Protein Lipidation

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Synthesis of Autophagosomal Marker Protein LC3-II under Detergent-Free Conditions**

*Yi-Chao Huang, Yi-Ming Li, Yang Chen, Man Pan, Yi-Tong Li, Li Yu, Qing-Xiang Guo, and Lei Liu**

Autophagy is a catabolic process for the bulk degradation of intracellular materials, such as abnormal protein aggregates and damaged organelles.[1] It normally occurs under nutrient deprivation, causing cytoplasmic components to be engulfed by the double-membrane-bound autophagosomes (Figure 1).

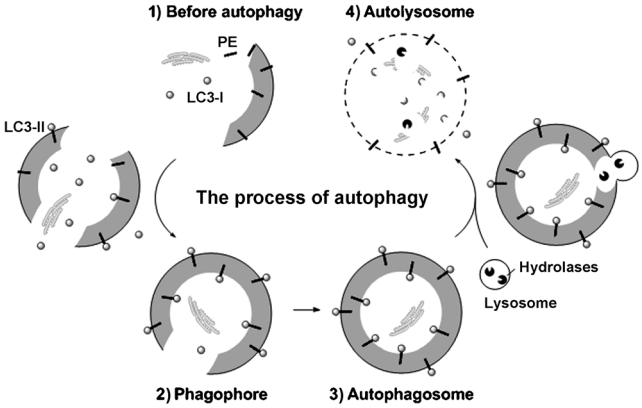

Figure 1. Inter-conversion of LC3-I and LC3-II during autophagy.

Autophagosomes then fuse with lysosomes to form autolysosomes where the sequestered materials are degraded by the lysosomal hydrolases. As a pivotal cellular housekeeping system, autophagy plays important roles in many physiological processes including bacterial and viral infection, cell death, and antigen presentation. Dysfunction of autophagy is involved in a number of pathologies such as hepatic inflammation, neurodegenerative diseases, and cancer.[2] Owing to these reasons, there has been growing interest to understand the mechanisms of autophagy at the molecular level.

The formation of autophagosomal membranes is a key event in autophagy. During this process, a cytosolic microtubule-associated protein 1A/1B-light chain 3 (LC3-I) is conjugated to 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) through an amide bond with the C-terminal residue, Gly120.[3] The resulting lipid-anchored protein is called LC3-II. It promotes the elongation of the phagophore (the autophagosome precursor) and engulfment of cytoplasmic materials targeted for degradation. Later on LC3-II is either enzymatically converted back to LC3-I by a cysteine protease, Atg-4, or degraded by the lysosomal proteases.[4] Previous studies have shown that the cellular LC3-II level can be connected to the autophagic activity.[5] Accordingly, LC3-II is a good marker for the study of the machinery of autophagy. Although traditional methods such as Western blotting can be used to monitor LC3-II,[6] more advanced studies on autophagy require preparative amounts of LC3-II, ideally with reporter groups and tags incorporated.

Previous attempts to produce LC3-II have relied on in vitro conjugation of LC3-I with PE using E1- and E2-like enzymes, Atg7 and Atg3.[7] Unfortunately, this enzymatic approach can only afford LC3-II in a protein–lipid mixture and in the low microgram scale. Herein, we report the first synthesis of homogeneous LC3-II in practical quantities, using the strategy of expressed protein ligation.[8] This study is part of our on-going efforts to elucidate the mechanism of autophagy regulation.[9] Previously chemical synthesis has been used for the preparation of lipidated proteins with attached isoprenoid lipids[10] and glycosylphosphatidylinositols.[11] However, we recognized that LC3-II presents an even more challenging synthetic target, because the PE tail is too hydrophobic for the previous detergent-assisted ligation methods. To solve this problem, we developed a new method for the synthesis of lipidated proteins using a removable solublizing side chain. With this approach, lipidated proteins can be prepared under detergent-free conditions, without the laborious screening of detergents and solvents. This strategy not only enables the preparation of LC3-II with good

[*] Y.-C. Huang,[+] M. Pan, Y.-T. Li, Prof. Dr. L. Liu
Tsinghua-Peking Center for Life Sciences, Key Laboratory of Bioorganic Phosphorus Chemistry and Chemical Biology (Ministry of Education), Department of Chemistry, Tsinghua University
Beijing 100084 (China)
E-mail: lliu@mail.tsinghua.edu.cn
Prof. Dr. Y.-M. Li[+]
School of Medical Engineering, Hefei University of Technology
Hefei, Anhui 230009 (China)
Dr. Y. Chen, Prof. Dr. L. Yu
School of Life Sciences, Tsinghua University
Beijing 100084 (China)
Prof. Q.-X. Guo
Department of Chemistry, University of Science and Technology of China
Hefei 230026 (China)
[+] These authors contributed equally to this work.

