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Forging Isopeptide Bonds Using Thiol–Ene Chemistry: Site-Specific Coupling of Ubiquitin Molecules for Studying the Activity of Isopeptidases

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Abstract

Chemical methods for modifying proteins can enable studies aimed at uncovering biochemical function. Herein, we describe the use of thiol–ene coupling (TEC) chemistry to report on the function of branched (also referred to as forked) ubiquitin trimers. We show how site-specific isopeptide (*Nε*-Gly-L-homothiaLys) bonds are forged between two molecules of Ub, demonstrating the power of TEC in protein conjugation. Moreover, we demonstrate that the *Nε*-Gly-L-homothiaLys isopeptide bond is processed to a similar extent by deubiquitinases (DUBs) as that of a native *Nε*-Gly-L-Lys isopeptide bond, thereby establishing the utility of TEC in the generation of Ub-Ub linkages. TEC is then applied to the synthesis of branched Ub trimers. Interrogation of these branched derivatives with DUBs reveals that the relative orientation of the two Ub units has a dramatic impact on how they are hydrolyzed. In particular, cleavage of K48C-linkages is suppressed when the central Ub unit is also conjugated through K6C, whereas cleavage proceeds normally when the central unit is conjugated through either K11C or K63C. The results of this work presage a role for branched polymeric Ub chains in regulating linkage-selective interactions.

Addition of thiyl radicals to alkenes, termed thiol–ene coupling (TEC), has the potential to serve as a powerful method for chemically modifying proteins.¹ The reasons are manifold. For instance, bimolecular rate constants on the order of $10^6 \text{ M}^{-1} \text{ s}^{-1}$ have been measured for the addition of thiyl radicals to alkenes,² which is ideal for carrying out reactions with

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ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, characterization of products, and structural analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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proteins at μM concentrations.³ TEC also allows for the use of recombinant proteins and offers the potential to forge stable thioether linkages that closely mimic amino acid side chains.⁴ Despite these advantages, TEC has seen limited use in the direct modification of Cys residues in proteins, presumably due to the number of side reactions a thiyl radical ($\text{CysS}^\bullet$) can undergo.⁵ Yet, there are examples of TEC with peptides and proteins, suggesting in the presence of an alkene side reactions based on $\text{CysS}^\bullet$ are not competitive.^{4,6} Inspired by these studies, we reasoned TEC could be exploited in the construction of isopeptide bonds, an abundant linkage established during the posttranslational modification of proteins with information-rich acyl groups such as ubiquitin (Ub) and Ub-like proteins.⁷

Many reports have recently emerged describing chemical approaches to the site-specific conjugation of Ub molecules through native *N* ϵ -Gly-L-Lys isopeptide linkages and various nonnative linkages.⁸ Indeed, some methods have elucidated important structural distinctions for Ub dimers linked through different lysines⁹ (K6, K27, K29, K33, K48, and K63) and enabled studies that uncovered how the structure and function of target proteins are altered upon Ub modification.¹⁰ Yet, many of the chemical approaches designed to recapitulate the *N* ϵ -Gly-L-Lys linkage suffer from drawbacks such as instability, the number of synthetic manipulations required, and the use of specialized recombinant DNA technologies for incorporating unnatural amino acids. Development of additional methods is therefore necessary to gain rapid access to a diverse range of Ub modified targets.

We hypothesized TEC would provide an alternative to known chemical approaches for forming isopeptide linkages, as standard recombinant proteins can be employed with minimal synthetic effort. In particular, Ub conjugation to a target protein requires a protein harboring a Cys residue in lieu of a Lys (a mutation introduced using site-directed mutagenesis) and Ub bearing a small alkene such as allylamine (AA) appended to the C-terminus. Upon TEC, an *N* ϵ -Gly-L-homothiaLys isopeptide bond would be furnished, which is only one bond longer than the native linkage; an alteration not expected to perturb function (Figure 1A).¹¹ Here, we describe the application of TEC in the concatenation of Ub molecules to afford dimers and trimers that exhibit similar behavior as those constructed enzymatically. We also show how TEC can be used to reveal biochemical details associated with a unique set of Ub oligomers referred to as branched chains, i.e., oligomers in which a single Ub unit is attached through multiple lysines to other Ub units.

