

Total Chemical Synthesis of ISGylated-Ubiquitin Hybrid Chain Assisted by Acetamidomethyl Derivatives with Dual Functions

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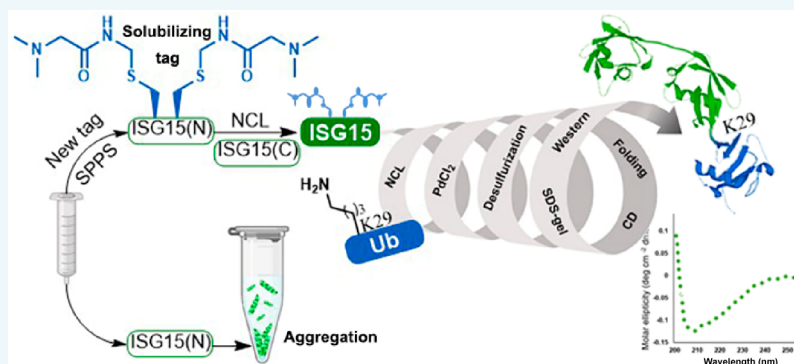
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ABSTRACT: Interferon-stimulated gene 15 (ISG15) is a member of the ubiquitin-like modifiers (ULM) family, which adopts a β -grasp fold domain(s) similar to ubiquitin (Ub) with only minor sequence homology. ISG15 consists of two Ub-like domains and aids the immune system in neutralizing infections by numerous pathogens and plays an important role in defending cells against many viruses including influenza A. Recently, Ub was found to be a substrate for ISG15, which can be ISGylated on Lys29 and Lys48, while the former is more dominant. The discovery of such hybrid ISG15-Ub chains brought forward various fundamental questions regarding the nature and effect of this conjugation. To further investigate the role of hybrid ISG15-Ub chains, the pure homogeneous material of these chains is needed in workable quantities. By applying advanced chemical strategies for protein synthesis, we report the total chemical synthesis of a 231-residue ISG15-Lys29-Ub hybrid chain. During the synthesis we encountered insoluble peptide fragments, and therefore we developed a new reversible AcM based solubilizing tag to efficiently tackle this hurdle. This new AcM tag was compared with the known Arg based AcM solubilizing tag and was found to be more reliable in terms of incorporation and efficiency as demonstrated in the synthesis of the native ISG15-Ub hybrid chain.

INTRODUCTION

Ubiquitin (Ub) is a 76 amino acid protein involved in many cellular events, e.g., trafficking, transcription, and proteasome degradation.^{1,2} The covalent attachment of Ub to a Lys residue of a target protein requires three enzymes, E1, E2, and E3, and is known as ubiquitination.³ Repeating this cycle on an ubiquitinated protein produces multiubiquitination either on different Lys residues or on the same site, eventually leading to different cellular signals.^{4,5} On the other hand, ubiquitin-like modifiers (Ubls) are a family of proteins similar in structure to Ub, yet different in sequence. This family includes SUMO 1/2/3/4 (small ubiquitin-like modifier), NEDD8 (neural precursor cell expressed developmentally downregulated protein 8), and ISG15 (interferon-stimulated gene 15).⁶ ISG15 has a similar structure to di-Ub, with two domains connected by a short linker.⁷ Additionally, the covalent attachment of ISG15 to a Lys residue of proteins (ISGylation) demands its own set of three enzymes E1, E2, and E3.^{8,9}

ISGylation is considered an essential element for neutralizing infections by numerous pathogens including influenza and

coronavirus.^{8,10,11} Moreover, ISGylation is involved in various other cellular functions including inhibiting proteasome-mediated degradation and protein translation; it controls actin cytoskeleton dynamics and is involved in translesion DNA synthesis.¹² Subsequently, researchers are interested in studying ISG15 to acquire deeper insight on the ISG15 role in numerous cellular processes. For instance, what is the fate of ISGylated protein compared to diubiquitinated proteins? And the effect of protein ISGylation on protein stability is unknown. Specifically, the role of ISGylated proteins and ISG15 in cancer development is also not yet understood.

Recently, a hybrid chain between Ub and ISG15 was discovered, where Ub was found to be a substrate for ISG15, which can be ISGylated at two sites, Lys29 and Lys48.¹³

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Furthermore, the study found that Lys29 was the major site for ISGylation, and this surprising interplay between the two post translational modifications (PTMs) can play a role in regulating the turnover of ubiquitinated proteins. However, the crosstalk between these two similar but distinct PTMs is yet to be investigated.

