

**Regular Article***Highlighted Paper selected by Editor-in-Chief***Hydrazide-Mediated Solubilizing Strategy for Poorly Soluble Peptides Using a Dialkoxybenzaldehyde Linker**Shoko Tanaka,<sup>a</sup> Tetsuo Narumi,<sup>a,b,c</sup> Nobuyuki Mase,<sup>a,b,c</sup> and Kohei Sato<sup>\*a,b,c</sup><sup>a</sup> Graduate School of Science and Technology, Shizuoka University; 3–5–1 Johoku, Hamamatsu 432–8561, Japan;<sup>b</sup> Department of Applied Chemistry and Biochemical Engineering, Faculty of Engineering, Shizuoka University; 3–5–1 Johoku, Hamamatsu 432–8561, Japan; and <sup>c</sup> Research Institute of Green Science and Technology, Shizuoka University; 3–5–1 Johoku, Hamamatsu 432–8561, Japan.

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Proteins modified in a controlled manner with artificial moieties such as fluorophores or affinity tags have been shown to be a powerful tool for functional or structural analysis of proteins. A reliable way to prepare proteins with a well-defined modification is protein synthesis. Although many successful syntheses have been reported, the poor aqueous solubility of synthetic intermediates causes difficulty in the chemical synthesis of proteins. Here we describe a solubilizing strategy for poorly soluble peptides which uses chemoselective incorporation of a hydrophilic tag onto a hydrazide in a peptide. We found that a hydrophilic tag possessing a dialkoxybenzaldehyde moiety can react with peptide hydrazides through reductive *N*-alkylation. No protecting groups are required for this reaction, and peptides modified in this way show enhanced solubility and consequently good peak separation during HPLC purification. The tag can be removed subsequently by treatment with trifluoroacetic acid to generate a free hydrazide, which can be converted in a one-pot reaction to a thioester for further modification. This method was validated by synthesis of a Lys<sub>63</sub>-linked ubiquitin dimer derivative. This late-stage solubilization can be applied in principal to any peptide and opens the possibility of the synthesis of proteins that have previously been considered inaccessible due to their poor solubility.

**Key words** chemical protein synthesis; peptide hydrazide; poorly soluble peptide; solubilizing tag; ubiquitin**Introduction**

Biological research often requires proteins to be modified with molecular labels such as fluorophores or affinity tags which can be used to probe the physiological functions of the proteins.<sup>1–4</sup> Chemical protein synthesis is a way to provide site-specific labeling and introduce multiple labels as well as post-translational modifications.<sup>5,6</sup>

The chemical synthesis of proteins involves solid-phase peptide synthesis (SPPS) and chemoselective assembly of peptides. Native chemical ligation (NCL) is the most widely used method for peptide ligation, and commonly involves intermolecular *S*-*S* acyl transfer between a peptide thioester and an *N*-terminal cysteinyl peptide followed by the intramolecular *S*-*N* acyl transfer to form the native peptide bond.<sup>7–9</sup> This reaction proceeds chemoselectively in an aqueous solution without any protecting groups and can also be applied to fragments created by cellular expression.<sup>10</sup> Successful protein synthesis depends on the solubility of fragments in aqueous solutions because the poor solubility of peptide fragments as starting materials or ligation intermediates hampers analysis or purification through reverse-phase HPLC or construction of fragment assemblies *via* NCL reactions.<sup>11</sup> For these reasons, a solubilizing strategy for poorly soluble peptides is required.

The introduction into peptides of solubilizing tags, consisting of a hydrophilic moiety and a cleavable linker, has been employed to improve the solubility of peptide fragments<sup>12</sup> (Chart 1A). Poly-arginine (Arg), poly-lysine (Lys), or polyethylene glycol (PEG) provide a hydrophilic character to the poorly soluble peptide through a covalent attachment to the peptide or protein. The hydrophilic tags must be removed

when the synthesis is complete and consequently, the linker should be designed to be a cleavable structure that can be removed in a chemoselective fashion. To date, many groups have developed solubilizing tags semi-permanently attached to the C-terminus,<sup>13,14</sup> side chain functionalities,<sup>15–25</sup> or backbone amides.<sup>26–29</sup>

Using hydrazide chemistry (Chart 1B), we recently reported a novel solubilizing tag.<sup>30</sup> In this method, the solubilizing tag, which consists of poly-Lys and a benzaldehyde moiety, can be attached to poorly soluble peptides synthesized as hydrazides through reductive *N*-alkylation. Such late-stage introduction of a solubilizing tag enables facile control of the new hydrophilic structure. In contrast, traditional solubilizing tag methods in which the tags are attached during SPPS require the resynthesis of fragments in cases where the solubility-enhancing property of the tag is inadequate. The fragments with the solubilizing tag were subjected to the subsequent NCLs followed by removal of the tag by Cu(II)-mediated oxidative hydrolysis of the alkyl hydrazide to produce the corresponding carboxylic acid. Since removal of the solubilizing tag generates a carboxylic acid however, this method is only applicable to the C-terminal peptide fragment. Modifying the linker to generate a hydrazide after removal of the linker should be helpful in further application of the late-stage solubilization using hydrazides to central or N-terminal fragments. This indicates that the ligation could be expanded to not only C-to-N directions but also to N-to-C directions and will offer broad synthetic options.

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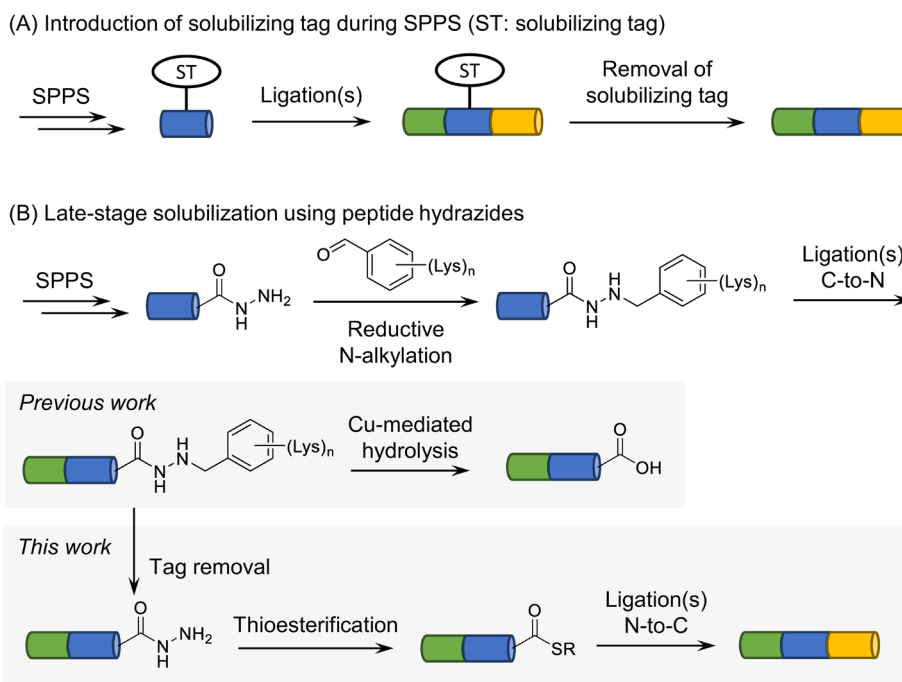


Chart 1. Solubilizing Tag Strategies for Poorly Soluble Peptides

## Results and Discussion

**N-Alkylation of Peptide Hydrazides with Different Substituents on the Benzene Ring** We envisioned the reaction sequence in Chart 1B for the late-stage solubilization of peptide hydrazides followed by regeneration of a free hydrazide to permit its use as a handle in further modifications. Benzyl-type protecting groups on a heteroatom are usually removed in peptide synthesis by treatment with trifluoroacetic acid (TFA), and the substituents on the benzene ring can tune the reactivity. TFA deprotection would be useful in the subsequent conversion to the thioester because a protocol has been developed to activate a hydrazide with the aid of  $\text{NaNO}_2$  in TFA which affords an acyl azide, which can react with a thiol to yield the corresponding thioester.<sup>31)</sup>