Our studies commenced with the installation of AA at the C-terminus of Ub. To accomplish this goal, we turned to a class of proteases referred to as Ub C-terminal hydrolases (UCHs). UCHs promote hydrolysis of Ub variants carrying C-terminal extensions through the formation of a Ub₁₋₇₆-S-UCH acyl-enzyme intermediate.¹² Based on this enzymatic logic along with the reversible nature of proteases, AA was added in large excess relative to a Ub variant harboring a C-terminal aspartate cap (UbD77) in the presence of the yeast UCH YUH1. Under these conditions, the AA adduct of Ub₁₋₇₆ (herein termed Ub-AA) could readily be obtained in ~30% yield (see Supporting Information). This procedure enables production of Ub-AA on a milligram scale.

With a method to generate Ub-AA, we examined conditions to carry out TEC with UbK48C obtained through site-directed mutagenesis. After screening a series of water-soluble free-

radical initiators, the lithium acyl phosphinate (LAP)¹³ photoinitiator proved to be the most effective in terms of the amount of Ub dimer formed (Figure 1B). Importantly, control experiments indicated that formation of dimer was dependent on the presence of each reaction component (Figure S2). Subsequent optimization studies revealed a marked improvement in the amount of dimer formed with higher LAP concentrations. Accordingly, 0.5 mM LAP furnished milligram quantities of the desired product after ion-exchange chromatography (Figure S4).

Extensive characterization of the K48C-linked dimer was conducted using high-resolution tandem mass spectrometry (MS/MS) on a Fourier-transform ion cyclotron resonance mass spectrometer (FT-ICR). Crude TEC reactions were monitored by MS analysis of intact proteins. Peaks corresponding to two distinct ionization states of the dimer ($z = 17$ and 18) are readily detected when TEC reactions are carried out with all components (Figure 2A). In addition, a peak 16 Da (Da) larger than that of the dimer was always observed, which might be attributed to the oxidation of the thioether linkage.¹⁴ The highly accurate mass measurement of the purified K48C-linked dimer (3.5 ppm between the experimental and theoretical molecular weights) provides strong evidence for the formation of the desired product (Figure 2B). To then verify modification of position-48, electron capture dissociation (ECD),¹⁵ a nonergodic MS/MS technique, was performed with a minimal trypsin digest of K48C-linked dimer. Minimal digest removes the C-terminal diGly motif leaving a Ub₁₋₇₄ unit with a Gly-Gly-AA (171 amu) appendage (Scheme S2); this greatly simplifies ECD analysis. ECD spectra report on the extensive fragmentation of N-terminal c ions and C-terminal z^* ions (Figure S10), and the fragmentation pattern surrounding position-48 unambiguously verified incorporation of the desired modification at this position (Figures 2C and D). Taken together, SDS-PAGE and MS data argue that TEC is a pragmatic method for site-specifically coupling two proteins and generating K48C-linked Ub dimers.

Encouraged by these results, we investigated the application of TEC in the synthesis of topoisomers, e.g., dimers linked through K6C, K11C, K27C, K29C, K33C, and K63C. Using similar conditions to those optimal for the K48C-linked dimer, K6C-, K11C-, and K63C-linked dimers were obtained in yields comparable to K48C (Figure S3). However, synthesis of K29C-, K33C-, and in particular the K27C-linked dimer proved more challenging. Specifically, high-resolution FT-ICR MS analysis of crude reaction mixtures containing 1:1 ratios of UbK29C/Ub-AA and UbK33C/Ub-AA showed dimers in low abundance relative to the monomeric substrates; in similar experiments with UbK27C, dimer formation was not observed. Based on these results, we reasoned the addition of CysS^{*} to Ub-AA could be suppressed by the steric bulk surrounding the radical species. To test this hypothesis, different concentrations of Ub-AA were employed. For UbK29C and UbK33C, MS analysis pointed to a clear trend in the relative amount of dimer to monomeric substrate; i.e., higher concentrations of Ub-AA led to an increase in the abundance of the dimer peak (Figures S18 and S19). Conversely, UbK27C remained refractory toward coupling even at UbK27C/Ub-AA ratios of 1:4. This result, however, was not surprising considering residue-27 is the least accessible according to the structure of Ub. Nevertheless, synthesis of six out of seven topoisomers without specialized techniques (e.g., unnatural amino acid incorporation and total chemical synthesis) highlights the generality of TEC.