Since it is hard to isolate a specific type of this hybrid chain in sufficient quantities from the heterogeneous pool of ISGylated-Ub chains, there is demand for producing sufficient homogeneous quantities of ISG15 and ISG15-Ub hybrid chains. To our best knowledge, there are no reports for the chemical synthesis or semisynthesis of ISG15-Ub hybrid chains. Producing ISG15 has been done only by expression,^{14,15} which also turned out to be challenging especially in handling the protein where mutations had to be introduced to enable their expression and perform biological studies with the protein.⁷

Herein, we describe our route toward the chemical synthesis of ISG15-Ub hybrid chains. Despite the structural similarities of ISG15 to Ub, the synthesis proved to be extremely challenging. Our initial efforts toward the synthesis of the target protein were hampered by its hydrophobic nature and high tendency for aggregation. These factors heavily affected SPPS leading to deletions and eventually complete termination of the growing peptide chain. In order to overcome all the synthetic difficulties, state of the art chemical methods and strategies have been employed.¹⁶ A key to our success was the development and application of a new Acm derivative to serve for cysteine protection and increase the peptide solubility.

RESULTS AND DISCUSSION

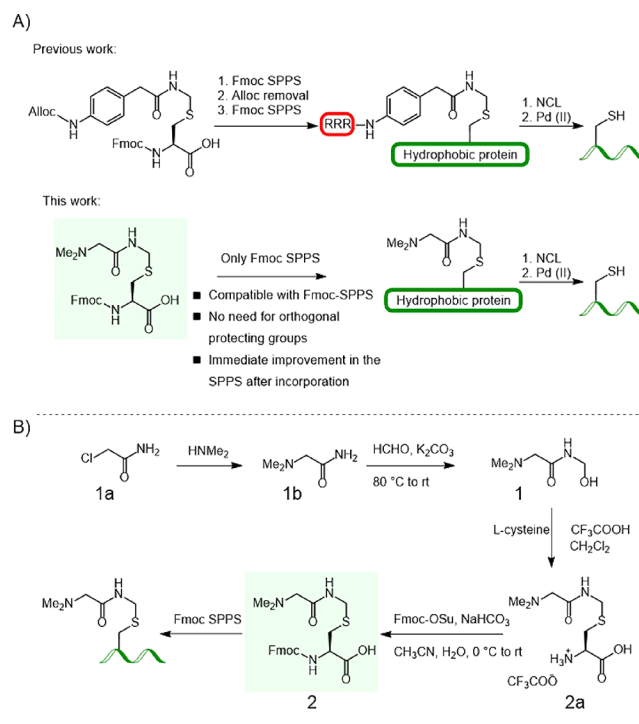
We envisioned the total chemical synthesis of the ISG15-Lys29-Ub protein equipped with a biotin tag, providing a useful tool for further studies. After analyzing the sequence of ISG15, made of 157 residues, we initially decided to divide it into three shorter peptide fragments, which can be prepared by Fmoc-SPPS. The synthesis of the second and third fragments (ISG15(29–59) and ISG15(60–165), Figures S1 and S4) proceeded without complications. We did not encounter problems in the synthesis or in the following purification step. Contrary to this, in the synthesis of the first fragment, ISG15(2–28), we found that after coupling the first 14 amino acids, the fragment was poorly soluble and the HPLC showed broad, tailing peaks, without a major peak that corresponded to the product. On the basis of our previous experiences in the synthesis of difficult proteins, installing a side chain solubilizing tag (e.g., attached through a phenylacetamidomethyl (Phacm) linker to a Cys residue) could significantly improve protein synthesis.^{17–19} Upon introduction of this solubilizing tag onto the temporary Ala11Cys mutation, we were glad to see that the synthesis of this fragment has improved, but to our disappointment, we found that this fragment (even with the solubilizing tag) was tailing in the column, rendering its separation and purification very challenging and its final isolation in a very low yield.

To overcome this issue, we thought to introduce a second solubilizing tag to further improve the solubility and reduce the tailing effect. However, since there are no additional positions suitable for its installation, we combined the first and second fragments and introduced a second solubilizing tag at the Ala40Cys temporary mutation site. The second solubilizing tag somewhat improved the purification step, but the SPPS itself still suffered from deletion and termination of the growing

peptide chain possibly due to aggregation on the polymer support (Figure S2), eventually leading to low yielding synthesis. Unfortunately, this issue cannot be strategically addressed as the beneficial effect of the used solubilizing tags materializes after the SPPS is completed but not during synthesis. This encouraged us to design a new solubilizing tag, which upon its installation would have an immediate effect on the solubility of the hydrophobic fragment, leading to a better tracking of the different coupling steps.

