Chapter 4

Creating Site-Specific Isopeptide Linkages Between Proteins with the Traceless Staudinger Ligation

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Abstract

Site-specific isopeptide linkages between the ε-amino group of a lysine residue in one protein and a carboxyl group in another are central to ubiquitin-mediated protein degradation and other cellular processes. These linkages are inaccessible with common recombinant DNA techniques. Here, we describe a method to link two proteins by an authentic isopeptide bond. The method unites three techniques at the forefront of molecular biology. An azidonorleucine residue is installed at a desired site in a substrate protein by non-natural amino acid incorporation, and a phosphinothioester is installed at the C terminus of a pendant protein by expressed protein ligation. Then, the traceless Staudinger ligation is used to link the substrate and pendant proteins via an isopeptide bond. This method facilitates the study of otherwise intractable protein structure–function relationships.

Key words Azidonorleucine, Expressed protein ligation, Isopeptide bond, Nonnatural amino acid incorporation, Traceless Staudinger ligation, Ubiquitin

1 Introduction

An isopeptide bond is an amide bond between an amino group of one amino acid and a carboxyl group of another amino acid in which at least one of these groups is not attached to the α-carbon. The most common isopeptide bonds are formed between the ε-amino group of a lysine residue in one protein and a carboxyl group in a second protein. These isopeptide bonds play many biological roles, which are generally classified as either signaling or structural in nature. These bonds are typically generated *in cellulo* by multiple enzyme-catalyzed reactions and are thus difficult to recapitulate in vitro.

Isopeptide bond-linked ubiquitin and ubiquitin-like proteins (ULPs) such as SUMO or Atg8 participate in a multitude of signaling pathways. These proteins become linked to substrate proteins through an isopeptide bond generated by the concerted actions of three groups of enzymes, known generally as E1, E2, and E3 ligases [1, 2].

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Moreover, these single appendages are often decorated further by ubiquitin or ULPs through isopeptide bonds to form chains. The proteins thus modified participate in a multitude of intracellular signaling pathways, depending upon the architecture of the modification [3]. Due to the complexity of these libraries, much of the encoded signals are not yet known. The preparation of chains of precise length and connectivity and attachment of these signals to target proteins could expedite the understanding of these various signals.

In contrast, the isopeptide bonds formed by transglutaminases typically serve structural roles. Enzymes of the transglutaminase family catalyze the formation of isopeptide bonds between the ε-amino group of lysine and the γ-carboxyl group of glutamic acid in a wide variety of proteins [4]. The ensuing cross-linked proteins play important roles in the extracellular matrix [5, 6], stabilization of tissue [7], and processes like wound healing and blood clotting [8]. The ability to control the extent and specificity of cross-linking could provide a more comprehensive understanding of the purpose of these species.

The creation of an isopeptide bond between two proteins in a site-specific manner has proven to be a major challenge to the field of chemical biology. Several synthetic strategies have been executed in recent years [9–15]. These strategies have limitations [16], due to highly specialized techniques, nonnative linkage products, harsh conditions, or low yields, predicating the need for additional methodological development.

Since its discovery in 2000 [17–19], the traceless Staudinger ligation has evolved into an important tool for the chemoselective production of peptide bonds [20–22]. The traceless Staudinger ligation uses a phosphine to reduce an azide via an iminophosphorane intermediate, which undergoes $S \rightarrow N$ acyl rearrangement [23]. Subsequent hydrolysis yields an amide linkage without any residual atoms or racemization [24]. This approach has proven useful in peptide chemistry, even allowing for the convergent synthesis of a whole protein [25, 26]. Here, we describe the use of the traceless Staudinger ligation to link two proteins through an authentic isopeptide bond. The chemical reactions occur under mild conditions in aqueous buffers, making them applicable to a wide variety of proteins.

The nitrogen of the nascent isopeptide bond derives from an azido group in the "substrate" protein (Fig. 1). The azido group can be introduced in the form of L-azidonorleucine by using a method for nonnatural amino acid incorporation developed by Tirell and coworkers [27]. This method requires the use of a modified methionyl-*t*RNA synthetase that incorporates azidonorleucine into AUG codons in methionine auxotrophic *Escherichia coli* cells grown in the absence of methionine.