Next, we tested the function of Ub dimers forged through TEC. To accomplish this goal, the hydrolytic cleavage of *N*ε-Gly-L-homothiaLys isopeptide linkages was investigated using isopeptidases (also referred to as deubiquitinases or DUBs).¹⁶ Several members of the DUB family of enzymes preferentially cleave specific linkages within a Ub oligomer. For instance, the ovarian tumor (OTU) domain-containing protein referred to as A20 prefers K48-linkages, whereas the DUB AMSH (associated molecule with the SH3 domain of STAM) cleaves K63-linkages.¹⁷ In many cases, and in particular with A20 and AMSH, linkage specificity arises from (i) the unique sequence context of each Ub Lys residue and (ii) direct contact with all atoms of the Lys side chain.¹⁶ Due to the discriminating features of linkage-selective DUBs, we surmised A20-OTU- and AMSH-catalyzed hydrolysis of K48C- and K63C-linked dimers would provide a stringent test for the ability of *N*ε-Gly-L-homothiaLys to mimic the native linkage (Figure 3A). For direct comparison, native K48- and K63-linked dimers were hydrolyzed alongside the dimers synthesized by TEC. Analysis of A20-OTU-catalyzed cleavage of *N*ε-Gly-L-homothiaLys indicated that while the K63C-linked dimer is not hydrolyzed (Figure 3B, lanes 10–12), the K48C-linked native dimer is almost completely converted to the respective monomeric units within 1 h (Figure 3B, lanes 4–6). These results are congruent with those obtained for dimers linked through the native isopeptide bond (Figure 3B, lanes 1–3 and 7–9). In the case of K63-linkage specific DUB AMSH, the K63C-linked dimers are rapidly hydrolyzed, whereas the K48C-linked dimers are not processed (Figure 3C, lanes 4–6 vs 10–12). It is important to note that although there is a small amount of each thioether dimer remaining after 1 h, the half-life of *N*ε-Gly-L-homothiaLys is nearly identical to that of *N*ε-Gly-L-Lys. These results indicate TEC, unlike the Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC),¹⁸ affords fully functional Ub dimers that can readily be obtained in two straightforward steps from standard recombinant proteins.

Given the ease with which functional Ub dimers can be forged, we surmised TEC could be applied to the synthesis of branched Ub chains, as this class of oligomers is currently unattainable using other methods such as amber stop codon suppression because of low incorporation efficiency of unnatural amino acids into multiple sites. Branched oligomers have been observed as products of specific pairs of E2 Ub-conjugating and E3 Ub-ligating enzymes, but their abundance and function *in vivo* remain unclear, in part, due to the inability to identify branched linkages from tryptic digests.¹⁹ Moreover, these oligomers display a low affinity for 26S proteasomes, and certain Ub-binding chaperones can prevent their formation thereby promoting protein degradation.²⁰ To gain more insight into the function of this class of oligomers, our established TEC protocol was used to synthesize three branched Ub₃ topoisomers starting from the Ub Lys-to-Cys double mutants: K6C, K48C; K11C, K48C; and K48C, K63C (Figure 4A). This particular set was chosen to systematically investigate the influence of an additional Ub unit on the hydrolysis of the K48C-linkage. Similar to the dimers, the trimers were purified using ion-exchange chromatography and characterized by ECD analysis of intact proteins minimally digested with trypsin (Figures S11–S16). Western blot analysis with a Ub antibody shows two bands for all trimers along with a faint band corresponding to a dimer (Figure 4B). The trimer bands can be ascribed to the presence of reduced and oxidized forms of the *N*ε-Gly-L-homothiaLys linkage as both are observed by MS analysis. A shift in electrophoretic

mobility between different forms of Ub oligomers is common as the number of units increases, which explains why distinct bands for the reduced and oxidized forms of the thioether linkage are not observed with dimers.²¹

