Protein Synthesis

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Total Chemical Synthesis of Di-ubiquitin Chains**

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Ubiquitination, the attachment of an ubiquitin (Ub) or polyubiquitin (polyUb) chain to a protein target, is involved in a wide range of cellular processes in eukaryotes.[1] This dynamic posttranslational modification utilizes three enzymes (E1, E2, and E3) to link the C-terminal Gly of Ub to the lysine side chain of a protein target through an isopeptide bond. In this process, the E1 enzyme activates the Ub terminal in an ATP-dependent manner; E2 is the conjugating enzyme, and E3 is the ubiquitin-protein ligase.[2] The conjugation of the Ub molecule can include either a monomer (monoubiqutination) or a chain of various lengths and linkage types (polyubiquitination). As a result, these modifications lead to a variety of molecular signals, for example, regulation of endocytosis and DNA repair, wherein the outcome depends upon the ubiquitination (polyubiquitination versus monoubiquitination). Polyubiquitination is a complex and diverse modification where the outcome of a molecular signal depends upon which one of the seven lysines in Ub (K6, K11, K27, K29, K33, K48, K63) is linked to consecutive Ub molecules.[3] The connectivity of the chain through K48 of Ub is called a typical chain, whereas connectivity through any one of the other six lysines is known as an atypical chain.[4] The latter can be homotypic in which a single type of lysine is used for the chain assembly, a mixed-linkage chain with several distinct lysines involved in the branching of the Ub chain, or a heterologue wherein Ub and Ub-like modifiers (e.g., SUMO = small ubiquitin-like modifiers) are utilized in the assembly of the chain.

The diversity in the ubiquitin chain is believed to lead to the formation of protein/Ub conjugates having a large conformational space, thus leading to a wide range of biological signals.[5,6] Besides recent molecular modeling studies exploring the linkage dependence of di-ubiquitin (di-Ub) conformations,[7] no structural data is available regarding the rest of the atypical ubiquitin chains. Moreover, despite the importance of these chains in vivo, their isolation in a large enough quantity for the in vitro biochemical studies and for providing structural information have been very challenging. The isolation is primarily hindered by the unavailability of the E2/E3 enzymes for reconstitution of the desired chain in vitro, as is the case with K48- and K63-Ub chains.

The importance of the functional and structural diversity of the Ub chain as related to the signals, and the fact that little is known about the various aspects of the atypical Ub chains has generated an urgent need for milligram quantities of homogeneous Ub chains with a high purity, which includes well-defined chain length and linkage type, to assist in structural, biochemical, and biophysical studies. Total chemical synthesis of proteins offers unique opportunities to achieve highly homogeneous posttranslationally modified proteins, as well as those with unusual connectivity because of the precise control over each atom in the protein structure.[8] In the ubiquitination front, chemical methods are devoid of using the E1/E3 enzymatic machinery in which their identification are considered to be the main challenge. In contrast, the unusual connectivity in Ub chains, that is the isopeptide bond, and their relatively large size presents a real challenge to an organic chemist as unprecedented chemistry must be developed to achieve the total chemical synthesis of such a natural product family.

Recently, several groups, including ours, have adopted chemical approaches to assemble the native isopeptide bond and allow the efficient synthesis of ubiquitinated peptides and proteins.[9] Specifically, our group introduced the δ mercaptolysine to mediate the transthioesterification step of Ub thioester and subsequent S–N acyl transfer to form the isopeptide bond.[9c] Subsequently, the thiol handle can be removed by applying the desulfurization reaction to furnish the unmodified isopeptide linkage. Moreover, we have also reported the synthesis of various analogues of the δ mercaptolysine to allow their use in Fmoc- and Boc-based solid-phase peptide synthesis (SPPS; Fmoc = 9-fluorenylmethoxycarbonyl, Boc = *tert*-butyloxycarbonyl), as well as in sequential ligation.[10] The ability to chemically synthesize Ub by applying native chemical ligation (NCL)[11,12] encouraged us to design a synthetic route to all of the di-Ub chains, in which the δ mercaptolysine is introduced at the desired position (i.e., K6, K11, K27, K29, K33, K48, and K63) to allow a site-specific attachment of the sequential Ub molecule. These analogues would be very useful for a variety of studies related to Ub biology. Herein we report the total chemical synthesis of the di-Ub chains using our developed chemical tools. Synthesis of the chains permitted an initial assessment of their behavior with the isopeptidase T (IsoT), a deubiquitinating enzyme (DUB) responsible for the disassembly of the majority of unanchored polyubiquitin in vivo.

