to minimize the number of disconnections without compromising efficiency, chemical yield, and purity.[72]

RESULTS AND DISCUSSION

Retrosynthetic Analysis of 2N4R Tau.

In the pursuit of the total synthesis of 2N4R Tau, we designed an approach where the protein was dissected into three major segments, each approximately 150 amino acids in length (Figure 2). Our rationale for choosing the G156–A157 junction for ligation was its unhindered nature. In addition, we selected K290–C291 as the ultimate ligation site due to its status as one of the two natural cysteine residues in the protein. These segments —designated as Tau(1–156), Tau(157–290), and Tau(291–441)would be subsequently assembled in the N to C direction. In order to retain the two natural cysteine residues, the final ligation was orchestrated between Tau(1–290) and Tau(291–441) at the K290–C291 junction.[50] The Tau(1–290) construct would be achieved by ligation, followed by desulfurization at G156–A157C involving Tau(1–156) and Tau(157–290). These major segments were assembled from smaller synthetic peptides encompassing approximately 30–50 residues (Figure 2B). We note that our objective of this study is to develop chemical synthesis of the longest 2N4R isoform that is found in the adult human brain and relevant to neurodegenerative diseases.[1,73] However, the presented chemical strategy is applicable to the other five isoforms with minimal modifications in terms of disconnections and chemistries used for segment couplings.

In designing the chemical synthesis of 2N4R Tau, we were mindful of the recent developments in preparative protein methodologies that have been extensively discussed else-where.[74–76] Several critical methods were employed to facilitate successful solid-phase peptide syntheses (SPPS) of Tau segments. One of the key strategies involved the preparation of synthetic peptides that contained fewer than 50 amino acids, facilitating a more manageable purification process.[77] Thioester synthesis needed for the successful native chemical ligation (NCL) also played a pivotal role in our synthesis.[78] We incorporated a technique originally developed by Dawson et al., which entailed the use of *o*-amino(methyl)-aniline (MeDbz) linker resin.[79] This technology proved invaluable for the preparation of *N*-acylurea (MeNbz) thioester surrogates directly from SPPS.[79] While

generally effective, certain cases revealed an incompatibility between the peptide chain and the activation step of MeNbz. To circumvent this, an alternative thioester strategy was adopted using the acid-sensitive HMPB linker resin, which selectively detached protected C-terminal acids from the resin with diluted TFA in CH_2Cl_2.[80] Furthermore, we utilized acyl hydrazides, instrumental in their subsequent conversion into thioesters.[81] Boc-protected Dbz linkers were employed for the synthesis of C-terminal Dbz residues, which can be converted into acyl-benzotriazoles using $NaNO_2$ acting as hydrazide substitutes.[82–84] Additionally, some segments, specifically Tau(41–76) and Tau(119–156), contained aspartimide-prone aspartic acid–glycine sequences, which could be effectively suppressed with the aid of DMB-protected dipeptides.[85]

Synthesis of Tau(1–156).

To implement the above-mentioned strategy, we first aimed to prepare individual oligopeptide fragments. Because the Tau(1–156) region lacks any native cysteine residues, we decided to use alanine ligation sites for the first Tau(1–156) fragment (Figure 2A).[86] A four-segment approach was deemed practical since this region contains numerous alanine residues suitable for segment coupling. For the first ligation site, we selected D40–A41 disconnection because the alternative disconnections at H14 and D65 would require peptide fragments over 50 amino acid long. The next alanine ligation site marked the halfway point after 35 amino acids at the T76–A77 junction, and the final ligation site was selected at the A118–A119 junction, which requires 41- and 37-mer fragments. We decided to merge the peptides Tau(1–76) and Tau(77–156) first and then couple these to obtain Tau(1–156) (Scheme 2). Furthermore, all cysteine mutations within this sequence could be converted back into alanine residues using a global metal-free desulfurization.[87] Before moving forward with the synthesis, we also considered the limitation of the proposed ligation at D40–A41 that could be potentially problematic due to the presence of a C-terminal aspartic acid residue in the Tau(1–40) segment. NCL reactions at C-terminal aspartic acid may result in a cyclic anhydride that is hydrolysis prone, and problematic β-branched ligation products are formed even with aryl thiols (4- mercaptophenylacetic acid (MPAA) and PhSH/BnSH) used as a catalyst.[88,89] Previous studies had shown that protection of aspartic acid inhibits cyclization of the side chain carboxylate with the thioester,[88,90,91] and this prompted us to the allyl ester for the side chain of D40.

With a feasible synthetic plan in hand, we set out to prepare the fragments using solid phase peptide synthesis (Scheme 1). The Tau(1–40) sequence **3** terminated with MeNBz was synthesized on the Fmoc-MeDbz-Rink Amide Chem-Matrix resin, but conversion into the *N*-acylurea (Nbz) thioester surrogate was unsuccessful because the peptide underwent oxidation, carbonylation, and dehydration upon treatment with the activating reagent (p-$NO_2C_6H_4OCOCl$). We reasoned that the length of the peptide fragment may be the source of this instability. Therefore, we dissected **3** into two smaller units Tau(1–14) and Tau(15–40), which both could be easily prepared by SPPS. Activation of the truncated peptides A15C–D40 **5a** did not suppress decomposition and neither did bulky Mpe derivative (Mpe, 3-(3-methyl)pentyl ester),[92] and only the N-terminal fragment M1–H14 could be efficiently converted into activated acyl donor MeDbz **4**.

To overcome this obstacle, we assembled peptide **6** on the HMPB linker resin and prepared the ethyl thioester in solution using PyBOP/EtSH (Scheme 2).[55] Assembly of Tau(77–118) **8** on Fmoc-MeDbz-Rink Amide Chem-Matrix resin proceeded smoothly, as well as activation of the MeDbz linker (this peptide contains a N-terminal thiazolidine as a cysteine protective group for orthogonal protection in NCL).[79,93,94] Finally, Tau(41–76) **7** and Tau(119–156) **9** were assembled in excellent yields on the Fmoc-Dbz(Boc)-Rink Amide Chem-Matrix resin, and the N-terminal cysteines were protected as ligation compatible disulfides.

Having accomplished the SPPS of the Tau(1–156) fragments, we began their assembly (Scheme 2). Ligation between Tau(1–40) **6** and Tau(41–76) **7** in the MPAA[55] buffer was complete within 2 h. We noted that products **10a** were unstable and over longer reaction times (6 h), cyclization to aspartimide occurred at the allyl ester side chain (41%; 15% aspartimide after 6 h, but complete conversion and hydrolysis of the aspartimide occur after 16 h resulting in a mixture of inseparable branched and epimerized isomers).[88] Therefore, it was critical to use efficient NCL and deallylation conditions to preserve the ligation product. A survey of the literature for allyl ester removal identified a Ru(II) catalyst that can deprotect allyl esters in aqueous Gnd·HCl buffers and uses residual MPAA to scavenge the allyl cations.[95] We were pleased to find that adding Cp*Ru(COD)Cl (30 mM) at the end of the NCL resulted in rapid allyl removal and furnished Tau(1–76) **10d** in 88% yield (Scheme 2, entry 4).