In our initial experiments, reductive *N*-alkylation of peptide hydrazide with benzaldehydes bearing different substituents was performed under the condition used previously<sup>30)</sup> (Table 1). Ac-LYRAG-NHNH<sub>2</sub> (**1**), a peptide hydrazide that has been widely used as a model sequence was reacted with 4-anisaldehyde (**2a**) or 2,4-dimethoxybenzaldehyde (**2b**) in acetic acid and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (50% AcOH-HFIP) for 1 h.<sup>32)</sup> Then, 20 equivalents of 2-picolineborane complex ( $\text{pic-BH}_3$ ) was added to the reaction mixture and reacted for an additional 1 h, affording the desired *N*-alkyl hydrazide (**3a**, **3b**) in 86% or 85% yield, respectively (entries 1 and 3). The same reaction proceeded even in 10 min to achieve reduction with high yields (entries 2 and 4). The reaction with 2,4,6-trimethoxybenzaldehyde (**2c**) afforded the *N*-alkyl hydrazide (**3c**) in 62% yield and 29% starting material remained after 1 h reaction whereas a shorter reaction time gave the product in higher yield (entries 5–7), suggesting that this may result from decomposition of the generated *N*-alkyl hydrazide under the slightly acidic reaction conditions. Indeed, the free hydrazide (**1**) was formed within 5 min when the isolated **3c** was treated with 50% AcOH-HFIP solution (Supplementary materials, Fig. S6).

Table 1. Reductive *N*-Alkylation of a Peptide Hydrazide<sup>a)</sup>

Entry	Aldehyde	Reduction time [min]	HPLC purity of <b>3</b> [%] <sup>b)</sup>
1	<b>2a</b>	60	86
2		10	88 (49) <sup>c)</sup>
3	<b>2b</b>	60	85
4		10	89 (48) <sup>c)</sup>
5	<b>2c</b>	60	62
6		10	88
7		5	87 (41) <sup>c)</sup>

<sup>a)</sup> Reactions were performed as follows: The peptide hydrazide **1** (1 mM) and aldehyde (**2a–2c**, 1.5 mM) were dissolved in 50% AcOH-HFIP and then incubated at 37 °C for 1 h. After the addition of  $\text{pic-BH}_3$  (20 equivalents) to the mixture, the reduction was performed at 37 °C. After removal of the solvent in an air stream, the crude material was washed with Et<sub>2</sub>O. The resulting precipitate was analyzed by HPLC. <sup>b)</sup> Detected at 220 nm. <sup>c)</sup> Isolated yield.

### Examination of the Reactivity of *N*-Alkyl Hydrazides

We then examined the reactivity and stability of *N*-alkyl hydrazides (Table 2). Deprotection of the monomethoxybenzyl derivative (**3a**) by TFA and triisopropylsilane (TIS) as a carbocation scavenger<sup>33)</sup> did not occur within 1 h while the di- or trimethoxybenzyl derivatives (**3b** and **3c**) were deprotected giving the free hydrazide (entries 1, 2 and 6). This trend can be explained in terms of the electron-donating ability of the benzene ring increasing the reactivity in acidic conditions as a result of the enhanced stability of the benzyl cation formed by the cleavage.<sup>34)</sup>

These benzyl moieties must remain attached during the protein synthesis reactions that include NCL, ring-opening of a thiazolidine (Thz) ring to be used in cysteine protection<sup>35)</sup> or desulfurization of cysteine (Cys) to enable peptide assembly at an alanine site.<sup>36)</sup> Dimethoxybenzyl hydrazide (**3b**) is stable in NCL reactions, Thz deprotection, and desulfurization conditions (entries 3–5), whereas hydrazides such as **3c** with a trimethoxybenzyl moiety are relatively unstable, and liable to decompose (entries 7–9). In light of these results, we decided in this study to use a dialkoxybenzyl linker to solubilize peptide hydrazides.

Table 2. Reactivity and Stability of *N*-Alkyl Hydrazide Peptides

Ac-LYRAG-NHNH-CH <sub>2</sub> Ar		conditions	Ac-LYRAG-NHNH <sub>2</sub>	
			1	
<b>3a</b> (Ar = C <sub>6</sub> H <sub>4</sub> (4-OMe))				
<b>3b</b> (Ar = C <sub>6</sub> H <sub>3</sub> (2,4-OMe))				
<b>3c</b> (Ar = C <sub>6</sub> H <sub>2</sub> (2,4,6-OMe))				
Entry	Substrate	Conditions	HPLC purity [%] <sup>a)</sup>	
			<b>3</b>	<b>1</b>
1	<b>3a</b>	TFA-TIS (95:5) <sup>b)</sup>	98	<1
2	<b>3b</b>	TFA-TIS (95:5)	<1	87
3		NCL <sup>c)</sup>	88	<1
4		Thz deprotection <sup>d)</sup>	97	<1
5		Desulfurization <sup>e)</sup>	81	3
6	<b>3c</b>	TFA-TIS (95:5)	<1	88
7		NCL	72	3
8		Thz deprotection	88	7
9		Desulfurization	63	12

a) Detected at 220 nm. b) TFA-TIS (95:5, v/v), 37 °C, 1 h. c) 6 M Gn·HCl, 0.2 M phosphate, 100 mM MPAA, 50 mM TCEP·HCl, pH 7.1, 37 °C, 12 h. d) 6 M Gn·HCl, 0.2 M phosphate, 100 mM MPAA, 50 mM TCEP·HCl, 200 mM MeONH<sub>2</sub>·HCl, pH 5.1, 37 °C, 12 h. e) 6 M Gn·HCl, 0.2 M phosphate, 250 mM TCEP·HCl, 150 mM MESNa, 50 mM VA-044, pH 6.2, 37 °C, 12 h.

**One-Pot Reaction for Tag Removal and Subsequent Azidation and Thiolysis** We attempted a one-pot conversion of the tag-linked hydrazide to the corresponding thioester which can be used as a handle in further modifications (Fig. 1). A peptide hydrazide with a dimethoxybenzyl linker (**3b**) was treated with a TFA-based deprotection cocktail (TFA-TIS-H<sub>2</sub>O-*m*-cresol-thioanisole = 80:2.5:2.5:5:10, v/v), which is an optimized condition for acyl azide formation,<sup>31)</sup> and the progress of the reaction was monitored by HPLC. The cleavage of the benzyl linker proceeded smoothly within 1 h to give the free hydrazide (**1**) (Figs. 1B, 1C). An aqueous solution of NaNO<sub>2</sub> (5 equivalents) was added to the resulting mixture, leading to the peptidyl azide intermediate. After a 20 min reaction at -10 °C followed by trituration with cold Et<sub>2</sub>O, the precipitate was dissolved in a buffer containing sodium 2-mercaptoethanesulfonate (MESNa). HPLC analysis of the thiolysis reaction mixture indicated that the desired thioester (**4**) had been formed without any significant amount of byproducts (Fig. 1D).

**Synthesis of a Lys<sub>63</sub>-Linked Di-Ubiquitin Derivative** A synthetic application of our strategy was confirmed through the synthesis of Lys<sub>63</sub>-linked di-ubiquitin (Ub), which is one of the Ub linkage types found in many cellular processes.<sup>37)</sup> Brik and colleagues reported the chemical synthesis of SUMO-2 linked to Lys<sub>63</sub>-di-Ub.<sup>38)</sup> They encountered synthetic difficulties due to the poor solubility of a synthetic intermediate, the Lys<sub>63</sub>-di-Ub thioester derivative. To address this problem, we used the method shown in Chart 2 to synthesize a Lys<sub>63</sub>-linked di-Ub derivative containing biotin.