Functional studies with the branched trimers revealed clear differences in the DUB-catalyzed hydrolysis of the K48C-linkage (Figure 4B). Cleavage of each trimer was examined with three different DUBs: IsoT, A20-OTU, and AMSH. IsoT hydrolyzes free polyUb chains, i.e., those not conjugated to a target protein, with little selectivity over linkage type.²² Given the presence of a free C-terminus in each trimer we anticipated that IsoT-catalyzed hydrolysis would rapidly furnish dimeric and monomeric products. Indeed, IsoT efficiently processed all three trimers as evidenced by Western blot analysis (Figure 4B). The most striking result, however, came while studying A20-OTU-catalyzed cleavage. That is, Western blot analysis indicated A20-OTU cleaved the K48C-linkage in K11C, K48C- and K48C, K63C-linked trimers, whereas the same linkage remained intact in the K6C, K48C-linked trimer (Figure 4B). Since other nonselective DUBs such as those in the USP (Ub-specific protease) family,²³ in particular USP7, trim K6C, K48C-linked Ub₃ down to the monomer (Figure S20), the results with A20-OTU suggest the additional Ub unit appended to position-6 abrogates hydrolytic cleavage by K48-linkage selective DUBs. In the context of other linkage selective DUBs such as AMSH, the presence of a Ub appendage at position 48 does not influence cleavage of the K63C-linkage as indicated by the formation of Ub₂ and Ub upon hydrolysis of the K48C, K63C-linked trimer (Figure 4B). Additional work is necessary to determine whether other branch points, e.g., K6C and K11C, affect hydrolysis of K63C-linkages.

Our systematic examination of branched trimer topologies suggests that branch points in a polyUb chain furnish a regulatory mechanism for linkage-selective interactions. Consistent with this analysis, K6-linkages are proposed to suppress degradation of target proteins by 26S proteasomes.^{19b,24} In principle, this could lead to the accumulation, and possibly aggregation, of the target protein, which, in turn, would set the stage for clearance by the lysosomal pathway.²⁵ If the latter is either unable or slow to process the aggregated proteins bearing polyUb chains, then toxic levels may begin to accrue in the cell: this is a hallmark of many neurodegenerative diseases. Interestingly, mixed K6-, K11-, and K48-linked polyUb chains have been observed in Tau aggregates isolated from brain tissue of individuals with Alzheimer's disease.²⁶