The carbon of the nascent isopeptide bond derives from a phosphinothioester in the "pendant" protein. A C-terminal phosphinothioester can be installed with expressed protein ligation, a method

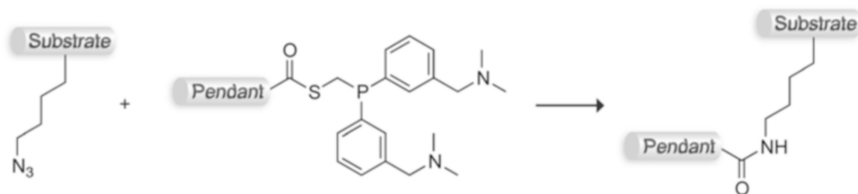

Fig. 1 Traceless Staudinger ligation between a substrate and pendant protein

developed by Muir, Cole, and coworkers [28]. In particular, the pendant protein is expressed as a C-terminal fusion protein with the *Mxe* GyrA intein and transthioesterification with a water-soluble phosphinothiol that generates the requisite C-terminal phosphinothioester.

Incubation of the substrate and pendant proteins engenders the traceless Staudinger ligation, generating an authentic isopeptide bond (Fig. 1). The application of this method to whole proteins allows access to heretofore unattainable protein conjugates, including those in the ubiquitin signaling pathways and products of transglutaminases. Hence, this method provides a useful tool for chemical biologists to explore the biological functions of proteins.

2 Materials

1. LB (Luria–Bertani medium): 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1.00 L ddH_2O (autoclaved).
2. TB (Terrific Broth): 12 g tryptone, 24 g yeast extract, 4 mL glycerol, 2.31 g KH_2PO_4, and 12.54 g K_2HPO_4 in 1.00 L ddH_2O (autoclaved).
3. LB agar plates: 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar in 1.00 L ddH_2O (autoclaved).
4. IPTG (isopropyl β-D-1-thiogalactopyranoside): Prepared as a 1 M stock solution in water. Final concentration for induction is 1 mM. Filter-sterilize, and store at −20 °C until use.
5. Chitin resin (New England Biolabs, Ipswich, MA, USA).
6. Intein lysis buffer (30 mM HEPES–NaOH buffer, pH 8.0, containing 0.30 M NaCl and 1.0 mM EDTA).
7. Intein wash buffer (30 mM HEPES–NaOH buffer, pH 8.0, containing 0.50 M NaCl and 1.0 mM EDTA).
8. Intein elution buffer (30 mM potassium phosphate buffer, pH 6.0, containing 0.20 M NaCl, 1.0 mM EDTA, and 0.10 M MESNA).
9. Phosphinothiol **1**, synthesized as described previously [29].

10. Dialysis tubing appropriate to protein size.
11. Spin concentrators appropriate to protein size.
12. Superdex G75 26/60 gel-filtration column or similar column appropriate to protein size.
13. 10× M9 salts (60 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl, and 10 g NH_4Cl in 1.00 L ddH_2O (autoclaved)).
14. 0.10 M $CaCl_2$ (autoclaved).
15. 1.0 M $MgSO_4$ (autoclaved).
16. 40 % w/v glucose: 40 g glucose in water to a total volume of 100 mL; filter-sterilize with a 0.2-μm filter.
17. 1,000× thiamine: 140 mg thiamine in 4.0 mL ddH_2O; filter-sterilize with a 0.2-μm filter. Store at −20 °C, protected from light until ready to use.
18. 19 AA solution: 1 g/L of each of the canonical amino acids, excluding methionine, in ddH_2O. Adjust the pH gradually with 1 M NaOH until all amino acids dissolve. Filter-sterilize with a 0.2-μm filter. Store at 4 °C until ready to use.
19. Azidonorleucine, synthesized as described previously [30].
20. Ni^{2+} Buffer A (30 mM HEPES–NaOH buffer, pH 8.0, containing 0.50 M NaCl and 20 mM imidazole).
21. Ni^{2+} Buffer B (30 mM HEPES–NaOH buffer, pH 8.0, containing 0.50 M NaCl and 0.40 M imidazole).
22. 5 mL HisTrap HP column (GE Healthcare Biosciences, Pittsburgh, PA, USA).
23. ProTEV protease, which is a modified tobacco etch virus protease (Promega, Madison, WI, USA).
24. 0.5 M HEPES–NaOH buffer, pH 8.0.