The sequence of Ub was disconnected to two peptide segments, Ub<sub>1-45</sub> and Ub<sub>46-76</sub>, and the Ala<sub>46</sub> was replaced by Cys to join these two peptides through an NCL reaction. The C-terminal segment can be synthesized as a branched form during SPPS. The Lys<sub>63</sub>-linked di-Ub<sub>46-76</sub> (**6**) as a hydrazide

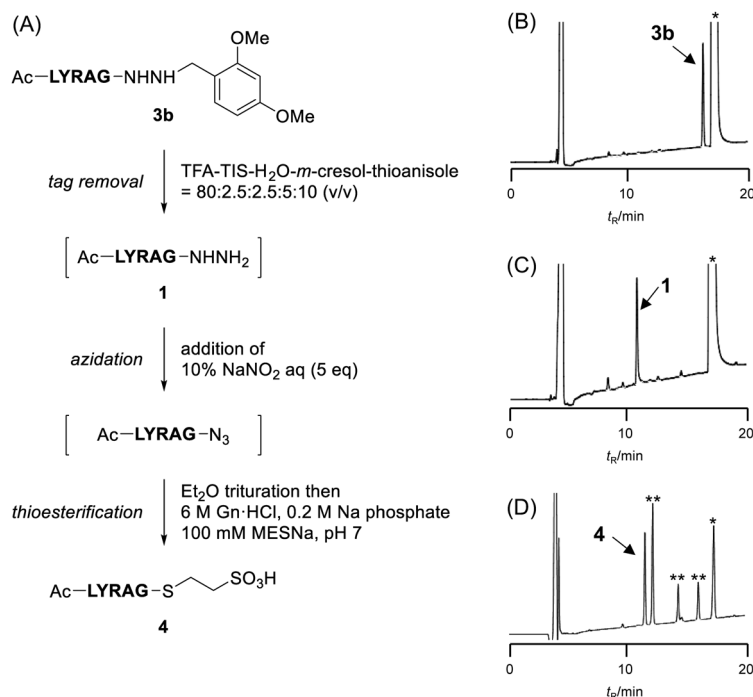
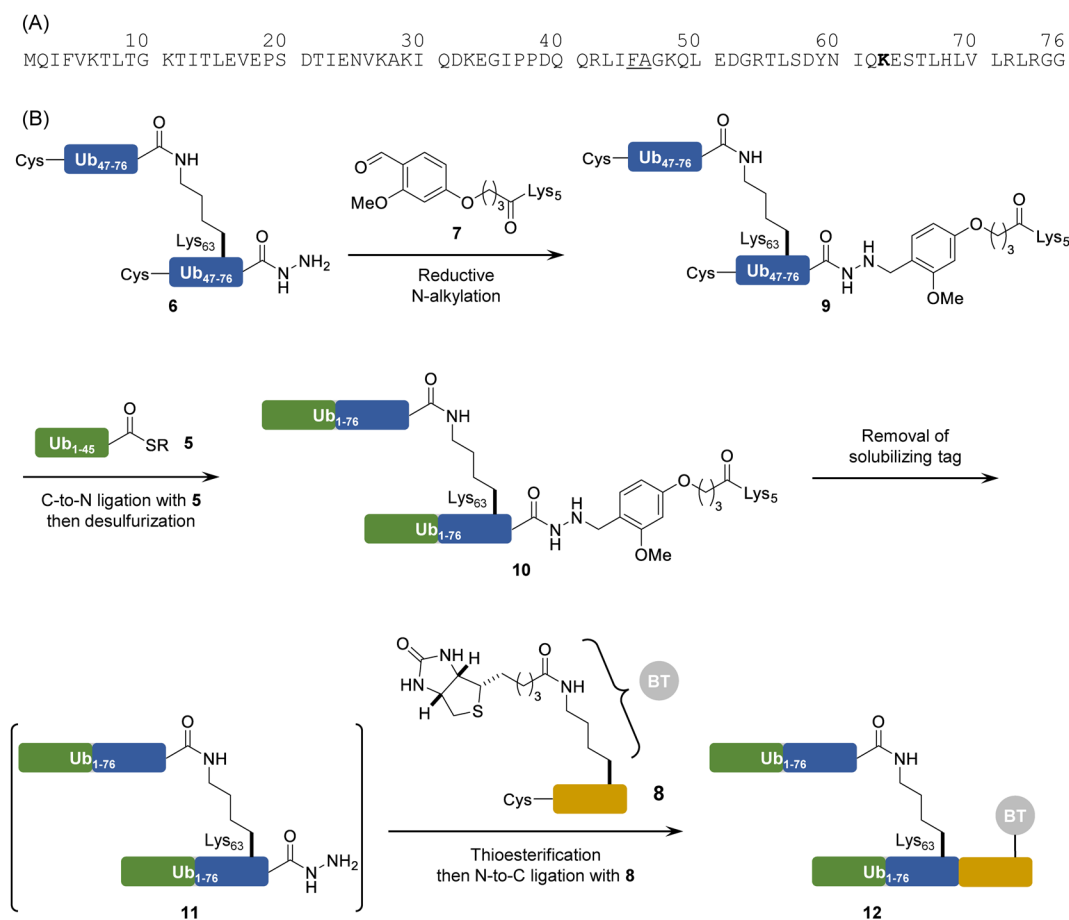


Fig. 1. One-Pot Conversion of Tag-Linked Hydrazides to Thioesters

HPLC chromatograms of: (B) tag removal (*t* = 0.5 min); (C) tag removal (*t* = 1 h); (D) thioesterification (*t* = 0.5 h). Asterisks in the HPLC chromatograms indicate *m*-cresol (\*), or non-peptidic compounds derived from reactions between NaNO<sub>2</sub> and scavengers (\*\*).



(A) Primary sequence of Ub. Ligation sites are underlined and Lys<sub>63</sub> are bold. (B) Synthetic Chart for Lys<sub>63</sub>-linked di-Ub derivative. See Chart S1 in supplementary materials for peptide fragment synthesis.

#### Chart 2. Synthesis of Lys<sub>63</sub>-Linked Di-Ub Derivative

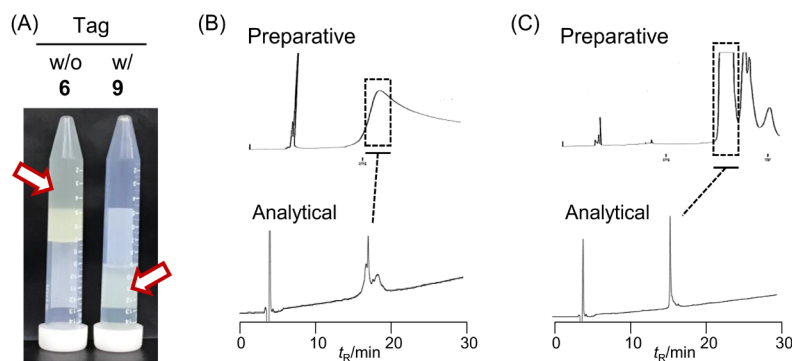


Fig. 2. Comparison of Properties with or without Solubilizing Tag

(A) 50  $\mu$ mol of peptide **6** (left) and peptide **9** (right) in 8 mL of 40% CH<sub>3</sub>CN-H<sub>2</sub>O containing 0.1% TFA. Preparative or analytical HPLC chromatograms of: (B) peptide **6**; (C) peptide **9**.

form should combine with a solubilizing tag possessing a dialkoxybenzyl linker (**7**) through reductive *N*-alkylation. A NCL reaction between two peptide segments (**5** and **9**) followed by desulfurization will afford Lys<sub>63</sub>-linked di-Ub<sub>1-76</sub> (**10**) containing the solubilizing tag. After removal of the tag and subsequent conversion of the alkyl hydrazide to a thioester, NCL of the resulting peptide with a biotin-containing peptide (**8**) that has Cys at the *N*-terminus should give the desired di-Ub derivative (**12**).