We designed a novel solubilizing-protecting group based on the widely used Acm group (Scheme 1A) modified with

Scheme 1. (A) Attachment of the Novel Acm-NMe₂ Solubilizing Group to Hydrophobic Peptides and (B) Preparation of Acm-NMe₂ Protected Fmoc-Cysteine Amino Acid



dimethylated amine moiety (Acm-NMe₂). The positively charged tertiary amino groups can simultaneously improve SPPS by lowering the tendency for aggregation on solid support and later in solution phase as well. Furthermore, it can positively contribute to the solubility of the peptide fragments and the later intermediates along the synthesis. The major advantage of this novel protecting group, compared to the previously reported side chain solubilizing groups,²⁰ is the immediate observed positive effect on the SPPS upon incorporation. Furthermore, the side chain deprotection and coupling steps of the Arg tag can be avoided. Finally, the Acm-NMe₂ group is less sterically hindered than the Phacm based solubilizing tag; thus the subsequent coupling cycles are expected to be more efficient.

We based the synthesis of the Fmoc-Cys(Acm-NMe₂)-OH building block on the inexpensive chloroacetamide that was reacted in the first step with dimethylamine and subsequently in the next step with formaldehyde, to provide the key intermediate **1** (Scheme 1B). This was attached to L-Cys analogously to the standard Acm protecting group as in the first reported synthesis.²¹ Finally, the Fmoc protecting group

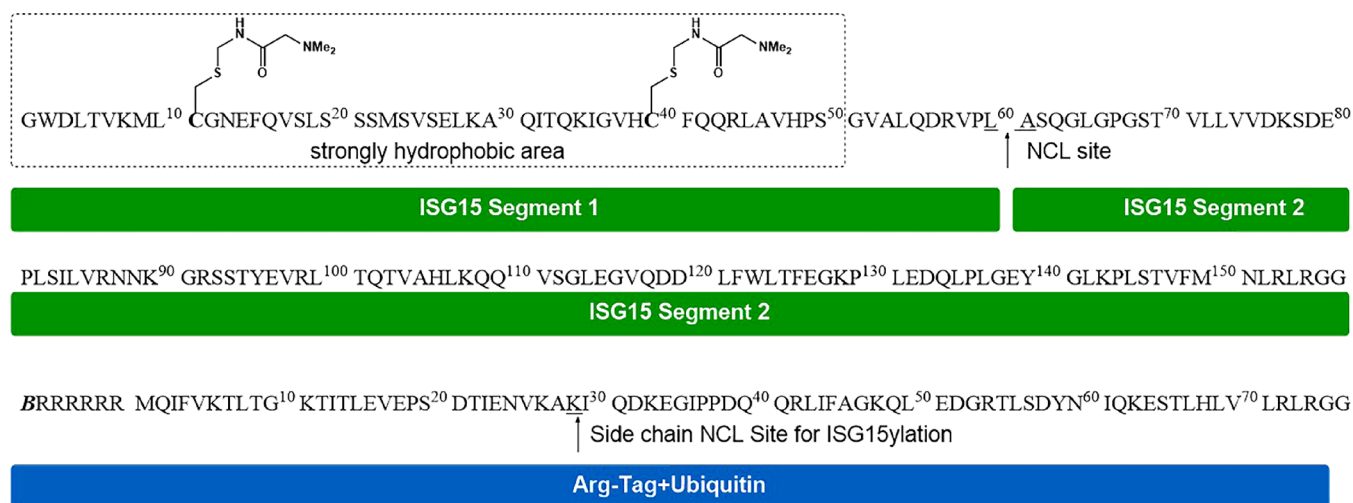


Figure 1. Synthesis plan toward ISGylated ubiquitin. The methionine residues are mutated to norleucine. B stands for biotin.

was installed, which gave upon purification the Fmoc-Cys(Acm-NMe₂)-OH **2** (Scheme 1B), in four steps with 59% overall yield (Scheme 1B).

With the newly developed Cys building block in hand we decided to assemble the ISG15 from two peptide segments obtained by Fmoc-SPPS employing NCL and ligate eventually the full length ISG15 onto the side chain of Lys29 of the Ub via a second NCL, utilizing the δ -mercaptolysine moiety (Figure 1).²² In order to address the extreme hydrophobicity of the N-terminal region of the ISG15, we decided to incorporate two Cys residues (both equipped with Acm-NMe₂ groups) instead of Ala11 and Ala41. These can be converted back to the native Ala residue upon palladium mediated deprotection and subsequent desulfurization.^{23,24} The ISG15(61–157) **2** (Figure 2A) could be prepared by standard Fmoc-SPPS without any major difficulty (Figure S4).