3 Methods

The methods detailed below describe the production of an isopeptide bond between two proteins using the traceless Staudinger ligation. The key reagents are a substrate protein with a site-specifically incorporated azidonorleucine and a pendant protein with a C-terminal phosphinothioester (Fig. 1). The preparation of these two proteins is described in Subheadings 3.1 and 3.2. They can be prepared concurrently and combined after purification to produce the final ligation product linked by an isopeptide bond.

3.1 Substrate Protein: Incorporation of Azidonorleucine

This section describes the incorporation of azidonorleucine into a specific site in the substrate protein, as directed by an AUG codon. The use of azidonorleucine allows for the formation of an authentic isopeptide bond and is accomplished by the co-expression of

the protein with a modified methionyl-*t*RNA synthetase in cells that are auxotrophic for methionine [27]. The methionyl-*t*RNA synthetase requires three substitutions (G13N, Y260L, H301L) to accommodate the azidonorleucine into its active site. The enzyme still maintains a preference for methionine, so depleting any methionine before inducing expression in the presence of azidonorleucine is necessary to maximize the incorporation of azidonorleucine.

The expression vector is a modified version of plasmid pQE60 (Qiagen, Venlo, the Netherlands). The gene is inserted into the open reading frame, with a His_6 tag between the initial start codon and the remainder of the protein. Within the protein-encoding sequence, any native methionine codons have been replaced with codons for other amino acids to prevent additional sites of azidonorleucine incorporation. The codon at the site of incorporation has been altered to AUG, to allow for the specific incorporation of azidonorleucine. The AUG start codon could be translated to azidonorleucine, which is undesirable in the final product. One of the two methods can be used to remove this additional site of incorporation. A leucine can be incorporated immediately following the first amino acid residue. Then, digestion with leucine aminopeptidase can remove the first two amino acid residues. Alternatively, a TEV protease cleavage site can be incorporated after a His_6 tag, which can be used for protein purification (Fig. 2). That method will be described here.

3.1.1 Protein Production

1. Day 1: Transform modified pQE60 vector into the M15MA *E. coli* or similar methionine auxotrophic strain, which will allow for recombinant expression. Spread transformed cells on plates of LB agar containing 200 mg/L ampicillin and 35 mg/L kanamycin (*see* **Note 1**).
2. Day 2: Prepare an overnight culture by adding 200 mg/L ampicillin and 35 mg/L kanamycin to 50 mL LB and inoculating with the transformed M15MA cells. Incubate overnight at 37 °C with shaking.
3. Day 3: Inoculate 2 L of LB with the overnight culture to an OD of 0.05 at 600 nm. Grow cultures in 37 °C shaker at 160 rpm for 4–5 h until the OD reaches 1.2–1.4 at 600 nm.

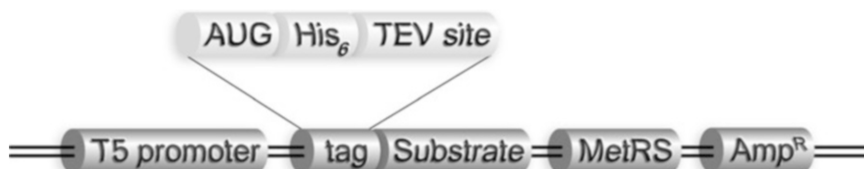

Fig. 2 Key features of the pQE60 vector that encodes the substrate protein

4. Prepare 4 L of 1× M9 media by combining (per liter of medium): 100 mL 10× M9 salts, 890 mL ddH_2O, 10 mL 40 % w/v glucose, 1.0 mL 0.10 M $CaCl_2$, 1.0 mL $MgSO_4$, and 1.0 mL 1,000× thiamine stock.
5. Collect cells by centrifugation at 5,000 ×*g* for 7 min. Resuspend in 2 L of 1× M9 media with 19 AA solution, and continue to grow at 37 °C with shaking for an additional 20 min.
6. Repeat the centrifugation step, and resuspend in M9 media with 1.0 mM azidonorleucine. Allow cells to continue to grow at 37 °C for 15 min, before adding 1.0 M IPTG to a final concentration of 1.0 mM. Allow cells to induce overnight at 25 °C with shaking.