The overall strategy for the synthesis of the di-Ub chains is depicted in Scheme 1. Our approach relies on three main steps to assemble the specific analogue. First is the use of NCL[13] to synthesize the proximal Ub molecule (Ub1) with the δ mercaptolysine at the desired position. To achieve this, the polypeptide sequence of Ub will be divided into two fragments (Figure 1 A), the C-terminal peptide Ub1C (Ub46-76) and the N-terminal peptide Ub1N (Ub1-45), to which the orthogonally protected thiazolidine δ mercaptolysine[10] will be incorporated at a specific location of the isopeptide bond (Scheme 1 A and C). Second, Ub1 will be ligated, through the δ mercaptolysine, with the Ub thioester (Ub2). The latter will be chemically synthesized, as reported previously,[12] from two peptides (Ub2C and Ub2N) wherein the C-terminal fragment Ub2C would bear the *N*-methylcysteine as a masked thioester group (Scheme 1 B). Finally, performing a ligation between Ub1 and Ub2 (Scheme 1 D) and a subsequent desulfurization step to convert the Cys46 residues in each Ub1–Ub2 analogue

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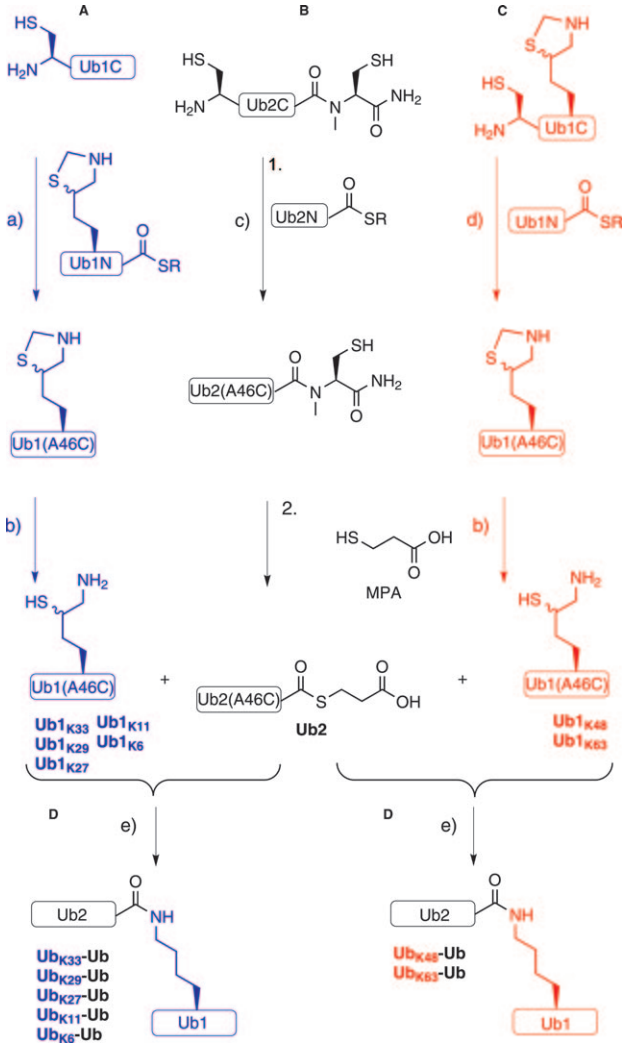