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efficiency, but also may provide a more general and user-friendly method for making diverse lipidated proteins.[12]

We began by searching for a ligation site suitable for expressed protein ligation in the C-terminal flexible region of LC3-I. The DNA sequences corresponding to the first 113, 114, 115, 116, or 118 amino acid residues of LC3-I were cloned into the pTXB1 bacterial expression vector. The truncated proteins fused to a C-terminal gyrA intein–chitin binding domain were expressed in *E. coli* ER2566 cells.[13] They were collected and loaded on a chitin affinity column, incubated overnight, and eluted with a MESNa (sodium 2-mercaptoethanesulfonic acid) cleavage buffer (100 mM, pH 7.8) to produce thioesters of the truncated protein. According to SDS-PAGE analysis (Supporting Information, Figures S2,S3) LC3-I[Met1–Ala114]-MESNa thioester was produced with a higher efficiency (5–6 mg L^{-1}) than the other thioesters. Thus we chose the Ala114-Ser115 bond as the ligation site, which required the mutation of Ser115 to Cys115 in the target protein.

We next synthesized the [Cys115–Gly120]-PE hexapeptide with a PE tail (Figure S4). For this purpose, we first used the 2-Cl-trityl resin to prepare the fully protected hexapeptide. This hexapeptide was then condensed with DSPE in chloroform using *N,N′*-diisopropylcarbodiimide (DIC) assisted by HOAt (1-hydroxy-7-azabenzotriazole). Subsequently, the protecting groups were removed using the standard trifluoroacetic acid (TFA) cocktail and the desired product (i.e. [Cys115–Gly120]-PE) was obtained through precipitation in cold diethyl ether. Unfortunately, the resulting lipopeptide was barely soluble in up to 80% aqueous acetonitrile containing 0.1% TFA, rendering its handling and purification very difficult. Moreover, this lipopeptide could not be dissolved in the solutions used for expressed protein ligation (aqueous buffers containing 6 M guanidinium chloride or 8 M urea mixed with 0–50% dimethyl sulfoxide or trifluoroethanol (TFE)). To solve this problem, a detergent-assisted ligation approach has been established for the synthesis of lipidated proteins. However, this strategy failed in the present case, because many commonly used detergents (e.g. sodium dodecyl sulphate,[14] β-octyl glucoside,[15] Triton X-114,[16] dodecylphosphocholine,[17] cetyl trimethylammonium bromide[18]) still gave almost no ligation. A possible explanation for the difficulty encountered with this ligation is that DSPE (with two $C_{17}H_{35}$ tails) is much more hydrophobic than previously used lipid chains (e.g. the farnesyl group with only one $C_{15}H_{25}$ tail). We therefore concluded that the detergent-assisted ligation strategy may not guarantee success for the synthesis of every lipidated protein.

To overcome this solubility problem, we decided to use a removable solubilizing side chain on the PE-modified lipopeptide. Because oligoarginine has often been used to improve the solubility of hydrophobic peptides[19] (mainly transmembrane protein fragments), we examined the attachment of oligoarginines of different lengths to the lipopeptide. To ease the synthesis of the compounds used for testing solubility, we initially attached three to five arginine residues (i.e. Arg3, Arg4, Arg5) at the side chain of Thr118 through a simple ester bond (Figure 2; Figure S5). Our experiments revealed that the PE-modified lipopeptide was still poorly soluble with an Arg3 tag (Figure S6). On the other hand, when an Arg4 or Arg5 tag was attached, the lipopeptide became soluble in 6 M guanidinium chloride buffer and could be ligated with LC3-I[Met1–Ala114]-MESNa thioester without the need for a detergent (Figures S6,S7).

