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Protein Synthesis

Total Chemical Synthesis of a 304 Amino Acid K48-Linked Tetraubiquitin Protein**

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Recent advances in chemical and semisynthesis of proteins have allowed for the efficient production of naturally occurring proteins, polymer–protein conjugates, and posttranslationally modified proteins for structural and functional analyses.[1] Among these examples are the recent developments of the non-enzymatic preparation of highly homogeneous ubiquitinated peptides and proteins, which are known to be crucial bioconjugates for the studies aiming at deciphering the effect of ubiquitination on cellular processes.[2] In this regard, various research groups, including ours, have reported innovative strategies for constructing the native isopeptide bond[3] and its mimetics,[4] which links the lysine side chain of a protein target to the C-terminus of ubiquitin (Ub). The chemical synthesis of highly homogeneous and naturally occurring ubiquitinated proteins is just beginning to have an impact on our understanding of various systems involving Ub.[4c,5] Of particular interest are the very recent reports on the semi- and chemical synthesis of diUb chains through native and nonnative isopeptide bond formation.[3d,4b,6] Notably, our group reported the total chemical synthesis of all seven Lys-linked diUb chains, thus paving the way for studying various aspects of these chains.[6b]

Unarguably, the diUb analogues have provided useful information on several aspects of Ub biology and will continue to contribute to the field in various studies. In this regard, several structural and biochemical studies have already been performed on the diUb chains linked thorough K63, K48, and more recently on the remaining chains.[3d,6,7] For most of the chains their optimal length for function is still unknown and this will have to be further investigated. For example, in the case of the K48 linkage, a chain of four Ub monomers is required for an efficient substrate recognition by the 26S proteasome.[8] Moreover, for structural information the diUb chains may not fully represent the native structure of the longer chain.[9] Finally, several in vitro studies have shown that most Ub binding domains bind to the Ub monomer, however, increasing the number of Ub units in a specific chain can be used both as a mechanism to increase binding and to enforce cellular specificity of deubiquitinases.[10] Hence, the preparation of longer Ub chains is crucial to address some of the open questions in the field. Earlier efforts involving the use of the enzymatic machinery (E1-E3) so far has been limited mainly to Ub chains linked through K48 and K63, which requires also mutations in the Ub sequence (e.g. Lys/Arg) and the formation of an isopeptide bond through thialysine.[11] Moreover, these methods are very limited to the natural sequence, thus rendering the introduction of a specific modification in a highly controlled manner unreachable. On the other hand, by using chemical synthesis, virtually unlimited variations could be performed and incorporated in a highly controlled manner into each Ub unit within the specific chain (e.g. specific labeling). This would lead to unraveling of the thus-far unattainable details of Ub biology.

Despite previous successes in applying chemical methods to prepare diUb chains, the synthesis of the tetraUb chain is much more challenging and requires new chemical methods to achieve such a formidable task. The tetraUb chain could, in principle, be synthesized through a linear approach or by applying a convergent strategy in which two diUb chains are synthesized separately and then linked to form the tetraUb chain (Scheme 1). The latter approach might be preferable, as we would expect it to proceed faster and give better yield. However, it also entails a challenge in that the **Ub4-Ub3** fragment must be prepared in the thioester form to allow ligation to **Ub2-Ub1** bearing δ-mercaptolysine group.

With this in mind, we initially tested the convergent approach wherein we first started to prepare the challenging **Ub4-Ub3** thioester (Scheme 2). To achieve this, we applied our recently developed strategy to chemically synthesize Ub thioester[12] to allow the incorporation of the δ-mercaptolysine group in the Ub sequence to enable further chain elongation. Thus, **Ub3** was first prepared from two fragments, Ub(1-45): **Ub-N**, and Ub(46-76): **Ub3-C**, wherein the latter was equipped with the 2-nitrobenzyl-protected *N*-methylcysteine and the δ-mercaptolysine groups in the thiazolidine form.[13] In this case, it was crucial to use the protected *N*-methylcysteine group because following **Ub3** assembly a treatment with methoxylamine is required to unmask the δ-mercaptolysine group. Under these reaction conditions (100 mM methoxylamine, pH 4) and when no protection was applied, the nucleophilic methoxylamine was found to attack the partially formed N–S acyl-transfer intermediate (25–30%). Meanwhile, if the protected *N*-methylcysteine group was used this side reaction was completely avoided. On the other hand, in the case of **Ub4**, which has no δ-mercaptolysine