Examining the reported cases for the expression of ISG15, it is established that Cys78 significantly contributes to the precipitation of the full ISG15 protein.^{7,25} Subsequently, we decided to introduce the Cys78Ser mutation in order to avoid the formation of the dimer form of the protein. This mutation is located in the loop region, connecting the two Ub like domains of ISG15; thus no impact on the biological activity is anticipated. The δ -mercaptolysine containing Ub was prepared according the previously reported procedures.²⁶

The incorporation of the new solubilizing tags enabled the synthesis of the challenging ISG15(2–60) **3** (Figure 2A, Figure S3) on multimilligram scale. The peptide was synthesized on hydrazide substituted Trt resin, and after resin cleavage the crude peptide was subsequently converted with 4-mercaptomethylacetic acid (MPAA) to the corresponding thioester according to a modified variant of the recently reported conditions by the Dawson group.²⁷ The second fragment of ISG15, **4** (Figure 2A), could be prepared without any synthetic difficulty bearing C-terminal hydrazide, using HCTU/HATU coupling reagents in NMP.²⁸

With sufficient amounts of the ISG15 fragments in hand, we proceeded to the first ligation (Figure 2A). During our initial trials, we observed that despite using standard 6 M guanidine hydrochloride (Gn·HCl) buffer (pH 7.2) with tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) and MPAA, the peptide fragments and the ligated product were very prone to aggregation and gel formation. This led to slow

reaction time and to inhomogeneous concentration of the reactants in the ligation mixture. We found that lowering the protein concentration to 1.7 mM and adding boric acid (25 mM) significantly improved the solubility and minimized the gel formation. Unfortunately, the ligated protein, ISG15(2–157) **5a** (Figure 2A), eluted at the same retention time as fragment **4**. In order to ensure full conversion, we used a substoichiometric amount of cysteine-fragment **4** and left the ligation to proceed overnight at 42 °C. After no more cysteine-fragment **4** could be observed by ESI-MS, the ligated protein ISG15(2–157) **5a**, with C-terminal hydrazide, was in situ transformed to the MPAA thioester by the addition of acetyl acetone (acac), TCEP·HCl, and MPAA, containing Gn·HCl buffer (pH 3), and overnight incubation at room temperature.²⁷ The full length ISG15 thioester protein **5b** (Figure 2A) was purified on semipreparative HPLC and gave the purified product in 28% isolated yield for one-pot NCL and thioester conversion.

With the ISG15 thioester **5b** in hand we proceeded to ligate it onto the Ub variant bearing δ -mercaptolysine (Lys29), and equipped with a biotin tag at it is the N-terminus, **6** (Figure 2A). In our preliminary study, we observed extremely poor recovery from HPLC and very low solubility even in 6 M Gn·HCl buffer of the final, 231 amino acid long ISG15-Ub protein. Thus, we decided to incorporate further charged residues (6 Arg moieties) between the N-terminus of the Ub protein and the biotin tag. The ligation between the ISG15 thioester, **5b**, and the Ub, **6**, was completed after 5 h. Despite the good conversion of the protein fragments to the ligated product, according the analytical HPLC, the full-length protein **7** (Figure 2A) could be isolated only with 27% yield. We reasoned that the moderate yield could be attributed to significant losses during purification, due to the hydrophobic nature of the protein which leads to tailing and sticking in the column resulting in the loss of material.

Next, we proceeded to remove the Acm-NMe₂ protecting groups from the ISGylated Ub and transform the Cys residues to the native alanine moieties by radical desulfurization in a one pot manner. The removal of the Acm-NMe₂ solubilizing groups drastically worsened the solubility properties of the protein and increased its hydrophobicity. The product could only be isolated on an analytical HPLC instrument using a heated C4 column. After the deprotection of the Cys residues