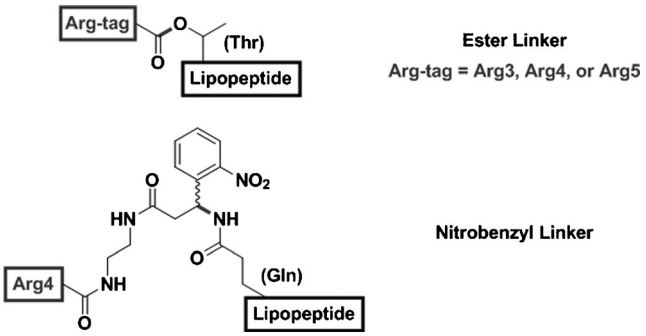
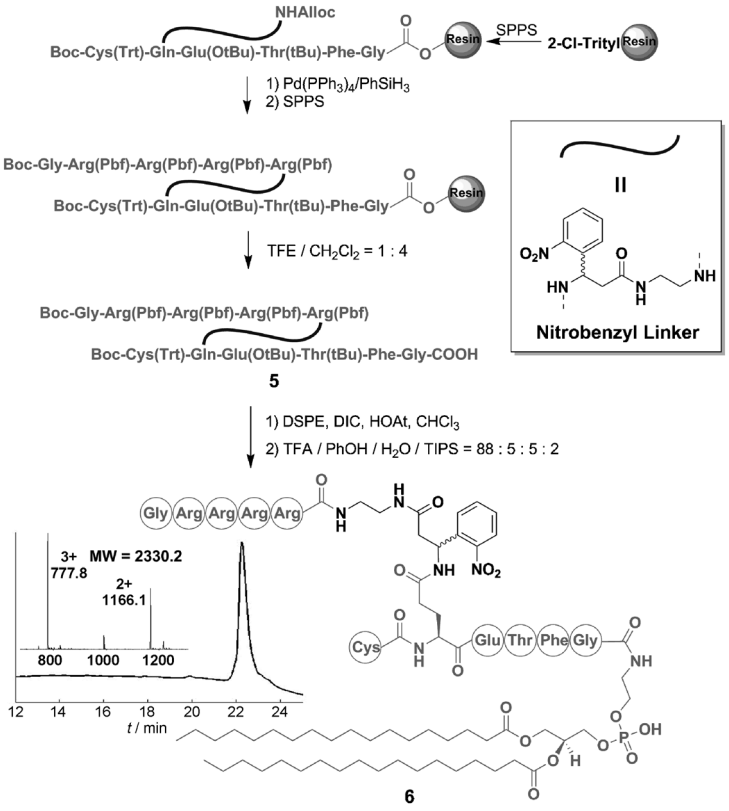


Figure 2. Development of a removable solubilizing side chain.

Unfortunately, the diacyl moiety of PE was destroyed under the saponification conditions used to cleave the ester linker. Therefore, we had to design an alternative linker to attach the Arg4 tag onto the side chain of the PE-modified lipopeptide. It is important to note that the linker must be



Scheme 1. Synthesis of light-activatable lipopeptide **6** with an observed ESI-MS of 2330.2 Da (calcd: 2330.4 Da). Trt = trityl protecting group; Alloc = allyloxycarbonyl protecting group; Pbf = pentamethyldihydrobenzofurane protecting group.

stable for both Fmoc-protected solid phase peptide synthesis (SPPS) and native chemical ligation, yet its removal should not affect the amide and ester bonds in the final lipidated protein. With this in mind, we designed a photosensitive nitrobenzyl linker that can be cleaved using ultraviolet light.[20]

The nitrobenzyl linker involved the use of an *ortho*-nitrobenzyl modified glutamine residue (compound **4**, see Supporting Information). It was synthesized from Fmoc-Glu-OMe (**1**) in three steps with an overall yield of 51% (Figure S1). We used the 2-Cl-trityl resin to assemble the fully protected hexapeptide through standard Fmoc SPPS. Without cleavage of the peptide from the solid support, the Alloc group on the side chain of **4** was removed using $Pd(PPh_3)_4/PhSiH_3$ in a dimethylformamide (DMF)/CH_2Cl_2 mixture. The liberated amino group was then used to assemble the Arg4 tag capped with a Boc-Gly-OH residue on the N-terminus, once again using Fmoc SPPS. Subsequently, the branched hexapeptides with their protecting groups were cleaved off the resin with a 1:4 mixture of TFE/CH_2Cl_2. This intermediate (**5**) was condensed with DSPE and treated with reagent B (TFA/phenol/H_2O/triisopropylsilane = 88:5:5:2) to give the final lipopeptide (**6**) suitable for the expressed protein ligation (Scheme 1). **6** could be easily handled, with good solubility in both 50% aqueous acetonitrile and 6 M guanidinium chloride buffer.