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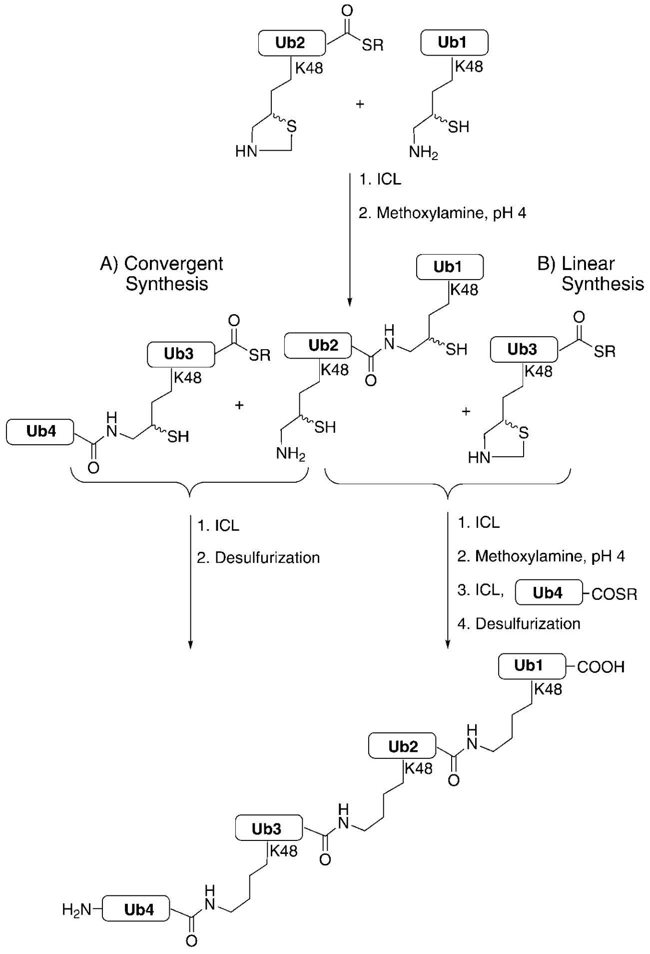
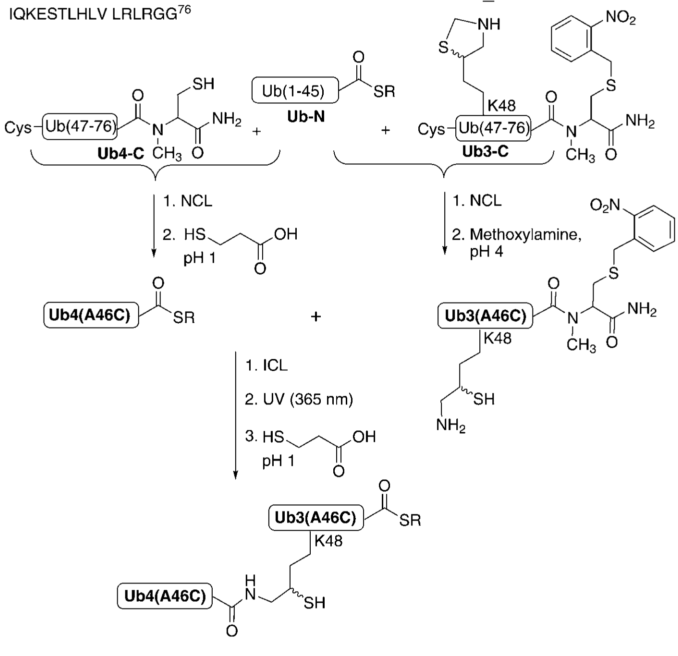


Scheme 1. Proposed convergent and linear strategies for the synthesis of tetraUb. ICL = isopeptide chemical ligation.

^{1}MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIF**A**GKQL EDGRTLSDYN IQKESTLHLV LRLRGG^{76}



Scheme 2. Chemical synthesis of **Ub4-Ub3** thioester, (R = CH_2CH_2COOH), Met1 was replaced with Nle to avoid oxidation during synthesis.

group, there was no need to use the protected form of *N*-methylcysteine.

