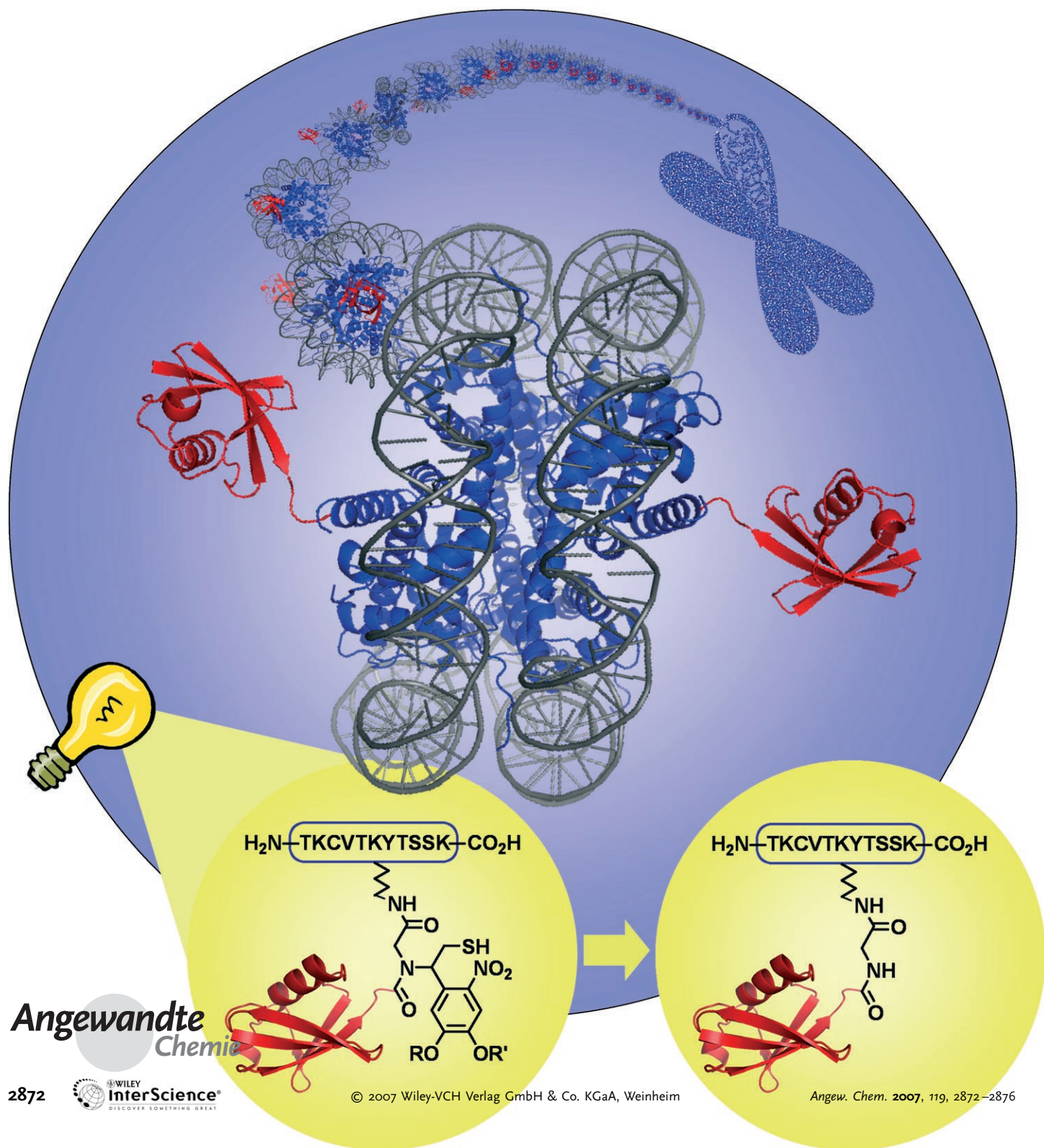


Auxiliary-Mediated Site-Specific Peptide Ubiquitylation**

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Dedicated to Professor Robert Ramage