We next investigated desulfurization of the A41C mutant of Tau(1–76). We found that *t*-BuSH (5% v/v) was the most effective H atom transfer regent, and the product was generated in 77% yield. Furthermore, using *t*-BuSH as opposed to other H atom donors (e.g., GSH) caused only minimal aspartimide formation (~10%) that could be separated at this stage by preparative HPLC. The resultant Tau(1–76) peptide was then subjected to Dbz activation with $NaNO_2$ and converted into MES thioester **10e**. To prepare Tau(77–156) **11**, coupling of Tau(77–118) **8** and Tau(119–156) **9** proceeded smoothly in MPAA buffer (200 mM) and was followed by one-pot thiazolidine removal with $MeONH_2$. This step required additional optimizations of the thiol catalyst—replacing MPAA with methyl thioglycolate (MTG, 200 mM)[96] or trifluoroethanethiol (TFET, 5% v/v)[97] resulted only in diminished yields (48 and 27%, respectively). Having accomplished the synthesis of Tau(77–156), we proceeded toward the assembly of Tau(1–156). To this end, ligation between Tau(1–76) **10e** and Tau(77–156) **11** proceeded uneventfully and provided the ligation product in 97%. Subsequent double desulfurization of A77 → C77 and A119 → C119 regenerated the native sequence in a good yield (89%). Finally, activation of Dbz and thiolysis afforded thioester intermediate Tau(1–156) **12** in excellent yield and purity. A dehydrated benzimidazole impurity was formed during this step, which is inseparable from the thioester but does not couple in subsequent ligations (benzimidazole formation has been observed previously at glycine).[98]

Synthesis of Tau(157–290).

Our next goal has been to prepare 133-mer Tau(157–290). Native cysteines are absent in Tau(157–290), but alanine residues in this segment are suitable for NCL desulfurization (Figure 2A). We have initially selected several potential disconnection sites such as Q165–

A166, N167–A168, P172–A173, P177–A178, V226–A227, S238–A239, and T245–A246. The G156–A157 junction that would be used for a large segment coupling (*vide infra*) is unhindered. Chemical ligations at C-terminal glutamine and asparagine are typically avoided because of a potential formation of cyclic imides and β-branched products.[99] However, subsequent studies that used MPAA to facilitate the NCL have shown that the amount of side products at the glutamine and, to a lesser extent, asparagine sites could be reduced.[89] With these considerations in mind, we have selected the Q165–A166 over the N167–A168 site. The Tau(157–290) region also contains two proline–alanine junctions, but these ligations are considered less favorable because they show slow reaction kinetics together with Xaa-Pro deletions due to diketopiperazine formation.[100–103] The following three disconnections, V226–A227, S238–A239, and T245–A426 were expected to proceed uneventfully. Finally, the T245–A246 site has also been deemed achievable because it uses a 45-mer peptide, which is sufficiently short to be produced in a homogeneous form, and this site has been used in a previous synthesis.[50]

With these considerations in mind, we initially focused on four peptide segments that would be assembled through ligations at the Q165–A166, V226–C227, and T245–C246 sites. We were able to synthesize Tau(227–290) in acceptable yields from two smaller segments Tau(246–290) and Tau(227–245), but attempts to produce a longer segment Tau(166–290) by NCL with Tau(166–226) were unsuccessful because the 60-mer peptide could not be purified to homogeneity. These problematic disconnections led us to consider an alternative approach using a threonine ligation.[104] Our plan was to dissect Tau(157–290) into three segments of lengths no more than 50 amino acids. After dissecting the region evenly, we decided to use the unhindered G204–T205 junction and serine–threonine ligation (STL).[104] The three required segments Tau(157–204), Tau(205–245), and Tau(246–290) were prepared by SPPS, and the key ligation between C-terminal salicylaldehyde ester Tau(157–204) and N-terminal threonine of Tau(205–245) was unsuccessful under basic, neutral, or acidic conditions.

The third approach to Tau(157–290) was based on the NCL ligation featuring a modified threonine (Scheme 3).[105] We selected a surrogate ligation at the unhindered G204–T205 site using mercaptothreonine, which would generate 47- and 40-residue segments.[105] We envisioned that the relevant fragments could be ligated in the C → N direction and that the thiol mutations would be removed through desulfurization. Initial attempts to generate Tau(205–245) on a thioester surrogate MeNbz resin were unsuccessful because γ-mercaptothreonine is incompatible with p-NO_2PhOCOCl activation and we decided to switch to the hydrazide surrogate Dbz linker.[79,83,84] This linker was protected with a BocDbz to help enable microwave-assisted SPPS and prevent over acylation.[82,98] After coupling of methyldisulfide **22a** onto **14**, the resin was subjected to global cleavage (TFA:H_2O:TA:TIS), which produced reduced thiol **15**. Further manipulations were challenging because mercaptothreonine **15** displayed self-splicing capabilities, likely through a retro-NCL producing **17**. This time-dependent cleavage had occured readily under acidic conditions (~20% splicing after 1 h), but thiol intermediate **15** could be preserved by the lyophilization of the HPLC fractions. To avoid further degradation, crude peptide thiol **15** was treated with MMTS to generate disulfide **16**,[106] which remained stable toward HPLC

purifications. The Tau(205–290) fragment was prepared by a one-pot ligation with hydrazide **23**, but the ligation product remained unstable in the reduced state, and to avoid degradation, MPAA was removed by desalting column followed by immediate treatment with MMTS to generate stable peptide **18** in 64% yield.

To complete the synthesis of an NCL-competent fragment, the Alloc group in **18** had to be removed, and two routes were investigated: deallylation/ligation/desulfurization (route A) and deallylation/trapping followed by ligation/desulfurization (route B). We attempted deallylation of **18** using Pd(II), TPPTS/MPAA followed by SEC and NCL with thioester **24a**, but under these conditions, only thioester **24a** remained (LCMS) indicating that the thiol sequestered the metal.[91,107] Similarly, the attempted trapping of the thiol with MMTS yielded no detectable product. Alloc removal with $PdCl_2$dppf/$PhSiH_3$ in DMSO followed by NCL with **24a** or trapping with MMTS showed the presence of the desired products albeit in low yields (11 and 7%, respectively). The Ru(II)-based method with Cp*Ru(COD)Cl/MPAA[95] produced **19** in almost quantitative yield, but **19** decomposed slowly likely through the intermolecular splicing as described above (route A). Attempts at the ligation of **19** with **24a**, followed by desalting to remove MPAA, and immediate desulfurization produced **20** but in irreproducible yields (0–55%). Additionally, thiol **19** could be trapped with MMTS, and pure disulfide **21** could be sent into the ligation–desalting–desulfurization sequence, but the yield was low (33%) and difficult to scale up (route B).