Having both **Ub3** and **Ub4** in hand, we then mixed these monomers in 6 M guanidine·HCl, 2% (v/v) benzyl mercaptan and thiophenol to give the ligation product, **Ub4-Ub3** methylcysteine. After isolation of the product, exposure to UV light for 1 hour released the 2-nitrobenzyl group and the addition of 20% of 3-mercaptopropionic acid (MPA) afforded the **Ub4-Ub3** thioester as the major product (Figure 1). However, under these conditions, we also observed about 20% cleavage of **Ub4-Ub3** at the isopeptide bond. It has been recently reported that peptides and proteins with a Gly-Cys junction can undergo cleavage of the peptide bond at acidic pH and elevated temperature, through N−S acyl-transfer intermediate.[14] Prior to desulfurization, our isopeptide bond in **Ub4-Ub3** resembles the Gly-Cys junction, hence a similar mechanism of cleavage could occur during thioester formation (40 °C, pH 1). These results emphasize the importance of developing an efficient N−S acyl-transfer method that operates under mild conditions.[15] Nevertheless, we were able to separate the desired **Ub4-Ub3** thioester from

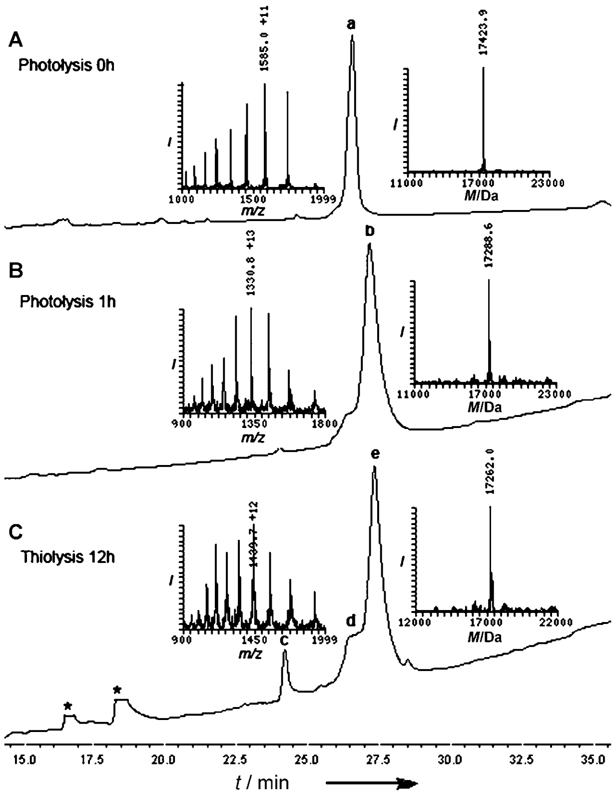


Figure 1. Conversion of **Ub4-Ub3** into **Ub4-Ub3** thioester; A) analytical HPLC traces/(ESI-MS) of **Ub4-Ub3** at 0 h: observed mass 17 423.9 Da (calcd 17 421.3 Da); B) Photolysis after 1 h: peak b corresponds to the unmasked product; C) Thiolysis after 12 h: peak e corresponds to the **Ub4-Ub3** thioester with the observed mass 17 262.0 Da (calcd 17 259.1 Da), while peak c and peak d correspond to the Ub thioesters corresponding to cleavage of isopeptide bond during thiolysis. The peak marked with * corresponds to thiol additives.

the side reaction products (**Ub3** thioester and **Ub4** thioester) in 14 % yield of isolated product (over three steps; Scheme 2). Having this synthetically challenging building block in hand, we then focused on the synthesis of **Ub2-Ub1**. The key step for this synthesis was the preparation of **Ub2** thioester bearing the protected δ-mercaptolysine group. This fragment was made in 25 % yield of isolated product by also applying the *N*-methylcysteine approach (see the Supporting Information). In this case, the unprotected *N*-methylcysteine form was used because the removal of the protecting group from δ-mercaptolysine unit was performed after the ligation step with **Ub1**. Subsequently, **Ub1** and **Ub2** thioester were ligated, as described above, and the product was isolated in 33 % yield after treatment with methoxylamine (see the Supporting Information).

