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Protein Synthesis

Synthesis of L- and D-Ubiquitin by One-Pot Ligation and Metal-Free Desulfurization

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Abstract: Native chemical ligation combined with desulfurization has become a powerful strategy for the chemical synthesis of proteins. Here we describe the use of a new thiol additive, methyl thioglycolate, to accomplish one-pot native chemical ligation and metal-free desulfurization for chemical protein synthesis. This one-pot strategy was used to prepare ubiquitin from two or three peptide segments. Circular dichroism spectroscopy and racemic protein X-ray crystallography confirmed the correct folding of ubiquitin. Our results demonstrate that proteins synthesized chemically by streamlined 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis coupled with a one-pot ligation–desulfurization strategy can supply useful molecules with sufficient purity for crystallographic studies.

Introduction

Both the total and semi-synthesis of proteins can produce homogeneous macromolecules with well-defined structures on the atomic level to pinpoint specific functions of target proteins.[1] Native chemical ligation (NCL) has become one of the leading methodologies in the field of chemical protein synthesis over the past two decades.[2] NCL entails a mild chemoselective Xaa–Cys (Xaa = any amino acid) amide-bond-forming reaction between a C-terminal Xaa thioester and an N-terminal Cys moiety in a neutral buffer at room temperature.[3] In 2001, Yan and Dawson extended the permitted ligation site of NCL from Xaa-Cys to Xaa-Ala by using a metal-assisted reductive desulfurization strategy.[4] Since then this ligation–desulfurization approach[5] has been expanded through the use of N-terminal residues that contain 1,2-aminothiol/selenol[6] or 1,3-aminothiol[7] units. Another important advancement, a highly efficient homogeneous radical desulfurization reaction, was reported by Wan and Danishefsky.[8] This metal-free desulfurization (MFD) reaction occurs in a buffered solution that contains the sulfur scavenger tris(2-carboxyethyl) phosphine (TCEP), radical initiator VA-044, and a hydrogen-donor compound, for example, an alkyl thiol species such as *t*BuSH,[8] glutathione,[6b] or sodium 2-mercaptoethanethiolate (MESNa).[9] This powerful stepwise ligation–desulfurization protocol has been widely employed by the chemical protein synthesis community to produce several challenging protein targets.[10]

To streamline sequential NCL and MFD reactions, a straightforward one-pot operation would be highly desirable to save time and labor costs (Figure 1 A). Unfortunately, the radical MFD process can be quenched by the aryl thiol catalyst[11,12] used for NCL, normally the gold-standard thiol additive 4-mercaptophenylacetic acid (MPAA, $pK_a = 6.6$).[11] Recently several methods, including ether extraction of the aryl thiol[7f] and solid-support capture of the aryl thiol[13] or ligation product,[14] have been used to separate MPAA from the one-pot reaction mixture. Another approach towards a one-pot NCL-MFD reaction is to use a catalytically potent alkyl thiol to replace MPAA. For example, alkyl thiols MESNa ($pK_a = 9.2$),[9] 2,2,2-trifluoroethanethiol (TFET, $pK_a = 7.3$),[15] and mercaptopropionylcysteine[16] have been successfully used to assist NCL. Among them, TFET was found to be as effective as MPAA and enabled one-pot NCL-MFD reactions to proceed in a satisfactory manner.[15] Although this thiol additive has been used in several reported cases,[6l,m,17] TFET is a malodorous compound, which might limit its usage on a larger synthetic scale.

Here we describe a one-pot ligation–desulfurization strategy assisted by a new thiol additive, methyl thioglycolate (MTG, $pK_a = 7.9$)[18] (Figure 1 B). This strategy has four notable features: 1) MTG possesses favorable reaction kinetics that are comparable to MPAA ($\approx$1.8-fold difference), 2) MTG (Sigma Aldrich, USD \$78.8/500 g) is a low-cost reagent, 3) MTG is more polar and has a lower extinction coefficient at $\lambda = 214$ nm than