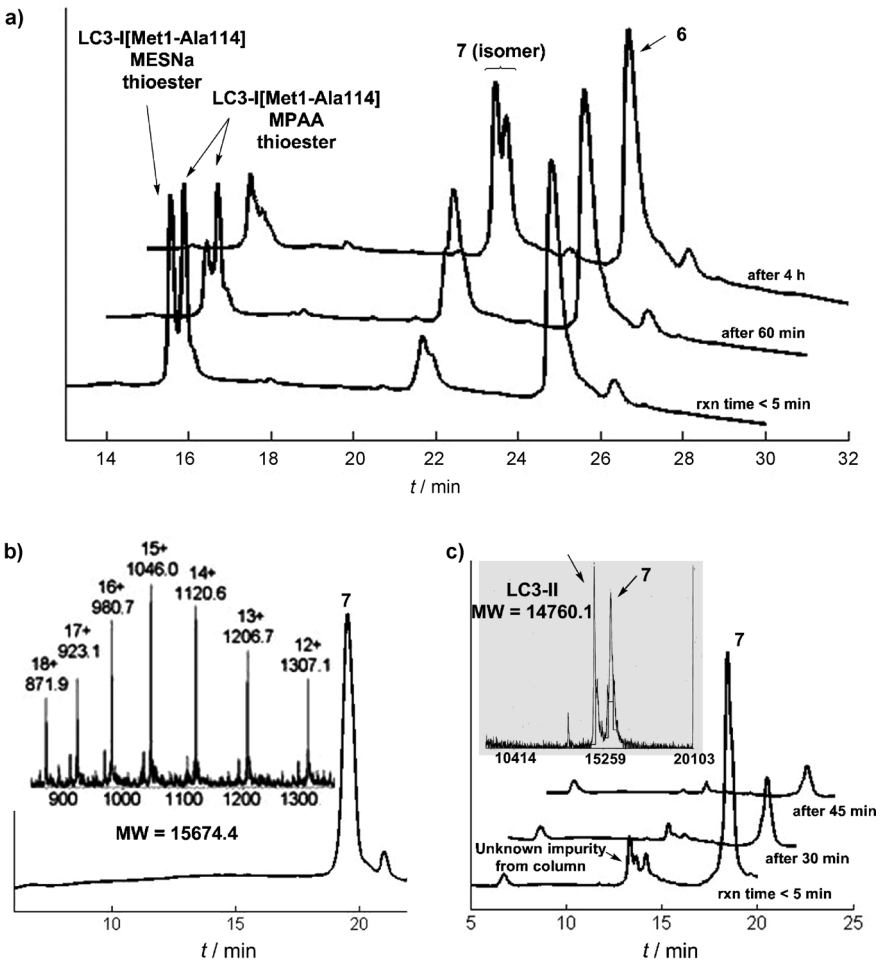


Figure 3. a) Analytical HPLC traces for the ligation of the LC3-I[Met1–Ala114]-MESNa thioester with lipopeptide **6**; b) Characterization of the purified LC3-II precursor **7** by analytical HPLC and ESI-MS (inset; obs: 15674.4 Da, calcd: 15674.6 Da); c) Characterization of LC3-II after photolysis under 365 nm UV light. Indet: the observed MALDI-TOF mass for synthetic LC3-II is 14760.1 Da (calcd [*M*+1]: 14759.6 Da).

