Protein Synthesis

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Chemical Synthesis of the Highly Hydrophobic Antiviral Membrane-Associated Protein IFITM3 and Modified Variants

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Abstract: *Interferon-induced transmembrane protein 3 (IFITM3) is an antiviral transmembrane protein that is thought to serve as the primary factor for inhibiting the replication of a large number of viruses, including West Nile virus, Dengue virus, Ebola virus, and Zika virus. Production of this 14.5 kDa, 133-residue transmembrane protein, especially with essential posttranslational modifications, by recombinant expression is challenging. In this report, we document the chemical synthesis of IFTIM3 in multi-milligram quantities (>15 mg) and the preparation of phosphorylated and fluorescent variants. The synthesis was accomplished by using KAHA ligations, which operate under acidic aqueous/organic mixtures that excel at solubilizing even the exceptionally hydrophobic C-terminal region of IFITM3. The synthetic material is readily incorporated into model vesicles and forms the basis for using synthetic, homogenous IFITM3 and its derivatives for further studying its structure and biological mode of action.*

Interferon-induced transmembrane protein 3 (IFITM3) is a 133 amino acid membrane-associated protein (MP) that inhibits the replication of pathogenic viruses. This protein was first named fragilis and reported by Saitou and co-workers in 2002,[1] but interest surged when its antiviral properties came to light during the 2009 H1N1 influenza pandemic. Everitt and co-workers[2] reported that patients lacking full IFITM3 were more quickly and severely infected, leading to a dramatic increase in mortality. Subsequent studies have shown that IFITM3 exhibits antiviral activity against a large number of viruses,[3,4] including West Nile virus,[5,6] Dengue virus,[5–7] Filoviruses (Ebola and Marburg viruses),[8] SARS Coronavirus,[8] Rift Valley fever virus,[9] HIV-1[10] and, most recently, Zika virus.[11]

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IFITM3 and its related proteins (IFITMs) contain two highly hydrophobic intramembrane domains: a conserved loop region, and a more polar *N*-terminal region that varies in sequence and length (Figure 1a). Several proposals for its structure, membrane-spanning topology, and mechanism of action have been reported but there is little consensus of how IFITM3 prevents viral replication.[12] Further studies, such as structure determination and investigation of its numerous posttranslational modifications,[13–16] are hampered in part by lack of access to the pure protein, especially homogeneous material containing the essential posttranslational modifications.

In this report, we document the production of milligram quantities of homogeneous IFITM3 and key post-translationally modified variants by total chemical synthesis by using α-ketoacid-hydroxylamine (KAHA) ligation.[17] Key to the success of this work is the unique nature of the KAHA ligation using 5-oxaproline, which operates under acidic conditions ideal for solubilizing hydrophobic peptide segments and delivers more soluble peptide esters compared to amide as the primary ligation product. The use of 5-oxaproline results in the introduction of homoserine, a non-canonical amino acid, at the ligation site. When carefully chosen, we have found that this is an innocuous mutation of many residues and have shown that it does not disturb folding or biological activity.[18] The synthetic route allows facile incorporation of key posttranslational modifications, including phosphorylation and the attachment of a fluorescent dye. This work establishes access to homogenous IFITM3 and will enable further studies on its structure and mode of action.

Our synthetic planning took into account the different structural regions of IFITM3 and took inspiration from a map of key residue constructed by Brass and co-workers.[7] We aimed to synthesize IFITM3 from three segments using two KAHA ligations. For the first ligation site, we chose Ser50Hse, since this is located in an exposed loop region that shows considerable variability among IFITM proteins. As the second ligation, we selected Thr95Hse, since this site was reported to be one of the few variable sites within an otherwise highly conserved region. This strategy called for the use of a valine α-ketoacid for the ligation, which although somewhat slower than less hindered residues, has proven to be an excellent choice for KAHA ligations.[18b]

The synthesis of segment 3, which contains the IM2 domain, proved to be the most challenging. Owing to its extreme hydrophobicity, we first attempted to synthesize it by tert-butyloxycarbonyl solid-phase peptide synthesis (Boc-SPPS), since Boc chemistry is known to be better suited than fluorenylmethyloxycarbonyl solid-phase peptide synthesis (Fmoc-SPPS) for the preparation of hydrophobic

