Published in final edited form as:

Methods Mol Biol. 2020 ; 2133: 293–312. doi:10.1007/978-1-0716-0434-2_14.

Facile semisynthesis of ubiquitylated peptides with the ligation auxiliary 2-aminooxyethanethiol

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Summary

The post-translational modification of cellular proteins by ubiquitin (Ub), called ubiquitylation, is indispensable for the normal growth and development of eukaryotic organisms. In order to conduct studies that elucidate the precise mechanistic roles for Ub, access to site-specifically and homogeneously ubiquitylated proteins and peptides is critical. However, the low abundance, heterogeneity, and dynamic nature of protein ubiquitylation are significant limitations toward such studies. Here we provide a facile expressed protein ligation method that does not require specialized apparatus and permits the rapid semisynthesis of ubiquitylated peptides by using the atom-efficient ligation auxiliary 2-aminooxyethanethiol.

Keywords

Ubiquitin; ligation; auxiliary; semisynthesis; peptide; ubiquitylation; sumoylation

1. Introduction

Ubiquitin (Ub) is a 76-amino acid long highly conserved protein that has been found in all eukaryotes studied to date [1]. Similar to chemical protein modifications such as phosphorylation, acetylation and methylation, Ub is best known as a modifier of protein side-chains. Cellular processes regulated by ubiquitylation (modification by Ub) include the proteasomal degradation of oxidatively damaged and terminally misfolded proteins, receptor trafficking [2], protein aggregation [3], DNA-damage repair and gene transcription [4]. In a vast majority of known ubiquitylated proteins, Ub is conjugated to specific lysine side-chain ϵ -amines by means of an isopeptide linkage through its C-terminal α -carboxylic acid. As Ub, and indeed most proteins contain multiple lysines, their site-specific ubiquitylation leads to a large diversity of modification states in eukaryotic cells [5]. Even within the same protein, ubiquitylation at different positions may be associated with differing functional outcomes [3]. In humans, a family of over 630 Ub-ligase enzymes (designated as E1–E3 proteins) and about 100 deubiquitylating enzymes (DUBs) are tasked with imparting substrate and lysine-site specificity to ubiquitylation, and in ensuring the synchronization of protein ubiquitylation states with varying cellular demands (Figure 1A) [6].

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Given its central role in eukaryotic biology, understanding the context-specific functions of protein ubiquitylation is a pressing goal for modern molecular biology. One significant challenge toward studies aimed at deciphering mechanistic roles for Ub is access to sufficient quantities of homogeneously and site-specifically ubiquitylated proteins for *in vitro* biochemical and biophysical assays [7]. The highly dynamic nature of protein ubiquitylation, coupled with the heterogeneity of protein post-translational modifications *in vivo*, has rendered access to uniformly ubiquitylated proteins limited to instances where the substrate-specific Ub ligases are known and found to be active *in vitro*. In some instances, sufficient quantities of the uniformly ubiquitylated protein may be isolated from cultured cells [8]. However, most studies seeking to ascribe mechanistic roles to site-specific ubiquitylation require significant quantities of Ub-modified proteins that are not readily accessible from cells, or from *in vitro* ubiquitylation reactions.

Here we present a chemical strategy that provides facile access to ubiquitylated peptides (Figure 1B), which in principle are readily extendable to protein substrates that are amenable to semisynthesis [9]. Key aspects of our methodology include (a) the synthesis of a ligation auxiliary, 2-aminooxyethanethiol, and its installation at the Lys site of ubiquitylation in peptide substrates [10], (b) expressed protein ligation of the auxiliary-bearing peptide with a C-terminally truncated Ub α -thioester [11], and (c) removal of the auxiliary from the ligation product by reduction with metallic Zn. The need for the 2-aminooxyethanethiol ligation auxiliary arises from the fact that the Ub C-terminus is conjugated to lysine side-chain ϵ -amines by means of an isopeptide linkage. This side-chain linkage cannot be generated by standard Cys-directed ligation techniques that, despite their tremendous utility, are limited to the generation of native peptide bonds [12].

2. Materials

2.1 Synthesis of the S-trityl protected ligation auxiliary

2.1.1 Equipment

1. 3 Å molecular sieves
2. Round bottom flask(s) (glass) of appropriate size
3. Magnetic stir bar(s) of appropriate size
4. Heated magnetic stir plate
5. Aluminum-backed silica gel thin-layer chromatography (TLC) plates with embedded fluorophore, e.g. TLC Silica gel 60 F₂₅₄
6. Pasteur pipettes and bulb
7. Aluminum foil
8. Medium-fritted glass filter funnel of appropriate size and rubber funnel adapter
9. Side-arm flask
10. Separatory funnel of appropriate size
11. Filter paper

12. Rotary evaporator
13. Dry ice
14. Rubber septa of appropriate size
15. 18 gauge needles connected to nitrogen and vacuum lines via adapter
16. Tygon vacuum tubing
17. 18 gauge double tipped needle (cannula)
18. Silica gel 60 (70–230 mesh)
19. Washed sand
20. Glass chromatography column of appropriate size, with glass frit

2.1.2 Chemical reagents

1. *N,N*-dimethylformamide (DMF)
2. *N*-hydroxyphthalimide
3. Triethylamine
4. 1,2-dibromoethane
5. Ethyl acetate
6. Hexane
7. Milli-Q pure water or distilled water
8. 1 N HCl
9. MgSO₄, anhydrous
10. Saturated NaCl (aqueous) or brine
11. 95% (v/v) ethanol (aqueous)
12. DMF, dried over molecular sieves
13. Sodium hydride, stored in dry glove box
14. Triphenylmethanethiol
15. N₂ gas
16. Chloroform
17. Hydrazine hydrate
18. Basified water. Distilled water adjusted to pH 8 with 1 M NaOH
19. MgSO₄, anhydrous

2.1.3 Chemical reagents for mass spectrometric characterization of *O*-(2-(tritylthio)ethyl)hydroxylamine (3)

1. DMF

2. *N,N*-diisopropylethylamine (DIEA)
3. Iodoacetamide
4. Acetonitrile (ACN)