In summary, the work described herein showcases the utility of TEC in the construction as well as the biochemical analysis of dimeric and trimeric Ub conjugates. With these new tools, future work will focus on understanding the abundance and function of branched Ub oligomers *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- (1). (a) Dondoni A. *Angew. Chem., Int. Ed.* 2008; 47:8995. (b) Hoyle CE, Bowman CN. *Angew. Chem., Int. Ed.* 2010; 49:1540.
- (2). Reddy SK, Cramer NB, Bowman CN. *Macromolecules.* 2006; 39:3673.
- (3). Kalia J, Raines RT. *Curr. Org. Chem.* 2010; 14:138. [PubMed: 20622973]
- (4). (a) Chalker JM, Bernardes GJ, Davis BG. *Acc. Chem. Res.* 2011; 44:730. [PubMed: 21563755] (b) Floyd N, Vijayakrishnan B, Koeppe JR, Davis BG. *Angew. Chem., Int. Ed.* 2009; 48:7798.
- (5). (a) Nauser T, Schoneich C. *J. Am. Chem. Soc.* 2003; 125:2042. [PubMed: 12590520] (b) Schoneich C. *Chem. Res. Toxicol.* 2008; 21:1175. [PubMed: 18361510]
- (6). (a) Dondoni A, Massi A, Nanni P, Roda A. *Chem.—Eur. J.* 2009; 15:11444. [PubMed: 19798718] (b) Lo Conte M, Staderini S, Marra A, Sanchez-Navarro M, Davis BG, Dondoni A. *Chem. Commun.* 2011; 47:11086. (c) Wittrock S, Becker T, Kunz H. *Angew. Chem., Int. Ed.* 2007; 46:5226. (d) Weinrich D, Lin PC, Jonkheijm P, Nguyen UT, Schroder H, Niemeyer CM, Alexandrov K, Goody R, Waldmann H. *Angew. Chem., Int. Ed.* 2010; 49:1252. (e) Aimetti AA, Feaver KR, Anseth KS. *Chem. Commun.* 2010; 46:5781.
- (7). (a) Pickart CM. *Annu. Rev. Biochem.* 2001; 70:503. [PubMed: 11395416] (b) Grabbe C, Husnjak K, Dikic I. *Nat. Rev. Mol. Cell Biol.* 2011; 12:295. [PubMed: 21448225]
- (8). (a) Fekner T, Li X, Chan MK. *ChemBioChem.* 2011; 12:21. [PubMed: 21181847] (b) Strieter ER, Korasick DA. *ACS Chem. Biol.* 2012; 7:52. [PubMed: 22196026]
- (9). (a) Virdee S, Ye Y, Nguyen DP, Komander D, Chin JW. *Nat. Chem. Biol.* 2010; 6:750. [PubMed: 20802491] (b) Castaneda CA, Spasser L, Bavikar SN, Brik A, Fushman D. *Angew. Chem., Int. Ed.* 2011; 50:11210.
- (10). (a) McGinty RK, Kim J, Chatterjee C, Roeder RG, Muir TW. *Nature.* 2008; 453:812. [PubMed: 18449190] (b) Fierz B, Chatterjee C, McGinty RK, Bar-Dagan M, Raleigh DP, Muir TW. *Nat. Chem. Biol.* 2011; 7:113. [PubMed: 21196936] (c) Chen J, Ai Y, Wang J, Haracska L, Zhuang Z. *Nat. Chem. Biol.* 2010; 6:270. [PubMed: 20208521] (d) Hejjaoui M, Haj-Yahya M, Kumar KS, Brik A, Lashuel HA. *Angew. Chem., Int. Ed.* 2011; 50:405.
- (11). Chatterjee C, McGinty RK, Fierz B, Muir TW. *Nat. Chem. Biol.* 2010; 6:267. [PubMed: 20208522]
- (12). Johnston SC, Riddle SM, Cohen RE, Hill CP. *EMBO J.* 1999; 18:3877. [PubMed: 10406793]
- (13). Fairbanks BD, Schwartz MP, Bowman CN, Anseth KS. *Biomaterials.* 2009; 30:6702. [PubMed: 19783300]
- (14). (a) Vogt W. *Free Radical Biol. Med.* 1995; 18:93. [PubMed: 7896176] (b) Xu G, Chance MR. *Chem. Rev.* 2007; 107:3514. [PubMed: 17683160] (c) Reddie KG, Carroll KS. *Curr. Opin. Chem. Biol.* 2008; 12:746. [PubMed: 18804173]
- (15). Zubarev RA, Kelleher NL, McLafferty FW. *J. Am. Chem. Soc.* 1998; 120:3265.
- (16). Komander D, Clague MJ, Urbe S. *Nat. Rev. Mol. Cell Biol.* 2009; 10:550. [PubMed: 19626045]
- (17). (a) Komander D, Barford D. *Biochem. J.* 2008; 409:77. [PubMed: 17961127] (b) Sato Y, Yoshikawa A, Yamagata A, Mimura H, Yamashita M, Ookata K, Nureki O, Iwai K, Komada M, Fukai S. *Nature.* 2008; 455:358. [PubMed: 18758443]
- (18). (a) Eger S, Scheffner M, Marx A, Rubini M. *J. Am. Chem. Soc.* 2010; 132:16337. [PubMed: 21033666] (b) Weikart ND, Sommer S, Mootz HD. *Chem. Commun.* 2012; 48:296.
- (19). (a) Kim HT, Kim KP, Lledias F, Kisselev AF, Scaglione KM, Skowrya D, Gygi SP, Goldberg AL. *J. Biol. Chem.* 2007; 282:17375. [PubMed: 17426036] (b) Ben-Saadon R, Zaaroor D, Ziv T, Ciechanover A. *Mol. Cell.* 2006; 24:701. [PubMed: 17157253]
- (20). Kim HT, Kim KP, Uchiki T, Gygi SP, Goldberg AL. *EMBO J.* 2009; 28:1867. [PubMed: 19387488]