To overcome the obstacles of stability and reproducibility, the synthesis of Tau(157–290) was redesigned. Because segment Tau(157–204) was difficult to prepare directly as a SEt ester or MeNbz due to rapid hydrolysis, we prepared Tau(157–204) **25** on the hydrolysis-resistant Dbz linker resin, which was later converted into a MES thioester using $NaNO_2$ (Scheme 4). Furthermore, this segment contained an aspartimide-prone region that formed during SPPS and under acidic conditions of HPLC purification (0.1% TFA). Fortunately, we were able to suppress this problematic dehydration by installing bulky Mpe protection at D193 followed by immediate lyophilizing. The synthesis of Tau(205–245) **26** proceeded uneventfully and feature mercaptothreonine building block **22b** with a more hindered disulfide (*t*-BuS-S vs MeS-S) introduced to reduce disulfide cleavage during global deprotection. The bulky disulfide was also resistant toward reduction with TIS, and the removal of TBDPS group was accomplished on resin with TBAF in DMF. The final segment Tau(246–290) **27** was prepared on a Dbz linker resin, but two pseudoprolines at T263 and S285 were required due to the enhanced hydrophobicity of this linker.

With these segments in hand, we proceeded toward the assembly of Tau(157–290) **28** as shown in Scheme 4.Mercaptothreonine ligation between Tau(157–204) **25** and Tau(205–245) **26** was rapid (2 h) with MPAA, but purification was challenging because of the previously discovered instability of γ-mercaptothreonine. These results prompted us to investigate a one-pot ligation–desulfurizaiton approach. Free-radical desulfurization is not compatible with aromatic thiols (MPAA and PhSH),[108,109] and aliphatic thiols such as *t*-BuSH,[86] GSH,[110] DTT, and MESNa[109] are used as H atom donors in the desulfurization step. To this end, the ligation between **25** and **26** was complete within 2 h using TFET[97] as the catalyst, and efficient one-pot desulfurization was accomplished upon adding desulfurization buffer with GSH (**28**). The Dbz group was then activated with $NaNO_2$ and

treated with MPAA/**27**, followed by monodesulfurization of C246 → A246 with GSH and Acm removal with $PdCl_2$ and $MgCl_2$ furnishing **29**.[111]

Synthesis of Tau(291–441).

Our next goal was to develop a synthesis of Tau(291–441). Since this region contains two natural cysteine residues (C291 and C322), the first ligation would use the K321–C322 junction. We also selected the G389–A390 site, which would require the 51-mer peptide segment Tau(390–441). Therefore, the immediate goal was to prepare Tau(322–389) using a surrogate ligation approach, and we selected the L357–D358 site for the mercaptoaspartic acid ligation.[112]

The first approach for assembling Tau(322–389) was based on the NCL at the L357–D358 junction with mercaptoaspartic acid **31** and Tau(322–357) **30**, which afforded product **32** in high yield (Scheme 5). The subsequent elaboration of **32** involved an NCL with Tau(390–441) **37** and homogeneous desulfurization of **33**, which produced a complex mixture of products. Alternatively, the metal-free desulfurization of mercaptoaspartic acid **32** was also problematic and resulted in a mixture of **34a**, **34c**, and **34d** (entries 1 and 2). Heterogeneous reduction with H_2 and Pd/Al_2O_3 was clean but generated a mixture of **34a** and **34e** (entry 3).[86] Other reducing conditions including phosphine–borane desulfurization (entry 4)[113] and increased reaction temperature with low pH (entry 5) were equally difficult.[112]

As an alternative, we investigated diselenide–selenoester ligation (DSL).[114] The reaction between diselenides and selenoesters proceeds under nonreducing conditions without an additive[115] and can be accompanied by a rapid one-pot deselenization.[116] In order to preserve the natural cysteines, we first prepared Tau(322–389) using DSL followed by ligations in the C to N direction. Tau(322–357) **35** features the N-terminal Acm protective group and C-terminal selenoester that was installed with $DPDS/PBu_3$ after protected cleavage from HMPB resin (Scheme 6). Tau(358–389) **36** was prepared with the Dbz linker at the C-terminus and PMB protected selenoaspartic acid at the N-terminus, which was converted into the reactive diselenide **36** using oxidation with DMSO/TFA. The 51-mer segment Tau(390–441) **37** was then prepared on a Wang resin. The Tau(291–321) fragment **38** featured N-terminal thiazoline and was prepared on HMPB resin followed by a solution-phase esterification with EtSH/PyBOP. To complete the synthesis of Tau(291–441), the DSL/deselenization reactions between the **36** and **37** proceeded well producing **39** cleanly, and the subsequent steps afforded Tau(291–441) **41** as a homogeneous material.

Assembly of Tau(1–441).

With all of the segments in hand, we were able to proceed to the completion of the synthesis of 2N4R Tau (Scheme 7). The ligation of Tau(1–156) **12** and Tau(157–290) **29** segments proceeded smoothly, and Tau(1–290) was isolated in 64%. Next, we removed the A157C mutation using metal-free desulfurization, and this step required HEPES (200 mM) and lysine (20 mM) in the desulfurization buffer to inhibit decomposition. We then converted Tau(1–290) to active MES thioester **42** using hydrazide activation chemistry. The final ligation step between Tau(1–290) **42** and Tau(291–441) **41** was carried out with lysine and MPAA buffer, and Tau(1–441) **1** was isolated in 41% yield (4.9 mg) after

preparative reversed-phase sample displacement chromatography.[117] To further corroborate the identity and purity of the synthetic material, we compared the synthetic **1** with recombinant Tau(2–441).[118,119] Although the material produced in *Escherichia coli* lacked the first methionine residue, both samples showed comparable HRMS spectra and purity on SDS-PAGE (Scheme 7C).

CONCLUSIONS

In summary, we have described the first total synthesis of the longest isoform of Tau using innovative protein chemistry methodologies. Our approach surpasses previous limitations of specificity and control in producing Tau and offers a reliable, scalable route to obtain pure, full-length Tau in multimilligram quantities. By facilitating the production of Tau proteins with specific modifications, our method provides the necessary tools for systematic studies that can shed light on the role of different Tau isoforms in the pathology of neurodegenerative diseases. The successful development of this synthetic strategy also sets the stage for future investigations of Tau posttranslational modifications. This enhanced understanding could, in turn, drive the development of novel therapeutic strategies for AD. Finally, the potential for our approach extends beyond tauopathies, and the approaches developed here could be adapted to the study of other proteins relevant to neurodegeneration.