Peptide syntheses covering the Ub sequence were per-

formed on a Trt ChemMatrix resin bound to hydrazine. An *N*-(2,4-dimethoxybenzyl)-glycine unit was introduced at Gly<sub>53</sub> to prevent aspartimide formation during the SPPS.<sup>39</sup> Pseudoproline units at the Leu<sub>8</sub>-Thr<sub>9</sub>, Ile<sub>13</sub>-Thr<sub>14</sub>, Leu<sub>56</sub>-Ser<sub>57</sub>, and Ser<sub>65</sub>-Thr<sub>66</sub> were used to improve the synthetic efficiency.<sup>40</sup> The *N*-terminal segment Ub<sub>1-45</sub> was synthesized as the hydrazide and converted to the MESNa thioester, affording the Ub<sub>1-45</sub> thioester (**5**). The *C*-terminal segment was prepared as the hydrazide, and the branched structure at Lys<sub>63</sub> was formed during SPPS using the orthogonal building block Lys(Alloc)

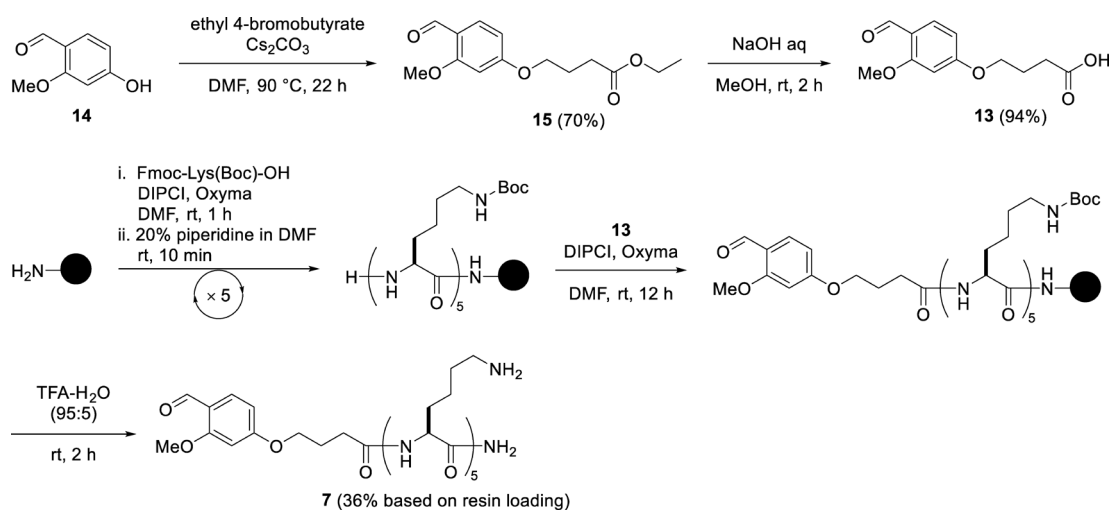
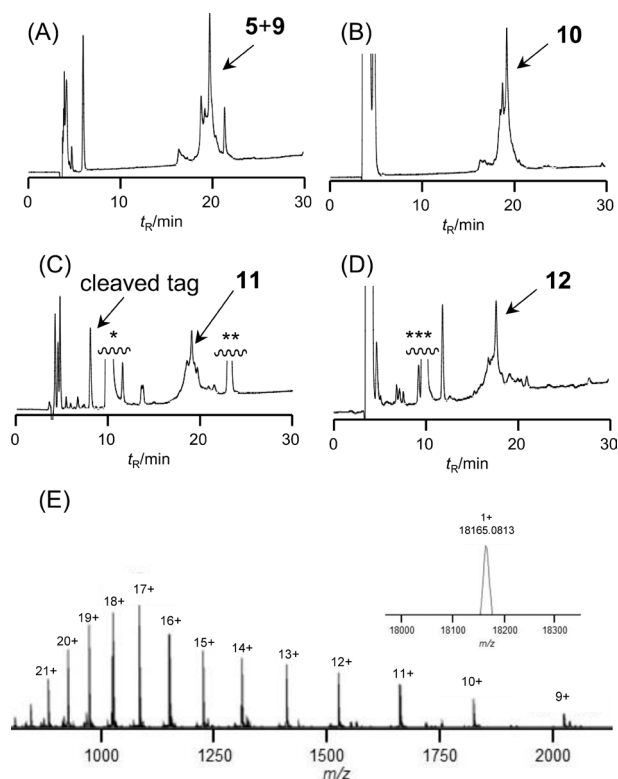


Chart 3. Synthesis of Solubilizing Tag 7

at this position. After elongation of the Boc-Ub<sub>46-76</sub>Ala<sub>46</sub>Cys peptide, the Alloc group at Lys<sub>63</sub> was removed by reaction with Pd(PPh<sub>3</sub>)<sub>4</sub> and PhSiH<sub>3</sub>, and the additional Ub<sub>46-76</sub>Ala<sub>46</sub>Cys sequence was elongated at the deprotected Lys<sub>63</sub> amino group to yield the branched peptide hydrazide (**6**). Although the N-terminal segment **5** was obtained successfully, the branched C-terminal segment **6** formed a gel when dissolving the crude material obtained after global deprotection in 40% aqueous CH<sub>3</sub>CN containing 0.1% TFA (Fig. 2A). The solvent was able to dissolve the crude material but imperfect peak separation was observed during HPLC purification (Fig. 2B), causing multiple purification steps and ultimately, an isolated yield of only 0.2%. Consequently, we tried to introduce the solubilizing tag prior to the purification steps.

The benzaldehyde derivative (**13**) was synthesized as shown in Chart 3. Alkylation of 4-formyl-3-methoxyphenol with ethyl 4-bromobutyrate followed by hydrolysis afforded the necessary formyl compound, 4-(4-formyl-3-methoxyphenoxy)butanoic acid (**13**) in 66% overall yield. As a hydrophilic moiety, Lys 5-mer was coupled with **13** on a Rink Amide AM resin, and this was followed by global deprotection, giving the solubilizing tag (**7**). The reductive *N*-alkylation of the hydrazide (**6**) as a crude material with the solubilizing tag (**7**) was performed in 50% AcOH-HFIP containing pic-BH<sub>3</sub> for 10 min to produce the tag-containing segment (**9**).<sup>41</sup> The resulting crude material showed improved solubility in 40% aqueous CH<sub>3</sub>CN containing 0.1% TFA without gel formation, leading to good peak separation in the HPLC purification (Fig. 2C). The isolated yield of segment **9** was 2%, ten times higher than the yield obtained in the absence of the solubilizing tag. Methyl thioglycolate-mediated NCL between **5** and **9** followed by desulfurization was performed in a one-pot reaction,<sup>42</sup> yielding the full-length protein containing the solubilizing tag (**10**) in 26% isolated yield without any difficulty associated with poor solubility (Fig. 3). The reaction sequence containing the removal of the solubilizing tag and azide formation by NaNO<sub>2</sub> in TFA resulted in a conversion of the alkyl hydrazide to the corresponding peptidyl azide. After being washed with Et<sub>2</sub>O to remove excess scavengers, the resulting precipitate was subjected to modification with the biotin-containing peptide H-CLPLTGGGK(biotin)-NH<sub>2</sub> (**8**) by NCL in a one-pot manner, yielding the Lys<sub>63</sub>-linked di-Ub with a biotin label from

Fig. 3. Synthesis of Lys<sub>63</sub>-Linked Di-Ub Derivative

HPLC chromatograms of: (A) NCL between **5** and **9** ( $t = 3$  h); (B) desulfurization of **5** + **9** ( $t = 18$  h); (C) tag removal ( $t = 1$  h), MS spectrum of the cleaved tag was identical to *m*-cresol adduct to the resulting benzyl cation; (D) NCL with **8** ( $t = 5$  h). Asterisks in the HPLC chromatograms indicate *m*-cresol (\*), thioanisole (\*\*), or MPAA(\*\*\*) (E) MS spectrum of Lys<sub>63</sub>-linked di-Ub derivative (**12**). The calculated mass is 18165.0 Da (average isotopes). Deconvolution of the mass spectrum yielded an observed mass of 18165.1 Da as a proton adduct.

the tag-containing segment (**10**) in 18% isolated yield.