Scheme 1. General strategy for the synthesis of all di-Ub chains. Reagents and conditions: a) Ub1N thioester (1.1 equiv) Gn·HCl (6 M), phosphate buffer (200 mM), pH 7, in the presence of thiophenol/benzylmercaptan (2% v/v); b) methoxylamine (200 mM), TCEP (30 equiv), pH 4, 12 h, 37 °C, 30 % yield of isolated product over two steps; c) 1. Ub2N thioester (1.0 equiv), Gn·HCl (6 M), phosphate buffer (200 mM), pH 7, in the presence of thiophenol/benzylmercaptan (2% v/v); 2. MPA (20%), pH 1, 16 h, 40 °C, 30% yield of isolated product over two steps; d) Ub1N thioester (1.1 equiv), Gn·HCl (6 M), phosphate buffer (200 mM), pH 7, in the presence of thiophenol/benzylmercaptan (2% v/v), 30% yield of isolated product; e) 1. Gn·HCl (6 M), phosphate buffer (200 mM), pH 7, in the presence of thiophenol/benzylmercaptan (2% v/v), 40% yield of isolated product; 2. H_2/Raney nickel, TCEP, 14—16 h, 60% yield of isolated product. Gn = guanidine, MPA = mercaptopropionic acid, TCEP = tris(2-carboxyethyl)phosphine.

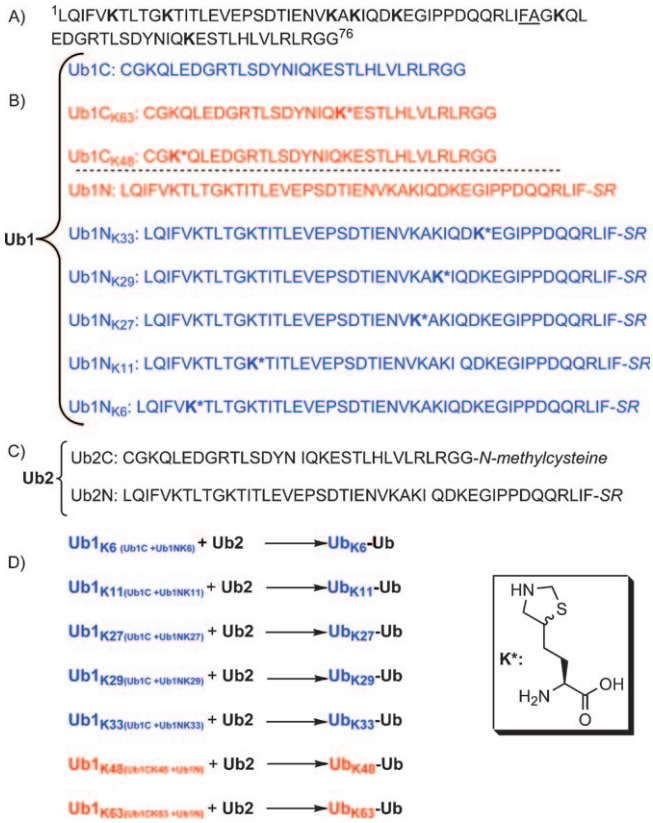

Figure 1. The synthesis of all di-Ub chains: A) Ub sequence (human) in which the seven Lys residues and the ligation junction are highlighted in bold. B) The specific peptide fragments that were used to prepare Ub1. C) The peptide precursors for the synthesis of Ub2. D) The assembly of all the di-Ub analogues and the structure of the protected δ mercaptolysine that was used in our study. All peptide fragments were prepared using Fmoc SPPS. Met1 was replaced with Leu to avoid oxidation (see the Supporting Information).

into their native Ala46 and remove the thiol handle from the δ mercaptolysine to furnish the unmodified isopeptide bond.

With this strategy in mind, we first synthesized the ten-peptide building blocks shown in Figure 1. The three C-terminal peptides (Figure 1 B), including the unmodified peptide Ub1C and the peptides bearing the protected δ mercaptolysine at positions 63 ($Ub1_{K63}$) and 48 ($Ub1_{K48}$), were prepared on Wang resin in 25–30 % yield (see the Supporting Information). In contrast, the N-terminal peptides, which are needed to complete the synthesis of the various analogues of Ub1 (Figure 1 B), were prepared by applying the N-acyl-urea-based chemistry.[14] The cleaved N-terminal peptides bearing the *N*-acyl-benzimidazolinone (Nbz) were converted into the thioester functionality by treatment with 5 % (v/v) methylmercaptopropionate for 20 minutes to give the desired pure thioester peptides in 30 % yield (see the Supporting Information). This set of peptides includes the thioazolidine-protected δ mercaptolysine at positions 33 ($Ub1_{K33}$), 29 ($Ub1_{K29}$), 27 ($Ub1_{K27}$), 11 ($Ub1_{K11}$), and 6 ($Ub1_{K6}$). For Ub2 synthesis, the unmodified peptides needed are Ub2C and Ub2N (Ub2N = Ub1N), which were prepared as described above (Figure 1 C).