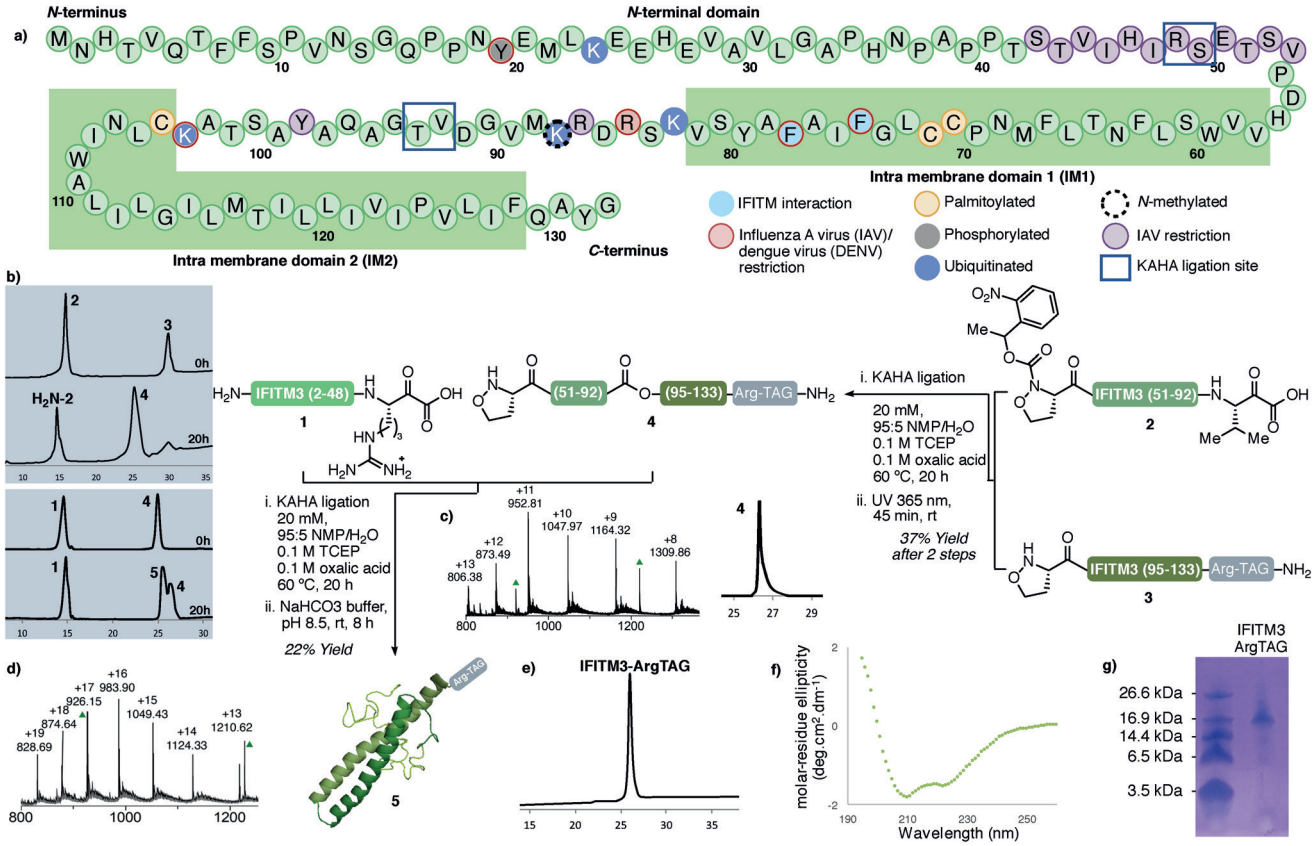

Figure 1. Synthesis of IFITM 3-ArgTAG. a) Map of IFITM3: The amino acids sequence and the important amino acid residues from the alanine scan performed by Brass and co-workers are highlighted, as well as the different posttranslationally modified residues[6] and the KAHA ligation site. b) Synthetic route to IFITM3-ArgTAG. HPLC traces show the progression of the ligation at 60 °C at $t = 0$ h and $t = 20$ h. c) HPLC trace at 60 °C and ESI-MS spectra of the isolated Opr-S2S3-ArgTAG segment (**4**). The green triangles highlight the calibration peaks. d) ESI-MS spectra of IFITM3-ArgTAG (**5**). e) HPLC trace of the purified **5** at 60 °C. f) CD spectra analysis of the reconstituted IFITM3-ArgTAG (**5**) in PC micelles. (50 μM in 1 mm quartz cell). g) SDS-PAGE/Coomassie staining of IFITM3-ArgTAG (**5**).

peptides.[19] Unfortunately, all attempts to purify the peptide obtained after HF cleavage were unsuccessful. We next attempted the synthesis of segment 3 by Fmoc-SPPS, however this was even less efficient, and after only 10 residues, the amount of impurities was greater than the desired product.

The addition of solubilizing tags or backbone protection have been reported to improve the synthesis of hydrophobic peptides.[20–23] We decided to employ an oligo-arginine tag (ArgTAG) similar to one described by Wade and co-workers,[24] which can be removed by basic treatment at room temperature. This tag presents two advantages: first, oligo-arginines have been shown to greatly increase the solubility of hydrophobic sequences, thus making the handling and purification of these segments easier. Second, this arginine tag is attached to the C terminus rather than to the backbone of the peptide, thus facilitating its synthesis and incorporation into the peptide. With this strategy, Fmoc-SPPS of the hydrophobic segment proceeded smoothly, and the cleaved peptide bearing the $(Arg)_7$ tag could be readily purified by reverse-phase HPLC. The synthesis on one gram of resin afforded 128 mg of the purified peptide **3** (7% overall yield) bearing an N-terminal 5-oxaproline. The central segment, bearing a C-terminal valine α-ketoacid, was prepared by Fmoc-SPPS, and the requisite photoprotected [(2-nitrophenyl)ethyl]carbamate-Opr (NpecOpr) was introduced as the final residue.[25]