3.1.2 Protein Purification

1. Day 4: Harvest the cells by centrifugation at 7,000 ×*g* for 20 min at 4 °C. Decant the medium.
2. Resuspend the cell pellets in ice-cold Ni^{2+} Buffer A.
3. Lyse cells using a French pressure cell or similar method.
4. Collect debris by centrifugation at 15,000 ×*g* for 45 min at 4 °C. Transfer the supernatant to a fresh tube. Filter the lysate through a glass fiber syringe filter.
5. Load the filtered lysate onto a 5 mL HisTrap HP column, and then wash the column with 5 column volumes of Ni^{2+} Buffer A.
6. Perform a gradient elution with Ni^{2+} Buffers A and B (0 → 100 % Ni^{2+} Buffer B) over 20 column volumes, collecting 4-mL fractions. Analyze the fractions with SDS–PAGE to determine which fractions contain the target protein (*see* **Note 2**).
7. Dialyze (2×) the protein into 4 L of 50 mM HEPES–NaOH buffer, pH 7.5, at 4 °C.
8. Cleave the initial peptide (Met–His_6–TEV) from the target protein with ProTEV protease by combining 5 μL of 20× ProTEV Buffer, 1 μL of 10 mM DTT, 20 μg of fusion protein, 1 μL of ProTEV protease, and water up to 100 μL. Incubate the reaction mixture at 30 °C for 1–8 h. Analyze cleavage efficiency by SDS–PAGE or mass spectrometry (*see* **Note 3**).
9. Dialyze (2×) the cleaved protein into 4 L of Ni^{2+} Buffer A at 4 °C.
10. Load the dialyzed protein onto a 5-mL HisTrap HP column, and collect the flow-through. The column will retain the cleaved peptide but not the protein. Analyze fractions by SDS–PAGE to identify those that contain the target protein.
11. Dialyze the target protein into 50 mM HEPES–NaOH buffer, pH 8.0. Concentrate the protein to 100–200 μM.
12. The extent of azidonorleucine incorporation can be estimated by mass spectrometry (*see* **Note 4**).

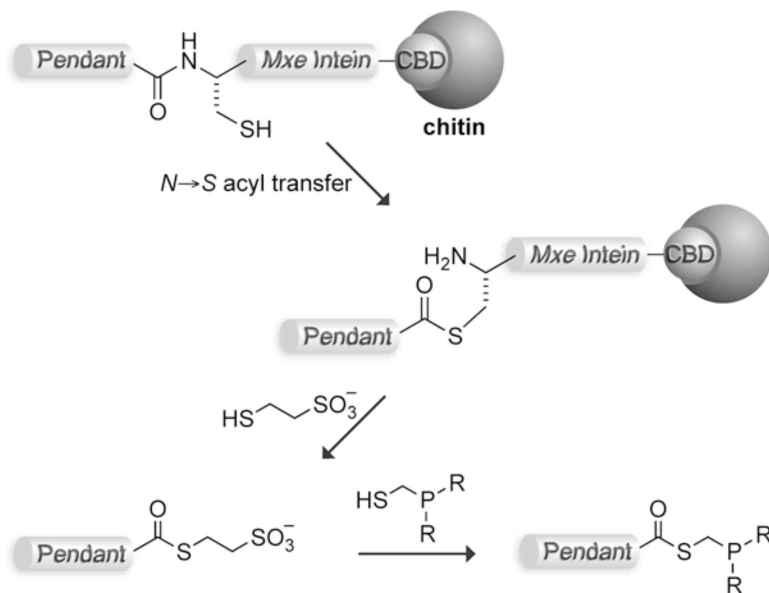

Fig. 3 Scheme for the installation of a phosphinothioester on the C terminus of a pendant protein. Phosphinothiol **1**: R = C_6H_4-*m*-$CH_2N(CH_3)_2$ [29]

3.2 Pendant Protein: Creation of the C-Terminal Phosphinothioester

This section describes the production and purification of a protein with a C-terminal phosphinothioester. This water-soluble phosphinothioester will react with the azido group of the substrate protein to produce an authentic isopeptide bond without any residual atoms. The pendant protein is produced as a C-terminal *Mxe* GyrA intein fusion protein with a chitin-binding domain (CBD) in plasmid pTXB1. The protein is then purified by chromatography on a chitin resin, and a pH shift in the presence of the sodium salt of mercaptoethanesulfonate (MESNA) induces an *N*→ *S* acyl transfer and entrapment of a C-terminal thioester (Fig. 3). Transthioesterification with a water-soluble phosphinothiol produces the final C-terminal phosphinothioester protein.