Ubiquitin (Ub) is a highly conserved 76-residue-long eukaryotic protein that plays an essential role in key cellular processes such as the proteosomal degradation of cytosolic and membrane proteins, endocytosis of membrane receptors, and the regulation of gene transcription.^[1,2] The discovery of Ub as one of the protein components necessary for ATP-dependent proteolytic activity in rabbit reticulocyte lysates^[3] paralleled its identification as a modification at the C termini of the histone nucleoproteins H2A^[4] and H2B.^[5] Interestingly, while proteosomal activity requires polyubiquitylation of proteins, histones primarily exhibit mono-ubiquitylation, which suggests a nondegradative role of Ub in the context of genomic DNA. Indeed, mono-ubiquitylation of H2A and H2B is associated with gene silencing^[6] and transcription elongation, respectively.^[7] Other ubiquitin-like proteins (Ubls), such as SUMO (small ubiquitin-like modifier) and Nedd8, that affect gene transcription, subcellular localization, and the activity of their target proteins have also been identified.^[8–10] SUMOylation of H2B, in contrast with ubiquitylation, is suggested to attenuate transcription of certain genes in *S. cerevisiae*.^[11]

A full understanding of the myriad processes initiated by tagging cellular proteins with Ub and Ubls is complicated by the difficulty associated with isolating, or generating, the modified proteins in quantities and purities suitable for biochemical and/or structural studies. Ubiquitin and SUMO are post-translationally attached to their respective targets by means of an isopeptide bond. This process is catalyzed by the E1–E3 group of enzymes.^[12] These enzymes employ an active site cysteine sulfhydryl group to activate Ub as a thioester at its C-terminal residue (Gly76) and catalyze its condensation with a lysine ϵ -NH₂ group of the target protein. While the uniformly ubiquitylated or SUMOylated proteins may be obtained by purification and in vitro reconstitution of the cognate ligases for each target,^[13,14] this process is limited by the requirement for identifying the ligases and their amenability to recombinant expression. Furthermore, enrichment of the modified targets from biological milieu may yield insufficient material owing to the transient nature of the Ub tag^[15] and may be complicated by multiple diverse post-translational modifications on a single protein. These considerations have led us to envision a readily adaptable and scalable approach for site-specific modification of target sequences with Ub and Ubls. The historical significance of histones in the discovery of protein ubiquitylation and the

crucial role of this modification in gene transcription prompted our choice of an 11 amino acid peptide from the C terminus of mammalian H2B (which includes the ubiquitylated Lys120 residue) for developing a semisynthetic ubiquitylation methodology.

The total chemical synthesis of Ub has been achieved using both stepwise solid-phase peptide synthesis (SPPS)^[16] and native chemical ligation.^[17] This has allowed the generation of various ubiquitin analogues including the attachment of Ub to the ϵ -NH₂ group of a single lysine residue.^[18] The extension of these synthetic protocols to the site-specific ubiquitylation of peptides and proteins is nontrivial. In thinking about this problem, we were influenced by the elegant work of Ploegh and co-workers on the semisynthesis of Ub analogues containing C-terminal electrophiles.^[19] Their approach involves direct aminolysis of recombinant Ub- α -thioesters generated from the corresponding Ub-intein fusions by thiolysis. We wondered whether such recombinant Ub- α -thioesters could be chemically ligated to the ϵ -NH₂ group of a specific lysine residue in a peptide using expressed protein ligation (EPL).^[20] The need to retain a native structure at and around the isopeptide linkage precludes the use of standard EPL strategy, as this would leave a nonnative Cys residue at the ligation junction. In principle, use of a ligation auxiliary group provides a solution to this problem. Several thiol-bearing auxiliaries have been reported that can be removed under acidic^[21] and photolytic conditions.^[22] However, they all suffer from the requirement for a sterically undemanding ligation site.^[23–25] We realized that the two C-terminal residues of Ub and many known Ubls are Gly-Gly, which could serve as a sterically acceptable junction for ligation. Scheme 1 shows our synthetic strategy, the key feature of which is dissection of the target ubiquitylated peptide between the last two glycine moieties of Ub, thereby allowing the use of an auxiliary-mediated EPL reaction with Ub(1–75)- α -thioester.

For the auxiliary, we chose photocleavable compound **1**,^[26] which is based on a ligation auxiliary described previously by the groups of Aimoto^[22] and Dawson.^[25] The ability to remove this group under mild conditions, in principle, makes it compatible with folded proteins.