With all of these peptides in hand, we turned our attention to the assembly of the different Ub1 units bearing the δ mercaptolysine at various positions (Scheme 1 A and C). The ligation reactions between the different peptides to assemble the Ub1 analogues were carried out under NCL conditions, that is, 6 M Gn·HCl, 200 mM phosphate buffer, pH 7, in the presence of 2 % (v/v) thiophenol/benzylmercaptan. The ligation reaction was monitored by HPLC and mass spectrometry analysis, which indicated a nearly complete ligation after 7 hours (see the Supporting Information).

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Subsequently, methoxylamine and TCEP were added and the reaction was left for an additional 12 hours at 37 °C, to unmask the δ mercaptolysine. The different positions of the δ mercaptolysine did not lead to differences in the ligation efficiency and all Ub1 analogues were isolated in approximately 30 % yield.

The Ub2 molecule was prepared according to Scheme 1 B and by applying our previous strategy for the chemical synthesis of the Ub thioester;[12] however there was an important modification to our original report. To reduce the number of steps in the synthesis of this precursor, we tested the ability of the *N*-methylcysteine device to function without the photolabile protecting group (2-nitrobenzyl). After successful testing with the model peptide (see the Supporting Information), the Ub2C was synthesized without the photolabile protecting group, and upon cleavage and lyophilization it was treated for 1–2 minutes in 6 M Gn·HCl, pH 7, and then purified by HPLC methods to give the desired peptide Ub2C in 25 % yield. After the ligation step with Ub2N, the ligated product was treated, without isolation, with 20 % mercaptopropionic acid (MPA) and stirred for 16 hours at 40 °C to afford the Ub2 in 30 % yield (see the Supporting Information). The absence of the photolabile protecting group in the *N*-methylcysteine moiety greatly simplified the synthesis of the Ub thioester by 1) allowing the use of the commercially available Fmoc-Cys(Trt)-OH instead of Fmoc-Cys(2-nitrobenzyl)-OH, which has to be prepared and 2) facilitating the thioester formation directly on the ligation product without the need of prior isolation, which was essential in the previous protocol to allow protecting group removal using photolysis and subsequent thioester formation.

To assemble all the di-Ub analogues, seven parallel ligation reactions (Figure 1 D) were carried out between each Ub1 analogue and Ub2 under the NCL conditions described above. In our previous studies with the model peptides bearing δ mercaptolysine and the Ub thioester, the ligation was completed within 4 hours.[9c] In this study, the ligation rate was slower and required 24 hours for completion (Figure 2 A). Apparently, the larger size of the Ub bearing the δ mercaptolysine slowed the ligation rate. Nevertheless, the desired products were obtained in 35–40 % yield upon isolation. Next, we attempted the desulfurization reaction to convert the two Cys units in the Ub molecules into their original Ala46 as well as remove the thiol handle from the δ mercaptolysine (Scheme 1 D). However, by applying the radical-free desulfurization conditions,[15] incomplete desulfurization resulted; that is, the removal of only two thiol groups (decrease of 64 Da) was observed after 12 hours. Despite our previous success using these conditions in different systems[9c,12] efforts to achieve full desulfurization by extending the reaction time as well as the addition of more reagents did not lead to a major improvement. Pleasantly, by applying the H_2/Raney nickel conditions,[16] in the presence of TCEP,[11] a complete desulfurization was achieved (14–16 h), and a mass decrease of 96 Da was observed, as expected for the loss of three sulfur atoms (Figure 2 B). All di-Ub analogues were desulfurized using these reaction conditions to give the desired products (Ub_{K6}-Ub, Ub_{K11}-Ub, Ub_{K27}-Ub, Ub_{K29}-Ub, Ub_{K33}-Ub, Ub_{K48}-Ub, Ub_{K63}-Ub) in 55–60 % yield.