3.2.1 Protein Production

1. Day 1: Transform the modified pTXB1 vector into BL21(DE3) *E. coli* or similar strain that allows for recombinant expression. Spread transformed cells on plates of LB agar containing 200 mg/L ampicillin.
2. Day 2: Prepare an overnight culture by adding 200 mg/L ampicillin to 50 mL LB and inoculating with the transformed cells. Incubate overnight at 37 °C with shaking.
3. Day 3: Inoculate 2 L of TB with the overnight culture to an OD_{600} of 0.05. Grow cultures in 37 °C shaker at 160 rpm for 4–5 h until the OD reaches 1.6–1.8 at 600 nm.
4. Induce the culture by adding IPTG to 1 mM and grow for 4 h to overnight at 25 °C with shaking.

3.2.2 Protein Purification

1. Day 4: Harvest the cells by centrifugation at 7,000 × *g* for 20 min at 4 °C. Decant medium.
2. Resuspend the cell pellet in intein lysis buffer.
3. Lyse cells using a French pressure cell or similar method.
4. Remove debris by centrifugation of the cell lysate at 15,000 × *g* for 45 min at 4 °C. Filter the lysate supernatant through a glass fiber syringe filter.
5. Equilibrate 20 mL chitin resin with 10 column volumes of intein lysis buffer in a plastic fritted column.
6. Resuspend chitin resin in the filtered cell lysate supernatant. Incubate on a tube tumbler for 3–4 h at 4 °C to allow for maximal binding of protein to the resin.
7. Transfer the resin–lysate slurry back to the fritted column. Collect a sample of the flow-through for subsequent analysis by SDS–PAGE.
8. Wash the resin with 2 column volumes of intein wash buffer.
9. Resuspend the resin in intein elution buffer. Place on tube tumbler at 4 °C for 2–3 days.
10. Transfer resin to the fritted column and collect the eluent. Wash resin with 1 column volume of intein elution buffer without thiol.
11. Transfer eluent to dialysis tubing appropriate for the size of the protein, and dialyze into a buffer appropriate for subsequent purification of the protein by gel-filtration chromatography (*see* **Note 5**).
12. Concentrate the dialyzed protein sample to <10 % of the gel-filtration column volume for maximal resolution.
13. Purify the cleaved protein thioester from the intein–chitin-binding domain and uncleaved fusion protein by gel-filtration chromatography using a Superdex G75 26/60 column or similar. Collect 5-mL fractions, and analyze with SDS–PAGE to determine which fractions contain target protein.
14. Concentrate the protein to 100–200 μM and exchange the buffer to 50 mM HEPES–NaOH buffer, pH 7.5, with a spin concentrator or analogous technique.

3.2.3 Transthioesterification

1. Add 100× molar excess of the phosphinothiol, and sparge the solution with N_2(g) for 10 min.
2. Stir the solution for 1–2 days at 4 °C to allow for transthioesterification.
3. Confirm the success of transthioesterification by mass spectrometry.
4. Dialyze (2×) the protein into 4 L degassed 50 mM HEPES–NaOH buffer, pH 8.0, at 4 °C to remove excess phosphinothiol (*see* **Note 6**).

3.3 Ligation

3.3.1 Ligation Reaction

1. Prepare the ligation reaction mixture by adding the two protein components in equimolar amounts, in 100 mM HEPES–NaOH buffer, pH 8.0. Place the reaction mixture on a nutator overnight at 37 °C, for 12–24 h. The reaction is complete when no pendant protein phosphinothioester is detected by mass spectrometry.
2. The theoretical yield for the ligation efficiency with equimolar substrate and pendant proteins can be calculated with the equation [31]:

$$\text{Yield} = \frac{kt[\text{protein}]_{t=0}}{1 + kt[\text{protein}]_{t=0}}$$

where the second-order rate constant for the fastest known traceless Staudinger ligation at room temperature is $k = 7.7 \times 10^{-3}\ \text{M}^{-1}\ \text{s}^{-1}$ [23] and [protein] refers to the concentration of substrate (or pendant) protein.