## Conclusion

A strategy based on a solubilizing tag has a potential to overcome synthetic difficulties when dealing with poorly soluble peptides. Late-stage solubilization used even after peptide synthesis should play a more significant role because this can easily tune the solubility-enhancing property and avoid

Table 3. Peptide Characterization Data

Comp. #	Yield (%)	HPLC		MS ( <i>m/z</i> )		
		Analytical		Preparative	Calcd	Found
		Gradient (%) (column used) <sup>a)</sup>	Retention time (min)	Gradient (%) (column used) <sup>b)</sup>		
<b>1</b>	50	10–60 (C8) <sup>c)</sup>	10.2	10–20 (C8) <sup>c)</sup>	635.4 [M + H] <sup>+</sup>	635.4
<b>3a</b>	49	15–50 (C8) <sup>c)</sup>	13.2	20–30 (C8) <sup>c)</sup>	755.4 [M + H] <sup>+</sup>	755.4
<b>3b</b>	48	15–50 (C8) <sup>c)</sup>	13.6	20–30 (C8) <sup>c)</sup>	785.4 [M + H] <sup>+</sup>	785.4
<b>3c</b>	41	15–50 (C8) <sup>c)</sup>	14.3	20–30 (C8) <sup>c)</sup>	815.4 [M + H] <sup>+</sup>	815.4
<b>4</b>	—	10–40 (C8) <sup>d)</sup>	11.6	—	745.3 [M + H] <sup>+</sup>	745.3
<b>5</b>	12	10–60 (PR) <sup>c)</sup>	20.3	27–37 (PR) <sup>e)</sup>	1309.7 [M + 4H] <sup>4+</sup>	1309.7
<b>6</b>	0.2	10–60 (PR) <sup>c)</sup>	18.9	27–37 (PR) <sup>e)</sup>	1399.7 [M + 5H] <sup>5+</sup>	1399.5
<b>7</b>	36	5–35 (C18) <sup>e)</sup>	16.1	10–20 (C18) <sup>e)</sup>	878.6 [M + H] <sup>+</sup>	878.6
<b>8</b>	19	10–60 (C18) <sup>e)</sup>	13.9	18–28 (C18) <sup>e)</sup>	1070.5 [M + H] <sup>+</sup>	1070.6
<b>9</b>	2	10–60 (PR) <sup>c)</sup>	18.4	27–37 (PR) <sup>e)</sup>	1572.3 [M + 5H] <sup>5+</sup>	1572.1
<b>10</b>	26	20–50 (PR) <sup>c)</sup>	19.3	28–38 (PR) <sup>e)</sup>	1199.5 [M + 15H] <sup>15+</sup>	1199.8
<b>11</b>	—	25–45 (PR) <sup>c)</sup>	19.1	—	1142.0 [M + 15H] <sup>15+</sup>	1142.2
<b>12</b>	18	25–45 (PR) <sup>c)</sup>	17.8	28–38 (PR) <sup>e)</sup>	1211.3 [M + 15H] <sup>15+</sup>	1211.4

a) C8: Cosmosil 5C<sub>8</sub>-AR-300 analytical column; C18: Cosmosil 5C<sub>18</sub>-AR-II analytical column; PR: Cosmosil Protein-R analytical column. b) C8: Cosmosil 5C<sub>8</sub>-AR-300 preparative column; C18: Cosmosil 5C<sub>18</sub>-AR-II preparative column; PR: Cosmosil Protein-R semi-preparative column. c) Gradient over 30 min. d) Gradient over 20 min. e) Gradient over 60 min.

the need to resynthesize peptides. A previous version of late-stage solubilization using hydrazides was only applicable to a C-terminal segment, limiting the NCL direction to only the C-to-N direction because the product formed by tag removal was converted to a carboxylic acid. By contrast, the expanded method described here has greater potential because it enables assembly in both C→N and N→C directions. The newly designed linker can be easily synthesized, and its hydrophilic property can be tuned depending on the nature of the peptide at hand. The synthesis of the Ub dimer derivative shows that the enhanced solubility could improve the isolated yield of poorly soluble peptide intermediates. This method will open the synthetic accessibility of proteins which due to their poor solubility, were thought to be synthesized with difficulty by a chemical method.

## Experimental

**General Information** All commercially available reagents and protected amino acids were used without further purification. Thin-layer chromatography was performed on Merck 60F254 precoated silica gel plates which were visualized by fluorescence quenching under UV light and by staining with 2,4-dinitrophenylhydrazine. Column chromatography was carried out using a silica gel 60N (Kanto Chemical, Tokyo, Japan).

<sup>1</sup>H-NMR (400 MHz) and <sup>13</sup>C-NMR (100 MHz) spectra were recorded using a Bruker AVANCE III HD spectrometer. Chemical shifts are reported in δ (ppm) relative to tetramethylsilane as an internal standard. Mass spectra were recorded on a Bruker Compact (ESI-Q-TOF) mass spectrometer. Melting points were measured using a CORNESCO Technologies MPA 100. IR spectra were recorded on a JASCO FT/IR 6300 with ATR and are reported in wavenumbers (cm<sup>-1</sup>).

HPLC analyses were carried out at 30 °C on a system using JASCO PU-2089 Plus, UV-2070 Plus, AS-2057 Plus, and CO-2060 Plus. A solvent system consisting of 0.1% TFA aqueous solution (v/v, solvent A) and 0.1% TFA in CH<sub>3</sub>CN (v/v, solvent B) was used for HPLC elution. Characterization data

of peptides are summarized in Table 3.

## General Procedure for 9-Fluorenylmethoxycarbonyl (Fmoc)-Based SPPS

### Typical Procedure

Side chain protecting groups of Fmoc amino acids are as follows: Boc: **H** and **K**; Ot-Bu: **D** and **E**; Pbf: **R**; *t*-Bu: **S**, **T** and **Y**; Trt: **C**, **N** and **Q**; Alloc: **K** for K<sub>63</sub> of proximal Ub or K<sub>9</sub> of peptide **8**. Fmoc-(Dmb)Gly-OH was prepared by a previously published procedure.<sup>43</sup> Fmoc SPPS was performed with an automated microwave-assisted synthesis or a manual synthesis. The microwave-assisted SPPS using a Biotage Initiator<sup>+</sup> Alstra peptide synthesizer with a 10 mL open-type vial was conducted as follows: coupling with amino acids except for Cys(Trt) or Arg(Pbf): Fmoc-protected amino acid, ethyl 2-cyano-2-(hydroxyamino)acetate (Oxyma), and *N,N'*-diisopropylcarbodiimide (DIPCI) (4.0 equivalents each) in *N,N*-dimethylformamide (DMF) (0.5 M), 5 min, 75 °C under microwave irradiation; coupling with Cys(Trt) or Arg(Pbf): Fmoc-protected amino acid, Oxyma and DIPCI (4.0 equivalents each) in DMF (0.5 M), 60 min, room temperature (r.t.); Fmoc removal: 20% (v/v) piperidine in DMF, 3 min, 50 °C under microwave irradiation. The reaction temperatures were monitored by an IR sensor. Except where specifically noted, the manual synthesis was conducted as follows: 1. (coupling) Fmoc-protected amino acid, Oxyma, and DIPCI (4.0 equivalents each) in DMF (0.3 M), 60 min, r.t.; 2. (Fmoc removal) 20% (v/v) piperidine in DMF, 10 min, r.t..