The fragments NpecOpr-S2-KA (**2**) and Opr-S3-ArgTAG (**3**) were joined by the first KAHA ligation. We were pleased to find that the segments were perfectly soluble in a 95:5 NMP/H_2O mixture at 20 mM. Despite the use of the hindered valine α-ketoacid, the ligation was almost complete after 10 h, by which time a gel had formed. The reaction mixture was diluted to 10 mM with NMP and left at 60 °C for an additional 10 h but no change was observed, thus indicating that the ligation reaction had reached maximum conversion. The ligation mixture was diluted with 1:1 CH_3CN/H_2O containing 0.1% TFA, DODT (2,2′-(Ethylenedioxy)diethanethiol) was added, and the resulting mixture was stirred under handheld UV light at 365 nm for 45 min to remove the Npec group. The reaction mixture was directly purified by HPLC to afford the desired ligated peptide **4** in an overall yield of 37% (Figure 1). The second and final ligation was performed between 1 equiv peptide **4** and 1.5 equiv H_2N-S1-KA **1** at 15 mM in a mixture of 95:5 NMP/H_2O. The ligation was somewhat slower than typically observed, and after 20 h a gel had formed and the ligation did not proceed beyond about 60% conversion. Despite the gel formation, the desired ligated product was cleanly formed. The mixture was diluted to 0.5 mM with

carbonate buffer (pH 8.5) and left for 8 h to effect the O→N acyl shift of the two homoserine esters.

After concentration and purification by HPLC, we isolated 16.3 mg (22% yield) of the desired IFITM3-ArgTAG (**5**; Figure 1). The synthetic IFITM3 bearing the C-terminal ArgTAG was characterized by HPLC, HRMS, and SDS-PAGE. For CD spectroscopy, the protein was incorporated into phosphatidylcholine (PC) lipid vesicles and the spectra showed a strong α-helix fingerprint, in accordance with the calculated 3D structure (Figure 1).[26] To release the IFITM3 protein from the C-terminal solubilizing tag, protein **5** was treated with 0.1M NaOH for 5 min. However, decomposition of the protein was observed, in part by hydrolysis of Pro40-Pro41.