With the two main building blocks in hands, we then performed the final ligation to assemble the K48-linked tetraUb. Thus, **Ub4-Ub3** thioester and **Ub2-Ub1** bearing the free δ-mercaptolysine group were treated in 6 M guanidine·HCl, 0.1 M phosphate, pH 7, at a final concentration of about 1 mM and in the presence of 2 % (v/v) of benzyl mercaptan/thiophenol. Pleasingly, as shown in Figure 2, the reaction progressed to give the desired tetraUb as the major product after 48 hours. Increasing the reaction time did not lead to further improvement in yield, thus the reaction was quenched and the product was isolated in 10 % yield (based on the fragment of **Ub4-Ub3** thioester). We also tested the efficiency of the linear strategy as depicted in Scheme 1 B. Thus, **Ub2-Ub1** was ligated to **Ub3** thioester followed by treatment with methoxylamine and final ligation with **Ub4** thioester to afford the tetraUb (see the Supporting Information).

At this stage, the tetraUb product synthesized by both strategies contained seven thiol groups, three of which are located near the isopeptide bonds and the remaining four thiol groups at position 46 in each Ub monomer (originally Ala46). These Cys residues were used for the native chemical ligation (NCL)[16] step to assemble each monomer unit. Thus, a desulfurization step was required to remove these thiol groups to furnish the native tetraUb. Unfortunately, despite several applying various desulfurization conditions, such as H_2/Ranny nickel[17] and the free-radical approach,[18] we were unable to achieve this task and an incomplete desulfurization was often the outcome. At this stage, we were also challenged by the low ionization properties of the undesulfurized tetraUb by ESI-MS (Figure 2 D) that which further complicated our analysis.

The results described above forced us to adopt an alternative approach wherein we thought to apply desulfurization during chain assembly, thus decreasing the number of thiol groups at the final stage of the synthesis. For this task we examined the linear approach along with desulfurization of as many thiol groups as possible before reaching the last step. Accordingly, three distinct Ub monomers were prepared for the synthesis of tetraUb. These include 1) **Ub1** with the acid functionality at the C-terminus and orthogonally protected δ-mercaptolysine, 2) **Ub2** and **Ub3** bearing thioester functionality and an orthogonally protected δ-mercaptolysine residue, and 3) **Ub4** thioester.

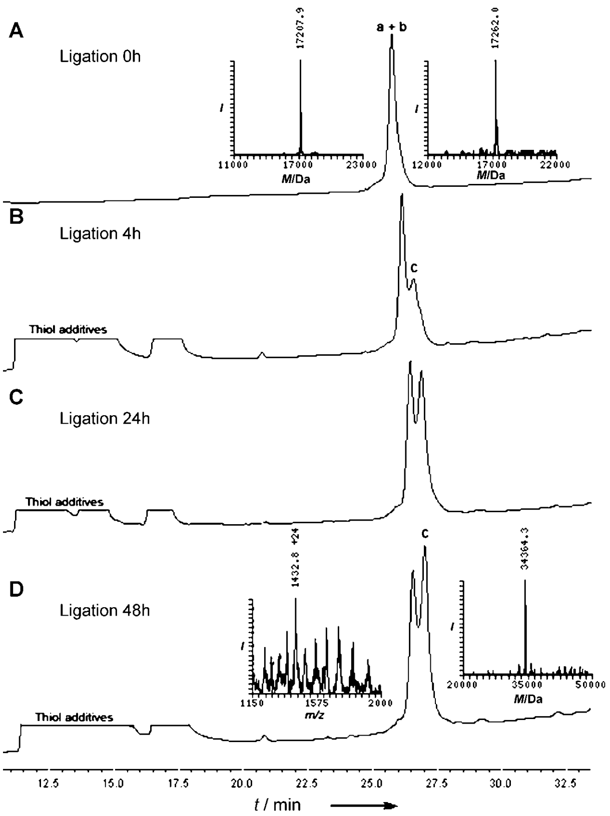


Figure 2. Synthesis of tetraUb using the convergent approach; A) analytical HPLC traces/(ESI-MS) of the ligation reaction between **Ub2-Ub1** and **Ub4-Ub3** thioester at 0 h; peak a + peak b corresponds to the mixture of **Ub2-Ub1** and **Ub4-Ub3** thioester; B) Ligation after 4 h; peak c corresponds to the ligation product with the observed mass 34 364.3 Da (calcd 34 357.8 Da); C) Ligation after 24 h; D) Ligation after 48 h.