Supplementary Material

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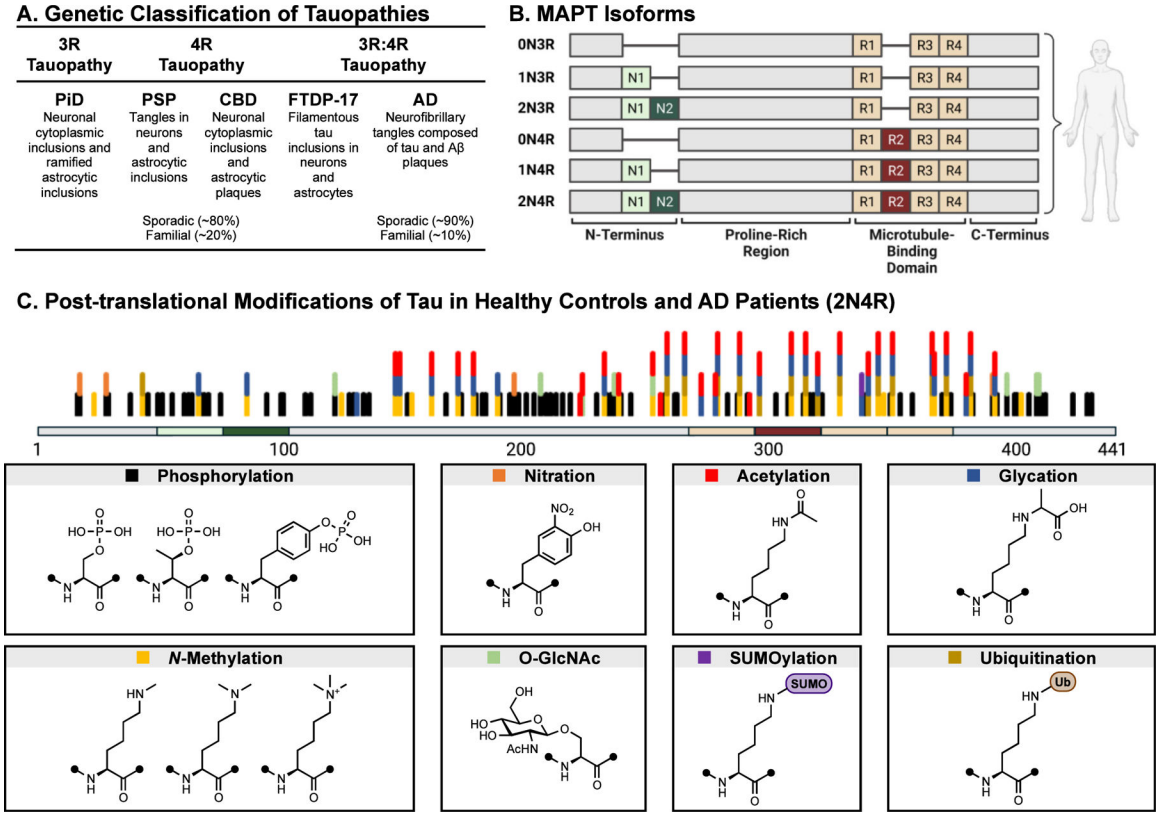


Figure 1.

(A) The *MAPT* gene, located in chromosome 17 arm 21, consists of 16 exons. Exons 1, 4, 5, 7, and 13, along with 9, 11, and 12, are consistently transcribed in the central nervous system (CNS). This gene gives rise to six different isoforms, ranging from 45 to 65 kDa. In a healthy adult brain, equal amounts of 4R to 3R tau isoforms are observed but this ratio varies in tauopathies.[1] Conditions such as AD and non-Alzheimer's tauopathy (e.g., frontotemporal dementia with parkinsonism) are classified as mixed 3R and 4R tauopathies. Tau isoforms exhibit also differences in aggregation properties (adapted from ref 11. (B) The 29 amino acid sequences in the N-terminal portion of Tau are followed by a basic proline-rich region. This N-terminal part known as the projection domain potentially interacts with other elements of the cytoskeleton and plasma membrane.[13] The C-terminal section, referred to as the repeat domains (R1–R4), comprises three (3R) or four copies (4R) of a highly conserved sequence of 18 amino acids. The microtubule-binding region is involved in microtubule assembly.[15] (C) The PTMs are distributed throughout the entire protein chain and are critical drivers of Tau aggregation.[11]