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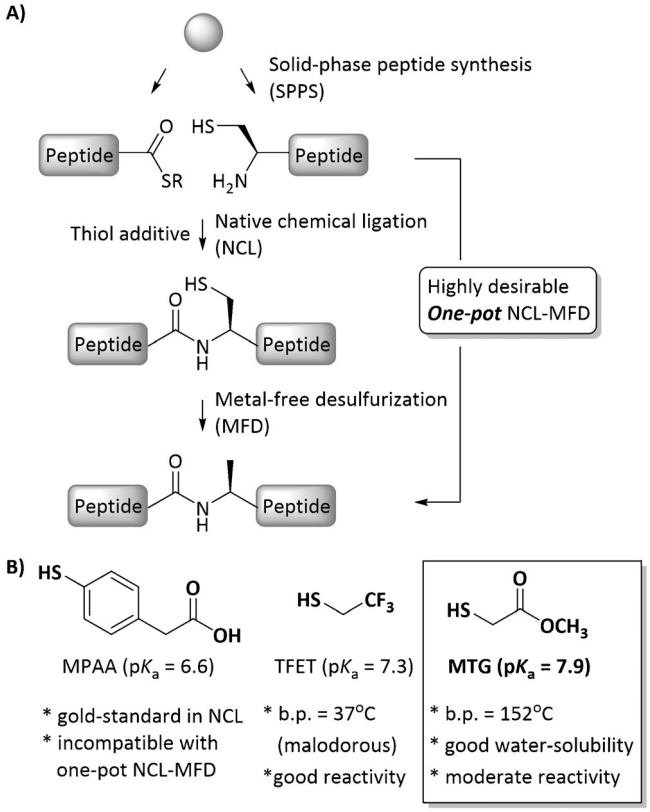


Figure 1. A) Native chemical ligation (NCL) and metal-free desulfurization (MFD); B) comparison of new thiol additive MTG with the gold-standard NCL catalyst MPAA and one-pot ligation–desulfurization additive TFET.

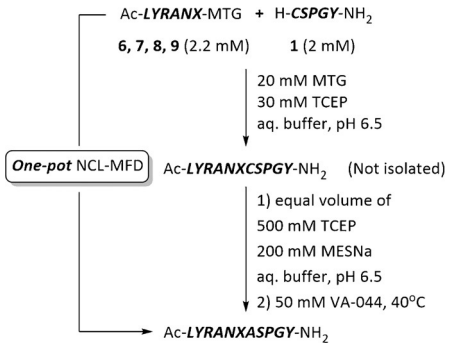
MPAA (it normally shows a small peak at the front of the HPLC profile (see Figures 4 and 5 below), whereas MPAA exhibits a broad peak[15] that interferes when MPAA coelutes with the reaction intermediates),[12a,15] and 4) MTG (b.p. 152 °C) is less volatile and malodorous than TFET (b.p. 37 °C) and has no negative effect on the desulfurization. To demonstrate the efficiency and robustness of our protocol, we synthesized monoubiquitin, a well-known model protein, in a one-pot ligation–desulfurization process on a 30 mg scale. The chemically synthesized monoubiquitin was characterized by high-resolution racemic X-ray crystallography. This synthetic ubiquitin demonstrates that both *tert*-butoxycarbonyl solid-phase peptide synthesis (Boc-SPPS)[21] and 9-fluorenylmethoxycarbonyl (Fmoc) SPPS combined with an NCL-MFD strategy can furnish proteins with sufficient homogeneity for X-ray crystallographic structural biology studies.

Results and Discussion

Our initial aim was to find an alkyl thiol that might become a useful alternative to TFET. We reasoned that the ideal thiol catalyst should: 1) be a good leaving group, 2) have good solubility in aqueous buffer, and 3) show good elution behavior for HPLC analysis (no significant peak overlapping). Because MTG is cheap and has a lower pK_a (7.9) than most alkyl thiols, we decided to assess MTG as the thiol additive for standard NCL reactions (see Figures S1–S4 in the Supporting Information). All reactions were conducted between the model peptide **1** (2 mM), which had an N-terminal Cys residue, and model peptides **2**–**5** (3 mM), which had a C-terminal thioester, in aqueous guanidine hydrochloride (GnHCl; 6 M) and Na_2HPO_4 (100 mM) buffer (pH 6.5) that contained MPAA or MTG (100 mM). Peptide MESNa thioesters were prepared by oxidation–thioesterification[19] of peptide hydrazides (see Scheme S1 in the Supporting Information) obtained by Fmoc SPPS.[20] To our delight, MTG performed almost equally as well as MPAA for ligations at sterically less-hindered residues (Gly, Ser, and Leu). All the reactions essentially finished within 6 h for both MTG and MPAA (see Figure S5 in the Supporting Information). In the case of kinetically less-favored ligation sites, such as Val, MTG was less efficient yet comparable to MPAA (≈1.8-fold kinetic difference). Reaction of the Val-terminated thioester **5** at 37 °C overnight completely consumed **1** in the presence of MPAA or MTG (see Figure S5 in the Supporting Information).