3.3.2 Product Identification and Purification

1. The ligation products can be identified by SDS–PAGE, mass spectrometry, or immunoblotting.
2. The ligation products can be separated by cation-exchange, anion-exchange, or gel-filtration chromatography, depending upon the attributes of the input proteins.

4 Notes

1. M15MA cells contain the pREP4 vector, which encodes lacI and kanamycin resistance. This vector limits leaky expression prior to induction.
2. At this stage the protein is quite pure, but can be purified further by cation- or anion-exchange chromatography.
3. The temperature and time of ProTEV cleavage can be modified based on the stability of the target protein. The cleavage efficiency will also depend upon how well exposed the cleavage site is which will vary from protein to protein.
4. The incorporation of azidonorleucine can be verified by performing a click reaction with a fluorescent alkyne in the presence of Cu(I). To do so, collect 300 μL of cells grown to OD 1.8 at 600 nm by centrifugation at 10,000 × *g* for 5 min. Resuspend the cell pellet in 100 μL of BugBuster Master Mix cocktail (Merck KGaA, Darmstadt, Germany), and place on a nutator at room temperature for 20 min. Remove debris by centrifugation at 14,000 × *g* for 10 min, and transfer the supernatant to a fresh tube. Combine 10–20 μL of cell lysate supernatant with 4 μL of 50 mM TCEP, 2 μL of 50 mM $CuSO_4$,

and 0.5 nmol of Alexa Fluor® 488 alkyne (Life Technologies, Grand Island, NY, USA) in DMF in 50 mM phosphate buffer, pH 8.0. Place on nutator in foil for 2–4 h. Analyze the completed reaction by SDS–PAGE using the fluorescent signal at 488 nm on a Typhoon scanner (GE Healthcare Biosciences) to assess the azidonorleucine incorporation efficiency.

5. This buffer will depend upon the stability of the protein.
6. The C-terminal phosphinothioester will hydrolyze slowly in solution, so it should be prepared as close to the time of use as possible. Typically, a batch will be sufficiently labeled for 1–2 weeks after preparation.

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References

1. Ciechanover A, Iwai K (2004) The ubiquitin system: from basic mechanisms to the patient bed. IUBMB Life 56:193–201
2. Li W, Ye Y (2008) Polyubiquitin chains: functions, structures, and mechanisms. Cell Mol Life Sci 65:2397–2406
3. Komander D (2009) The emerging complexity of protein ubiquitination. Biochem Soc Trans 37:937–953
4. Griffin M, Casadio R, Bergamini CM (2002) Transglutaminases: nature's biological glues. Biochem J 368:377–396
5. Gentile V, Thomazy V, Piacentini M, Fesus L, Davies PJ (1992) Expression of tissue transglutaminase in Balb-C 3T3 fibroblasts: effects on cellular morphology and adhesion. J Cell Biol 119:463–474
6. Jones RA, Nicholas B, Mian S, Davies PJ, Griffin M (1997) Reduced expression of tissue transglutaminase in a human endothelial cell line leads to changes in cell spreading, cell adhesion and reduced polymerisation of fibronectin. J Cell Sci 110:2461–2472
7. Greenberg CS, Birckbichler PJ, Rice RH (1991) Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. FASEB J 5:3071–3077
8. Ariëns RAS, Lai T-S, Weisel JW, Greenberg CS, Grant PJ (2002) Role of factor XIII in fibrin clot formation and effects of genetic polymorphisms. Blood 100:743–754
9. El Oualid F, Merkx R, Ekkebus R, Hameed DS, Smit JJ, de Jong A, Hilkmann H, Sixma TK, Ovaa H (2010) Chemical synthesis of ubiquitin, ubiquitin-based probes, and diubiquitin. Angew Chem Int Ed 49:10149–10153
10. Eger S, Scheffner M, Marx A, Rubini M (2010) Synthesis of defined ubiquitin dimers. J Am Chem Soc 132:16337–16339
11. Bavikar SN, Spasser L, Haj-Yahya M, Karthikeyan SV, Moyal T, Ajish Kumar KS, Brik A (2011) Chemical synthesis of ubiquitinated peptides with varying lengths and types of ubiquitin chains to explore the activity of deubiquitinases. Angew Chem Int Ed 50:758–763
12. Virdee S, Kapadnis PB, Elliott T, Lang K, Madrzak J, Nguyen DP, Riechmann L, Chin JW (2011) Traceless and site-specific ubiquitination of recombinant proteins. J Am Chem Soc 133:10708–10711