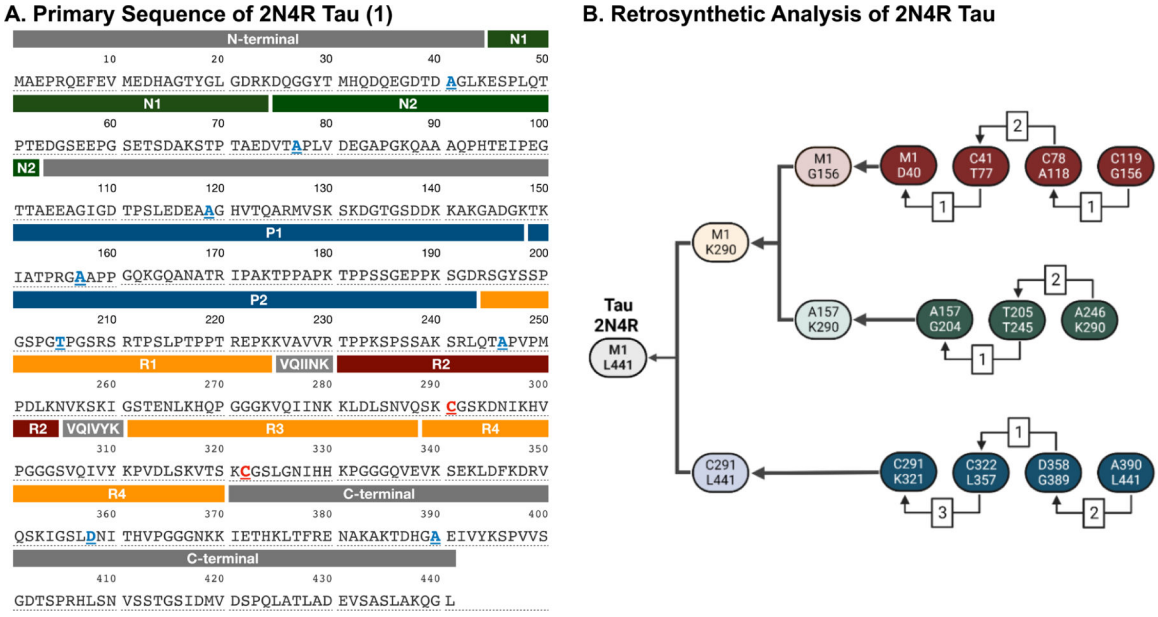
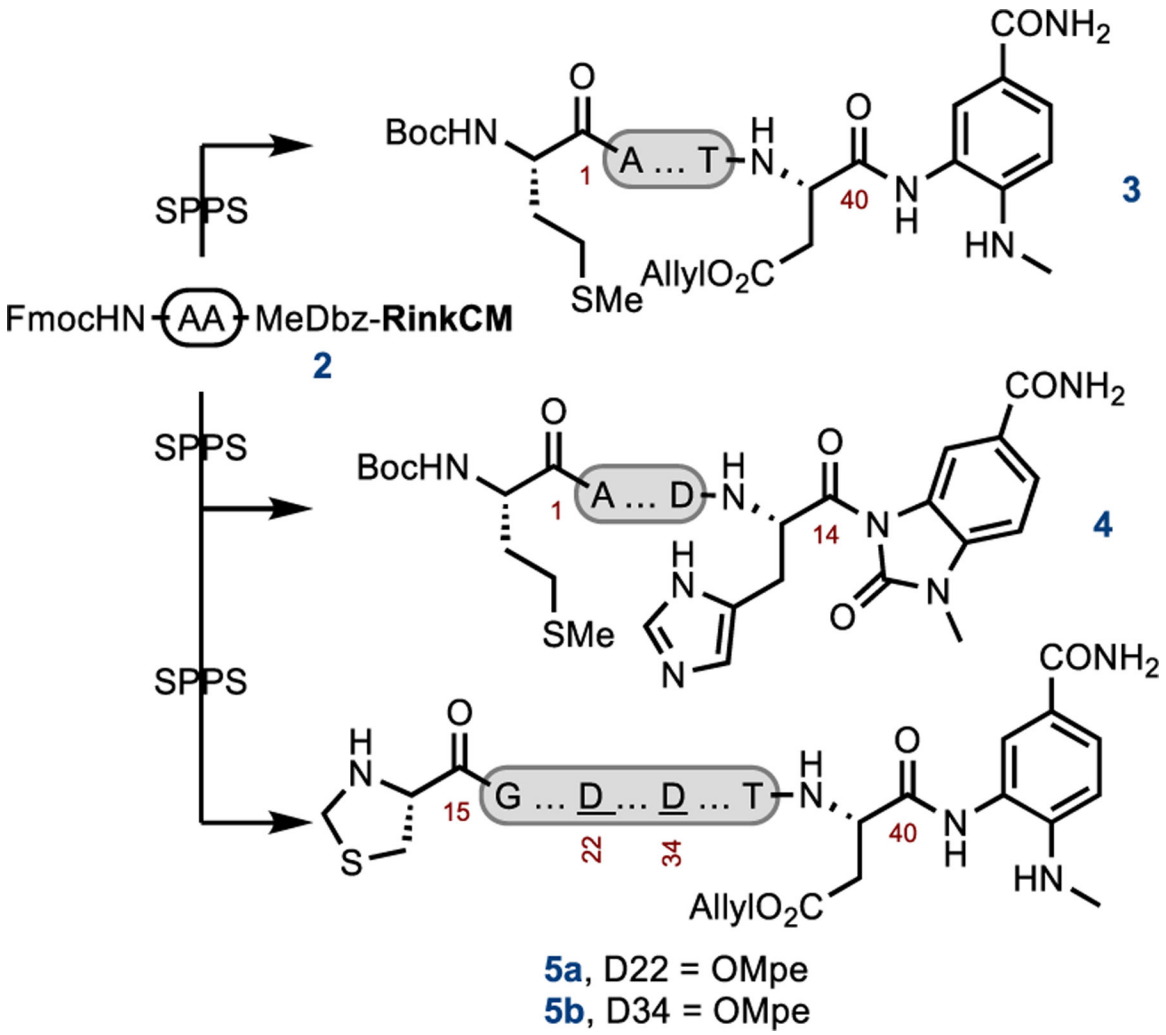
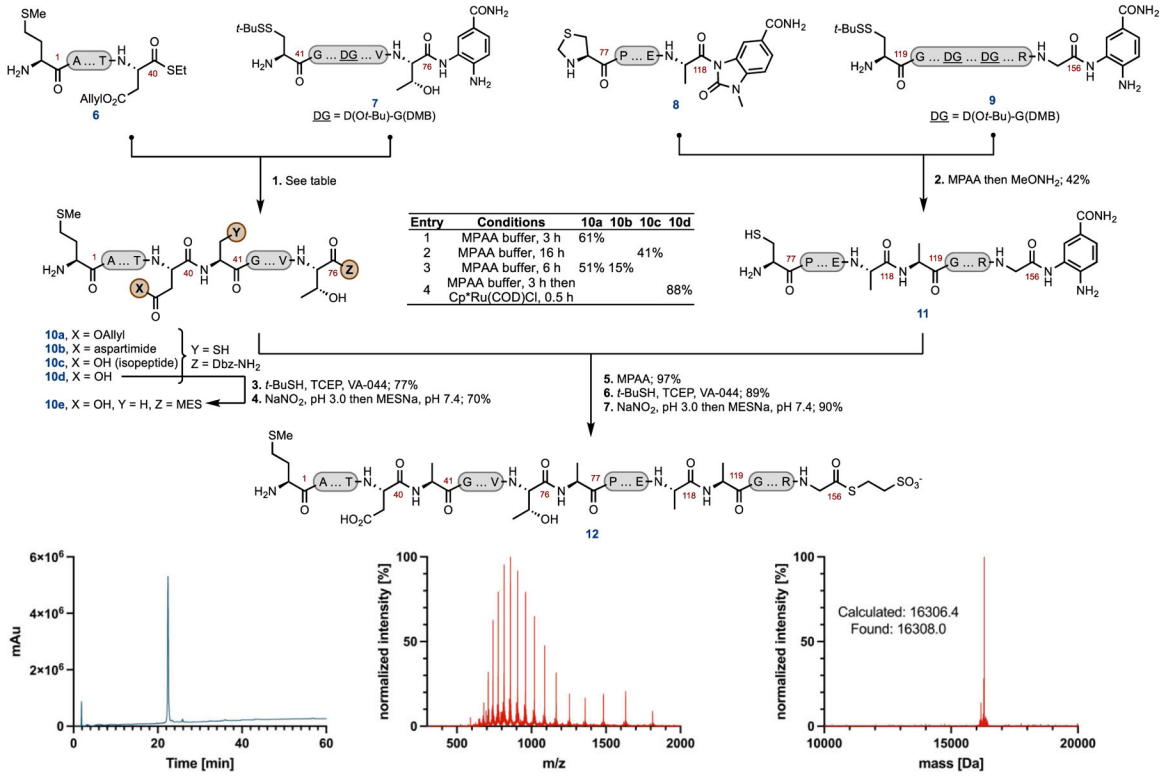


Figure 2.

Chemical strategy for the synthesis of 2N4R Tau isoform (1). (A) Natural cysteine sites (C291 and C322) are highlighted in red. Additional ligation sites to produce the native alanine (A41, A77, A119, A157, A246, and A390), threonine (T205), and aspartic acid (D358) residues are highlighted in blue. (B) Individual oligopeptides (11 fragments) are merged into three major segments (M1–G156, A157–K290, and C291–L441) through NCL-desulfurization followed by assembly of M1–K290 and the NCL with C291–L441 to produce the native protein.