2.1.4 Deuterated solvents for compound characterization by NMR

1. Chloroform (CDCl₃)
2. DMF (DMF-D₇)
3. Dimethylsulfoxide (DMSO-D₆)

2.2 Solid Phase Peptide Synthesis

2.2.1 Equipment

1. Reaction vessel
2. Rubber vacuum tubing
3. Solvent wash bottle(s) with spout cap
4. Glass scintillation vial, 20 mL
5. Bench-top shaker
6. Refrigerated centrifuge and microcentrifuge

2.2.2 Chemical reagents

1. Rink-amide resin (100–200 mesh size, 0.72 mmol/g)
2. DMF
3. Methylene chloride (dichloromethane, DCM)
4. Methanol
5. 9-fluorenylmethoxycarbonyl (Fmoc) deprotection solution: 20% (v/v) piperidine in DMF
6. 4-methyltrityl (Mtt) group deprotection solution: 1% (v/v) triisopropylsilane (TIS), 1% (v/v) trifluoroacetic acid (TFA) in DCM
7. DMSO
8. DIEA
9. *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU)
10. *N,N'*-diisopropylcarbodiimide (DIC)
11. Ethyl-2-cyano-2-(hydroxyimino)acetate (Oxyma)
12. 9-fluorenylmethoxycarbonyl (Fmoc) N^α-protected amino acids with standard, acid-labile protecting groups

13. Fmoc-Lys(Mtt)-OH
14. Acetic anhydride
15. Di-*tert*-butyl dicarbonate (Boc₂O)
16. Bromoacetic acid
17. N₂ gas
18. Kaiser test solution 1: 4/1 (w/v) phenol/ethanol
19. Kaiser test solution 2: 0.2 mM KCN in pyridine
20. Kaiser test solution 3: 0.28 M ninhydrin in ethanol
21. 60% (v/v) ethanol (aqueous)
22. Cleavage cocktail: 92.5:2.5:2.5:2.5 (v/v) TFA: TIS: H₂O: anisole
23. Reagent K cleavage cocktail: 82.5:5.0:5.0:5.0:2.5 (v/v) TFA: thioanisole: H₂O: phenol: 1,2-ethanedithiol.
24. Diethyl ether

2.3 Recombinant Ub(1–75)MES and SUMO(1–91)MES α-thioester preparation

2.3.1 Equipment

1. Chitin resin
2. Glass chromatography column, 50 mL
3. Autoclave
4. Glass culture flask, 2.8 L
5. Sterilizing syringe filter (0.22 μm pore size)
6. Centrifuge bottles, 500 mL and 50 mL
7. Refrigerated ultracentrifuge
8. Ice
9. Sonifier Cell Disruptor
10. 0.45 μm syringe filter
11. Nutator at 4 °C

2.3.2 Chemical and biological reagents

1. *E. coli* BL21(DE3) cells
2. pTXB1 vector
3. Plasmids pUb(1–75) and pSUMO3(1–91). These plasmids were generated from the pTXB1 vector and contain truncated forms of the human ubiquitin and human SUMO-3 genes that are missing the terminal Gly residue (Gly76 for Ub and Gly92 for SUMO-3). The *ub(1–75)* and *sumo-3(1–91)* genes are fused in-

frame and directly N-terminal to a mutant gyrase A intein from *Mycobacterium xenopi*.

4. Luria-Bertani medium, sterile
5. 100 mg/mL ampicillin stock solution, sterile-filtered
6. 1 M isopropyl β -D-1-thiogalactopyranoside (IPTG) stock solution (aqueous), protected from light
7. Cell lysis buffer: Phosphate Buffered Saline (PBS), pH 7.2, containing 1 mM sodium 2-mercaptoethanesulfonate (MESNa)
8. Wash buffer: PBS, pH 7.75
9. Thiolysis buffer: PBS, pH 7.75, containing 100 mM MESNa

2.4 Expressed protein ligation and auxiliary removal

2.4.1 Equipment

1. Microcentrifuge tubes of appropriate size
2. Liquid N₂
3. 18 gauge needles connected to nitrogen and vacuum lines via needle adapter
4. Tygon vacuum tubing
5. Nutator at 25 °C
6. Nutator at 37 °C

2.4.2 Chemicals

1. Ligation buffer: 6 M guanidinium hydrochloride (Gn-HCl), 0.1 M Na₂HPO₄, 10 mM tris(2-carboxyethyl)phosphine (TCEP), pH 7.5
2. 0.1% formic acid (aqueous)
3. 1 M NaOH
4. Freshly activated Zn dust.
5. N₂ gas
6. 1 N HCl
7. Auxiliary removal solution: 6 M Gn-HCl, pH 3.0, degassed
8. Zn wash solution: 6 M Gn-HCl, 50 mM Ethylenediaminetetraacetic acid (EDTA), pH 3.0

2.5 Protein and peptide purification and characterization

1. C18 preparative reverse-phase high-performance liquid chromatography (RP-HPLC) column (10 micron particle size, 250 × 22 mm)
2. HPLC solvent A: H₂O, 0.1% (v/v) TFA

3. HPLC solvent B: 90:10 (v/v) ACN: H₂O, 0.1% (v/v) TFA
4. Collection tubes of appropriate size
5. Electrospray ionization mass spectrometer (ESI-MS)
6. C18 LC-ESI-MS column (3.5 micron particle size, 100 × 2.1 mm)
7. LC-ESI-MS solvent A: 5:95 (v/v) ACN:H₂O, 1% (v/v) acetic acid.
8. LC-ESI-MS solvent B: ACN, 1% (v/v) acetic acid

3. Methods

3.1 Synthesis of the *S*-trityl protected ligation auxiliary, *O*-(2-(tritylthio)ethyl)hydroxylamine (3) (Figure 2).

In this section we describe the synthesis of the ligation auxiliary, 2-aminooxyethanethiol, in the *S*-trityl protected form. The protected ligation auxiliary may be directly incorporated into any desired unprotected lysine side-chains on the solid-phase using the Zuckerman submonomer approach [13]. The facile three-step synthetic procedure we describe requires only a single chromatographic purification step, and may be undertaken at multi-gram scales with no significant changes in overall yield.