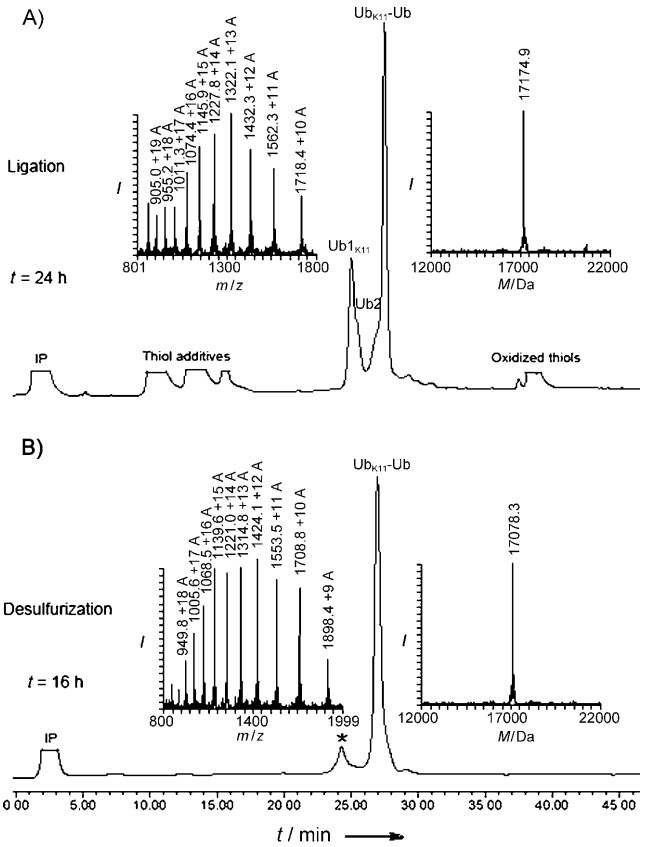

Figure 2. Representative data for the di-Ub chains Ub_{K11}-Ub which was prepared from $Ub1_{K11}$ and Ub2. A) Analytical HPLC analysis of the ligation reaction after 24 h; peaks: remaining $Ub1_{K11}$ having the observed mass 8610.4 Da (calcd 8611.7 Da), hydrolyzed Ub2 having the observed mass of 8578.6 Da (calcd 8579.8 Da), and the ligation product Ub_{K11}-Ub having the observed mass of 17174.9 (calcd 17172.8 Da). B) Analytical HPLC analysis of the crude material from the desulfurization reaction; note the *m*/*z* values and deconvoluted ESI mass spectrum of desired the product Ub_{K11}-Ub (total protein) with the observed mass of 17078.3 Da (calcd 17076.6 Da); the peak denoted with an * represents a by-product with an unidentified mass. The progress of the reaction was analyzed using RP/HPLC (C_4 column) methods with a gradient of 5–50 % B over 40 min (B = 1 % TFA in acetonitrile). TFA = trifluoroacetic acid.

The circular dichroism (CD) spectra of the di-Ub chains were also obtained and compared to the commercially available Ub. Figure 3 shows CD spectra for the different analogues and the similarities in the spectra show that each Ub monomer in these chains retains its globular fold, thus indicating correct folding of the synthesized Ub chains. The small differences in the CD spectra of the different di-Ub chains may reflect differences in the packing and intersubunit interactions of the Ub monomers. X-ray crystallography and NMR studies will be carried out to decipher the chain topology relative to the linkage type and provide insight into the molecular basis underlying the role of Ub in cellular processes.