The ligation of LC3-I[Met1–Ala114]-MESNa thioester (2.4 mg) with **6** (3.1 mg) was carried out in 200 μL ligation buffer (6 M guanidinium chloride, 100 mM NaH_2PO_4, 50 mM 4-mercaptophenylacetic acid (MPAA), pH 7.4), with final reaction concentrations of approximately 0.9 mM and 6.7 mM, respectively. Again, no detergents were needed for this ligation. The desired ligation product **7** (which is the LC3-II precursor still carrying an Arg4 tag) was detected within five minutes by HPLC (Figure 3a) and we stopped the reaction after four hours. **7** appeared as a double peak in the HPLC profile because the nitrobenzyl linker was prepared as an isomeric mixture. **7** could be easily purified with a semi-preparative reverse-phase HPLC column to afford 2.1 mg of white powder with a yield of the isolated product of 75%. The purity and identity of **7** was characterized by analytical HPLC and ESI-MS (Figure 3b). The use of less than four equivalents of **6** significantly slowed down the ligation reaction and led to much lower ligation yields. This may indicate that **6** still aggregates in the solution despite the attachment of an Arg4 tag. Fortunately, the excess **6** could be easily recovered following the ligation. This is an advantage of the detergent-free method for the synthesis of lipidated proteins, because the use of detergents in the previous methods often made it difficult to purify the product by reverse-phase HPLC or to recover the reactants.[21]

To remove the Arg4 tag in the synthetic protein, we dissolved **7** (to a final concentration of 0.5 mg mL^{-1}) in the photolysis buffer (pH 5.5) containing 6 M guanidinium chloride, 100 mM NaH_2PO_4, 300 mM $MeONH_2$·HCl, and 10 mM sodium ascorbate. The solution was irradiated with 365 nm UV light and monitored by HPLC (Figure 3c). Almost all of compound **7** was consumed after 45 minutes irradiation, but the desired product LC3-II was too hydrophobic to be eluted off of the C4 column. This observation indicated that it is difficult, if not impossible, to purify LC3-II using HPLC. To solve this problem, we chose to successively dialyze the photolysis buffer against renaturation buffers containing 6 M, 4 M, 2 M, 0 M urea, according to the standard method.[22] Using this approach, we expected to separate all the small molecules from the desired product. We then characterized LC3-II using

MALDI-TOF MS after ESI-MS was unsuccessful (Figure 3c). In the MALDI-TOF spectrum, the major peak corresponded to LC3-II but a peak of **7** still appeared. One possible explanation for this is that the ionization of **7** was better than that of LC3-II, owing to the Arg4 tag.

To further characterize the synthetic LC3-II, we performed urea/SDS-PAGE experiments (Figure 4a). We found that LC3-II migrated faster (at approximately 15 kD) on urea/SDS-PAGE as compared to LC3-I (at approximately 16 kD).

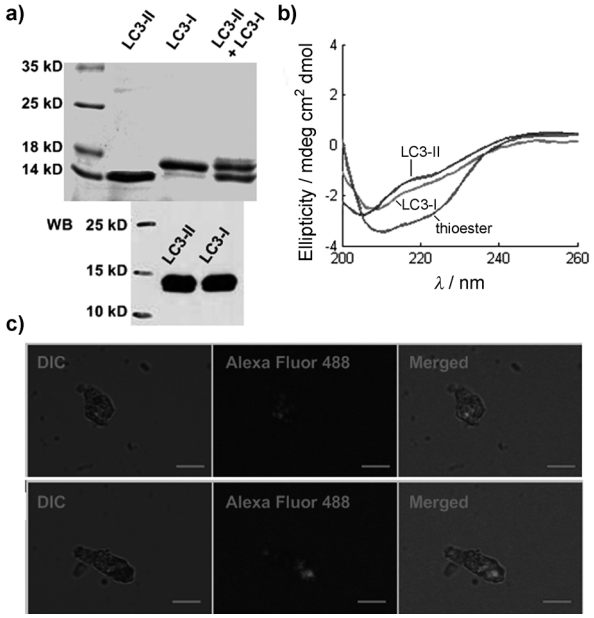
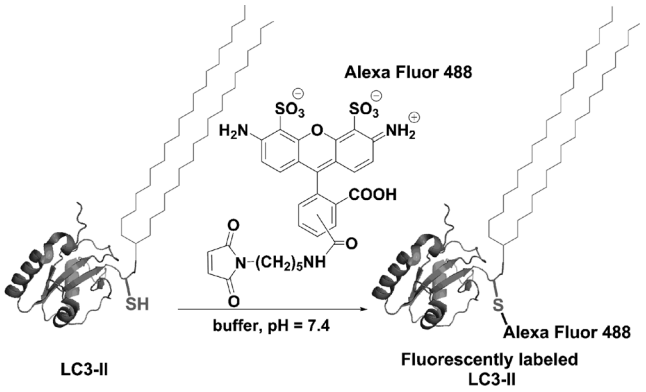