### Deprotection of Alloc Group on Solid Support

The peptidyl resin was swollen in dichloromethane (DCM) and solutions of Pd(PPh<sub>3</sub>)<sub>4</sub> (0.24 equivalents), then PhSiH<sub>3</sub> (24 equivalents) in DCM were added to the reaction mixture which was then stirred at r.t. under an argon atmosphere for 2 × 30 min. After filtration, the resulting resin was washed three times each with the following solvents: tetrahydrofuran (THF), DMF, DCM, 0.5% (v/v) *N,N*-diisopropylethylamine (DIPEA) in DCM, 0.02 M sodium diethyldithiocarbamate in DMF, DMF and DCM.<sup>44</sup>

### Preparation of Hydrazide-Incorporated Resin

Trityl OH-ChemMatrix resin was swollen in dry DCM and treated with thionyl chloride (10 equivalents) at r.t. for 2h. After being washed with dry DCM and dry DMF, the resulting resin was again swollen with dry DCM. A solution of 9-fluorenylmethyl carbazate (4.0 equivalents) and DIPEA (10 equivalents) in dry DMF was added dropwise to the resin at 0°C. The reaction mixture was shaken at r.t. overnight, then MeOH was added to cap extra sites. After 10min, the resin was filtered and washed successively with DMF, water, DMF, MeOH and DCM. The loading was confirmed by quantification of the Fmoc group.

### Examinations Using Model Peptides

#### Synthesis of Model Peptide Hydrazide **1**

Model peptide Ac-LYRAG-NHNH<sub>2</sub>**1** was elongated on the hydrazine-incorporated resin by the microwave-assisted synthesis and the resulting resin was treated with TFA-TIS-H<sub>2</sub>O (95:2.5:2.5 (v/v), 50 μL/1 mg resin) at r.t. for 2h. The resin in the reaction mixture was removed by filtration and then the filtrate was concentrated in a stream of air. Cold Et<sub>2</sub>O was added to the resulting filtrate to give a precipitate. After having been repeatedly washed with Et<sub>2</sub>O, the precipitated crude peptides were purified by preparative HPLC.

#### Reductive *N*-Alkylation of a Peptide Hydrazide

The peptide hydrazide **1** (0.10 μmol) was reacted with aldehyde **2a**, **2b** or **2c** (1.5 equivalents) in 50% (v/v) AcOH-HFIP (100 μL) at 37°C. After 1h reaction, pic-BH<sub>3</sub> (20 equivalents) was added, and the mixture was incubated at 37°C. After the appropriate time shown in Table 1, TFA (2 μL) was added to the reaction mixture. The resulting solution was concentrated in a stream of air and then washed with Et<sub>2</sub>O. The precipitate that was formed was dissolved in 50% (v/v) aqueous CH<sub>3</sub>CN (100 μL) and the solution was analyzed by HPLC. For isolation, hydrazide **1** (5.0 μmol) was used and the product was purified by preparative HPLC.

#### Examination of Reactivity and Stability of *N*-Alkyl Hydrazide Peptides

The *N*-alkyl hydrazide (**3a–3c**, 0.10 μmol) was dissolved in each of the following solutions and incubated at 37°C for the appropriate time shown in Table 2. 1. (Tag removal condition) 5% (v/v) TIS-TFA (100 μL); 2. (NCL condition) 6M guanidine (Gn)·HCl, 0.2M Na<sub>2</sub>HPO<sub>4</sub>, 100mM 4-mercaptobenzoic acid (MPAA), 50mM tris(2-carboxyethyl)phosphine (TCEP)·HCl, pH 7.1; 3. (Thz deprotection) 6M Gn·HCl, 0.2M Na<sub>2</sub>HPO<sub>4</sub>, 100mM MPAA, 50mM TCEP·HCl, 200mM MeONH<sub>2</sub>·HCl, pH 5.1; 4. (Desulfurization) 6M Gn·HCl, 0.2M Na<sub>2</sub>HPO<sub>4</sub>, 150mM MESNa, 250mM TCEP·HCl, 50mM 2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044), pH 6.2.

#### One-Pot Conversion of Alkyl Hydrazides to Thioesters

The *N*-alkyl hydrazide (**3b**, 0.10 μmol) was treated with TFA-TIS-H<sub>2</sub>O-*m*-cresol-thioanisole (80:2.5:2.5:5:10, (v/v), 0.1 μmol/100 μL) and the mixture was allowed to react for 1h at 37°C. An aqueous solution of NaNO<sub>2</sub> (5 μL, 5 equivalents) was added to the mixture at -10°C, and the solution was incubated at -10°C for 20min. To the reaction mixture was added cold Et<sub>2</sub>O to give a precipitate which was collected by centrifugation and thoroughly washed with Et<sub>2</sub>O to afford a peptide azide. The crude product was dissolved in 100 μL of buffer (6M Gn·HCl, 0.2M Na phosphate, 100mM MESNa, pH 7.0), and the solution was incubated at 37°C to yield the thioester (**4**).

### Synthesis of the Solubilizing Tag

#### Compound **15**

4-Formyl-3-methoxyphenol (**14**, 0.50 g, 3.3 mmol) was dissolved in DMF (2 mL), then Cs<sub>2</sub>CO<sub>3</sub> (0.76 g, 3.9 mmol) was added in one portion followed by the addition of neat ethyl 4-bromobutyrate (0.57 mL, 3.9 mmol). The temperature was then increased to 90°C and maintained there for 22h and then cooled to r.t. and filtered. The filtrate was concentrated under reduced pressure. The residue was dissolved in EtOAc (20 mL), the organic phase was washed with aqueous 1M HCl (3 × 5 mL) and aqueous 1M NaOH (3 × 1 mL), and then dried over Na<sub>2</sub>SO<sub>4</sub>. After filtration and subsequent removal of the solvent under reduced pressure, chromatographic purification on silica gel (*n*-hexane/EtOAc = 3:1, (v/v)) gave compound **15** in 70% isolated yield (0.61 g) as a white solid. <sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ: 1.27 (3H, t, *J* = 7.1 Hz), 2.14 (2H, tt, *J* = 7.2, 6.2 Hz), 2.52 (2H, t, *J* = 7.2 Hz), 3.9 (3H, s), 4.09 (2H, t, *J* = 6.2 Hz), 4.16 (2H, q, *J* = 7.1 Hz), 6.45 (1H, d, *J* = 2.2 Hz), 6.53 (1H, dd, *J* = 8.7, 2.2 Hz), 7.79 (1H, d, *J* = 8.7 Hz), 10.29 (1H, s); <sup>13</sup>C-NMR (CDCl<sub>3</sub>) δ: 14.2, 24.4, 30.6, 55.6, 60.6, 67.2, 98.3, 106.3, 119.1, 130.8, 163.6, 165.4, 173.0, 188.3; mp: 47°C; IR (ATR) cm<sup>-1</sup>: 1730 (CO), 1600 (CO); ESI-MS *m/z*: 289.0959 (Calcd for C<sub>14</sub>H<sub>18</sub>NaO<sub>5</sub>: 289.1046).

#### Compound **13**

Compound **15** (0.61 g, 2.3 mmol) in MeOH (10 mL) was treated with 2M NaOH aqueous solution (2.3 mL). After being stirred at r.t. for 2h, the reaction mixture was concentrated *in vacuo* to obtain a crude material. A solution of this material in H<sub>2</sub>O (1 mL) was acidified with aqueous 6M HCl to pH 1–2 to afford a precipitate which was collected by filtration, washed with H<sub>2</sub>O, and dried to give compound **13** in 94% isolated yield (0.51 g) as a white solid. <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>) δ: 1.97 (2H, tt, *J* = 7.3, 6.5 Hz), 2.4 (2H, t, *J* = 7.3 Hz), 3.91 (3H, s), 4.12 (2H, t, *J* = 6.5 Hz), 6.64 (1H, dd, *J* = 8.6, 2.1 Hz), 6.69 (1H, d, *J* = 2.1 Hz), 7.65 (1H, d, *J* = 8.6 Hz), 10.17 (1H, s), 12.19 (1H, s); <sup>13</sup>C-NMR (DMSO-*d*<sub>6</sub>) δ: 24.5, 30.4, 56.5, 67.8, 99.1, 107.6, 118.6, 130.3, 163.9, 165.8, 174.5, 187.7; mp: 130°C; IR (ATR) cm<sup>-1</sup>: 3523 (OH), 1731 (CO), 1588 (CO), 1174 (CO); ESI-MS *m/z*: 237.0909 (Calcd for C<sub>12</sub>H<sub>13</sub>O<sub>5</sub>: 237.0768).