3.1.1 Synthesis of *N*-(2-bromoethoxy)phthalimide (1)

1. Prepare a room temperature solution of *N*-hydroxyphthalimide (0.85 M in DMF, 41 mL, 34.9 mmol) in a round-bottom flask equipped with a magnetic stir-bar (see Note 1).
2. To this stirring solution, add triethylamine (10.7 mL, 76.7 mmol) dropwise.
3. To this stirring solution, add 1,2-dibromoethane (12.6 mL, 146.2 mmol) dropwise.
4. Stir the resulting mixture at room temperature. Keep protected from light by wrapping the flask with aluminum foil (see Note 2).
5. Monitor reaction progress by TLC (see Note 3) in a 70:30 (v/v) mixture of hexane and ethyl acetate.
6. Upon disappearance of starting material (about 18 h), remove the solids formed by vacuum-filtration on a medium-fritted glass filter. The product remains dissolved in the DMF filtrate.
7. Precipitate the product by adding 350 mL water, then vacuum-filter on a medium-fritted glass filter and wash with 100 mL of ice-cold water.
8. Dissolve the precipitated solids in 200 mL ethyl acetate and extract with 1 N HCl (2 × 100 mL), water (1 × 100 mL), and saturated NaCl (1 × 100 mL).
9. Dry the resultant organic layer over anhydrous MgSO₄, and remove ethyl acetate *in vacuo* at 25 °C on a rotary evaporator.

10. Purify the crude product by recrystallization from 95% EtOH (aq) to give *N*-(2-bromoethoxy)phthalimide, **1**, as white, needle-like crystals (5.7 g, 64%).
11. Confirm product identity and purity by NMR and ESI-MS. ¹H NMR (300 MHz, CDCl₃): δ 7.81 (m, 4H), 4.47 (t, 2H, *J* = 6.88 Hz), 3.64 (t, 2H, *J* = 6.88 Hz). ¹³C NMR (499 MHz, CDCl₃): δ 163.49, 134.81, 128.79, 123.78, 77.27, 26.89. ESI-MS: *m/z* [M+Na]⁺ 291.96 Da.

3.1.2 Synthesis of *N*-(2-(tritylthio)ethoxy)phthalimide (2**)**—This reaction is moisture-sensitive and should be performed under N₂ atmosphere. Glassware should be flame-fried under vacuum prior to use.

1. In a round-bottom flask equipped with a magnetic stir-bar, combine triphenylmethanethiol (1.23 g, 4.45 mmol) and sodium hydride (0.133 g, 5.54 mmol) (see Note 4).
2. To this mixture, add anhydrous DMF (18 mL) dropwise and continue to stir until the evolution of bubbles is no longer observed. The solution will become bright yellow in color as the deprotonated triphenylmethanethiolate sodium salt forms.
3. In a separate round-bottom flask equipped with a magnetic stir-bar, prepare a solution of *N*-(2-bromoethoxy)phthalimide (0.2 M in anhydrous DMF, 18 mL, 3.70 mmol).
4. To the stirring solution of *N*-(2-bromoethoxy)phthalimide, add the triphenylmethanethiolate solution by cannula. Monitor the reaction by TLC using a solvent system of 90:10 (v/v) hexane:ethyl acetate.
5. When no further reaction progress is observed, prepare and add a second identical solution of triphenylmethanethiolate (see Note 5).
6. After starting material has been consumed (about 1.5 h), quench the reaction with water until no further bubbling is observed (see Note 6).
7. Remove the precipitate by vacuum filtration and concentrate under high vacuum.
8. Purify the residue by silica gel column chromatography using a solvent system of 90:10 (v/v) hexane:ethyl acetate to give compound *N*-(2-(tritylthio)ethoxy)phthalimide, **2**, (1.43 g, 79%).
9. Confirm product identity and purity by NMR and ESI-MS. NMR: ¹H NMR (301 MHz, DMF-D₇): δ 7.89 (m, 4H), 7.44–7.16 (15H), 3.83 (t, 2H, *J* = 7.30 Hz), 2.65 (t, 2H, *J* = 7.30 Hz). ¹³C NMR (499 MHz, DMF-D₇): δ 163.51, 144.90, 135.10, 129.72, 129.17, 128.34, 127.16, 123.48, 76.45, 67.23, 30.11. ESI-MS *m/z* [M+Na]⁺ 488.13 Da.

3.1.3 Synthesis of *O*-(2-(tritylthio)ethyl)hydroxylamine (**3**)

1. Prepare a room temperature solution of *N*-(2-(tritylthio)ethoxy)phthalimide, **2**, (58.9 mM in CHCl₃, 27 mL, 1.59 mmol) in a 100 mL round-bottom flask equipped with a magnetic stir-bar.⁴

2. To this stirring solution, add hydrazine hydrate (0.3 mL, 4.81 mmol) (see Note 7).
3. Allow the reaction to proceed for 2 h at room temperature with vigorous stirring. Monitor reaction progress by TLC using a solvent system of 80:20 (v/v) hexane: ethyl acetate.
4. After 2 h, remove solids formed in the reaction mixture by filtration on a medium-fritted glass filter.
5. Wash the CHCl_3 filtrate with basified water (pH 8.0, 3×30 mL) in a 250 mL separation flask, and dry over anhydrous MgSO_4 . Remove solvent *in vacuo* to yield compound **3** (0.48 g, 90%).
6. Confirm product identity and purity by NMR and ESI-MS. NMR: ^1H NMR (301 MHz, CDCl_3): δ 7.41–7.03 (15H), 5.23 (s, 2H), 3.46 (t, 2H, $J = 6.32$ Hz), 2.36 (t, 2H, $J = 6.32$ Hz). ^{13}C NMR (500 MHz, $\text{DMSO}-d_6$): δ 144.49, 129.10, 128.01, 126.70, 72.59, 65.93, 31.36. ESI-MS: Due to the poor ionization of **3** during ESI-MS, it is best observed as its acetamide derivative following reaction with 2-Iodoacetamine and *N,N*-diisopropylethylamine (DIEA) in DMF. The ion of the derivatized compound is m/z $[\text{M}+\text{Na}]^+ 415.5$ Da.