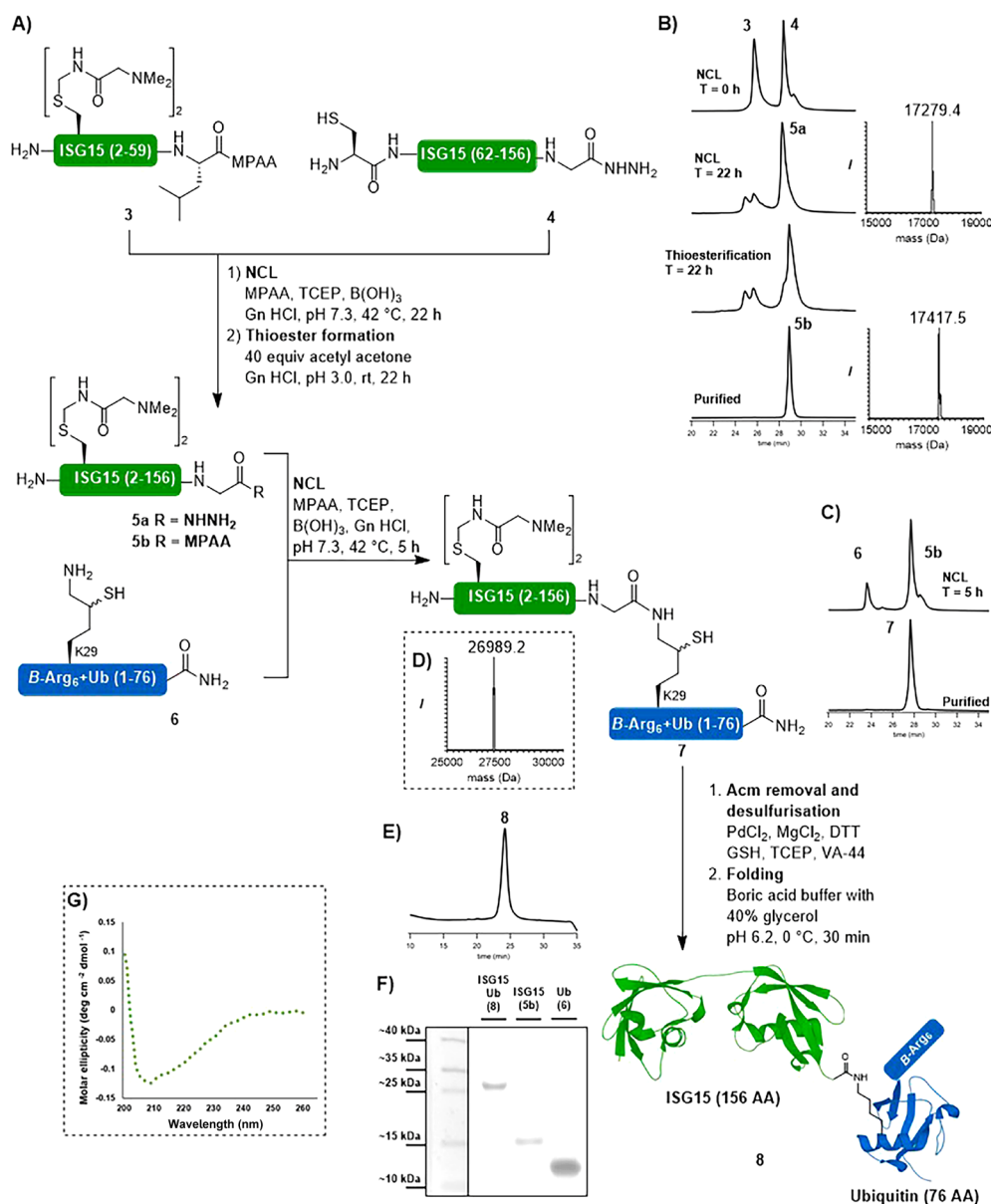


Figure 2. (A) Synthesis of ISGylated ubiquitin variant. (B) HPLC and ESI-MS traces following of NCL between the ISG segments. (C) HPLC following of the isopeptide chemical ligation between the full length ISG15 (5b) and Ub (6). (D) ESI-MS trace of peptide 7. (E) HPLC trace of the final product 8. (F) SDS-PAGE/comassie staining showing the bands of the Ub (6) and ISG15 (5b) intermediates and final ISGylated Ub (8). (G) CD spectra of the folded protein. B stands for biotin, and Arg6 stands for 6xArg tag.

and the desulfurization steps, all our efforts to obtain ESI-MS spectra failed. Similar phenomena were reported in cases of some highly hydrophobic proteins.^{29,30}

The isolated final protein was analyzed on SDS-PAGE with the Ub fragment, 6, and the full ISG15-thioester, 5b (Figure 2F). The SDS gel showed that the isolated final protein has a mass that ranged between 25 and 35 kDa, which corresponds to our final ISG15-Ub hybrid chain molecular weight. Moreover, we did not identify any band that corresponds to the presence of the Ub or ISG15 fragments along with the isolated final protein. Consequently, we performed Western blotting against both anti-Ub and anti-ISG15 antibody to further provide evidence for the purity of the final protein and show the recognition of antibodies toward our synthesized ISG15-Lys29-Ub hybrid chain (Figure S8).