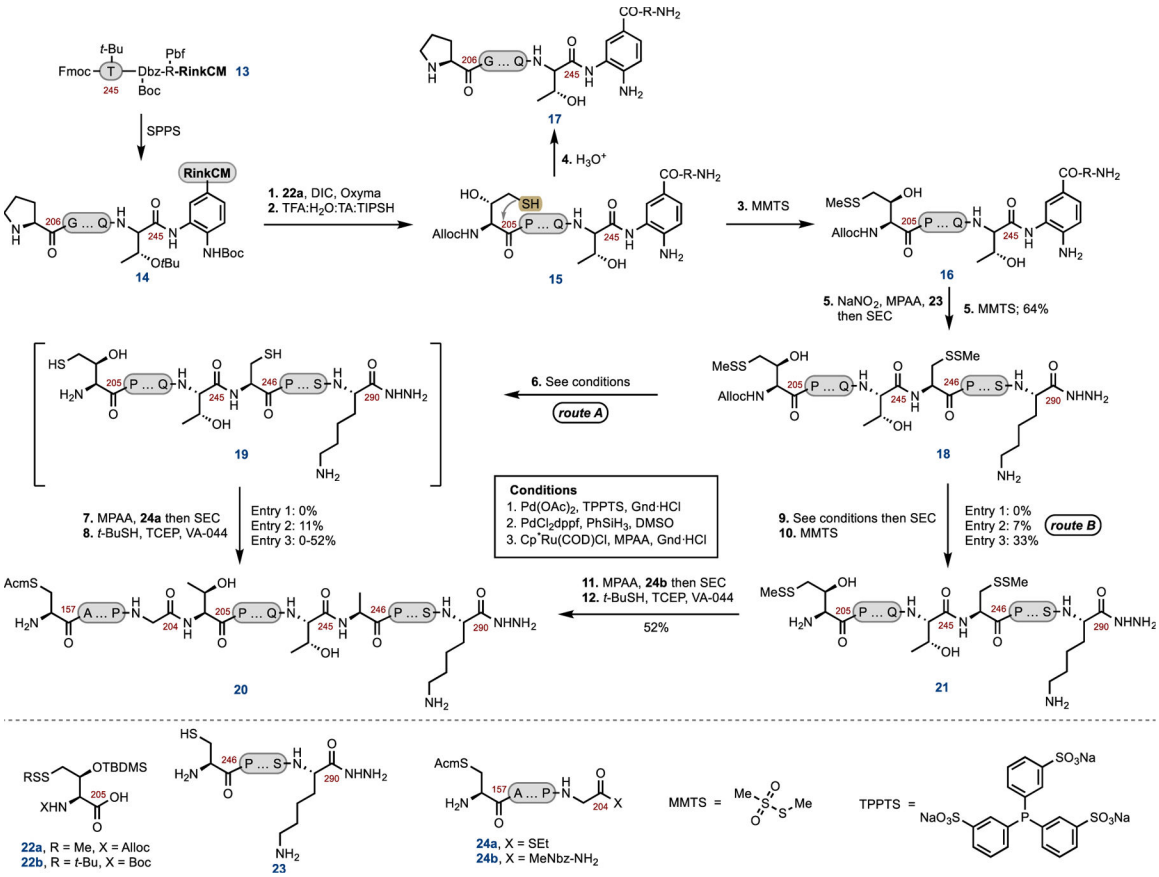
Scheme 1.
Attempted Synthesis of Tau(1–40) Fragments



Scheme 2.

Synthesis of Tau(1–156) Thioester[a]

[a]Reagents and conditions: 1. MPAA (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), TCEP·HCl (20 mM), pH 7.0, 23 °C, 3 h; then Cp*Ru(COD)Cl (30 mM), 37 °C, 1 h; 88% (entry 4). 2. MPAA (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), TCEP·HCl (20 mM), pH 7.1, 37 °C, 6 h then $MeONH_2$·HCl (200 mM), Gnd·HCl (6 M), NaH_2PO_4 (200 mM), TCEP·HCl (20 mM), pH 4.0, 23 °C, 4 h; 42%. 3. *t*-BuSH (5% v/v), TCEP·HCl (175 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), VA-044 (2 mM), pH 7.1, 37 °C, 18 h; 77%. 4. $NaNO_2$ (10 equiv), Gnd· HCl (6 M), NaH_2PO_4 (100 mM), pH 3.0, −15 °C, 25 min, addition of MESNa (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), pH 7.4, −15 to 23 °C, 2 h then adjusted to TCEP·HCl (50 mM); 70%. 5. MPAA (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), TCEP·HCl (20 mM), pH 7.1, 23 °C; 97%. 6. *t*-BuSH (8% v/v), TCEP·HCl (175 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), VA-044 (3 mM), pH 7.0, 37 °C, 24 h; 89%. 7. $NaNO_2$ (20 equiv), Gnd·HCl (6 M), NaH_2PO_4 (100 mM), pH 3.1, −15 °C, 20 min then MESNa (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), pH 7.1, −15 to 23 °C, 1.5 h then adjusted to TCEP·HCl (30 mM); 90%. Abbreviations: *t*-Bu, *tert*-butyl; COD, 1,5-cyclooctadiene; Cp*, pentamethylcyclopentadienyl; DMB, 2,4-dimethoxybenzyl; Gnd·HCl, guanidine hydrochloride; Me, methyl; MESNa, mercaptoethanesulfonic acid sodium salt; MPAA, 4-mercaptophenylacetic acid; TCEP·HCl, tris(2-carboxyethyl)phosphine hydrochloride; VA-044, 2,2′-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride.

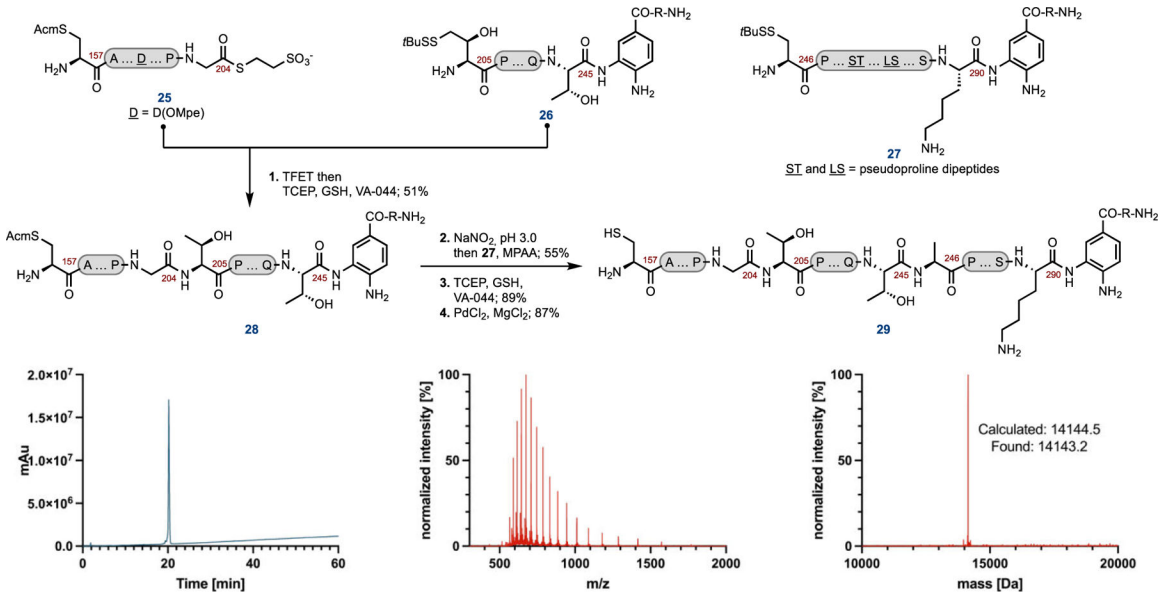


Scheme 3.