Notably, we found that **Ub2-Ub3** thioester with the protected δ-mercaptolysine group was stable under the free-radical desulfurization conditions (*t*BuSH, tris(2-carboxyethyl)phosphane hydrochloride (TCEP), VA-044)[18a] and we were able to desulfurize Cys46, within 30 minutes, leaving the protected δ-mercaptolysine and thioester units completely intact (see the Supporting Information). Moreover, we have recently shown that the use of 2-mercaptoethanesulfonate (MES) allows in situ NCL and the desulfurization reaction.[19] Thus, the three different Ub monomers were prepared along with the desulfurization of Cys46 in each monomer (see the Supporting Information). To assemble the tetraUb from the new Ub monomers, we first ligated **Ub1** to **Ub2** thioester followed by removal of the δ-mercaptolysine residue in **Ub2** to allow subsequent ligation with **Ub3** thioester. After methoxylamine treatment of **Ub3-Ub2-Ub1**, ligation with **Ub4** thioester gave the tetraUb bearing only three thiol groups. Each ligation step proceeded efficiently and gave the product in 30–40 % yield of isolated product. Moreover, the presence of fewer thiol groups, in particular at the final stage, improved both the ligation efficiency as well as the ionization properties of tetraUb in comparison to the use of the same approach, in which all the thiol groups remain until the last step. At this stage, we were very pleased to see that by applying the optimized free-radical desulfurization conditions (VA-044, glutathione, TCEP, 40 °C)[18b] the desired tetraUb was obtained and isolated in 5% yield for the five steps starting from **Ub1** (Figure 3).

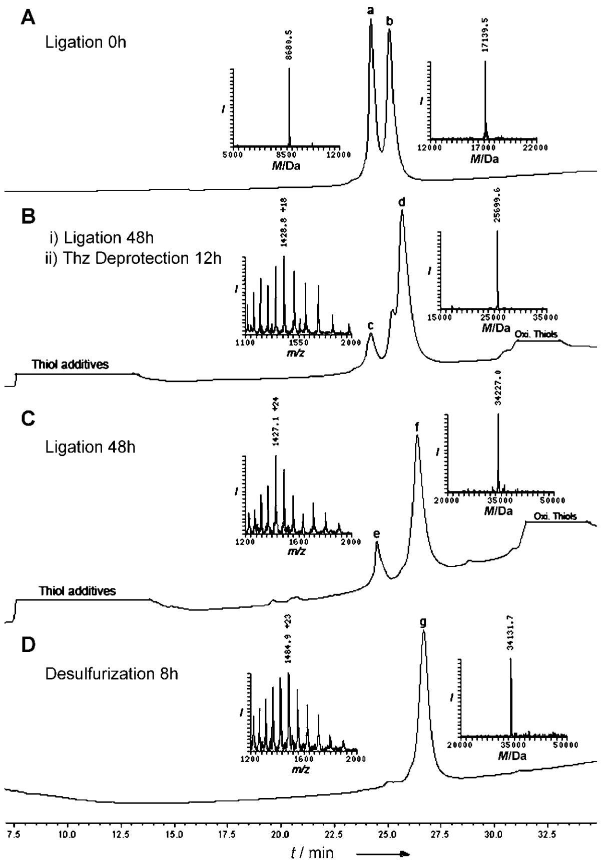


Figure 3. Synthesis of tetraUb using the linear approach; A) analytical HPLC traces/(ESI-MS) of the ligation reaction between **Ub2-Ub1** (peak b) and **Ub3** thioester (peak a) at 0 h, B) Ligation after 48 h and methoxylamine treatment after 12 h; peak c corresponds to the hydrolyzed **Ub3** thioester and peak d corresponds to the ligation product **Ub3-Ub2-Ub1** after methoxylamine treatment with the observed mass 25 699.6 Da (calcd 25 700.6 Da); C) Ligation after 48 h between **Ub3-Ub2-Ub1** and **Ub4** thioester; peak e is the hydrolyzed **Ub4** thioester and peak f corresponds to the ligation product **Ub4-Ub3-Ub2-Ub1** with the observed mass 34 227.0 Da (calcd 34 230 Da) D) Desulfurization after 8 h showing the desired tetraUb with the observed mass 34 131.7 Da (calcd 34 133.2 Da).