In order to prove the identity of the final protein, we performed sequence analysis by tryptic digestion and LC-MS/

MS analysis of the digested fragments (Figure 3). This procedure gave 81.51% coverage of the full protein. The purity of the protein was confirmed by analytical HPLC (Figure 2E).

With the full-length ISGylated Ub in hand, we proceeded to the folding step. First, the folding precursor was dissolved in 6 M Gn·HCl buffer (typically 0.1 mg in 2 μ L of buffer) at pH 6.2 and kept at 37 °C for 1 h in order to fully denature the protein. The folding experiments were performed using rapid dilution as well as slow dialysis. Several buffers were tested (phosphate buffers with various additives, at different pH values), but the ISGylated Ub rapidly precipitated from all the buffers (Supporting Information). Subsequently, we found that 40 v/v % glycerol is necessary to stabilize the protein in solution. Furthermore, the addition of boric acid and low amount of Gn·HCl into the folding buffer is also required to minimize the protein's tendency for aggregation. Finally, the protein was folded in 25 mM boric acid buffer with 0.6 M Gn·HCl at pH

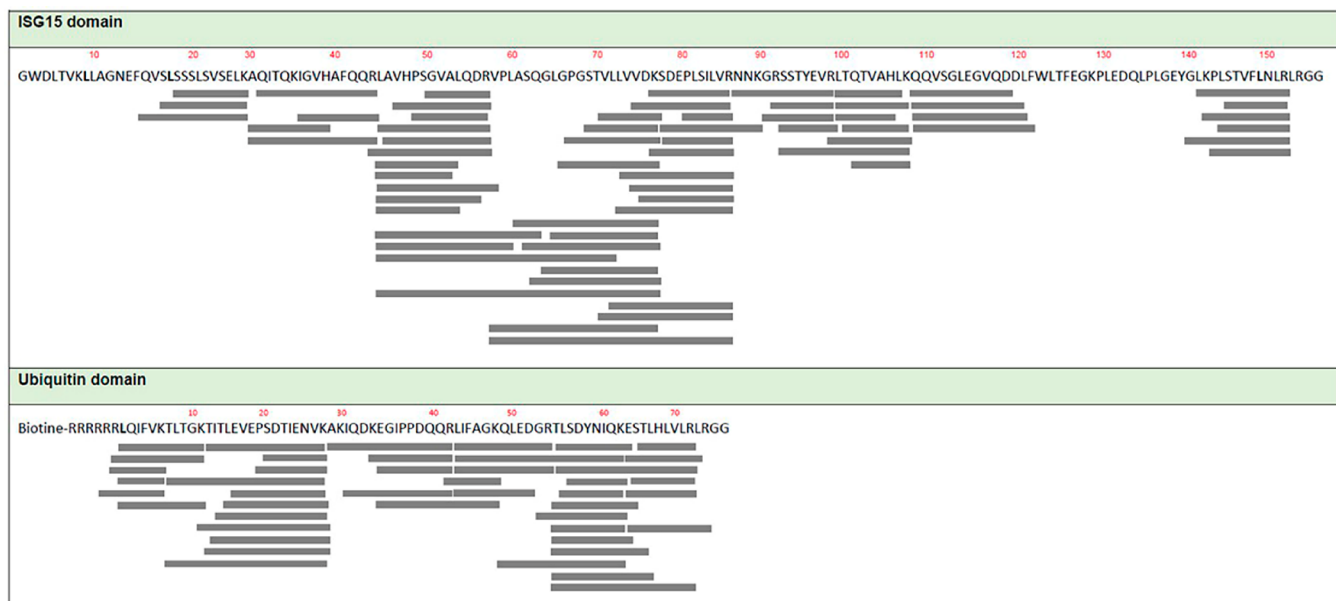


Figure 3. Tryptic digestion and LC–MS/MS analysis show 81.51% coverage of the ISGylated Ub sequence. The gray bars represent the detected peptide fragments after digestion. L stands for the methionine residues that were mutated to norleucine.