Having demonstrated the satisfactory reaction kinetics of MTG-catalyzed NCL reactions, we tested the one-pot ligation–desulfurization process. First, ligations between N-terminal Cys peptide **1** (2 mM) and a slight excess of peptide thioesters **6**–**9** (2.2 mM) were conducted. HPLC analysis confirmed almost quantitative conversion for all of the ligation steps (see Figures S6–S9 in the Supporting Information). Afterwards, the reaction solution was mixed with an equal volume of GnHCl (6 M) and Na_2HPO_4 (100 mM) desulfurization buffer (pH 6.5) that contained TCEP (500 mM) and MESNa (200 mM). VA-044 (50 mM) was then added as the radical initiator. The desulfurization reaction (pH 6.5) was incubated at 40 °C overnight to ensure complete transformation (Table 1). The peptide products were purified by semipreparative HPLC in good isolated yields (44–69 %).

Table 1. One-pot ligation–desulfurization of MTG thioester peptides **6**–**9** and an N-Cys peptide.



Entry	Peptide thioester	Ligation time [h]	HPLC conversion [%][a]	Isolated yield [%][b]
1	**6**, Ac-***LYRANG***-MTG	1.0	>95	66
2	**7**, Ac-***LYRANS***-MTG	4.0	>95	69
3	**8**, Ac-***LYRANL***-MTG	4.0	>95	67
4	**9**, Ac-***LYRANV***-MTG	8.0	90	44

[a] Conversion estimated by integration of the UV trace. [b] The isolated yield after one-pot NCL-MFD was determined by the weight of the lyophilized product.

Next we used the MTG-assisted one-pot ligation–desulfurization sequence to synthesize monoubiquitin, an important model protein that consists of 76 amino acid residues. Ubiquitin has been successfully synthesized by three-segment ligation,[21] two-segment ligation,[22] and direct Fmoc SPPS.[23] To test the robustness of our one-pot protocol, we adopted a three-segment (1–27+28–45+46–76) approach to assemble full-length ubiquitin. All of the thioester and non-thioester peptides were prepared by air-bath heated Fmoc SPPS.[24] Met1 was replaced by an isosteric norleucine (Nle) residue to prevent methionine oxidation.[10a] Ala28 and Ala46 were mutated to Cys during Fmoc SPPS and were converted back to Ala after desulfurization. For the racemization-prone dipeptide motif Asp52–Gly53,[25] a Dmb (2,4-dimethoxybenzyl) or Hmb (2-hydroxy-4-methoxybenzyl) backbone protecting group was introduced. In our first method, Fmoc-(Dmb)Gly-OH and Fmoc-Asp(O*t*Bu)-OH were coupled as usual (Figure 2A). In another