To overcome this problem, we pursued an alternative approach by removing the $(Arg)_7$ tag prior to the final ligation (Figure 2). After the first ligation and UV irradiation, the reaction mixture was diluted with a 0.25M NaOH solution until a concentration of 0.1M of NaOH was reached. After 5 min, the solution was cooled in an ice bath and slowly quenched with a solution of 50% TFA in water and directly purified by HPLC to afford the desired Opr-S2S3-OH **6** in 24% yield after 3 steps (Figure 2). Removal of the ArgTAG under basic conditions gave a single peak on HPLC.

The final ligation, now without the solubilizing group, was performed using 95% NMP with 5% aqueous oxalic acid. Despite the hydrophobic segment, the ligation proceeded relatively well. Following dilution of the ligation mixture to 0.5 mM with a carbonate buffer (pH 8.5) and stirring for 8 h at 23°C to effect O→N shift, 8.1 mg of unmodified IFITM3 (**7**) was isolated by preparative HPLC on a heated C4 column (Figure 2).

CD spectra of IFITM3 **7** were measured following the same procedure as before. The CD spectra showed a strong fingerprint for α-helix conformation. The two CD spectra were nearly identical, thus showing that the C-terminal ArgTAG does not influence the 3D structure (Figure 2). Our first attempts at measuring the MS of the IFITM3 protein lacking the solubilizing tag were unsuccessful. A similar problem was noted by Liu and co-workers during their synthesis of the multidrug-resistance transporter EmrE, another membrane protein,[21] and can be rationalized by the highly hydrophobic nature of the protein and paucity of ionizable sites. We succeeded in characterizing the protein by digestion under acidic conditions and analysis of the resulting fragments by MALDI-MS. This procedure gave more than 95% sequence coverage of IFITM3, confirming the synthesis of the full protein (Figure 2).

Several studies have established that posttranslational modifications of IFITM3 are essential for its antiviral activity.[13,16] The role of these numerous modifications remains a subject of debate, and the inability to prepare and isolate homogeneous protein complicates further efforts. A key advantage of chemical protein synthesis is the ability to