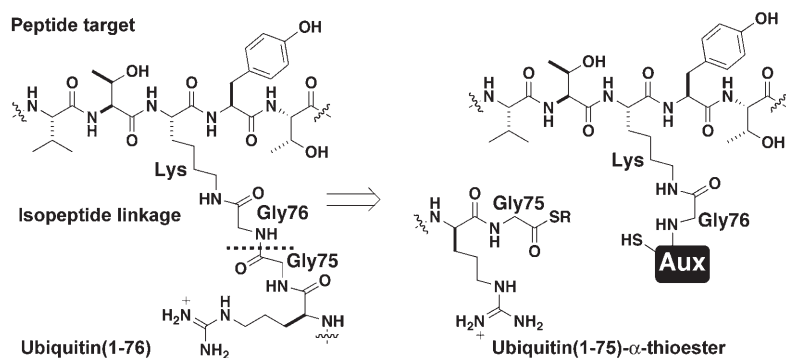
The peptide, H₂N-TKCVTKYTSSK-OH, corresponding to residues 115–125 of human H2B and containing an Ala117Cys mutation, was synthesized by SPPS employing the 9-fluorenylmethoxycarbonyl (Fmoc) strategy (Scheme 2). The Cys mutation was included as it provides a convenient handle for further derivatization of the final ubiquitylated product.

Lys6 in peptidyl resin **2**, which is homologous with Lys120 of H2B, was orthogonally protected with a hydrazine-labile ivDde group.^[27] Removal of this protecting group followed by coupling of bromoacetic acid to the lysine ϵ -NH₂ group afforded peptidyl resin **3**. Subsequent coupling of **3** with the ligation auxiliary **1** afforded peptidyl resin **4**. Compound **1** was synthesized in eight steps from vanillin following reported protocols^[26] with minor modifications, including an additional step to convert the side-chain carboxylic acid into the methylamide (Supporting Information). This was necessitated by the nucleophilicity of the carboxylate group toward

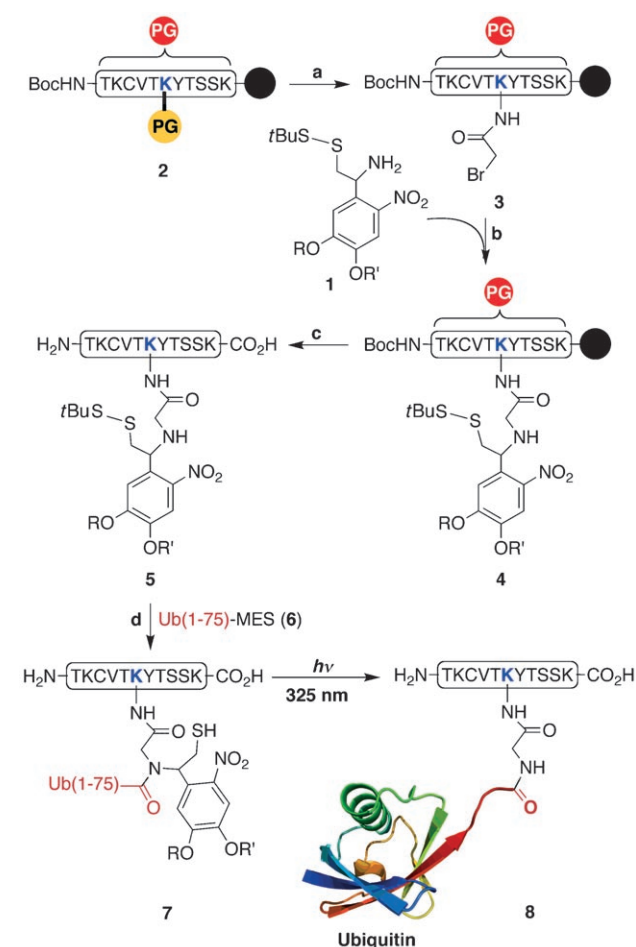
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Scheme 1. Retrosynthetic analysis of a ubiquitylated peptide. The partial amino acid sequence of ubiquitylated H2B C-terminal peptide **8** is used to demonstrate our general disconnection approach. Aux = ligation auxiliary.