13. Castañeda C, Liu J, Chaturvedi A, Nowicka U, Cropp TA, Fushman D (2011) Nonenzymatic assembly of natural polyubiquitin chains of any linkage composition and isotopic labeling scheme. J Am Chem Soc 133:17855–17868
14. Weikart ND, Sommer S, Mootz HD (2012) Click synthesis of ubiquitin dimer analogs to interrogate linkage-specific UBA domain binding. Chem Commun 48:296–298
15. Valkevich EM, Guenette RG, Sanchez NA, Chen Y-c, Ge Y, Strieter ER (2012) Forging isopeptide bonds using thiol–ene chemistry: site-specific coupling of ubiquitin molecules for studying the activity of isopeptidases. J Am Chem Soc 134:6916–6919
16. Martin LJ, Raines RT (2010) Carpe diubiquitin. Angew Chem Int Ed 49:9042–9044
17. Nilsson BL, Kiessling LL, Raines RT (2000) Staudinger ligation: a peptide from a thioester and azide. Org Lett 2:1939–1941
18. Saxon E, Armstrong JI, Bertozzi CR (2000) A "traceless" Staudinger ligation for the chemoselective synthesis of amide bonds. Org Lett 2:2141–2143
19. Pickart CM, Cohen RE (2004) Proteasomes and their kin: proteases in the machine age. Nat Rev Mol Cell Biol 5:177–187
20. Nilsson BL, Soellner MB, Raines RT (2005) Chemical synthesis of proteins. Annu Rev Biophys Biomol Struct 34:91–118
21. Tam A, Raines RT (2009) Protein engineering with the traceless Staudinger ligation. Methods Enzymol 462:25–44
22. Schilling CI, Jung N, Biskup M, Schepers U, Bräse S (2011) Bioconjugation via azide-Staudinger ligation: an overview. Angew Chem Int Ed 40:4840–4871
23. Soellner MB, Nilsson BL, Raines RT (2006) Reaction mechanism and kinetics of the traceless Staudinger ligation. J Am Chem Soc 128:8820–8828
24. Soellner MB, Nilsson BL, Raines RT (2002) Staudinger ligation of α-azido acids retains stereochemistry. J Org Chem 67:4993–4996
25. Nilsson BL, Hondal RJ, Soellner MB, Raines RT (2003) Protein assembly by orthogonal chemical ligation methods. J Am Chem Soc 125:5268–5269
26. Tam A, Soellner MB, Raines RT (2007) Water-soluble phosphinothiols for traceless Staudinger ligation and integration with expressed protein ligation. J Am Chem Soc 129:11421–11430
27. Tanrikulu IC, Schmitt E, Mechulam Y, Goddard WA, Tirrell DA (2009) Discovery of *Escherichia coli* methionyl-*t*RNA synthetase mutants for efficient labeling of proteins with azidonorleucine *in vivo*. Proc Natl Acad Sci U S A 106:15285–15290
28. Muir TW, Sondhi D, Cole PA (1998) Expressed protein ligation: a general method for protein engineering. Proc Natl Acad Sci U S A 95:6705–6710
29. Tam A, Raines RT (2009) Coulombic effects on the traceless Staudinger ligation in water. Bioorg Med Chem 17:1055–1063
30. Link AJ, Vink MKS, Tirrell DA (2004) Presentation and detection of azide functionality in bacterial cell surface proteins. J Am Chem Soc 126:10598–10602
31. McGrath NA, Raines RT (2011) Chemoselectivity in chemical biology: acyl transfer reactions with sulfur and selenium. Acc Chem Res 44:752–761