3.2 Solid-phase synthesis of peptide substrates for ubiquitylation

In this section we describe the synthesis of short peptide substrates for ubiquitylation using solid-phase peptide synthesis (SPPS). Following peptide assembly using either manual or automated SPPS, the desired lysine site of ubiquitylation is selectively deprotected and the *S*-trityl protected auxiliary is introduced by the submonomer approach [13]. Selective deprotection of one or more targeted lysines may be accomplished by their incorporation as either Lys(Mtt) [14] or Lys(ivDde) [15, 16] side-chain protected forms. Non-ubiquitylated lysines are incorporated with standard Lys(Boc) side-chain protection that precludes incorporation of the ligation auxiliary at these residues.

3.2.1 Synthesis of Ac-QKE-CONH₂ on Rink-amide AM resin—The peptide Ac-QKE-CONH₂ may be manually synthesized on a 0.5 mmol scale employing standard 9-fluorenylmethoxycarbonyl (Fmoc)-based N^α -deprotection chemistry [17] and custom glassware (Adams and Chittenden Scientific Glass, catalog number 942167). The synthesis vessel comprises a 25 mL or 50 mL cylindrical reservoir with a medium-fritted glass filter at the bottom equipped with a two-way stopcock and spout for attaching rubber tubing. This facilitates the application of either vacuum to draw out solvent, or nitrogen to mix solutions during deprotection/coupling, by switching the tubing as needed (see Note 8). Standard black vacuum rubber tubing (1/4" $\times$ 5/8" I.D. $\times$ O.D., 3/16" wall-thickness) is sufficient for both vacuum and nitrogen application. Starting with commercially available Rink-amide resin (100–200 mesh size, 0.70 g, 0.72 mmol/g) couple each amino acid in 4-fold molar excess based on initial resin loading (see Note 9).

1. Deprotect the Fmoc- group with 20% piperidine in DMF (v/v) (5 mL, 3×10 min).

2. Perform each coupling reaction for a minimum of 1 hour and maximum of 4 hours with a mixture of Fmoc-amino acid (2.0 mmol), *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU, 1.97 mmol) and *N,N*-Diisopropylethylamine (DIEA, 4.0 mmol) in 4 mL DMF. Coupling efficiency may be monitored by the Kaiser test [18] and additional couplings performed until a negative test is obtained (see Note 10). Overnight couplings, if required, should be performed under base-free conditions with a mixture of Fmoc-amino acid (2.0 mmol) *N,N'*-diisopropylcarbodiimide (DIC, 4.0 mmol) and ethyl-2-cyano-2-(hydroxyimino)acetate (Oxyma, 2.0 mmol) [19]. Employ Fmoc-Lys(Mtt)-OH at the desired site of ubiquitylation, as it contains the acid-sensitive 4-methyltrityl (Mtt) protecting group at the ϵ -NH₂ [14].
3. After completion of the peptide and following Fmoc-deprotection, acetylate the N-terminal Gln by reaction with DIEA (20-fold molar excess) and acetic anhydride (40-fold molar excess) for 3 × 15 min.
4. To confirm identity of the peptide, remove a 25–50 mg test portion of the resin and dry *in vacuo* after extensive washing with DMF, DCM and finally MeOH (see Note 11).
5. Cleave the peptide from the resin with 300 μ L of freshly-prepared cleavage cocktail for 1 hour at room temperature.
6. Remove the resin beads by filtration and precipitate the peptide from the filtrate by the addition of 5–10 volumes of ice-cold dry ether (see Note 12).
7. Centrifuge briefly to pellet the precipitate and decant the ether layer. Wash the pellet twice with ice-cold diethyl ether and dry under a slow stream of nitrogen in the fume-hood.
8. Purify the crude peptide C18 analytical RP-HPLC with a gradient of 0–40% B over 30 min, and characterize by ESI-MS. ESI-MS: m/z [M+H]⁺ 445.2 Da.

3.2.2 Synthesis of H₂N-KAKI-CONH₂ on Rink-amide AM resin—The peptide H₂N-KAKI-CONH₂ is synthesized by a similar method as that for Ac-QKE-CONH₂ with key differences being a second Lys and free N-terminal amine. Toward this, the N-terminal Lys is protected with *tert*-butoxycarbonyl (Boc-) protecting groups on both the α - and ϵ -amines (see Note 13). The interior Lys, which is the desired site of ubiquitylation, is orthogonally protected with the Mtt protecting group.

1. Following peptide assembly, a test cleavage of the resin should be performed as in section 3.2.1.
2. Purify the peptide by C18 analytical RP-HPLC with a gradient of 0–73% B over 30 min, and characterize by ESI-MS. ESI-MS: m/z [M+H]⁺ 680.4 Da.

3.2.3 Introduction of O-(2-(tritylthio)ethyl)hydroxylamine (3) in peptide substrates.

1. Treat each resin-bound protected peptide, that is confirmed to have the correct sequence by test-cleavage, with a solution of 1% TFA and 1% TIS in DCM (v/v) to remove the Mtt protecting group and expose the Lys ϵ -amine (see Note 14).
2. Neutralize the protonated ϵ -amine by washing with 10% DIEA in DMF (v/v).
3. Couple the Lys ϵ -amine with 8 equivalents of Bromoacetic acid and 8 equivalents of DIC (based on initial resin-loading) in DMF for 45 min at room temperature. Repeat the coupling once.
4. Cleave a test portion of the resin to confirm complete coupling of bromoacetic acid to the side-chain.
5. Wash the bromoacetylated peptide resin extensively with DMF, DCM, and MeOH, then dry *in vacuo*.
6. Transfer the dry peptidyl resin to a scintillation vial and suspend in solution containing 9 equivalents of *S*-trityl protected auxiliary **3** (1 M in DMSO) (see Note 15).
7. Gently shake the mixture for 24 hours at room temperature on a bench-top shaker occluded from light.
8. Cleave a test portion of the resin to determine the degree of bromine displacement by **3** (Figure 3).
9. When the test cleavage shows complete conversion of the bromoacetylated peptide, remove and recover the solution containing unreacted auxiliary. This solution can be lyophilized and stored at -80°C for reuse (see Note 16). Wash the resin extensively with DMF.
10. Deprotect the N-terminal Fmoc of the KAKI peptide with 20% (v/v) piperidine in DMF (5 mL, 2×15 min).
11. Wash the resin extensively with DMF, DCM, and finally MeOH. Dry *in vacuo*.
12. Cleave each peptide from resin by reaction with Reagent K [20] cleavage cocktail (20 $\mu\text{L}/\text{mg}$ resin) for 1.5 hours at room temperature.
13. Remove the resin beads by filtration and precipitate the peptide from the filtrate by the addition of 5–10 volumes of ice-cold dry ether.
14. Centrifuge briefly to pellet the precipitate and decant the ether layer. Wash the pellet twice with ice-cold diethyl ether and dry under a slow stream of nitrogen in the fume-hood.
15. Dissolve the peptides in RP-HPLC buffer A and purify by C18 analytical RP-HPLC with a gradient of 0–40% B over 30 min.