Scheme 2. Synthesis of the auxiliary-containing peptide **5** and ligation with ubiquitin(1-75)-α-thioester **6**. a) 1. 2% NH_2NH_2 in DMF, 2. $\text{BrCH}_2\text{CO}_2\text{H}$, DIC, DMF; b) auxiliary **1** ($\text{R} = \text{CH}_3$, $\text{R}' = \text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{N}(\text{CH}_3)\text{H}$), DIEA, DBU, DMF; c) TFA/ H_2O /TIS/anisole (92.5:2.5:2.5:2.5); d) **6**, 300 mM NaP_i , 50 mM MESNa, 25 mM TCEP, 3 M Gn-HCl, pH 7.5. PG = protecting group, Boc = *tert*-butoxycarbonyl, DMF = *N,N*-dimethylformamide, DIC = 1,3-diisopropylcarbodiimide, DIEA = *N,N*-diisopropylethylamine, DBU = 1,8-diazabicyclo-[5.4.0]undec-7-ene, TFA = trifluoroacetic acid, TIS = triisopropylsilane, NaP_i = sodium phosphate, Gn = guanidinium.

the bromoacetyl group under basic conditions, which leads to an undesired ester linkage with the peptide. Following attachment of the auxiliary moiety, peptide **5** was cleaved from the support, subsequently purified, and isolated in 4% overall yield based on the initial loading of the resin. A recombinantly expressed Ub(1-75)-α-thioester protein **6** required for the EPL reaction was obtained by thiolysis of the corresponding GyrA intein fusion with mercaptoethanesulfonic acid (MES) using standard procedures.^[19,20]

Peptide **5** was reduced with tris(2-carboxyethyl)phosphine (TCEP) to remove the S*t*Bu protecting group, immediately prior to the addition of protein **6**, and the time course of ligation at pH 7.5 was followed by analytical reverse-phase (RP)-HPLC. The auxiliary-mediated ligation reaction was significantly slower than typical EPL reactions with N-terminal Cys-containing peptides and was allowed to proceed for 5 days (Figure 1). The ligation

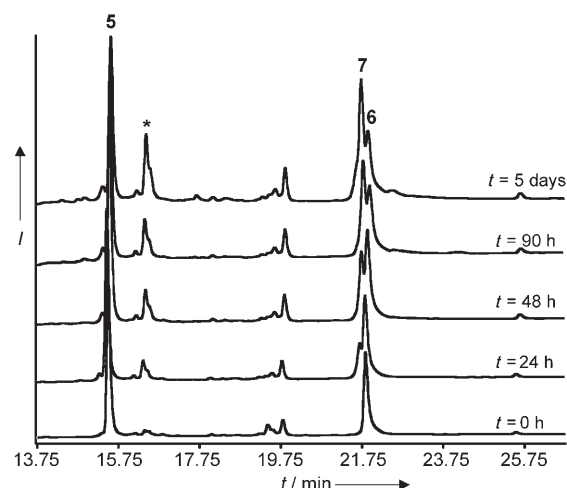


Figure 1. Ligation of ubiquitin(1-75)-MES thioester **6** with the peptide **5**. Formation of the ligation product **7** was followed over 5 days by C18 analytical RP-HPLC. An asterisk indicates formation of the mixed disulfide of **5** and MES after prolonged incubation.

mixture was then reduced and purified by RP-HPLC to obtain the auxiliary-containing branched protein **7**. To remove the auxiliary, protein **7** was dissolved in a buffer containing 50 mM Tris-HCl and 150 mM NaCl at pH 7.5 and irradiated with a He-Cd laser at 325 nm. Three to four short (5 s) pulses led to almost complete conversion of **7** into the final ubiquitylated protein **8** as judged by analytical RP-HPLC (Figure 2a) and ESI mass spectrometry (Figure 2b).

Next, we determined whether ubiquitylated protein **8** acts as a substrate for a ubiquitin-dependent hydrolase. UCH-L3 belongs to the low-molecular-weight Ub C-terminal hydrolase family of enzymes and is proposed to generate free ubiquitin from polymeric ubiquitin gene products and to recover ubiquitin from small C-terminal adducts.^[28] UCH-L3 has also been shown to hydrolyze isopeptide conjugates of