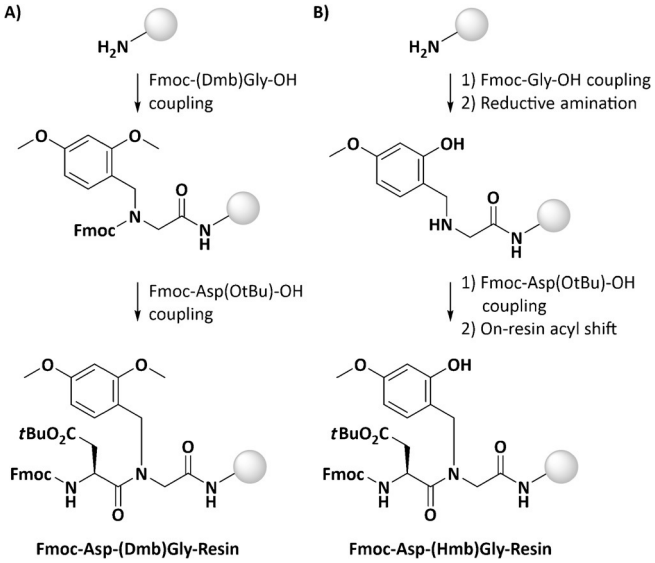


Figure 2. The racemization-prone Asp–Gly dipeptide motif was incorporated into the peptide chain with A) Dmb or B) Hmb backbone protection.

method, Fmoc-Gly-OH was coupled to the resin, then the Hmb group was introduced by reductive amination of the deprotected amine with 2-hydroxy-4-methoxybenzaldehyde. Fmoc-Asp(O*t*Bu)-OH was then coupled onto the phenol hydroxyl substituent of Hmb and transferred to the secondary amine by an on-resin six-membered-ring acyl shift (Figure 2B). Both methods worked well to overcome the formation of aspartimide[25] during Fmoc SPPS according to LC-MS analysis.

For the three-segment iterative NCL-MFD approach, the key operation was the release of protected N-terminal Cys in the middle segment. Thz (Figure 3), the most-popular N-terminal Cys protecting group, comes from the commercially available Boc- or Fmoc-protected 1,3-thiazolidine-4-carboxylic acid.[26] Unfortunately, we and others found that Thz was unstable under the hydrazide oxidation conditions, and Pentelute's group reported that an unproductive nitric oxide adduct might form.[27] Although several N-terminal Cys protecting

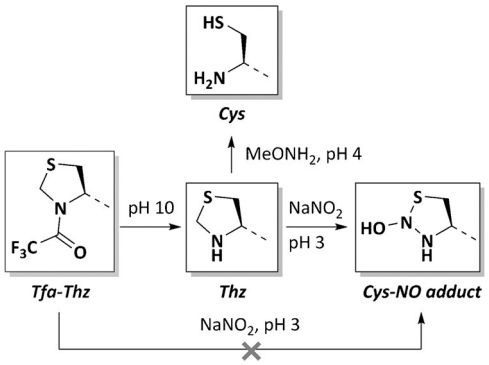


Figure 3. A new protecting group Tfa-Thz undergoes mild two-step deprotection to release Thz and N-terminal Cys.

groups have been reported to replace Thz, some of them are unstable to TCEP (a routine reductant in NCL),[28,29] whereas others require long multistep syntheses.[30,31] Luckily, we noticed that trifluoroacetyl (Tfa) protected primary or secondary amines could be mildly deprotected by treatment with aqueous base.[32] Therefore, Tfa-Thz (Figure 3) could replace Thz during the hydrazide oxidation.

First we synthesized Tfa-Thz-OH (3 g scale) in one step (see Scheme S2 in the Supporting Information). Tfa-Thz-OH was then incorporated into the middle peptide segment UbM (Tfa-Thz28~Phe45-$NHNH_2$) by *N*,*N*′-diisopropylcarbodiimide (DIC)/Oxyma coupling.[33] Next, we prepared the N-terminal segment UbN (H-Nle1~Lys27-$NHNH_2$) and the C-terminal segment UbC (H-Cys46~Gly76-NH_2). The UbN and UbM hydrazides were transformed into their respective MTG thioesters then purified. In the first step of the one-pot three-segment ligation, UbM MTG thioester (2.1 mM) was incubated with the UbC fragment (2.0 mM) in GnHCl (6 M) and Na_2HPO_4 (100 mM) ligation buffer (pH 6.5) that contained MTG (20 mM) and TCEP (30 mM). After 4 h, the first ligation was complete and partial Tfa deprotection was observed by analytical HPLC. Clean Tfa deprotection (pH 10.0, 30 min) followed by Thz deprotection (pH 4.0, 2 h) was performed by addition of methyoxyamine (0.2 M) to give the UbM+C peptide. The second ligation was initiated at pH 6.5 by the addition of the UbN MTG thioester (2.2 mM) and pH re-adjustment. After 16 h, an equal volume of desulfurization buffer that contained TCEP (500 mM), MESNa (200 mM), and VA-044 (40 mM) was poured into the ligation buffer (final pH = 6.5). The reaction mixture was incubated at 40 °C for 12 h. HPLC and ESI-MS analysis indicated complete reaction to afford full-length ubiquitin (Figure 4). However, in our hands, semipreparative HPLC purification was complicated by overlap of the UbN hydrolysis peak (≈10% based on the peak-area ratio from the analytical HPLC data) with the product peak. Therefore, unfortunately this three-segment assembly strategy had some practical difficulties at the purification stage.