#### SPPS for Solubilizing Tag **7**

The Lys 5-mer was elongated on a Rink Amide AM resin (0.28 g, 0.20 mmol, 0.70 mmol/g) using a manual protocol to produce a protected peptidyl resin. After removal of the final Fmoc group, compound **13** (0.19 g, 0.80 mmol) was coupled in the presence of DIPCI (4 equivalents) and Oxyma (4 equivalents) in DMF. The coupling proceeded to completion within 12h. Cleavage of the solubilizing tag from the resin was performed using TFA-H<sub>2</sub>O (95:5 (v/v), 50 μL/1 mg resin) at r.t. for 2h. The resin in the reaction mixture was filtered off and then the filtrate was concentrated in a stream of air. The crude material was dissolved in 0.1% TFA-containing H<sub>2</sub>O (3 mL) and washed with Et<sub>2</sub>O (3 × 5 mL). This was purified by preparative HPLC.

### Synthesis of Lys<sub>63</sub>-Linked Di-Ub Derivative

#### Synthesis of Biotin Peptide **8**

Fmoc-Lys(Alloc)-OH was coupled to Rink Amide ChemMareix resin (0.42 mmol/g, 0.12 g, 0.05 mmol) and the peptide was elongated using microwave-assisted Fmoc SPPS. Alloc group was deprotected as described above and then biotin (48 mg, 0.20 mmol, 4 equivalents) was coupled to the resulting amino group using 1-[bis(dimethylamino)methylene]-

1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate, (HATU) (72 mg, 0.19 mmol, 3.8 equivalents) and DIPEA (70  $\mu$ L, 0.40 mmol, 8 equivalents) in 50% (v/v) DMSO–DMF for 30 min at r.t. Cleavage of the peptide was performed using TFA–TIS–H<sub>2</sub>O (95:2.5:2.5, (v/v), 50  $\mu$ L/1 mg resin) at r.t. After 2 h cleavage, the reaction mixture was filtrated off and then the filtrate was concentrated in a stream of N<sub>2</sub>. Cold Et<sub>2</sub>O was added to the resulting filtrate to give a precipitate. After successive washing with Et<sub>2</sub>O, the crude product was purified by preparative HPLC.

#### Synthesis of the Peptide Thioester (**5**)

On hydrazine incorporated resin (0.31 mmol/g), the peptide was elongated using microwave-assisted Fmoc SPPS. Fmoc-Leu-Thr( $\Psi^{Me,Me}$ pro)-OH and Fmoc-Ile-Thr( $\Psi^{Me,Me}$ pro)-OH were used for the Leu<sub>8</sub>-Thr<sub>9</sub> and Ile<sub>13</sub>-Thr<sub>14</sub> sites. The resin was treated with TFA–TIS–H<sub>2</sub>O–*m*-cresol-thioanisole (80:2.5:2.5:5:10, (v/v), 50  $\mu$ L/1 mg resin) at r.t. After 2 h, 10% (w/w) NaNO<sub>2</sub> aq (1  $\mu$ L/1 mg resin) was added to the mixture at –10 °C and the solution was incubated at –10 °C for 20 min. To the reaction mixture was added cold Et<sub>2</sub>O giving a precipitate. The precipitate was collected by centrifugation and thoroughly washed with Et<sub>2</sub>O to afford a crude peptide azide. The crude product was dissolved into 5% (w/w) MESNa in buffer (6 M Gn·HCl, 0.2 M Na phosphate, pH 7.3, 5 mL). After 30 min at r.t., the solution was acidified to pH 2–3 by the addition of TFA and purified by preparative HPLC.

#### Synthesis of Peptide Hydrazides **6** and **9**

Peptide **6** was elongated on a hydrazine incorporated resin (338 mg, 0.10 mmol, 0.31 mmol/g) using a manual SPPS procedure. On this resin, standard Fmoc SPPS (coupling: 3.0 equivalents of amino acid using HATU (2.8 equivalents) and DIPEA (6.0 equivalents) in DMF, 10 min, r.t.; Fmoc removal: 20% (v/v) piperidine/DMF, 10 min, r.t.) was performed for the chain elongation to give a protected peptide resin. Fmoc-Leu-Ser( $\Psi^{Me,Me}$ pro)-OH, Fmoc-Ser(*t*-Bu)-Thr( $\Psi^{Me,Me}$ pro)-OH and Fmoc-(Dmb)Gly-OH were used for the Leu<sub>56</sub>–Ser<sub>57</sub>, Ser<sub>65</sub>–Thr<sub>66</sub> and Gly<sub>53</sub> sites, respectively. After elongation of the Boc-Ub<sub>46-76</sub>Ala<sub>46</sub>Cys peptide, the Alloc group at Lys<sub>63</sub> was removed. The remaining sequence was further elongated at the resulting Lys side chain. The resulting resin (666 mg, 50  $\mu$ mol) was treated with TFA–TIS–H<sub>2</sub>O (95:2.5:2.5 (v/v), 50  $\mu$ L/1 mg resin) at r.t. for 2 h. After filtration of the resin in the reaction mixture, the filtrate was removed in an air stream and the addition of cooled Et<sub>2</sub>O to the residue afforded a precipitate. After successively washed with Et<sub>2</sub>O, the crude peptide was purified by preparative HPLC. Four times purification was required to attain a pure product **6** due to the low peak separation.

The crude material obtained from cleavage of the resin (662 mg, 50  $\mu$ mol) was dissolved in 50% AcOH–HFIP (10 mL). The solubilizing tag **7** (110 mg, 73  $\mu$ mol, 1.5 equivalents) was added, and the mixture was allowed to react for 2 h at 37 °C. Following the addition of Pic–BH<sub>3</sub> (100 mg, 970  $\mu$ mol, 20 equivalents), the mixture was incubated for additional 10 min at 37 °C. After the addition of TFA (370  $\mu$ L) to the solution, the solvent was removed in an air stream, affording the crude material (**9**). The residue was dissolved in H<sub>2</sub>O–CH<sub>3</sub>CN (5:3 (v/v), 8 mL) and purified by preparative HPLC. In this case two purification steps were required to purify the product (**9**).

#### NCL between **5** and **9** Followed by Desulfurization

The N-Cys peptide (**9**, 0.90  $\mu$ mol, 1.0 equivalents) and pep-

ptide thioester (**5**, 1.85  $\mu$ mol, 2.05 equivalents) were dissolved in 900  $\mu$ L of ligation buffer (6 M Gn·HCl, 0.2 M Na phosphate, 50 mM methyl thioglycolate, 30 mM TCEP·HCl, pH 7.3), and the solution was incubated at 37 °C. After completion of the ligation in 3 h, desulfurization buffer (6 M Gn·HCl, 0.2 M Na phosphate, 500 mM TCEP·HCl, 200 mM MESNa, pH 6.6, 900  $\mu$ L) and VA-044 (31 mg, 90  $\mu$ mol, 100 equivalents) as a solid were added. The mixture was allowed to react at 37 °C for 18 h. After pH adjustment to 1–2 by addition of TFA, the desulfurized product (**10**) was purified by preparative HPLC.