The unavailability of the naturally occurring atypical chains has also hampered the efforts to provide a full picture on the activity of these conjugates with the DUBs such as the

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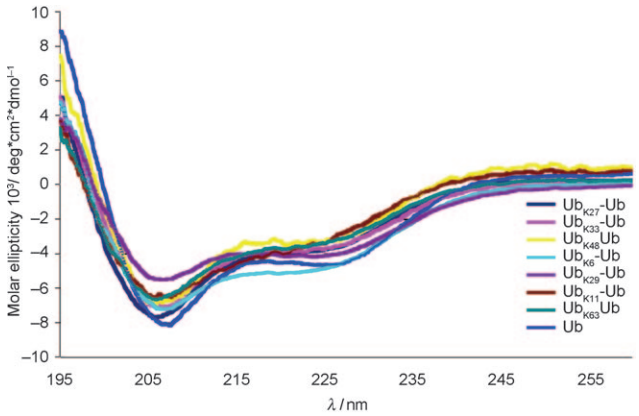

Figure 3. CD analyses of all the di-Ub chains showing the comparison to Ub.

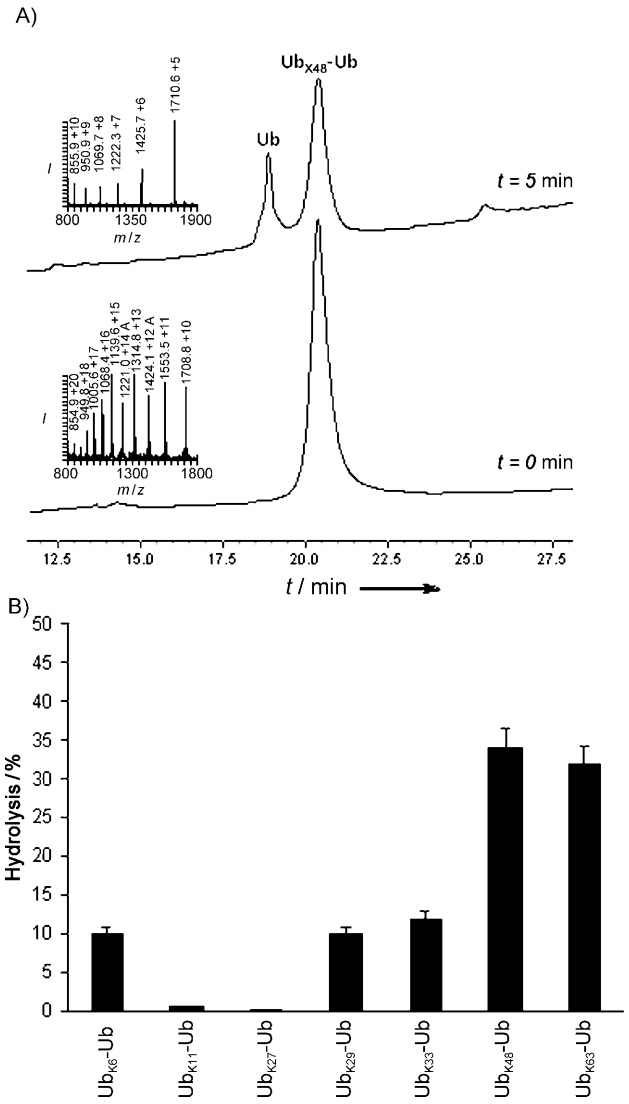

Figure 4. Enzymatic hydrolysis of all the di-Ub chains with IsoT. Conditions: di-Ub (5 μM) was treated with IsoT (10 nM) in 50 mM Tris/HCl, pH 7.6, 37 °C. A) analytical HPLC analysis of the time course for the hydrolysis reaction of Ub$_{K48}$-Ub at time 0 and after 5 min. The starting material Ub$_{K48}$-Ub and Ub, the hydrolysis product with the observed mass of 8549.0 Da (calcd 8547.8 Da). The progress of the reaction was analyzed using a RP/HPLC (C$_4$ analytical column) method employing a gradient of 5–55% B over 35 min (B = 1% TFA in acetonitrile). B) The percent hydrolysis as determined for all the di-Ub chains using identical reaction conditions. Error bars correspond to the standard deviation of three measurements.