16. Perform ESI-MS to verify identity and purity of the peptides. Ac-QK^{auxE}-CONH₂ (4) m/z [M+H]⁺ 578.3 Da. H₂N-KAK^{auxI}-CONH₂ (5) m/z [M+H]⁺ 591.4 Da.

3.3 Intein-mediated generation of Ub(1–75)- and SUMO-3(2–91)- α -thioesters

The plasmids pUb(1–75) and pSUMO3(1–91) contain truncated forms of the human ubiquitin and human SUMO-3 genes that are missing the terminal Gly residue (Gly76 for Ub and Gly92 for SUMO-3) [10]. The *ub(1–75)* and *sumo-3(1–91)* genes are fused in-frame and directly N-terminal to a mutant gyrase A intein from *Mycobacterium xenopi*. This mutant intein is only capable of catalyzing an N-to-S acyl shift of the amide bond between any N-terminal protein and itself, but cannot undertake the subsequent steps of intein-mediated ligation (pTXB1 vector, New England Biolabs). A C-terminal chitin-binding domain (CBD) present in the final fusion protein facilitates its purification from other *E. coli* proteins by selective binding to a chitin column.

1. Transform chemically-competent (CaCl₂ method) *Escherichia coli* BL21(DE3) cells by heat shock with the plasmids pUb(1–75) and pSUMO3(1–91) (see Note 17).
2. Pick a single colony from Luria-Bertani (LB) agar plates containing 100 μ g/mL of Ampicillin (Amp) and grow the transformed cells in small, 10 mL starter cultures in LB-Amp at 37 °C with shaking at 250 rpm for 14 h.
3. Inoculate six flasks containing 1 L LB-Amp medium with 10 mL started culture. Incubate at 37 °C with shaking at 250 rpm.
4. Once the OD₆₀₀ of the culture reaches ~0.6–0.8, induce overexpression of the desired fusion proteins by adding 0.3 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). Continue to culture the cells for an additional 6 h at 25 °C.
5. Harvest the cells by centrifugation at 7,000 $\times g$ for 15 min. Discard the supernatant media.
6. Resuspend the cell pellet in lysis buffer consisting of phosphate buffered saline (PBS) at pH 7.2 containing 1 mM 2-mercaptoethanesulfonic acid sodium salt (MESNa) (see Note 18).
7. Lyse cells by pulsed sonication on ice, 3 $\times$ 5 min, with 5 min between each sonication step.
8. Centrifuged at 20,000 $\times g$ for 15 min to remove insoluble cellular debris.
9. Pass the lysate supernatant through a 0.45 μ m syringe-filter and apply to a 30 mL chitin column pre-equilibrated with 10 column volumes (CV) of lysis buffer.
10. Bind proteins to the column over a period of 12 h at 4 °C with gentle shaking on a nutator.
11. Wash the column with 20 CV of lysis buffer followed by 2 CV of PBS, pH 7.75.
12. Cleave the Ub(1–75)-MESNa and SUMO-3(2–91)-MESNa thioesters from their respective intein-CBD fusions by incubation with 1.5 CV of PBS, pH 7.75

containing 100 mM MESNa for 72 h at 4 °C (Figure 4, also see Note 19). We observe that the N-terminal Met of SUMO-3 (but not of Ub) is consistently processed by Met aminopeptidases *in vivo*, leading to the SUMO-3(2–91)- α -thioester product.

13. Purify the eluted Ub(1–75)- and SUMO-3(2–91)- α -thioesters by C18 preparative RP-HPLC employing a gradient of 30–60% B over 60 min. Identify fractions containing the desired thioesters by ESI-MS. a. Ub(1–75)-MES (**6**) m/z [M+H]⁺ 8,632.8 Da. b. SUMO-3(2–91)-MES (**7**) m/z [M+H]⁺ 10,461.6 Da.

3.4 Expressed protein ligation of auxiliary-containing peptides and protein α -thioesters

In this section we describe expressed protein ligation of peptides containing the ligation auxiliary, 2-aminoxyethanethiol, with protein α -thioesters. Due to our incorporation of the auxiliary into peptides using the submonomer approach, the terminal Gly of Ub and SUMO-3 are already attached to the target lysine. Therefore, C-terminally truncated α -thioesters missing Gly76 in Ub, Ub(1–75) - α -thioester, and Gly92 in SUMO-3, SUMO-3(2–91)- α -thioester, are employed in the expressed protein ligation reaction.