- (21). Dong KC, Helgason E, Yu C, Phu L, Arnott DP, Bosanac I, Compaan DM, Huang OW, Fedorova AV, Kirkpatrick DS, Hymowitz SG, Dueber EC. *Structure*. 2011; 19:1053. [PubMed: 21827942]
- (22). Reyes-Turcu FE, Shanks JR, Komander D, Wilkinson KD. *J. Biol. Chem.* 2008; 283:19581. [PubMed: 18482987]
- (23). Faesen AC, Luna-Vargas MP, Geurink PP, Clerici M, Merckx R, van Dijk WJ, Hameed DS, El Oualid F, Ovaa H, Sixma TK. *Chem. Biol.* 2011; 18:1550. [PubMed: 22195557]
- (24). (a) Sato K, Hayami R, Wu W, Nishikawa T, Nishikawa H, Okuda Y, Ogata H, Fukuda M, Ohta T. *J. Biol. Chem.* 2004; 279:30919. [PubMed: 15184379] (b) Wu-Baer F, Lagrazon K, Yuan W, Baer R. *J. Biol. Chem.* 2003; 278:34743. [PubMed: 12890688] (c) Yu X, Fu S, Lai M, Baer R, Chen J. *Genes Dev.* 2006; 20:1721. [PubMed: 16818604]
- (25). Pandey UB, Nie Z, Batlevi Y, McCray BA, Ritson GP, Nedelsky NB, Schwartz SL, DiProspero NA, Knight MA, Schuldiner O, Padmanabhan R, Hild M, Berry DL, Garza D, Hubbert CC, Yao TP, Baehrecke EH, Taylor JP. *Nature*. 2007; 447:859. [PubMed: 17568747]
- (26). Cripps D, Thomas SN, Jeng Y, Yang F, Davies P, Yang AJ. *J. Biol. Chem.* 2006; 281:10825. [PubMed: 16443603]

Figure 1.

Site-specific conjugation of Ub molecules using TEC. (A) Structures of *Nε*-Gly-L-Lys, the native isopeptide linkage, and *Nε*-Gly-L-homothiaLys, the linkage forged through TEC. (B) Overview of TEC mechanism showing the generation of K48C-linked Ub₂ after conjugating Ub-AA and UbK48C using the free-radical photoinitiator LAP.

Figure 2.

Representative mass spectrometric analysis of K48C-linked Ub dimer. (A) FT-ICR MS analysis of crude reaction mixture shown in (C); * corresponds to the mass of Ub-AA plus the phosphinate portion of LAP (Scheme S3). (B) An individual charge state ($z = 18$ or M^{18+}) of purified K48C-linked Ub dimer. The isotopic distribution represents intact mass of full-length dimer. Red circles correspond to the theoretical distribution of isotopic abundance. (C,D) Representative ECD spectra of a minimal trypsin digest of K48C-linked Ub dimer indicating the installation of a Gly-Gly-AA motif at Ub residue 48. The c ions are shown for fragments containing the first 47 residues (C) and the first 48 residues (D).

Figure 3.

DUB-catalyzed hydrolysis of dimers forged through TEC. (A) General scheme for the DUB-catalyzed hydrolysis of K48C- and K63C-linked Ub dimers. (B) Silver-stained SDS-PAGE analysis of cleavage reactions with the K48-linkage selective A20-OTU domain. At $t = 0$ min, the DUB is absent from the reaction mixture. (C) Silver-stained SDS-PAGE analysis of cleavage reactions with K63-linkage selective AMSH.

Figure 4.

Structure and function of branched Ub₃ derivatives. (A) The three branched Ub₃ derivatives synthesized in this study: K6C, K48C-; K11C, K48C-; and K48C, K63C-linked. The central Ub unit conjugated to two Ub molecules is shown in blue. (B) Western blots developed with a Ub antibody (P4D1) showing the extent to which different DUBs (IsoT, A20-OTU, and AMSH) hydrolyze the three branched Ub₃ derivatives. Hydrolysis is indicated by the formation of dimers (Ub₂) and monomers.