human isopeptidase T (IsoT or USP5).[17] This enzyme is responsible for the cleavage between adjacent Ub units in the unanchored K48-linked chains in vivo, a process which is essential for avoiding their accumulation and possible inhibition of the proteasome.[18] Extensive studies of the IsoT structure and biochemical investigations have shown that IsoT nonprocessively disassembles the unanchored K48-chain, with a rate similar to that for the cleavage of branched di-, tri-, and tetra-Ub$_{K48}$, thereby releasing one ubiquitin at a time from the proximal end of the chain.[18] Moreover, IsoT was found, in addition to K48-Ub$_4$, the K48-linked tri-and di-Ub, to cleave K63-Ub$_4$, the mixed chain K29/6-Ub$_4$, and linear polyUb.[19] Recent structural data of this enzyme has shed more light on the promiscuity of IsoT with respect to different types of polyUb chains.[20] However, the substrate activity of the remaining atypical Ub chains (i.e., K6, K11, K27, K29, K33) is still unknown. Having the seven analogues of the di-Ub chains in hand we were able to perform a first assessment to determine if the atypical chains are potential substrates for the IsoT. Thus, each di-Ub (5 μM) was treated with IsoT (10 nM) in 50 mM Tris/HCl, pH 7.6, 37 °C.

The progress of each reaction was monitored by HPLC analysis, in which the appearance of the hydrolyzed product (Ub) elutes earlier on the C$_4$ column relative to the starting material. Initially, we analyzed the hydrolysis of the Ub$_{K48}$-Ub, which is known to act as a substrate for the IsoT, and found that the reaction reached a maximum conversion (35–40%) within 5 minutes of being treated with IsoT (Figure 4A).[21] Analysis of the rest of the di-Ub chains under identical conditions clearly validated the reactivity of IsoT with Ub$_{K48}$-Ub and Ub$_{K63}$-Ub. Interestingly, IsoT is able to also hydrolyze Ub$_{K6}$-Ub, Ub$_{K29}$-Ub, and Ub$_{K33}$-Ub, whereas very low to no detectable activity was observed with Ub$_{K27}$-Ub and Ub$_{K11}$-Ub (Figure 4B). These results support the notion that IsoT is promiscuous; however some selectivity towards certain chains is also noticeable.

To examine the selectivity of the IsoT towards one of these chains, we screened the hydrolysis reaction with a higher enzyme/substrate ratio (1:1000). Under these conditions, we found that the selectivity towards the K48-chain became more noticeable, as the percent of hydrolysis was slightly affected (reduced by 7%) compared to that of the other chains where the decrease in hydrolysis was much higher (30–50%). These results indicate that the K48-chain is the preferred substrate for IsoT. In contrast, screening Ub$_{K27}$-Ub and Ub$_{K11}$-Ub chains at a higher enzyme/substrate ratio (1:100) led to an increase in the hydrolysis of the Ub$_{K11}$-Ub chain (15%) but not for the Ub$_{K27}$-Ub chain. Furthermore, the UCH-L3 enzyme from the ubiquitin C-terminal hydrolase family showed no detectable activity with any of these chains. These results with UCH-L3 are consistent with the structural

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data of this specific enzyme in which folded polyUb chains cannot be accommodated in the active site to allow hydrolysis.[22]

In summary, we have reported the first total chemical synthesis of all the di-Ub chains by utilizing our tools consisting of δ mercaptolysine and the chemically synthesized Ub thioesters. These analogues displayed similar folding to the Ub monomer and were tested for their activities with the IsoT. The analogues of Ub_{K48}-Ub, Ub_{K63}-Ub, Ub_{K33}-Ub, Ub_{K29}-Ub, Ub_{K11}-Ub, and Ub_{K6}-Ub were found to be substrates of this enzyme, thus supporting the proposed promiscuous nature of the IsoT. However, our study shows that Ub_{K48}-Ub is the preferred substrate of this enzyme. Little is known regarding the recognition and processing of atypical Ub chains. The availability of all the di-Ub chains should assist in unraveling several aspects of these chains with respect to other DUBs. Moreover, it should enable a comprehensive structural analysis study, which would shed more light on how the diversity of Ub signaling is achieved. Our synthetic approach should also allow complete control over the atomic structure of each Ub and the possible introduction of unnatural elements such as specific chromophores for various biochemical and structural studies.

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