1. Dissolve the purified auxiliary-conjugated peptides **4** or **5** (0.35 mg, 0.6 μ mol) and protein α -thioesters **6** or **7** (1.0 mg, 0.12 μ mol) separately dissolved in 120 μ L of ligation buffer each. Combine the two solutions and gently shake at room temperature for 24 h.
2. To monitor the kinetics of ligation, remove 3 μ L aliquots from the reaction mixture at specific time-points. Dilute the aliquots into 27 μ L H₂O, 0.1% formic acid and analyze by LC-ESI-MS.
3. To monitor the extent of the S-to-N acyl shift (leading to the desired amide-linked product), remove 3 μ L aliquots from the reaction mixture at specific time points. To each aliquot, add 3 μ L 1M NaOH and incubate on ice for 30 sec. These conditions lead to the hydrolysis of non-rearranged thioester-linked ligation products. Quench the hydrolysis reaction with 25 μ L H₂O, 0.1% formic acid, and analyze by LC-ESI-MS employing a gradient of 0–50% D, 40 min. The ratio of peak heights in ESI-MS of the hydrolyzed Ub(1–75)-COOH product to the desired ligation product was considered an indicator of S-to-N acyl rearrangement.
4. Purify the final rearranged ligation products (see Note 20) by C18 analytical RP-HPLC employing a gradient of 30–60% B over 30 min. For each ligation, typical conversion to product is greater than 70%, with the major side product being the hydrolyzed Ub(1–75)-COOH, or SUMO-3(2–91)-COOH protein. Identify fractions containing the desired thioesters by ESI-MS. a. QK^{Ub(aux)}E m/z [M+H]⁺ 9,068.0 Da. b. KAK^{Ub(aux)}I m/z [M+H]⁺ 9,081.1 Da. c. QK^{Su(aux)}E m/z [M+H]⁺ 10,896.8 Da. d. KAK^{Su(aux)}I m/z [M+H]⁺ 10,909.9 Da.

3.5 Auxiliary removal by reductive cleavage of the N-O bond with activated Zn

In this section we describe the facile removal of the ligation auxiliary from the final expressed protein ligation product upon treatment with activated Zn under acidic conditions. We have found this method to be highly compatible with proteins that may be refolded from the denatured state. Although denaturants are not strictly required when cleaving the N-O bond in peptides, the expressed protein ligation product with Ub or SUMO does require the inclusion of a denaturing agent to enable effective electron transfer from activated Zn to the N-O bond.

1. Freshly activate 5 mg of metallic Zn by rinsing with 1 N HCl, then extensively wash with water[21]. Dry the activated Zn *in vacuo*.
2. Add the activated Zn to 200 μL of degassed 6 M Gn-HCl, pH 3.0 containing 0.2 mg purified ligation product.
3. Degas the mixture by 3 freeze-thaw cycles under N_2 . This step is necessary to reduce the generation of oxidized side products.
4. Allow the reduction to proceed at 37 °C under N_2 with gentle shaking for 24 h.
5. Briefly centrifuge the reaction at $15,000 \times g$ to pellet the Zn dust and remove the supernatant containing reduced products.
6. Wash the pelleted Zn twice with 150 μL of 6 M Gn-HCl, 50 mM EDTA, pH 3.0.
7. Reduce the combined supernatant and washes with 10 mM TCEP at pH 7.5 (see Note 21) and purify by C18 analytical RP-HPLC with a gradient of 30–60% B over 30 min. Identify fractions containing the desired thioesters by ESI-MS. a. $\text{QK}^{\text{UbE}} m/z [\text{M}+\text{H}]^+ 8,992.0$ Da. b. $\text{KAK}^{\text{UbI}} m/z [\text{M}+\text{H}]^+ 9,005.1$ Da. c. $\text{QK}^{\text{SuE}} m/z [\text{M}+\text{H}]^+ 10,820.8$ Da. d. $\text{KAK}^{\text{SuI}} m/z [\text{M}+\text{H}]^+ 10,833.9$ Da.

Notes

1. Freshly distilled triethylamine stored over 3 Å molecular sieves gives the best yields. Triethylamine should be stored under argon and protected from light that can cause unwanted oxidation.
2. When working with brominated compounds, rigorously occluding light reduces their decomposition and hence the number of side-products observed.
3. Aluminum-backed silica gel plates are easier to use than glass-backed TLC plates as they require less solvent and can be easily cut to desired sizes. They can be bent to be slightly convex along the longitudinal axis to ensure an even solvent front.
4. Sodium hydride is very moisture sensitive and reacts violently with water. It should be stored in a glove box at all times and aliquots weighed out in capped scintillation vials as needed. All solvents to be used with sodium hydride should be dried over 3 Å molecular sieves for 48 h.

5. The steric bulk of the trityl group hinders nucleophilic attack by the thiolate and slows reaction kinetics. Due to undesirable competing reactions, such as disulfide formation, we have found that adding a second equivalent of freshly prepared thiolate gives higher yields than starting with a large excess of the thiolate.
6. A small excess of sodium hydride (1.25 equivalents) is employed to deprotonate the trityl thiol and we have never observed violent reactions upon quenching, however, the solvent used to quench sodium hydride (water or methanol may be used) must be added in a slow dropwise manner.
7. Hydrazine hydrate is corrosive and toxic and must be handled with care. Wear personal protective equipment including gloves and safety glasses. Work with this chemical in a functioning fume-hood.
8. In order to prevent cross-contamination of the vacuum and nitrogen delivery tubes, we employ a reaction vessel with one spout and switch the lines as needed. However, alternate versions of the synthesis vessel with three-way stopcocks and dedicated lines for nitrogen and vacuum may be used. Peptides containing orthogonally protected lysines may also be synthesized by automated microwave-assisted peptide synthesis using the 1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl (ivDde) protecting group [15], which is stable to heating at 75 °C for 5 min that is employed by commercial synthesizers (for example, the Liberty Blue synthesizer from CEM corporation).
9. Rink amide aminomethyl (AM) or 4-methylbenzhydrylamine (MBHA) solid-phase resins may be used with no significant difference for short peptides <10 amino acids long. For longer peptides, MBHA is recommended [22]. Wang resin pre-loaded with the first Fmoc-amino acid (EMD Millipore or Anaspec) should be employed if the C-terminal carboxylic acid form is desired instead of the C-terminal amide.
10. Although methods for quantitative Ninhydrin have been reported [23], for routine tests of coupling efficiency the resin beads may be visually evaluated after heating at 95–100 °C for 5 min with Ninhydrin reagents. A few blue beads (<10%) in a majority of yellow beads and a yellow solution is considered to be a successful coupling reaction, and it is acceptable to proceed with the next step of deprotection. Incomplete couplings, which require additional rounds of coupling, are judged by the presence of >10% blue beads and a bluish solution during the Kaiser test. If the Kaiser test remains unchanged after three rounds of coupling to the same deprotected amino acid, the resin is capped with acetic anhydride before proceeding to the next step of deprotection.
11. In order to conserve peptidyl-resin and increase final yields, test-cleavages may be performed with as little as 10 mg of dry resin. However, care must be taken in the precipitation step to add diethyl ether dropwise as only a small amount of white precipitate is formed and may not be visible in a large volume of ether. The

precipitate is centrifuged in a microfuge at 13,500 rpm for 5 min and washed twice with ether, then taken up in 100 μ L of HPLC buffer for analysis.