#### Tag Removal from **10** Followed by NCL with Peptide **8**

The peptide (**10**, 0.23  $\mu$ mol) was treated with TFA–TIS–H<sub>2</sub>O–*m*-cresol-thioanisole (80:2.5:2.5:5:10, (v/v), 0.1  $\mu$ mol/100  $\mu$ L) and the mixture was allowed to react for 3 h at 37 °C. Then, NaNO<sub>2</sub> aq (5  $\mu$ L, 5 equivalents) was added at –10 °C, and the solution was incubated at –10 °C for 30 min. Cold Et<sub>2</sub>O was added to the reaction mixture to give a precipitate which was collected by centrifugation and thoroughly washed with Et<sub>2</sub>O to afford a peptide azide. The crude product was dissolved in 400  $\mu$ L of ligation buffer (6 M Gn·HCl, 0.2 M Na phosphate, 100 mM MPAA, pH 7.3) containing peptide **8** (0.46  $\mu$ mol, 2 equivalents), and the solution was incubated at 37 °C. The reaction was monitored by HPLC and essentially completed within 5 h. After adjustment to pH 1–2 by addition of TFA, the ligated product (**12**) was isolated and purified by preparative HPLC.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** This article contains supplementary materials.

#### References and Notes

- 1) Toseland C. P., *J. Chem. Biol.*, **6**, 85–95 (2013).
- 2) Liu J., Cui Z., *Bioconjugate Chem.*, **31**, 1587–1595 (2020).
- 3) Shimogawa M., Petersson E. J., *Curr. Opin. Chem. Biol.*, **64**, 57–66 (2021).
- 4) Vogl D. P., Conibear A. C., Becker C. F. W., *RSC Chem. Biol.*, **2**, 1441–1461 (2021).
- 5) Conibear A. C., Watson E. E., Payne R. J., Becker C. F. W., *Chem. Soc. Rev.*, **47**, 9046–9068 (2018).
- 6) Conibear A. C., *Nat. Rev. Chem.*, **4**, 674–695 (2020).
- 7) Dawson P. E., Muir T. W., Clark-Lewis I., Kent S. B. H., *Science*, **266**, 776–779 (1994).
- 8) Kulkarni S. S., Sayers J., Premjee B., Payne R. J., *Nat. Rev. Chem.*, **2**, 1–17 (2018).
- 9) Agouridas V., Mahdi O. E., Diemer V., Cargoët M., Monbaliu J.-C. M., Melnyk O., *Chem. Rev.*, **119**, 7328–7443 (2019).
- 10) Muir T. W., Sondhi D., Cole P. A., *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 6705–6710 (1998).
- 11) Mueller L. K., Baumruck A. C., Zhdanova H., Tietze A. A., *Front. Bioeng. Biotechnol.*, **8**, 162 (2020).
- 12) Zhao D.-D., Fan X.-W., Hao H., Zhang H.-L., Guo Y., *Curr. Org. Chem.*, **23**, 2–13 (2019).
- 13) Harris P. W. R., Brimble M. A., *Pept. Sci.*, **94**, 542–550 (2010).
- 14) Paradis-Bas M., Tulla-Puche J., Albericio F., *Org. Lett.*, **17**, 294–297 (2015).



- 15) Huang Y.-C., Li Y.-M., Chen Y., Pan M., Li Y.-T., Yu L., Guo Q.-X., Liu L., *Angew. Chem. Int. Ed.*, **52**, 4858–4862 (2013).
- 16) Tsuda S., Masuda S., Yoshiya T., *ChemBioChem*, **20**, 2063–2069 (2019).
- 17) Giesler R. J., Spaltenstein P., Jacobsen M. T., Xu W., Maqueda M., Kay M. S., *Org. Biomol. Chem.*, **19**, 8821–8829 (2021).
- 18) Tan Z., Shang S., Danishefsky S. J., *Proc. Natl. Acad. Sci. U.S.A.*, **108**, 4297–4302 (2011).
- 19) Jacobsen M. T., Petersen M. E., Ye X., Galibert M., Lorimer G. H., Aucagne V., Kay M. S., *J. Am. Chem. Soc.*, **138**, 11775–11782 (2016).
- 20) Karas J. A., Noor A., Schieber C., Connell T. U., Separovic F., Donnelly P. S., *Chem. Commun.*, **53**, 6903–6905 (2017).
- 21) Tsuda S., Nishio H., Yoshiya T., *Chem. Commun.*, **54**, 8861–8864 (2018).
- 22) Maity S. K., Mann G., Jbara M., Laps S., Kamnesky G., Brik A., *Org. Lett.*, **18**, 3026–3029 (2016).
- 23) Brailsford J. A., Stockdill J. L., Axelrod A. J., Peterson M. T., Vadola P. A., Johnston E. V., Danishefsky S. J., *Tetrahedron*, **74**, 1951–1956 (2018).
- 24) Tsuda S., Mochizuki M., Ishiba H., Yoshizawa-Kumagaye K., Nishio H., Oishi S., Yoshiya T., *Angew. Chem. Int. Ed.*, **57**, 2105–2109 (2018).
- 25) Liu J., Wei T., Tan Y., Liu H., Li X., *Chem. Sci.*, **13**, 1367–1374 (2022).
- 26) Zheng J.-S., Yu M., Qi Y.-K., Tang S., Shen F., Wang Z.-P., Xiao L., Zhang L., Tian C.-L., Liu L., *J. Am. Chem. Soc.*, **136**, 3695–3704 (2014).
- 27) Bello C., Wang S., Meng L., Moremen K. W., Becker C. F. W., *Angew. Chem. Int. Ed.*, **54**, 7711–7715 (2015).
- 28) Zheng J.-S., He Y., Zuo C., Cai X.-Y., Tang S., Wang Z. A., Zhang L.-H., Tian C.-L., Liu L., *J. Am. Chem. Soc.*, **138**, 3553–3561 (2016).
- 29) Huang D.-L., Montigny C., Zheng Y., Beswick V., Li Y., Cao X.-X., Barbot T., Jaxel C., Liang J., Xue M., Tian C.-L., Jamin N., Zheng J.-S., *Angew. Chem. Int. Ed.*, **59**, 5178–5184 (2020).
- 30) Sato K., Tanaka S., Wang J., Ishikawa K., Tsuda S., Narumi T., Yoshiya T., Mase N., *Org. Lett.*, **23**, 1653–1658 (2021).
- 31) Sato K., Tanaka S., Yamamoto K., Tashiro Y., Narumi T., Mase N., *Chem. Commun.*, **54**, 9127–9130 (2018).
- 32) Narita M., Honda S., Umeyama H., Obana S., *Bull. Chem. Soc. Jpn.*, **61**, 281–284 (1988).
- 33) Pearson D. A., Blanchette M., Baker M. L., Guindon C. A., *Tetrahedron Lett.*, **30**, 2739–2742 (1989).
- 34) James I. W., *Tetrahedron*, **55**, 4855–4946 (1999).
- 35) Bang D., Kent S. B. H., *Angew. Chem. Int. Ed.*, **43**, 2534–2538 (2004).
- 36) Wan Q., Danishefsky S. J., *Angew. Chem. Int. Ed.*, **46**, 9248–9252 (2007).
- 37) Komander D., Rape M., *Annu. Rev. Biochem.*, **81**, 203–229 (2012).
- 38) Bondalapati S., Eid E., Mali S. M., Wolberger C., Brik A., *Chem. Sci.*, **8**, 4027–4034 (2017).
- 39) Cardona V., Eberle I., Barthélémy S., Beythien J., Doerner B., Schneeberger P., Keyte J., White P. D., *Int. J. Pept. Res. Ther.*, **14**, 285–292 (2008).
- 40) Wöhr T., Wahl F., Nefzi A., Rohwedder B., Sato T., Sun X., Mutter M., *J. Am. Chem. Soc.*, **118**, 9218–9227 (1996).
- 41) Shorter reduction time can suppress the excess installation of the tag on the N-terminal cysteines. See ref. 30.
- 42) Huang Y.-C., Chen C.-C., Gao S., Wang Y.-H., Xiao H., Wang F., Tian C.-L., Li Y.-M., *Chem. Eur. J.*, **22**, 7623–7628 (2016).
- 43) Hart P., Kleijn L. H. J., Bruin G. D., Oppedijk S. F., Kemmink J., Martin N. I., *Org. Biomol. Chem.*, **12**, 913–918 (2014).
- 44) Leelasatanakij L., Aldrich J. V., *J. Pept. Res.*, **56**, 80–87 (2000).