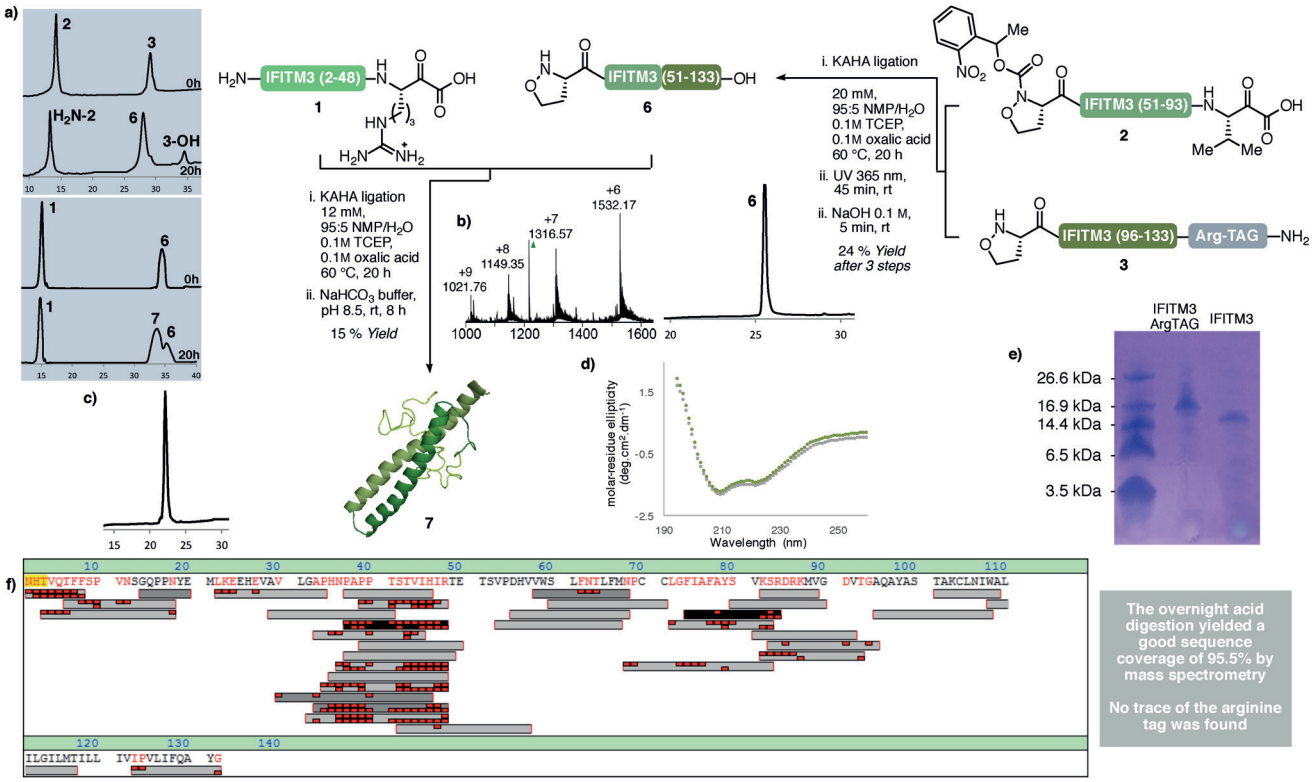

Figure 2. Synthesis of IFITM3. a) Synthetic route to IFITM3. HPLC traces show the progression of the ligation at 60°C. b) HPLC trace at 60°C and ESI-MS spectra of the isolated Opr-S2S3-OH fragment (**6**). The green triangles highlight the calibration peaks. c) HPLC trace of the purified IFITM3 (**7**) at 60°C. d) CD spectra analysis of the reconstituted **5** in PC micelles (grey) and CD spectra analysis of the reconstituted IFITM3 (**7**) in PC micelles (green) (50 μM in 1 mm quartz cell). e) SDS-PAGE/Coomassie staining of IFITM3. f) Sequence analysis by MALDI-MS of overnight acid digested IFITM3 shows a sequence coverage of 95%.