12. An alternate strategy for peptide cleavages is to directly evaporate the filtrate under a stream of nitrogen in the fume hood. This typically works well for small-scale cleavages and does not require careful addition of diethyl ether to generate small amounts of precipitates. However, the increased concentration of reactive cations, derived from the side-chain protecting groups employed, may generate covalent adducts on the final peptide. Therefore, this alternate procedure must be evaluated at the test-scale for each new peptide.
13. Although the Boc- group is useful for protecting the α -amine of an N-terminal Lys, it is labile to extended washing with 1% TFA that may be required to completely remove the Mtt- group. Therefore, an N-terminal Fmoc- protection strategy may also be employed. For short peptides where the Mtt- group is readily deprotected, an N-terminal Boc- group is sufficient.
14. One problem with deprotecting the Mtt- group is the persistence of yellow color on the resin even after several washes with the 1% TFA, 1% TIS mixture in DCM. However, we find that 4–6 flow washes for 1 min each are sufficient to remove the Mtt group in most peptides, even if the beads remain somewhat yellow. A flow wash involves spraying solvent from a Nalgene wash bottle in a continuous stream on top of the resin while simultaneously draining solvent from the bottom. This ensures that the level of solvent on top of the resin remains largely constant and only a few millimeters above the resin bed. This prevents the creeping of reagents along the sides of the reaction vessel and leads to cleaner final products. Longer batch washes of the resin may lead to deprotection of Boc- protecting groups and should be undertaken cautiously, with the optimal duration empirically determined for each new peptide.
15. It is important to use completely dry resin and solvents for this reaction as the presence of water leads to displacement of -Br by -OH. Drying the resin overnight on a lyophilizer and the solvents over 3 Å molecular sieves for 48 h is sufficient to prevent unwanted reactions at this step.
16. The recovered auxiliary solution in DMSO can be reused up till four times without significant deterioration in displacement yields. However, the addition of 1 equivalent of DIEA is necessary after each use, in order to deprotonate the auxiliary hydrobromide salt. Dissolving the auxiliary at 1 M in DMSO requires gentle heating, and it is prone to precipitation at temperatures below 25 °C. If this is observed repeatedly, a 0.5 M solution of the auxiliary in DMSO may be employed with no appreciable reduction in yields.
17. Heat shock transformations were undertaken as described by Sambrook and Russell [24]. Briefly, chemically competent *E. coli* DH5 α or BL21(DE3) cells stored at –80 °C were thawed on ice for 15 min. To each 40 μ L aliquot of competent cells, 1 pg–100 ng of plasmid DNA (1–5 μ L) was added and mixed by gentle pipetting. The cells were placed on wet ice for 2 min, followed by heat

shock in a water bath at 42 °C for 45 s. The heat shocked cells were diluted with 950 μ L of room temperature LB or SOC media and grown for an additional 1 h at 37 °C. Finally 200–300 μ L of cells were plated on LB-Agar plates with the appropriate antibiotic for selection and grown for 12–14 h at 37 °C. Single well-resolved colonies on the plate were selected by using a sterile pipette tip and grown in liquid media for protein expression,

18. Tris-based buffers may be used for intein-mediated thioester production. However, in some instances the protein- α -thioester reacts with the primary amine of Tris to generate the C-terminal amide, which is inert toward native chemical ligation. This is indeed a problem for the SUMO-3(2–91)- α -thioester but not for the Ub(1–75)- α -thioester. This can be pre-empted by employing phosphate buffer whenever possible.
19. Lower temperatures prevent hydrolysis of the protein- α -thioester, but also slow down the rate of trans-thioesterification of the target-intein-fusion with MESNa. Hence, for proteins that do not readily cleave from the intein with 200 mM MESNa at 4 °C, a good compromise is to perform thioesterification at pH 7.5 and 25 °C.
20. Most ligations where one fragment is in at least 5-fold excess appear to reach completion by 24 h, with some ligations complete in as little as 12 h. Therefore, it is acceptable to skip early time-points and simply test the ligation mixture after incubation for one day.
21. Although activated Zn was reported to reduce peptide disulfide bonds [25], we have not observed this effect. This necessitates a quick reduction of ubiquitylated/sumoylated products with TCEP prior to purification. Treatment with 10 mM TCEP at pH 7.5 on ice for 30 min is usually sufficient to reduce disulfides. Extended treatment with TCEP at higher temperatures often results in desulfurization of Cys residues, which can be avoided by reducing the time of incubation or equivalents of TCEP employed.

Acknowledgements

C.C. is thankful for support from the Department of Chemistry and the University of Washington Royalty Research Fund, NIH/NIGMS R01GM110430 and NSF-MCB 1715123. C.E.W. gratefully acknowledges support from an NSF GRFP (grant number DGH-1256082) and an ARCS foundation fellowship.