Figure 4. a) Urea/SDS-PAGE (top) and Western blot (bottom) of recombinant LC3-I and synthetic LC3-II. b) CD spectra of the synthetic LC3-II as compared with that of recombinant LC3-I and the LC3-I[Met1–Ala114]-MESNa thioester. The protein concentration was approximately 0.2 mg mL^{-1}. c) Liposomes anchored with Alexa Fluor 488 labeled LC3-II. Differential interference contrast (DIC) images showed the presence of liposomes (first column), whereas images in the second column were measured by confocal microscopy using a 488 nm laser. Images from the first and second lines show two independent windows under confocal microscopy. For colored pictures, see the Supporting Information. Scale bars = 10 μm.

This extraordinary behavior was in agreement with the previous observations for LC3-II generated in vivo,[3] which may be explained by the sticking of negatively charged SDS molecules to the PE tail of LC3-II. Moreover, a Western-blot assay showed that our synthetic LC3-II could be detected by the antibody against LC3 (Figure 4a). Note that the Western-blot assay was performed with SDS-PAGE instead of urea/SDS-PAGE, in which the migrations of LC3-I and LC3-II were similar to each other. To confirm the proper folding of synthetic LC3-II, we conducted circular dichroism (CD) measurements. Synthetic LC3-II exhibited the absorptions of both α-helices and β-sheet structures, similar to the recombinant LC3-I (Figure 4b). On the other hand, the recombinant LC3-I[Met1–Ala114]-MESNa thioester showed a slightly different CD spectrum from LC3-I and LC3-II, possibly owing to the absence of the C-terminal residues.



Scheme 2. Generation of fluorescently labeled LC3-II.

To test the biological activity of synthetic LC3-II, we labeled it with a fluorescent dye by conjugating Alexa Fluor 488 maleimide to the Cys115 residue (Scheme 2). This conjugation reaction was completed in two hours at 4 °C in aqueous buffer (10 mM NaH_2PO_4, pH 7.4). Meanwhile, we prepared liposomes as in vitro mimics of the autophagosome using the previous method of mixing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, and phosphatidylinositol in a molar ratio of 30:55:15.[23] Without the addition of LC3-II, the prepared liposomes were approximately 400 nm in diameter, as measured by transmission electron microscopy. Following incubation of the fluorescently labeled LC3-II with the above liposomes, we found that the synthetic LC3-II co-localized with the liposomes, as shown by confocal fluorescence microscopy (Figure 4c). The LC3-II anchored liposomes formed irregularly shaped cluster-like structures with increased diameters to several micrometers. This observation was consistent with Ohsumi and co-workers previous finding that Atg8-PE (the yeast homologue of LC3-II), prepared by way of enzymatic conjugation, was also caused clustering of liposomes in vitro.[23] Thus, our synthetic LC3-II was successfully anchored to the liposome membranes leading to the fusion of membranes to create larger liposome clusters. This experiment confirmed the correct biological activity of the synthetic LC3-II. Moreover, synthetic LC3-II labeled with a small fluorophore could provide a useful replacement for green fluorescent protein (GFP)-fused LC3.[6b]

To summarize, phosphatidylethanolamine conjugated LC3-II is an important lipidated protein that is needed for mechanistic studies on autophagy. Our investigation of the synthesis of LC3-II revealed that this particular protein posed a unique challenge for the existing technology of expressed protein ligation owing to the very high hydrophobicity of the PE moiety. To solve this problem, we developed a new method to use a light-activatable solubilizing side chain to assist the ligation of the lipopeptides. This strategy allowed for the synthesis of lipidated proteins under detergent-free conditions without laborious screening of the solvents and additives. It also made the handling and separation of the synthetic intermediates by the standard reverse-phase HPLC methods easier. We showed that LC3-II could be readily prepared using this newly developed method. Physicochem-

ical and biological tests verified that synthetic LC3-II had the desired structure and activity. Synthetic LC3-II equipped with reporter groups or tags is expected to be useful for studying the regulation of autophagy. We also expect that the light-activatable solubilizing side chain strategy may provide a general approach for the synthesis of lipidated proteins.

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