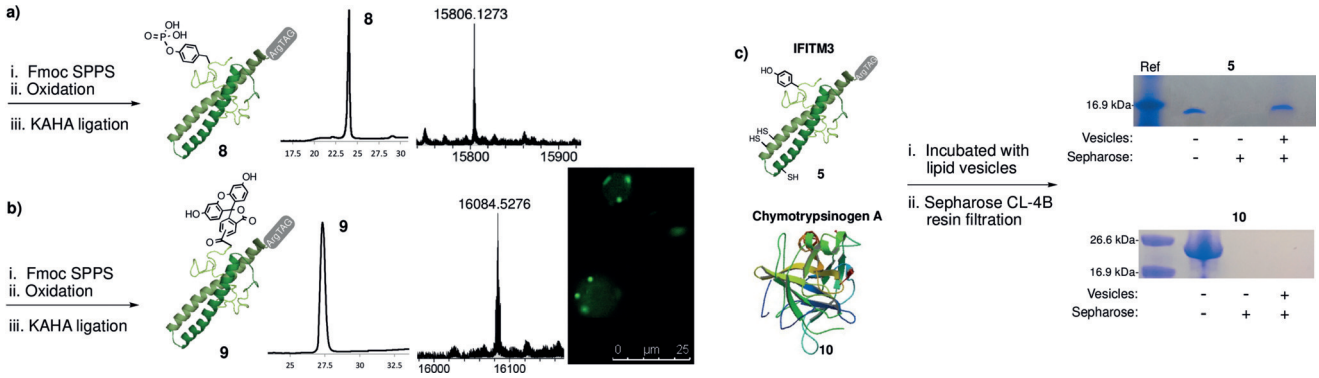

Figure 3. Modified variants of IFITM3. a) HPLC trace at 60 °C and ESI-MS spectra of the purified Tyr20Phospho-IFITM3 (**8**). The Phosphorylated tyrosine is perfectly stable to the oxidation and KAHA ligation conditions b) HPLC trace at 60 °C and ESI-MS spectra of the purified carboxyfluorescein IFITM3 **9**. Fluorescent microscopy of **9** incorporated into lipid vesicles. c) Proteins were incubated with lipid vesicles and passed through size-exclusion resin. SDS-PAGE of the filtrates showed that IFITM3 **5** is incorporated into the vesicles.

precisely control the presence or absence of posttranslational modifications, as well as the power to incorporate probes such as affinity tags or fluorescent dyes to facilitate biochemical and biophysical studies.[27,28] In our initial efforts at using our synthetic route to IFITM3 derivatives, we focused on one of the most important posttranslational modifications: phosphorylation of tyrosine 20. In addition, we prepared a fluorescently labeled IFITM3 for studies on its incorporation into membrane vesicles. We had not previously attempted KAHA ligations with phosphorylated peptides. Our strategy required that the phosphotyrosine be stable to the oxidative conditions for conversion of the phosphorylated segment 1 cyanosulfurylide to the corresponding α-ketoacid, as well as to the acidic conditions of the KAHA ligation. For the preparation of segment 1 containing the phospho-Tyr, we choose O-benzyl-protected phospho-Tyr for Fmoc-SPPS, which affords the unprotected residue upon TFA cleavage. The segment synthesis proceeded smoothly and phospho-Tyr was stable to both sulfurylide oxidation and KAHA ligation, affording Tyr20Phos-IFITM3 (**8**; Figure 3).

Several reports have attributed the antiviral activity of IFITM3 to its ability to block the fusion of viral membranes in the endosomes.[4,29] The activity of IFITM3 is therefore dependent on its ability to localize into the cell membranes. In order to use IFITM3 and its variants to probe the mode of action, we wished to incorporate the protein into a membrane mimetic and we chose egg phosphatidylcholine as a starting point. Synthetic IFITM3 was incubated with vesicles and then passed through Sepharose CL-4B exclusion resin. If IFITM3 is inserted into the membrane vesicles, it should elute with the larger vesicles; the smaller, non-incorporated proteins will be retained. The flow-through was lyophilized and analyzed by SDS-PAGE. Chymotrypsinogen A was used as a negative control, as shown in the work of Francis and co-workers.[30] We were pleased to observe that IFITM3 was incorporated into the lipid membrane as expected.

For further confirmation that synthetic IFITM3 was incorporated into the membrane, we prepared a H_2N-S1-KA segment with an N-terminal 5(6)-carboxyfluorescein by Fmoc-SPPS. Resin cleavage, oxidation of the cyanosulfurylide, and ligation with Opr-S2S3-ArgTAG (**4**) afforded the protein with the fluorescent dye. The fluorophore-modified IFITM3 (**9**) was incorporated into medium-sized vesicles, passed through the size-exclusion resin, and observed by fluorescence microscopy. As shown in Figure 3, IFITM3 is clearly localized in the membrane of the vesicles, as expected from the previous experiment.

In conclusion, we have developed an efficient chemical synthesis of pure IFITM3 on a multi-milligram scale that makes use of KAHA ligation. This approach provides facile access to IFITM3 bearing natural posttranslational modification, as well as other derivatives that are not possible to prepare with the current recombinant approaches. The unique feature of KAHA ligation, including its ability to operate under acidic conditions, its tolerance of organic co-solvents, and the formation of more soluble depsipeptides at the ligation site, make it an ideal method for the chemical synthesis of membrane proteins. This works also establishes that KAHA ligation is compatible with phosphorylated peptide segments and provides access to important posttranslational modifications. The materials produced by this route will be used for ongoing studies on the role of IFITM3 and its variants on restricting entry of the influenza A virus.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: chemical protein synthesis · IFITM3 · KAHA ligation · membrane proteins · protein modifications

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