References

- [1]. Sharp PM, Li W-H (1987) Molecular evolution of ubiquitin genes. *Trends Ecol Evol* 2:328–332. [PubMed: 21227875]
- [2]. Terrell J, Shih S, Dunn R, Hicke L (1998) A function for monoubiquitination in the internalization of a G protein-coupled receptor. *Mol Cell* 1:193–202. [PubMed: 9659916]
- [3]. Meier F, Abeywardana T, Dhall A, Marotta NP, Varkey J, Langen R, Chatterjee C, Pratt MR (2012) Semisynthetic, site-specific ubiquitin modification of α -synuclein reveals differential effects on aggregation. *J Am Chem Soc* 134:5468–5471. [PubMed: 22404520]
- [4]. Hicke L (2001) Protein regulation by monoubiquitin. *Nat Rev Mol Cell Biol* 2:195–201. [PubMed: 11265249]

- [5]. Komander D, Rape M (2012) The ubiquitin code. *Ann Rev Biochem* 81:203–229. [PubMed: 22524316]
- [6]. Hershko A, Ciechanover A (1998) The ubiquitin system. *Ann Rev Biochem*. 67:425–479. [PubMed: 9759494]
- [7]. Weller CE, Pilkerton ME, Chatterjee C (2014) Chemical strategies to understand the language of ubiquitin signaling. *Biopolymers* 101:144–155. [PubMed: 23576160]
- [8]. Davies N, Lindsey GG (1994) Histone H2B (and H2A) ubiquitination allows normal histone octamer and core particle reconstitution. *Biochim Biophys Acta* 1218:187–193. [PubMed: 8018719]
- [9]. Weller CE, Dhall A, Ding F, Linares E, Whedon SD, Senger NA, Tyson EL, Bagert JD, Li X, Augusto O, Chatterjee C (2016) Aromatic thiol-mediated cleavage of N-O bonds enables chemical ubiquitylation of folded proteins. *Nat Commun* 7:12979. [PubMed: 27680493]
- [10]. Weller CE, Huang W, Chatterjee C (2014) Facile synthesis of native and protease-resistant ubiquitylated peptides. *ChemBioChem* 15:1263–1267. [PubMed: 24838693]
- [11]. Muir TW, Sondhi D, Cole PA (1998) Expressed protein ligation : A general method for protein engineering. *Proc Nat Acad Sci USA* 95:6705–6710. [PubMed: 9618476]
- [12]. Dawson PE, Muir TW, Clark-Lewis I, Kent SBH (1994) Synthesis of proteins by native chemical ligation. *Science* 266:776–778. [PubMed: 7973629]
- [13]. Zuckermann RN, Kerr JM, Kent SBH, Moos WH (1992) Efficient method for the preparation of peptoids [Oligo(N-Substituted Glycines)] by submonomer solid-phase synthesis. *J Am Chem Soc* 114:10646–10647.
- [14]. Sax B, Dick F, Tanner R, Gosteli J (1992) 4-Methyltrityl (Mtt): a new protecting group for the side chain protection of Asn and Gln in solid-phase peptide synthesis. *Pept Res* 5:245–246. [PubMed: 1421811]
- [15]. Chhabra SR, Hothi B, Evans DJ, White PD, Bycroft BW, Chan WC (1998) An appraisal of new variants of Dde amine protecting group for solid phase peptide synthesis. *Tet Lett* 39:1603–1606.
- [16]. Chatterjee C, McGinty RK, Pellois J-P, Muir TW (2007) Auxiliary-mediated site-specific peptide ubiquitylation. *Angew Chem Int Ed Eng* 46:2814–2818.
- [17]. Carpino LA, Han GY (1972) 9-Fluorenylmethoxycarbonyl Amino-Protecting Group. *J Org Chem* 37:3404–3409.
- [18]. Kaiser E, Colescott RL, Bossinger CD, Cook PI (1970) Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal Biochem* 34:595–598. [PubMed: 5443684]
- [19]. Subiros-Funosas R, Prohens R, Barbas R, El-Faham A, Albericio F (2009) Oxyma: An efficient additive for peptide synthesis to replace the benzotriazole-based HOBt and HOAt with a lower risk of explosion. *Chem-Eur J* 15:9394–9403. [PubMed: 19575348]
- [20]. King DS, Fields CG, Fields GB (1990) A cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis. *Int J Peptide Protein Res* 36:255–266. [PubMed: 2279849]
- [21]. Canne LE, Bark SJ, Kent SBH (1996) Extending the applicability of native chemical ligation. *J Am Chem Soc* 118:5891–5896.
- [22]. Matsueda GR, Stewart JM (1981) A P-Methylbenzhydrylamine resin for improved solid-phase synthesis of peptide amides. *Peptides* 2:45–50.
- [23]. Sarin VK, Kent SB, Tam JP, Merrifield RB (1981) Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. *Anal Biochem* 117:147–157. [PubMed: 7316187]
- [24]. Sambrook J, Russell DW, *Molecular Cloning: A Laboratory Manual*, Third Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 2001.
- [25]. Erlandsson M, Hallbrink M (2005) Metallic zinc reduction of disulfide bonds between cysteine residues in peptides and proteins. *Int J Pept Res Ther* 11:261–265.

Figure 1. Biological and chemical protein ubiquitylation.

(A) Site-specific ubiquitylation of protein lysine side-chains by the E1–E3 family of ubiquitin ligases. The deubiquitinase (DUB) family of proteases remove Ub from mono- and polyubiquitylated target proteins. (B) Chemical strategy for site-specific ubiquitylation of peptides employing the temporary ligation auxiliary, 2-aminoxyethanethiol, at the desired site of ubiquitin attachment in substrates.

Figure 2. Synthesis of the S-trityl protected ligation auxiliary for incorporation into peptides on the solid phase.

Et₃N= Triethylamine, Trt= Trityl group, NaH= Sodium hydride, DMF=*N,N*-dimethylformamide, CHCl₃= Chloroform, N₂= Nitrogen gas.

Figure 3. Synthesis of peptides containing the ligation auxiliary 2-aminoxyethanethiol. Fmoc= Fluorenylmethoxycarbonyl group, Boc= *tert*-Butyloxycarbonyl group, Mtt= 2-methyltrityl group, TFA= Trifluoroacetic acid, TIS= Triisopropylsilane, DIC= *N,N'*-Diisopropylcarbodiimide.

Figure 4. Intein-mediated generation of Ub(1-75)-MES (6).

Lane 1, Pre-stained protein molecular weight standards (NEB). Lane 2, cell lysate supernatant. Lane 3, column wash with PBS. Lane 4, residual protein on chitin beads after 72 h of treatment with intein-cleavage buffer. Lane 5, column eluate after 72 h of treatment with intein-cleavage buffer. MES= 2-mercaptoethanesulfonic acid.