First-Generation Approach to Tau(157–290)[a]

[a]Reagents and conditions: 1. SPPS then 22a, DIC (10 equiv), Oxyma (5 equiv), DMF. 2. TFA:H_2O:TA:TIPSH (85:5:5:5), 3 h. 3. MMTS (100 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), pH 7.3, 23 °C, 2 h; 9%. 3. H_2O/CH_3CN, TFA (0.1%). 5. $NaNO_2$ (10 equiv), Gnd·HCl (6 M), NaH_2PO_4 (100 mM), pH 3.1, −15 °C, 25 min addition of MPAA (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), pH 7.2, −15 to 23 °C, 60 min addition of Tau(246–290)-$NHNH_2$ 23 followed by TCEP·HCl (50 mM), 23 °C, 6 h; desalting over Sephadex LH-20. 5. MMTS (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), pH 7.4, 23 °C, 3 h; 64%. 6. Conditions 1–3. 7. Cp*Ru(COD)Cl (0.3 equiv), MPAA (100 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), pH 7.0, 37 °C, 3 h, then adjusted to TCEP·HCl (20 mM), addition of Tau(157–204)-SEt **24a**, 37 °C, 3 h, then Sephadex LH-20 (entry 3). 8. *t*-BuSH (5% v/v), TCEP·HCl (175 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), VA-044 (2 mM), pH 7.0, 37 °C, 18 h; 33%. 9. Cp*Ru(COD)Cl (30 mM), MPAA (200 mM), Gnd·HCl (6 M), Na_2HPO_4(200 mM), pH 7.0, 37 °C, 3 h, then adjusted to TCEP·HCl (20 mM), then desalted over Sephadex LH-20 (entry 3). 10. MMTS (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), pH 7.2, 23 °C, 2 h; 33%. 11. Tau(157–204)-MeNbz-NH_2 **24b**, MTG (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), TCEP·HCl (20 mM), pH 7.0, 37 °C, 5 h, then Luna C4 desalting. 12. *t*-BuSH (5% v/v), TCEP·HCl (175 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), VA-044 (2 mM), pH 7.0, 37 °C, 18 h; 52%. Abbreviations: Alloc, allyloxycarbonyl; DMSO, dimethyl sulfoxide; MMTS, methyl

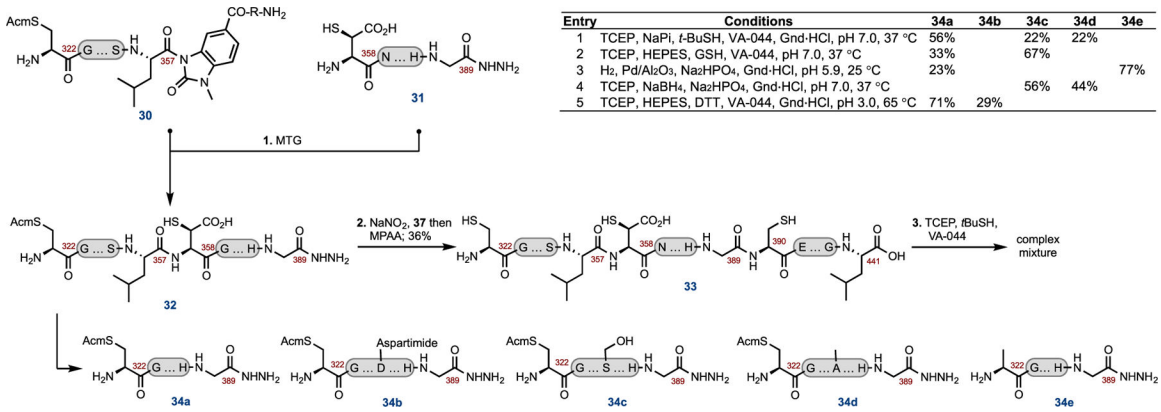
methanethiosulfonate; Oxyma, ethyl cyano-(hydroxyimino)acetate; SEC, size-exclusion chromatography; TA, thioanisole; TFA, trifluoroacetic acid; TIPSH, triisopropylsilane; TPPTS, 3,3′,3″-phosphanetriyltris(benzenesulfonic acid) trisodium salt.



Scheme 4.

Assembly of Tau(157–290) Fragment[a]

[a]Reagents and conditions: 1. TFET (2% v/v), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), TCEP·HCl (20 mM), pH 7.3, 37 °C, 2 h then adjusted to TCEP·HCl (200 mM), GSH (50 mM), VA-044 (20 mM), 37 °C, 3 h; 51%. 2. $NaNO_2$ (20 equiv), Gnd·HCl (6 M), NaH_2PO_4 (100 mM), pH 3.1, −15 °C, 20 min addition of MPAA (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), pH 7.3, −15 to 23 °C, 20 min addition of Tau(246–290)-Dbz-R-NH_2 **27** followed by TCEP·HCl (30 mM), 23 °C, 4 h; 55%. 3. TCEP·HCl (175 mM), GSH (50 mM), Gnd·HCl (6 M), HEPES (200 mM), VA-044 (20 mM), pH 7.1, 37 °C, 48 h; 89%. 4. $PdCl_2$ (15 equiv), $MgCl_2$ (50 equiv), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), pH 7.1, 37 °C, 2 h; 87%. Abbreviations: GSH, reduced glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mpe, 3-(3-methyl)pentyl ester; TFET, 2,2,2- trifluoroethanethiol.