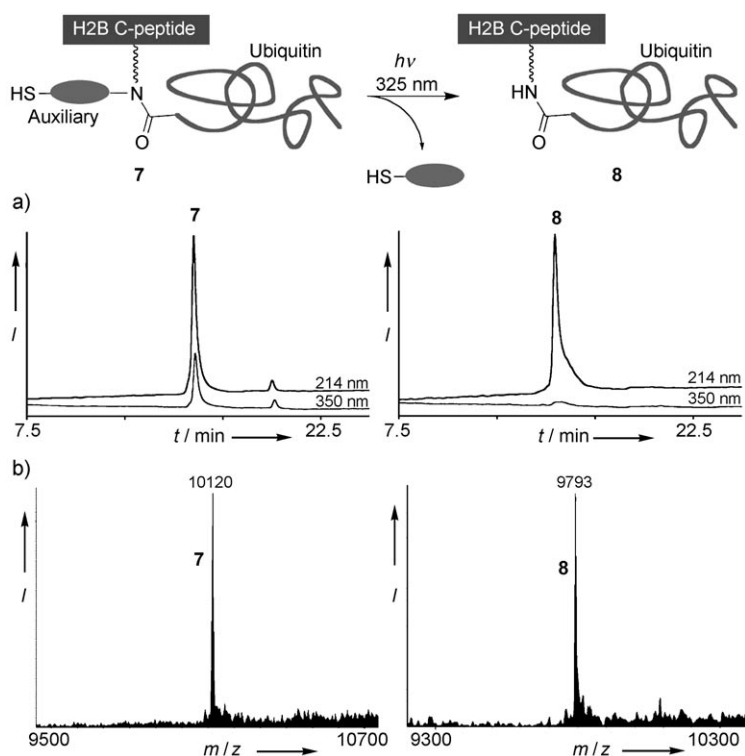


Figure 2. Photolytic removal of the ligation auxiliary: a) RP-HPLC elution profiles of branched ligation product **7** and a crude photolysis mixture containing ubiquitylated product **8**. b) Corresponding deconvoluted ESI mass spectra of **7** (calcd m/z 10120 Da) and **8** (calcd m/z 9793 Da).

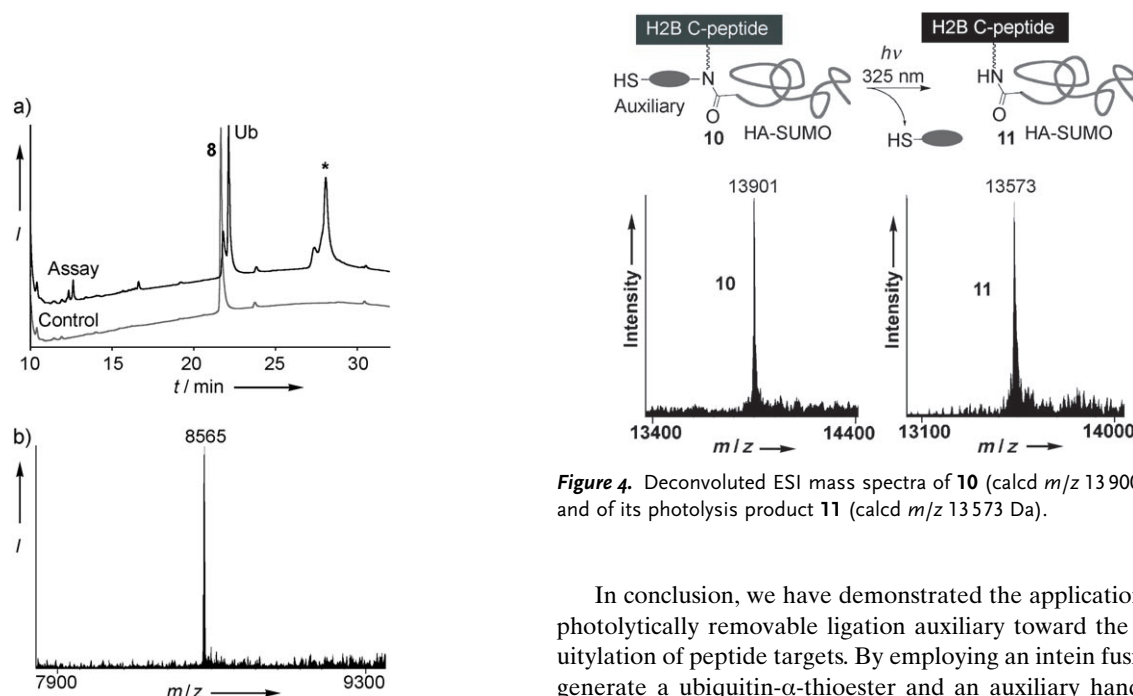


Figure 3. a) RP-HPLC chromatogram of **8** reacted with the enzyme UCH-L3 for 8 h at 37°C. Note that almost complete hydrolysis of **8** to give free ubiquitin is observed. An asterisk denotes the peak corresponding to UCH-L3. b) Deconvoluted ESI mass spectrum of ubiquitin(1-76) produced by UCH-L3 hydrolysis (calcd m/z 8566 Da). The ch2B peptide was detected by LTQ ion-trap ESI-MS (Supporting Information).