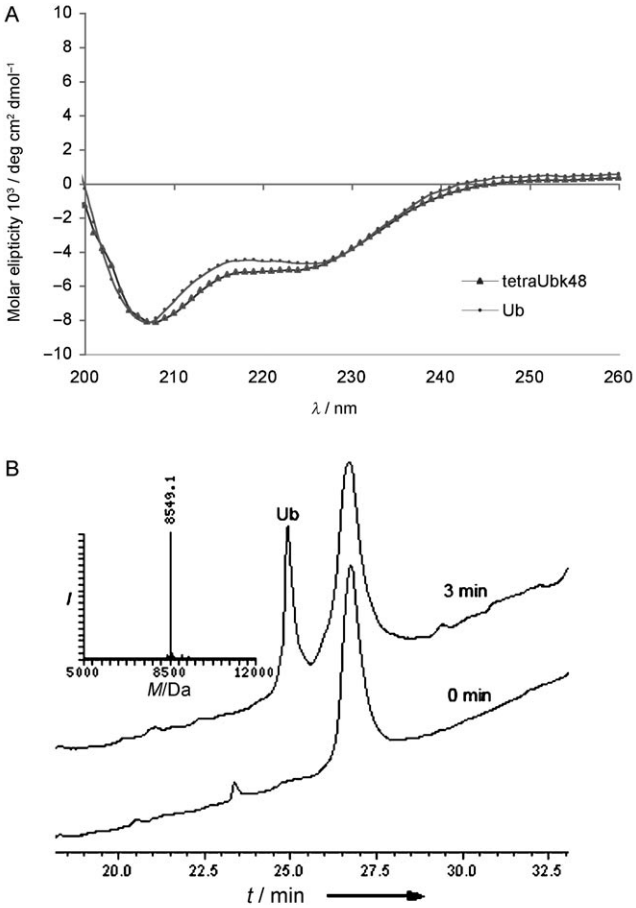


Figure 4. Characterization of synthetic K48-linked tetraUb: A) CD analysis of tetraUb in comparison to monoUb. B) Analytical HPLC of the time course for the hydrolysis reaction of tetraUb after 3 min showing the hydrolysis product, Ub, with the observed mass of 8549.1 Da (calcd 8547.8 Da). The broad peak is composed of a mixture of masses, which correspond to other cleavage products (di- and triUb) that co-elute with the starting material.

To further characterize the synthetic tetraUb we carried out circular dichroism (CD) analysis and chain disassembly with a known deubiquitinating enzyme. The CD spectra of the synthetic tetraUb resembled the commercial monoUb and the previously synthesized diUb chains.[6b] This result supports correct folding since the Ub molecule retains its globular folding regardless of the chain's type and length (Figure 4 A). Finally, the folded tetraUb was treated with IsoT, a deubiquitinating enzyme responsible for the disassembly of the K48-linked polyubiquitin in vivo. After 3 minutes, the monoUb hydrolysis product started to appear in a significant amount along with the di- and triUb (Figure 4 B). This observation was also supported by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) analysis that showed a similar behavior to the hydrolysis of the enzymatically prepared tetraUb.[20] These results support the fact that our synthetic version is well folded and active with the specific deubiquitinase, thus paving the way for future studies with the K48-linked tetraUb and the other chains.

In summary, the total chemical synthesis of K48-linked tetraUb chain was achieved for the first time. Generally, chemical synthesis of proteins enables the preparation of targets composed of 50–150 residues. Our tetraUb comprises 304 residues with three isopeptide bonds, thus representing a new size record for chemical synthesis of naturally occurring proteins,[21] thereby testifying to the power of chemical synthesis and our developed synthetic tools in the preparation of proteins with unusual structures. Our strategy, when combined with the recent advances in the direct synthesis of Ub[6d] and the one-pot-based ligation method,[22] should expedite the synthetic process and increase significantly the yield of the product. Such an optimized strategy will enable the chemical synthesis of other tetraUb chains and their analogues for structural and functional analyses. Ultimately, this approach should assist in the ongoing efforts in fully understanding how the different chains achieve the remarkable diversity of Ub signaling.

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