6.2 containing 40 v/v % glycerol at 0 °C for 30 min. Despite all the additives, some precipitation was observed. This was separated by centrifugation. The CD spectra were recorded from the folding buffer. To our best knowledge no CD spectra of ISGylated Ub have been reported yet. Expressed ISG15 (Cys78Ser) was characterized by CD to show one distinct minimum at 208 nm, similar to our recorded spectra (Figure 2 G).⁷

CONCLUSIONS

In this study, we described the first, total chemical synthesis of ISG15-Lys29-Ub hybrid chain. During the attempted synthesis we encountered major handling and purification problems. The key to successfully overcome these challenges was the development and incorporation of our novel Acm based solubilizing-protecting group (Acm-NMe₂). This synthetic handle aided the SPPS, the assembly of the peptide fragments, and the HPLC purification steps along the protein synthesis. The final ISG15-Lys29-Ub hybrid chain was characterized by trypsin digestion, SDS–gel, and Western blotting, and then the CD measurement of the folded native hybrid chain was obtained. The current study and our new Acm solubilizing tag establish a straightforward platform for generating a range of ISGylated Ub variants and open new horizons for studying the role of the hybrid chains. Moreover, the characterization, folding, and synthetic lessons learned here could assist in the synthesis of other challenging proteins.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.bioconjchem.0c00026>.

Experimental methods, HPLC and mass spectrometry analyses of synthetic peptides and proteins, folding procedure, Western blots, and ¹H NMR and ¹³C NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Spasser, L., and Brik, A. (2012) Chemistry and biology of the ubiquitin signal. *Angew. Chem., Int. Ed.* 51, 6840.
- (2) Komander, D., and Rape, M. (2012) The ubiquitin code. *Annu. Rev. Biochem.* 81, 203.