To overcome this problem, we tested a two-segment approach with UbN+M hydrazide (H-Nle1~Phe45-$NHNH_2$) and UbC (H-Cys46~Gly76-NH_2) as the starting materials. The UbN+M hydrazide was first converted to the UbN+M MTG thioester. The purified thioester (1.0 mM) was then treated with UbC (1.0 mM) in the ligation buffer. After 12 h the reac-

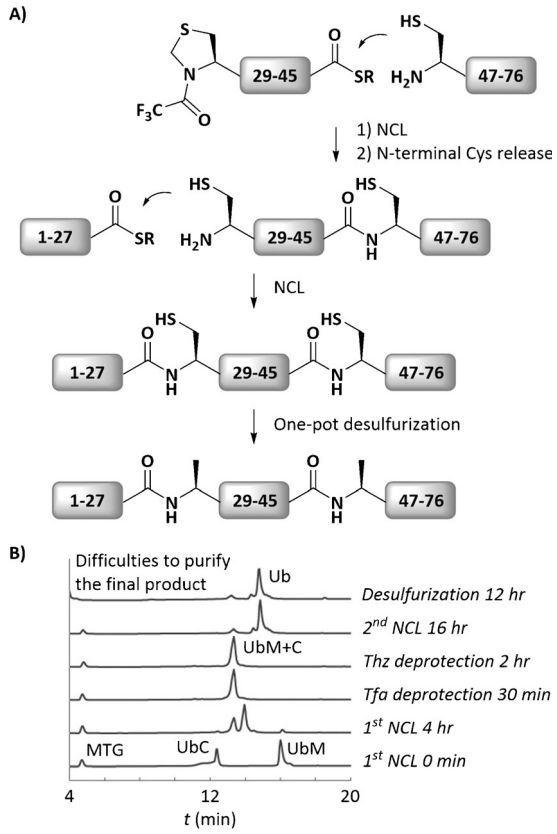


Figure 4. A) A one-pot three-segment ligation–desulfurization approach to ubiquitin (RSH = MTG); B) monitoring the reaction by HPLC.

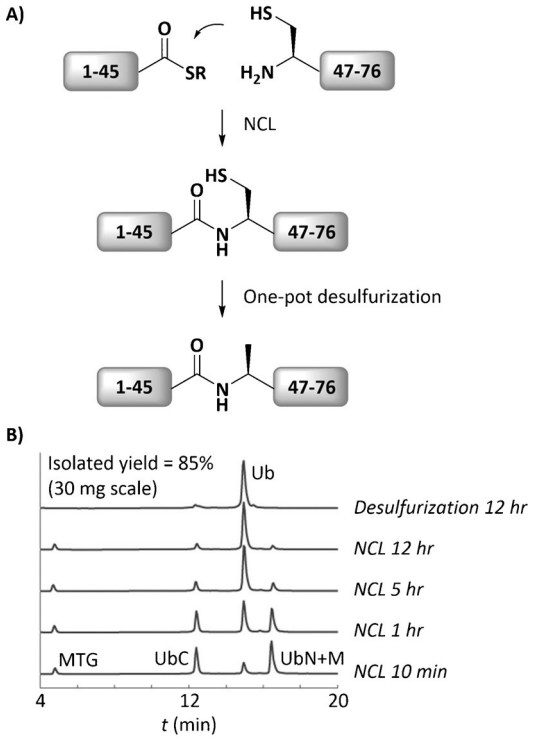


Figure 5. A) A one-pot two-segment ligation–desulfurization approach to ubiquitin (RSH = MTG); B) monitoring the reaction by HLPC.

tion mixture was subjected to the desulfurization protocol described above (Figure 5). Semipreparative HPLC purification afforded full-length ubiquitin in an excellent isolated yield of 85% (30 mg scale).