Ub.^[29,30] Recognition and hydrolysis of **8** by UCH-L3 would provide good evidence that the Ub in our construct had assumed its native fold and that the isopeptide linkage with Lys6 in the peptide was present. Incubation of ubiquitylated peptide **8** with UCH-L3 led to the generation of two hydrolysis products (Figure 3a), which were confirmed to be ubiquitin(1-76) and the H2B peptide by ESI-MS (Figure 3b and Supporting Information). This result indicates that the Ub within semisynthetic product **8** comprises a native fold in Tris-buffered solutions and validates the utility of our semisynthetic approach for generating biologically active ubiquitin–peptide conjugates.

To demonstrate the generality of our approach for conjugating UbIs, peptide **5** was also ligated with recombinantly expressed HA-tagged SUMO-(1-97)- α -thioester **9**. The time course of this ligation reaction was similar to that for Ub, and the reaction was at least 60% complete after 7 days (Figure S5 in the Supporting Information). The sluggish nature of the ligation is presumably a result of the sterically hindered S-to-N acyl shift onto a disubstituted amine.^[31] Following purification, the auxiliary group was successfully removed by photolysis of the ligation product **10** to give the SUMOylated peptide product **11** (Figure 4).

Figure 4. Deconvoluted ESI mass spectra of **10** (calcd m/z 13900 Da), and of its photolysis product **11** (calcd m/z 13573 Da).

In conclusion, we have demonstrated the application of a photolytically removable ligation auxiliary toward the ubiquitylation of peptide targets. By employing an intein fusion to generate a ubiquitin- α -thioester and an auxiliary handle to direct site-specific ubiquitylation at one of three Lys residues in a target peptide, we have essentially mimicked the functions of the E1 and E2/E3 enzymes that activate ubiquitin and catalyze substrate-specific ubiquitylation, respectively. This methodology is also readily adaptable to other UbI proteins such as SUMO. The mild conditions for removal of the auxiliary after ligation do not affect the functional

properties of the final product, which is promising for studies of the downstream effects of protein ubiquitylation. Efforts to extend this methodology to the ubiquitylation of full-length H2B and H2A are currently underway in our laboratories.

Experimental Section

Protein semisynthesis: See Supporting Information for full experimental details. Briefly, Ub(1-75)-MES and HA-SUMO(1-97)-MES thioesters **6** and **9**, respectively, were obtained by intein-mediated cleavage from the respective GyrA-CBD fusion proteins by employing 50 mM MESNa as the thiol nucleophile. The auxiliary-attached H2B C-terminal peptide **5** was synthesized on the solid phase and purified by C18 semipreparative RP-HPLC. Ligation of **6** or **9** with peptide **5** was undertaken in a buffer consisting of 300 mM NaP_i, 50 mM MESNa, 25 mM TCEP, and 3 M Gn-HCl at pH 7.5. Ligations were typically allowed to proceed for 5 days at 4°C and quenched by acidification with TFA. Following reduction with TCEP, the ligation mixtures were purified by RP-HPLC and fractions containing the ligation product **7** or **10** were identified by ESI-MS. Purified ligation products **7** and **10** were photolyzed by UV irradiation at 325 nm to yield ubiquitylated and SUMOylated proteins **8** and **11**, respectively.

Ubiquitin C-terminal hydrolase assay: Purified protein **8** was dissolved in an assay buffer consisting of 50 mM Tris, 150 mM NaCl, and 1 mM DTT at pH 7.5 at 25°C to a final concentration of about 100 µM. Commercially available UCH-L3 (4.5 µg) was pre-reduced for 20 min at 25°C in a buffer containing 50 mM Tris, 150 mM NaCl, and 12 mM DTT at pH 8.0, then **8** (78 µL) was added to the reduced enzyme, and the mixture was incubated for 8 h at 37°C. A similar control assay without enzyme was also conducted. The assays were quenched by acidification with 1 µL TFA, and the products were analyzed by C18 analytical RP-HPLC. A gradient of 0–73% solvent B (B: 90% CH₃CN, 0.1% TFA in H₂O) over 30 min was employed, and Ub was observed by its absorbance at 214 nm (*R*_t = 22.1 min). As a result of its low absorbance at 214 nm, the cH2B peptide was detected by LC-MS employing an LTQ ion-trap mass spectrometer.

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