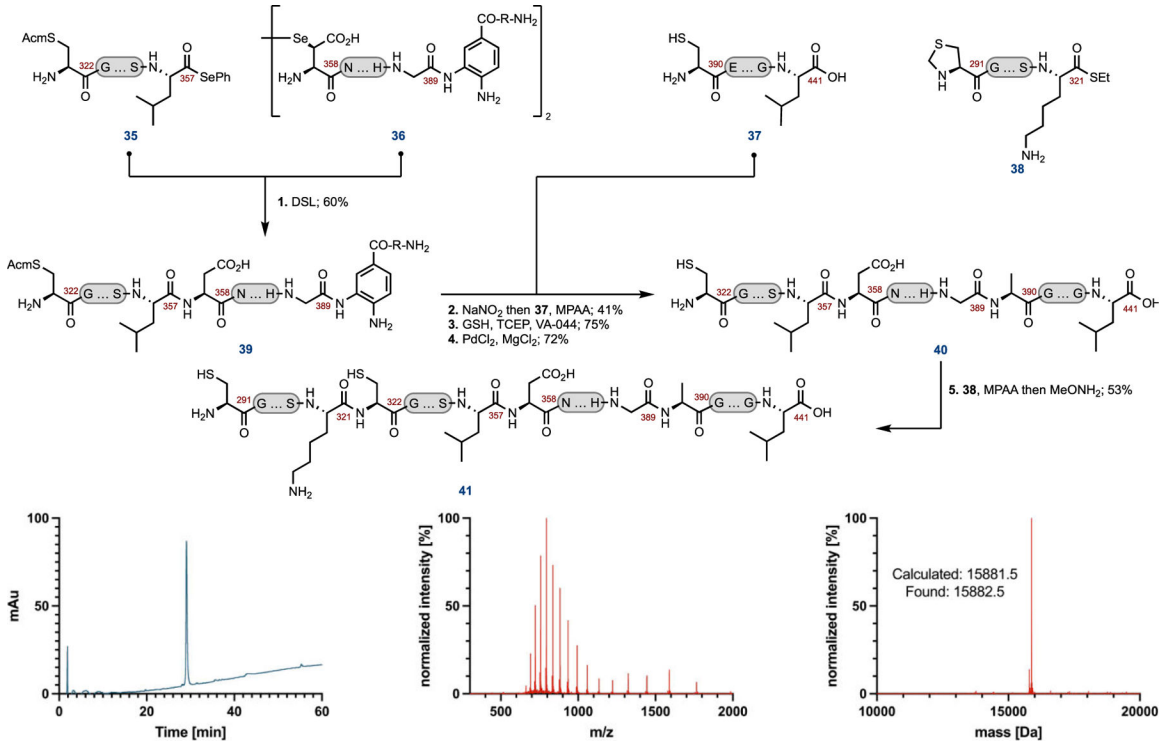


Entry	Conditions	34a	34b	34c	34d	34e
1	TCEP, NaPi, *t*-BuSH, VA-044, Gnd·HCl, pH 7.0, 37 °C	56%		22%	22%	
2	TCEP, HEPES, GSH, VA-044, pH 7.0, 37 °C	33%		67%		
3	H_2, Pd/Al_2O_3, Na_2HPO_4, Gnd·HCl, pH 5.9, 25 °C	23%				77%
4	TCEP, $NaBH_4$, Na_2HPO_4, Gnd·HCl, pH 7.0, 37 °C			56%	44%	
5	TCEP, HEPES, DTT, VA-044, Gnd·HCl, pH 3.0, 65 °C	71%	29%			

Scheme 5.

Studies on the Tau(322–441) Segment[a]

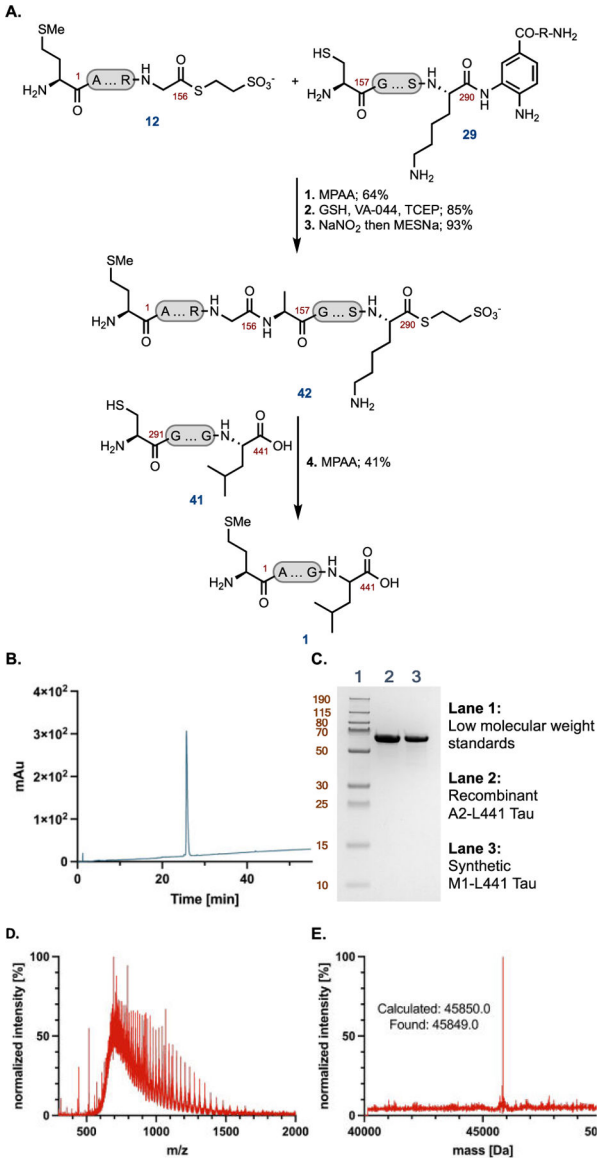
[a]Reagents and conditions: 1. MTG (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), TCEP·HCl (20 mM), pH 7.0, 37 °C, 3 h; 51%. 2. Tau(390–441)–OH 37, $NaNO_2$ (10 equiv), Gnd·HCl (6 M), NaH_2PO_4 (100 mM), pH 3.2, −15 °C, 20 min addition of MPAA (200 mM), Gnd· HCl (6 M), Na_2HPO_4 (200 mM), pH 7.2, −15 to 23 °C, 2 h followed by TCEP·HCl (60 mM), 23 °C, 5 h; 36%. 3. *t*-BuSH (5% v/v), TCEP·HCl (175 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), VA-044 (3 mM), pH 7.0, 37 °C, 24 h; decomposes. Abbreviations: Acm, acetamidomethyl; MTG, methyl thioglycolate.



Scheme 6.

Assembly of the Tau(291–441) Fragment[a]

[a]Reagents and conditions: 1. Gnd·HCl (6 M), Na_2HPO_4 (100 mM), pH 6.2, 23 °C, 1 h; extraction with hexanes then adjustment to NH_2NH_2 (2% v/v), pH 7.4, 23 °C, 30 min, adjustment to TCEP·HCl (83 mM), DTT (8 mM), pH 5.3, 45 min; 60%. 2. $NaNO_2$ (15 equiv), Gnd·HCl (6 M), NaH_2PO_4 (100 mM), pH 3.0, −15 °C, 20 min then addition of MPAA (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), pH 7.3, −15 to 23 °C, 5 min then addition of Tau(390–441)–OH, adjustment to TCEP·HCl (30 mM), 23 °C, 4 h; 41%. 3. TCEP·HCl (175 mM), GSH (50 mM), Gnd· HCl (6 M), HEPES (200 mM), VA-044 (20 mM), pH 7.0, 37 °C, 16 h; 75%. 4. $PdCl_2$ (15 equiv), $MgCl_2$ (50 equiv), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), pH 7.1, 37 °C, 2 h; 72%. 5. MPAA (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), TCEP·HCl (20 mM), pH 7.0, 23 °C, 4 h then adjustment to $MeONH_2$·HCl (200 mM), pH 4.0, 23 °C, 4 h; 53%.



Scheme 7.

Completion of Chemical Synthesis of Tau(1–441) and Biophysical Characterizations[a]

[a]Reagents and conditions: 1. MPAA (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), TCEP·HCl (20 mM), lysine (20 mM), pH 7.0, 23 °C, 5 h; 64%. 2. TCEP·HCl (450 mM), GSH (100 mM), Gnd· HCl (6 M), HEPES (200 mM), VA-044 (20 mM), lysine (20 mM), pH 7.0, 37 °C, 20 h; 85%. 3. $NaNO_2$ (20 equiv), Gnd·HCl (6 M), NaH_2PO_4 (100 mM), pH 3.0, −15 °C, 20 min then addition of MESNa (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), pH 7.2, −15 to 23 °C, 1 h then adjust to TCEP·HCl (20 mM); 93%. 4. MPAA (200 mM), Gnd·HCl (6 M), Na_2HPO_4 (200 mM), TCEP· HCl (20 mM), lysine (20 mM), pH 7.0, 23 °C, 5 h; 41%.