- (3) Pickart, C. M. (2001) Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* 70, 503.
- (4) Gopinath, P., Ohayon, S., Nawatha, M., and Brik, A. (2016) Chemical and semisynthetic approaches to study and target deubiquitinases. *Chem. Soc. Rev.* 45, 4171.
- (5) Swatek, K. N., and Komander, D. (2016) Ubiquitin modifications. *Cell Res.* 26, 399.
- (6) Cappadocia, L., and Lima, C. D. (2018) Ubiquitin-like protein conjugation: structures, chemistry, and mechanism. *Chem. Rev.* 118, 889.
- (7) Narasimhan, J., Wang, M., Fu, Z., Klein, J. M., Haas, A. L., and Kim, J. J. (2005) Crystal structure of the interferon-induced ubiquitin-like protein ISG15. *J. Biol. Chem.* 280, 27356.
- (8) Zhang, D., and Zhang, D. E. (2011) Interferon-stimulated gene 15 and the protein ISGylation system. *J. Interferon Cytokine Res.* 31, 119.
- (9) Skaug, B., and Chen, Z. (2010) Emerging role of ISG15 in antiviral immunity. *Cell* 143, 187.
- (10) Morales, D. J., and Lenschow, D. J. (2013) The antiviral activities of ISG15. *J. Mol. Biol.* 425, 4995.
- (11) Lenschow, D. J., Lai, C., Frias-Staheli, N., Giannakopoulos, N. V., Lutz, A., Wolff, T., Osiak, A., Levine, B., Schmidt, R. E., Garcia-Sastre, A., Leib, D. A., Pekosz, A., Knobeloch, K. P., Horak, I., and Virgin, H. W. t. (2007) IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. *Proc. Natl. Acad. Sci. U. S. A.* 104, 1371.
- (12) Villarroya-Beltri, C., Guerra, S., and Sanchez-Madrid, F. (2017) ISGylation—a key to lock the cell gates for preventing the spread of threats. *J. Cell Sci.* 130, 2961.
- (13) Fan, J. B., Arimoto, K., Motamedchaboki, K., Yan, M., Wolf, D. A., and Zhang, D. E. (2015) Identification and characterization of a novel ISG15-ubiquitin mixed chain and its role in regulating protein homeostasis. *Sci. Rep.* 5, 12704.
- (14) D'Cunha, J., Knight, E., Jr., Haas, A. L., Truitt, R. L., and Borden, E. C. (1996) Immunoregulatory properties of ISG15, an interferon-induced cytokine. *Proc. Natl. Acad. Sci. U. S. A.* 93, 211.
- (15) Zhao, C., Denison, C., Huibregtse, J. M., Gygi, S., and Krug, R. M. (2005) Human ISG15 conjugation targets both IFN-induced and constitutively expressed proteins functioning in diverse cellular pathways. *Proc. Natl. Acad. Sci. U. S. A.* 102, 10200.
- (16) Bondalapati, S., Jbara, M., and Brik, A. (2016) Expanding the chemical toolbox for the synthesis of large and uniquely modified proteins. *Nat. Chem.* 8, 407.
- (17) Bondalapati, S., Eid, E., Mali, S. M., Wolberger, C., and Brik, A. (2017) Total chemical synthesis of SUMO-2-Lys63-linked diubiquitin hybrid chains assisted by removable solubilizing tags. *Chem. Sci.* 8, 4027.
- (18) Tsuda, S., Mochizuki, M., Ishiba, H., Yoshizawa-Kumagaye, K., Nishio, H., Oishi, S., and Yoshiya, T. (2018) Easy-to-Attach/Detach Solubilizing-Tag-Aided Chemical Synthesis of an Aggregative Capsid Protein. *Angew. Chem., Int. Ed.* 57, 2105.
- (19) Jacobsen, M. T., Petersen, M. E., Ye, X., Galibert, M., Lorimer, G. H., Aucagne, V., and Kay, M. S. (2016) A helping hand to overcome solubility challenges in chemical protein synthesis. *J. Am. Chem. Soc.* 138, 11775.
- (20) Maity, S. K., Mann, G., Jbara, M., Laps, S., Kamnesky, G., and Brik, A. (2016) Palladium-assisted removal of a solubilizing tag from a Cys side chain to facilitate peptide and protein synthesis. *Org. Lett.* 18, 3026.
- (21) Veber, D. F., Milkowski, J. D., Varga, S. L., Denkwalter, R. G., and Hirschmann, R. (1972) Acetamidomethyl. A novel thiol protecting group for cysteine. *J. Am. Chem. Soc.* 94, 5456.
- (22) Ajish Kumar, K. S., Haj-Yahya, M., Olschewski, D., Lashuel, H. A., and Brik, A. (2009) Highly efficient and chemoselective peptide ubiquitylation. *Angew. Chem., Int. Ed.* 48, 8090.
- (23) Maity, S. K., Jbara, M., Laps, S., and Brik, A. (2016) Efficient Palladium-Assisted One-Pot Deprotection of (Acetamidomethyl)-Cysteine Following Native Chemical Ligation and/or Desulfurization To Expedite Chemical Protein Synthesis. *Angew. Chem., Int. Ed.* 55, 8108.
- (24) Dawson, P. E. (2011) Native chemical ligation combined with desulfurization and deselenization: a general strategy for chemical protein synthesis. *Isr. J. Chem.* 51, 862.
- (25) Akutsu, M., Ye, Y., Virdee, S., Chin, J. W., and Komander, D. (2011) Molecular basis for ubiquitin and ISG15 cross-reactivity in viral ovarian tumor domains. *Proc. Natl. Acad. Sci. U. S. A.* 108, 2228.
- (26) Haj-Yahya, N., Haj-Yahya, M., Castaneda, C. A., Spasser, L., Hemantha, H. P., Jbara, M., Penner, M., Ciechanover, A., Fushman, D., and Brik, A. (2013) Modifying the vicinity of the isopeptide bond to reveal differential behavior of ubiquitin chains with interacting proteins: organic chemistry applied to synthetic proteins. *Angew. Chem., Int. Ed.* 52, 11149.
- (27) Flood, D. T., Hintzen, J. C. J., Bird, M. J., Cistrone, P. A., Chen, J. S., and Dawson, P. E. (2018) Leveraging the Knorr Pyrazole Synthesis for the Facile Generation of Thioester Surrogates for use in Native Chemical Ligation. *Angew. Chem., Int. Ed.* 57, 11634.
- (28) Zheng, J. S., Tang, S., Qi, Y. K., Wang, Z. P., and Liu, L. (2013) Chemical synthesis of proteins using peptide hydrazides as thioester surrogates. *Nat. Protoc.* 8, 2483.
- (29) Harmand, T. J., Pattabiraman, V. R., and Bode, J. W. (2017) Chemical Synthesis of the Highly Hydrophobic Antiviral Membrane-Associated Protein IFITM3 and Modified Variants. *Angew. Chem., Int. Ed.* 56, 12639.
- (30) Zheng, J. S., He, Y., Zuo, C., Cai, X. Y., Tang, S., Wang, Z. A., Zhang, L. H., Tian, C. L., and Liu, L. (2016) Robust chemical synthesis of membrane proteins through a general method of removable backbone modification. *J. Am. Chem. Soc.* 138, 3553.