By using the same one-pot NCL-MFD protocol, we completed the one-pot total synthesis of D-ubiquitin. Synthetic L- and D-ubiquitin were both characterized by HPLC and direct-injection ESI-MS (Figure 6A). Spontaneous folding was conducted by directly dissolving lyophilized ubiquitin in aqueous buffer or double-distilled water (ddH_2O). The circular dichroism (CD) spectra of L- and D-ubiquitin were compared to that of expressed L-ubiquitin and confirmed the well-folded secondary structure in ddH_2O (0.8 mg mL^{-1}) (Figure 6B). To further characterize the homogeneity of our synthetic products, we employed racemic X-ray crystallography, which was pioneered by Yeates and Kent.[34] Lyophilized L- and D-ubiquitin were both dissolved in Tris buffer (10 mM; pH 8.0) to obtain the protein solution (8 mg mL^{-1}) as determined by UV A280 (Nanodrop). Crystallization trials with L- or D-ubiquitin alone were unsuccessful at low protein concentration (< 20 mg mL^{-1}), with or without $CdCl_2$ as a precipitating additive, for one month.[21] This was consistent with previous observations that direct crystallization of monoubiquitin was difficult without seeding.[21] Crystals were obtained overnight at 18 °C under almost half of the conditions when L- and D-ubiquitin were mixed and underwent crystallization screening with commercially available Hampton Research PEG/Ion Screen in the presence of $CdCl_2$.

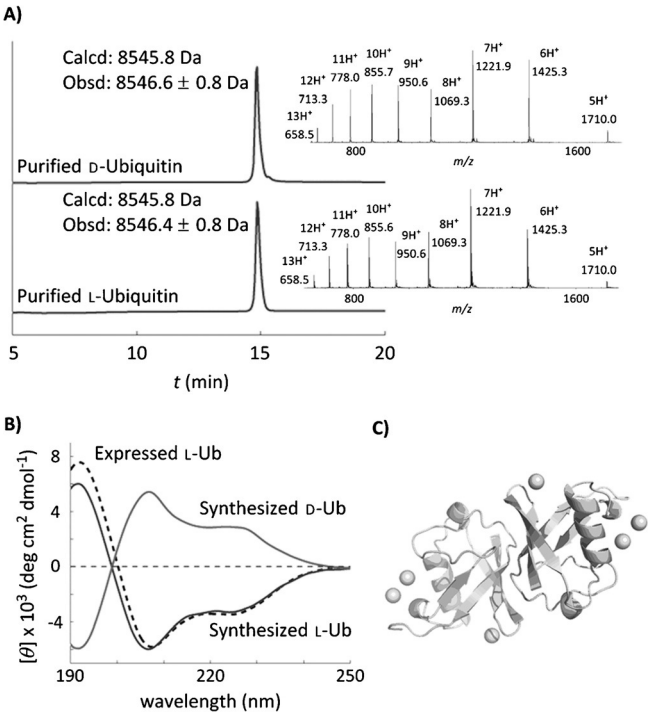


Figure 6. A) Characterization of the synthetic ubiquitins by analytical HPLC and direct-injection ESI-MS; B) CD spectra of the synthetic and expressed ubiquitins; C) representation of racemic L-ubiquitin and D-ubiquitin (see the Supporting Information (Figure S46) for a colored version of the image).

Diffraction-quality single crystals could be reproducibly gathered by mixing the racemic protein solution (1 μL; L-ubiquitin

(4 mg mL^{-1})/D-ubiquitin (4 mg mL^{-1})/Tris buffer (10 mM)/$CdCl_2$ (20 mM); pH 8.0) and well solution (1 μL; lithium acetate dihydrate (0.2 M)/polyethylene glycol 3350 (20% *w/v*); pH 7.9). X-ray diffraction data were collected on the Tsinghua Protein X-ray Crystallography Platform. The structure of synthetic ubiquitin was solved to 1.95 Å by using a molecular replacement method with the structure of extracted ubiquitin (PDB:1UBQ) as the search model. As anticipated, the synthetic ubiquitin had the same molecular shape as previously reported, which includes an α-helix and five β-strands (Figure 6C).[35]

Conclusion

We have developed an efficient strategy for protein synthesis that relies on one-pot NCL-MFD. Key to this technical advance is the use of MTG as the thiol additive for the ligation process. MTG is easily accessible and displays sufficient reactivity. Our streamlined synthesis of ubiquitin (85% isolated yield, 30 mg scale) demonstrates that this strategy has the potential to efficiently generate large-scale high-quality proteins. Racemic X-ray crystallography was employed to characterize the synthetic ubiquitin. We hope that the utility of this one-pot NCL-MFD approach can be demonstrated in the chemical synthesis of post-translational modified proteins[30,36] (e.g., glycosylated proteins,[37] lipidated proteins,[38] ubiquitinated proteins,[39] and histone derivatives)[40] in future studies.

Experimental Section

Air-bath heated SPPS

Screw-cap glass SPPS reaction vessels were obtained from commercial sources. The SPPS glassware was immobilized on a rotary shaker (130 rpm) during the reaction.

Fmoc-protected Rink amide AM or hydrazide resins were swollen in 1:1 CH_2Cl_2/DMF for 10 min before use.

Fmoc deprotection was conducted by treatment with 20% piperidine in DMF (containing 0.1 M Oxyma) twice (1 min + 9 min). The resin was washed with DMF (×5), CH_2Cl_2 (×5), and then DMF (×5).

Arg coupling was initiated by pouring a preactivated (30 s at RT) solution of Fmoc-Arg(Pbf)-OH (0.1 M in DMF; 4 equiv), *O*-(6-chlorobenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HCTU; 3.6 equiv), and *N*,*N*-diisopropylethylamine (DIEA; 8 equiv) onto the resin. The reaction mixture was kept at RT for 45 min. The coupling of amino acids other than Arg was initiated by pouring a preactivated (5 min at RT) solution of protected amino acid (0.1 M in DMF; 4 equiv) and *N*,*N*′-diisopropylcarbodiimide (DIC; 4 equiv) in Oxyma (4 equiv) onto the resin. The His(Trt) coupling was kept at RT for 45 min. The Cys(Trt) coupling was kept at 50 °C (air-bath heating) for 20 min. Other residue couplings were kept at 75 °C (air-bath heating) for 20 min.

At the end of the SPPS, the peptide was cleaved from the resin with a modified Reagent K: H_2O (5% v/v), thioanisole (5% v/v), and 1,2-ethanedithiol (2.5% v/v) in trifluoroacetic acid (TFA). The combined TFA eluents were concentrated under a flow of nitrogen gas. The crude peptides were obtained by precipitation with ice-cold ether and centrifugation (5000 rpm, 4 °C, 2 min). The peptide pellet was dissolved in 1:1 CH_3CN/H_2O that contained TFA (0.1%) and was characterized by analytical HPLC and ESI-MS. The peptide was purified, if necessary, by semipreparative HPLC then lyophilized.

One-pot ligation-desulfurization protocol

Ac-***LYRANX***-MTG (2.2 mM; X = G, S, L, V) and H-***CSPGY***-NH_2 (2 mM) were dissolved in GnHCl (6 M) and Na_2HPO_4 ligation buffer (100 mM) that contained MTG (20 mM) and TCEP (30 mM). The mixture was adjusted to pH 6.5 and incubated at RT. When the ligation was complete, the reaction mixture was combined with an equal volume of ligation buffer that contained TCEP (500 mM) and MESNa (200 mM). The mixture was adjusted to pH 6.5. After blending VA-044 (50 mM) was added to the mixture as radical initiator. The desulfurization reaction was incubated at 40 °C overnight to ensure complete transformation. The desulfurization products were purified by semipreparative HPLC then lyophilized.

Acknowledgements

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Keywords: desulfurization · methyl thioglycolate · native chemical ligation · proteins · X-ray diffraction

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